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Autoimmune Hemolytic Anemias: Challenges in Diagnosis and Therapy

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Warm autoimmune hemolytic anemia · Cold agglutinin disease · Rituximab · Direct anti-globulin test · Complement system · Thrombosis · Infection

Abstract
Background: Autoimmune hemolytic anemia (AIHA) is a rare disease due to increased destruction of erythrocytes by autoantibodies, with or without complement activation. ***Summary:*** AIHA is usually classified in warm AIHA (wAIHA) and cold agglutinin disease (CAD), based on isotype and thermal amplitude of the autoantibody. The direct antiglobulin test (DAT) or Coombs test is the cornerstone of AIHA diagnosis, as it is positive with anti-IgG in wAIHA, and with anti-C3d/IgM antisera plus high titer cold agglutinins in CAD. Therapy is quite different, as steroids and rituximab are effective in the former, but have a lower response rate and duration in the latter. Splenectomy, which is still a good option for young/fit wAIHA, is contraindicated in CAD, and classic immunosuppressants are moving to further lines. Several new drugs are increasingly used or are in trials for relapsed/refractory AIHAs, including B-cell (parsaclisib, ibrutinib, rilzabrutinib), and plasma cell target therapies (bortezomib, daratumumab), bispecific agents (ianalumab, obexelimab, povetacicept), neonatal Fc receptor blockers (nipocalimab), and complement inhibitors (sutimlimab, riliprubart, pegcetacoplan, iptacopan). Clinically, AIHAs are highly heterogeneous, from mild/compensated to life-threatening/fulminant, and may be primary or associated with infections, neoplasms, autoimmune diseases, transplants, immunodeficiencies, and drugs. Along with all these variables, there are rare forms like mixed (wAIHA plus CAD), atypical (IgA or warm IgM driven), and DAT negative, where the diagnosis and clinical management are particularly challenging. ***Key Messages:*** This article covers the classic clinical features, diagnosis, and therapy of wAIHA and CAD, and focuses, with the support of clinical vignettes, on difficult diagnosis and refractory/relapsing cases requiring novel therapies.

Introduction

Autoimmune hemolytic anemia (AIHA) is a rare disease with an estimated incidence of 1–3 per 100,000/year and is caused by increased destruction of red blood cells (RBC) by autoantibodies, with or without complement activation [1–3]. Based on the isotype and thermal characteristics of the autoantibody two main types of AIHA are recognized: classic warm AIHA (wAIHA) and cold agglutinin disease (CAD), identified by the direct antiglobulin test (DAT) or Coombs test. wAIHA is sustained by an IgG that may weakly activate complement (especially the IgG2 and IgG4 subclasses), while CAD is due to an IgM, that usually binds to erythrocytes at temperatures <20°C and strongly activates complement. More rare forms are the mixed forms (wAIHA plus CAD), the atypical ones (IgA or warm IgM driven, and DAT negative) and the ultra-rare paroxysmal cold hemoglobinuria [1]. AIHA may be primary in about half of cases or associated to several conditions such as neoplasms (chronic lymphocytic leukemia, lymphoma, ovarian teratoma, thymoma, Castleman disease), autoimmune diseases (systemic lupus erythematosus,

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systemic sclerosis, vasculitis, Sjogren syndrome, antiphospholipid syndrome, autoimmune lymphoproliferative syndrome, autoimmune bowel disease, autoimmune hepatitis), solid organ and hematopoietic stem cell transplants, numerous drugs (including immune checkpoint inhibitors anti-PD-1, PD-L1, and CTLA-4), congenital immunodeficiencies and infections (Parvovirus B19, mycoplasma pneumonia, mycobacterium tuberculosis, brucellosis, syphilis, EBV, CMV, hepatotropic virus, HIV, and SARS-CoV-2) [1, 2]. Of note, infections may be either triggers for the onset of the disease or complications of an established AIHA, generally due to heavy therapeutic immunosuppression or underlying immunodeficiency [4–6]. Clinically, the disease is highly heterogeneous, from mild/compensated to life-threatening/fulminant cases. Hemolytic markers (unconjugated bilirubin, LDH, haptoglobin, reticulocytes) may be variably altered and have several confounders (Gilbert syndrome, hypohaptoglobinemia, tissue necrosis or increased turnover, vitamin or iron deficiency, liver and renal disease, blood loss, and many others). The DAT is neither 100% specific nor sensitive, and several technical issues should be considered for a proper interpretation. Additionally, therapy is poorly evidence-based and mainly funded on consensus of experts [1, 7, 8]. However, several new drugs have been investigated or are under investigation in prospective/controlled trials offering new therapeutic options for this disease. In this review, we will address the diagnostic and therapeutic challenges in AIHA, as well as the unmet needs for rare forms and relapsed/refractory cases.

Diagnosis

The direct antiglobulin test (DAT) is the cornerstone for the diagnosis of AIHA. It is commonly performed with polyspecific antisera that are unable to distinguish wAIHA and CAD and provide a semiquantitative positivity. Therefore, for a proper diagnosis and therapy, monospecific antisera anti-IgG, -IgA, -IgM, and -complement (C3d) are required: in wAIHA the DAT is positive for IgG and in a fraction of cases IgG plus (weakly) C3d, and in CAD the DAT is positive for C3d or (rarely) for IgM. In fact, C3d can be considered a substitute of IgM positivity, since after complement activation the IgM usually detaches from erythrocytes during washing procedures or even *in vivo*. The diagnosis of CAD may be suspected when spontaneous agglutination occurs at room temperature and is confirmed by a serum titer of cold agglutinins >1/64. IgA-associated AIHA is very rare, as these autoantibodies are commonly associated with IgG [9]. A diagnostic flowchart is provided in Figure 1. Of note, Hb values may be highly variable, from less than 3 g/dL to normal values; reticulocytes are usually increased if an effective bone marrow compensation occurs, but reticulocytosis may be absent in about 20% of adults and up to 40% of children. Unconjugated hyperbilirubinemia is a typical marker of extravascular hemolysis occurring in spleen, liver, and lymphoid organs (predominant in classic wAIHA and chronic CAD), while increased LDH is a hallmark of intravascular hemolysis (typical of wAIHA with complement involvement, acute crisis of CAD, mixed forms, and paroxysmal cold hemoglobinuria [10–12]. Finally, haptoglobin is the most sensitive hemolytic marker and may be reduced even after complete recovery. The diagnosis of an underlying disease may be particularly complex, as it involves several conditions, including hematologic and non-hematologic neoplasms, systemic and organ-specific autoimmune diseases, congenital immunodeficiencies, infections, solid organ and hematopoietic stem cell transplants, numerous drugs, and novel biological treatments [6]. Among associated conditions it is worth mentioning Evans syndrome, defined by the simultaneous or subsequent presence of AIHA, immune thrombocytopenia, and rarely autoimmune neutropenia, which has a severe clinical course, high relapse rate, infectious and thrombotic complications, and fatal outcome [13, 14].

Clinical Vignette of a Complex DAT-Negative AIHA

A 62-year-old woman was referred to our center for worsening moderate hemolytic anemia since about 5 years, along with some "autoimmune" features (arthralgias, photosensitivity, positive antinuclear, and antithyroglobulin antibodies) and DAT persistently negative. She had been treated intermittently with steroids, with partial response, and heavy side effects (hypertension, diabetes, osteoporosis). A careful anamnesis revealed anemia in the mother, never investigated, and no drugs that could be temporally associated with the appearance/worsening of hemolytic anemia. At presentation, she had Hb 9.8 g/dL, unconjugated bilirubin 2.7 mg/dL, LDH 2.3 upper limit of normal (ULN), reticulocytes 80×10^9/L (clearly inadequate to compensate anemia). Liver and kidney function, serum and urine electrophoresis, vitamins and iron, serum IgG, IgA and IgM levels, lymphocyte subpopulations by cytometry were normal as well. Extensive evaluation of congenital forms (Hb electrophoresis - HPLC, membrane and enzyme activity, EMA-binding, ektacytometry, and NGS panel) and total body CT scan were negative. The DAT with monospecific antisera anti-IgG, -IgA, -IgM, -C3, and specialized DAT tests such as microcolumn, solid phase, washes with cold or LISS, Donath-Landsteiner test were negative; at variance, mitogen-stimulated DAT that can disclose a latent autoimmunity through mitogen stimulation in vitro, was positive [15]. Additionally, flow-cytometry revealed a small paroxysmal nocturnal hemoglobinuria (PNH) clone (5%), described in about 1/3 of AIHAs [16], that

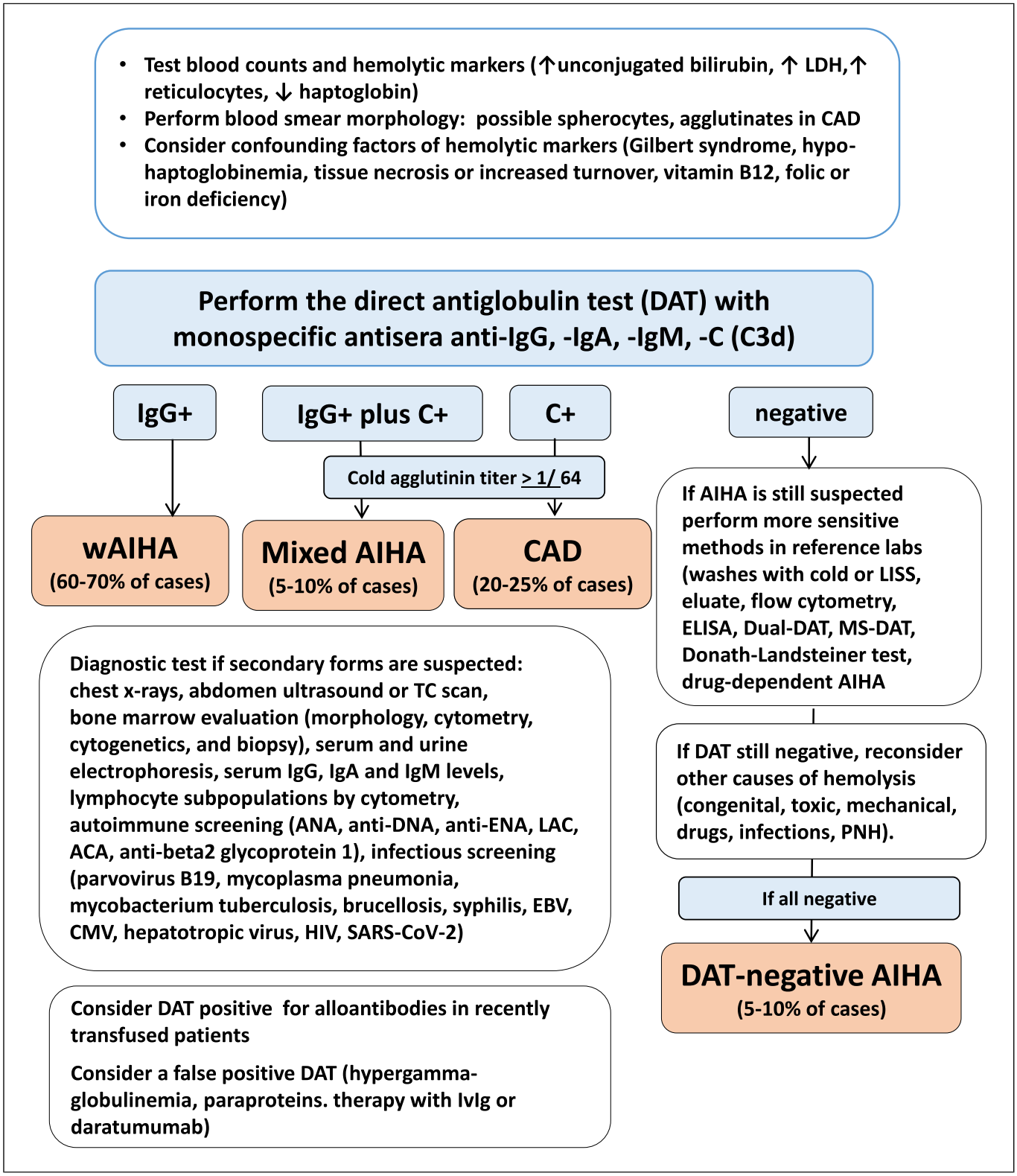

Fig. 1. Diagnostic algorithm for warm autoimmune hemolytic anemia (wAIHA) and cold agglutinin disease (CAD). LISS, low-ionic salt solutions; MS-DAT, mitogen-stimulated DAT; ANA, anti nuclear antibody; anti-ENA, anti-extractable nuclear antigens; LAC, lupus anticoagulant; ACA, anti-cardiolipin antibody; EBV, Epstein Barr virus; CMV, cytomegalovirus.

may justify the disproportional increase of LDH (a feature of intravascular hemolysis). Bone marrow evaluation (morphology, cytometry, and biopsy) showed reduced cellularity by age and features of myelodysplasia, with normal cytogenetics and negative NGS myeloid panel. The final diagnosis was DAT-negative AIHA with possible hypoplastic myelodysplastic syndrome and small PNH clone. While bone marrow features may explain the inadequate reticulocyte response and suggest the use of recombinant erythropoietin, other therapeutic options for AIHA (i.e., rituximab) will require careful evaluation and close follow-up of the patient. Overall, this case underlines the importance of a comprehensive diagnostic evaluation of the several hemolytic conditions that may puzzle the diagnosis of AIHA.

wAIHA: Clinical Features and Therapy

wAIHA patients are usually middle-aged females, and, given the thermal characteristics of warm autoantibodies being active at 37°C, generally present with an acute and very severe disease requiring hospital admission and transfusion support [1–3]. Extremely severe anemia (Hb <6 g/dL) is in fact more frequent in wAIHA as compared to CAD, and virtually all patients require treatment. As stated above, wAIHA is generally sustained by IgG autoantibodies (DAT positive for IgG), that opsonize erythrocytes and lead to IgG mediated extravascular hemolysis in the reticuloendothelial system. However, complement activation may also occur in up to 50% of patients (DAT IgG+C3d+) and contributes to anemia severity and refractoriness to available treatments [1–4, 6, 10]. Notably, rarer wAIHAs include IgA+, and "warm" IgM+ forms that are more difficult to diagnose and may be fatal in accordance with their ability to fix complement [4]. These subtypes of wAIHA may deserve specific therapeutic approaches.

It is widely established that >80% of wAIHAs respond to prednisone 1 mg/kg/day (or equivalent (methyl)prednisolone doses) within about 1–2 weeks of treatment. Despite a slow steroid tapering in 4–6 months, 1/3 of patients relapse and require a further therapy line. The anti-CD20 monoclonal antibody rituximab has become the preferred 2nd line being able to induce 70–80% of responses. It can be added as early second line in refractory cases or reserved for relapses after steroid tapering. Two schedules may be used: a low fixed dose of 100 mg/week for 4 weeks, preferred for moderate cases with IgG+ DAT only, or standard dose of 375 mg/sqm/week for 4 weeks in IgG+C3d+ DAT positive wAIHAs and very severe forms. Splenectomy is also effective with up to 70% of responses lasting more than 5 years [1–4, 10, 11, 17]. It may be considered in young low comorbid patients after vaccination against capsulated organisms, and accounting for a life-long increase in infectious and thrombotic risk. For patients who are refractory to or relapsing after rituximab and not candidate for splenectomy, cytotoxic immunosuppressants, such as azathioprine, cyclophosphamide, mycophenolate mofetil, and cyclosporine may be a therapeutic option [1, 17, 18]. However, these drugs have limited efficacy and known toxicities and may be reserved for patients with wAIHA associated with systemic connective tissue diseases [18].

Supportive measures are pivotal, particularly transfusions are generally advised for Hb <7 g/dL or at higher cut-offs in comorbid, symptomatic patients [1, 10, 11, 19]. Up to 20% of patient may develop alloantibodies, impacting on transfusion efficacy and risk of febrile reactions. Therefore, a careful matching is recommended along with a close collaboration with transfusion medicine experts [19]. The pivotal role of bone marrow compensatory reticulocytosis has been already mentioned and may be particularly relevant during acute wAIHA crises. In this instance, BM may be object of the immune attack, "shocked" by the acuteness of the event, or compromised by a concomitant sepsis/associated condition. Stimulation with recombinant erythropoietin (i.e., epoetin alpha 40,000 IU/week subcutaneously) may boost erythroid response in >70% of patients, improving anemia and reducing transfusion need [20, 21]. Finally, thrombotic complications (mainly pulmonary embolism) may occur in up to 20% of cases, being associated with anemia severity, hemolytic markers alteration, and prothrombotic comorbidities (particularly anti-phospholipid antibodies). LDH increase by >1.5 fold the upper normal limit appears as a good proxy for thrombotic risk and prophylaxis with low molecular weight heparin has been shown to abate thrombosis occurrence in these patients [22–24].

In the recent years, several new drugs and ongoing trials have enriched the therapeutic armamentarium of wAIHA (Table 1), including the use of off-label plasma-cell targeting agents (i.e., the proteasome inhibitor bortezomib or the anti-CD38 daratumumab and isatuximab). Anti-plasma cells inhibitors aim at targeting long-lived plasma cells that do not express CD20 and may be rituximab refractory [25, 26]. Regarding bortezomib, 2 of 4 severe refractory wAIHA patients responded after several cycles of this drug [27], and 6/8 multi-refractory wAIHA responded to 6 cycles of bortezomib 1.3 mg/sqm [28] in combination with dexamethasone. Responses lasted a median of 14 (6–36) months. However, 5 subjects experienced significant adverse events, including grade 2 peripheral neuropathy in 2, requiring treatment discontinuation in one of them; finally, 88% of 7 wAIHA patients responded to the combination of low-dose rituximab, bortezomib, and dexamethasone [29]. Some case reports/

Table 1. Novel drugs for warm autoimmune hemolytic anemia (wAIHA)

Drug	Phase/status	Target	Comments
B-cell/plasma cell targeting agents			
Parsaclisib	Phase 2/3	PI3K inhibitor	64% response rates in a phase 2 study of rel/ref wAIHA and CAD. Phase 3 study withhold
Ibrutinib/ rilzabrutinib	Phase 2	BTK inhibitor	Effective on reducing AIHA flares in CLL patients. Phase 2 of rilzabrutinib in primary rel/ref wAIHA ongoing
Bortezomib	Phase 2/case reports	Proteasome inhibitor	88% response in combination with low dose rituximab and dexamethasone in rel/ref wAIHA
Daratumumab/ Isatuximab	Case reports/ phase 1	Anti-CD38 MoAb	Case reports of daratumumab efficacy in severe multi-refractory wAIHA A phase 1 trial of isatuximab withhold
Ianalumab	Phase 3	Anti-BAFF MoAb	/
Obexelimab	Phase 2/3	Anti-CD19 and FcγRIIb bispecific MoAb	/
Povetacicept	Phase 3	Double inhibitor of BAFF and APRIL	/
Complement inhibitors			
Pegcetacoplan	Phase 2	C3 inhibitor	About 1/3 of wAIHA patients with IgG+C3d+ DAT responded in a phase 2 study
ANX005	Phase 2	Anti-C1q MoAb	The study included CAD and IgG+C3d+ wAIHA withhold
Inhibitors of IgG mediated extravascular hemolysis			
Fostamatinib	Phase 2/3	SyK inhibitor	45% of patients responded in phase 2; 35.6% of fostamatinib treated patients achieved a durable Hb response >10 g/dL at week 24 versus 26.7% on placebo
Nipocalimab	Phase 3	Anti-FcRn MoAb	Phase 3 study for rel/ref wAIHAs, no results posted

series also exist about the anti-CD38 monoclonal antibody daratumumab, given at doses similar to multiple myeloma with heterogeneous schedules [25]. Finally, a phase 1 study of the anti-CD38 isatuximab is ongoing in wAIHA patients (NCT04661033). Further novel drugs under development for wAIHA include B-cell targeting agents as the oral B-cell receptor inhibitors parsaclisib (NCT03538041 and NCT05073458), ibrutinib (NCT03827603), and rilzabrutinib (NCT05002777) that are being studied in clinical trials. Parasclisib (NCT03538041) induced a 64% response rate in relapsed/refractory wAIHA and CAD in phase 2 and is now being evaluated in a randomized phase 3 trial in wAIHA (NCT05073458). Other investigational drugs include the anti-B-cell activating factor (BAFF) MoAb ianalumab (NCT05648968), the engineered double inhibitor of BAFF and APRIL povetacicept (NCT05757570), and the bispeciphic Moab obexelimab, which simultaneously binds CD19 and FcγRIIb (NCT05786573). Complement inhibitors, particularly the C3 inhibitor pegcetacoplan, although more suitable for CAD, have shown some efficacy in IgG+C3d+ wAIHA patients (NCT03226678), and a phase 2 study of the anti-C1q ANX005 MoAb is ongoing (NCT04691570). Novel strategies aimed at inhibiting IgG-mediated extravascular hemolysis encompass the spleen tyrosine kinase inhibitor fostamatinib. The drug induced 45% responses in phase 2 study of rel/ref wAIHA and 35.6% in phase 3, so that the primary endopoint was not met [30]. Finally, blocking the neonatal Fc receptor (FcRn) by MoAbs such as nipocalimab is an interesting strategy to dampen IgG recycling, thus increasing the clearance of pathogenic autoantibodies. Intravenous nipocalimab is being evaluated in a phase 3 trial (NCT03075878).

Some very severe acute wAIHA cases may require admission to the intensive care unit. Mortality is up to 13% after a median of 3.5 days from admission [31]. Very limited options are available for these patients, including transfusions, high dose steroids, and plasma exchange, stressing the need for further novel agents.

Clinical Vignette of a Multi-Refractory wAIHA Associated with Immune Thrombocytopenia (Evans Syndrome)

A 23-year-old boy presented to the emergency room due to fever from 6 days not responsive to amoxicillin/clavulanate and worsening fatigue. At physical examination jaundice, mild splenomegaly and enlarged laterocervical lymph nodes were noted. Laboratory work-up showed very severe anemia (Hb 2.4 g/dL) with unconjugated hyperbilirubinemia, normal transaminases, and negative viral serologies including Epstein-Barr virus. Further testing highlighted extremely elevated LDH and haptoglobin consumption. DAT turned strongly positive for IgG and the patient was transfused with 2 red cell units and started on methylprednisolone 1 mg/kg/day. After 1 week and further 2 transfusions, platelet counts dropped to 2×10^3/L and intravenous immunoglobulin

0.4 g/kg/day were administered for 5 days. Platelets completely recovered and Hb stabilized around 8 g/dL. CT scan confirmed mildly enlarged spleen (15 cm) and lymph nodes (maximal diameter 2.1 cm latero-cervical only), while bone marrow evaluation, performed due to bi-cytopenia, was unremarkable. The patient obtained a complete hematologic recovery after 3 weeks and steroid tapering was started. Three months later a further thrombocytopenia relapse occurred, clearly configuring a case of Evans syndrome. Hypogammaglobulinemia was noted while screening for rituximab, and common variable immunodeficiency was confirmed by lymphoid subpopulation studies. The patient was put on IVIG replacement therapy with full platelet recovery. A further wAIHA relapse occurred 12 months later and was managed with standard dose rituximab with full response. Six months after the patient experienced a further relapse; splenectomy was not considered due to infectious risk, and enrolment in a clinical trial was evaluated, but all trials excluded Evans syndrome or patients with hypogammaglobulinemia, so that the patient was given steroids and kept on IVIG maintenance. This case illustrates how secondary wAIHA patients and those associated with other immune cytopenias (namely Evans syndrome) are particularly challenging to treat given the higher rate of relapse and the potential risks linked to wide-spectrum immunosuppressive therapies.

Cold Agglutinin Disease: Clinical Features and Therapy

CAD is an established clonal B-cell lymphoproliferative disorder of the bone marrow (MYD88 L265P negative), recently included into the World Health Organization classification of hematolymphoid neoplasms, distinct from other malignant lymphomas [32]. Accordingly, most of the cold agglutinins are monoclonal usually of the IgM class with κ light chain restriction and are responsible of both chronic extravascular complement-mediated RBC destruction (mainly in the liver) and acute intravascular hemolysis in case of massive complement activation (cold exposure, infections, major trauma, surgery). Moreover, cold agglutinins are responsible for the circulatory symptoms typical of CAD (acrocyanosis, Raynaud-like phenomenon, ulcerations up to gangrene) in the acral parts of the body, where temperatures reach the optimal range for antigen-antibody reaction. As for other AIHAs secondary forms (identified as cold agglutinin syndrome or secondary CAD) should be carefully investigated, as the association with infections, lymphoproliferative disorders and tumors is particularly frequent [1–4]. Of note, bone marrow re-examination by a centralized expert center greatly increased the proportion of CAD diagnosis versus other indolent lymphoproliferative disorders [12]. In the largest retrospective study including 232 CAD with a median follow-up of 6 years, median age at diagnosis was 68 years (range 33–96) with a 60% of patients diagnosed within 1 year of clinical onset but with the longest time to diagnosis of 32 years. Median hemoglobin at onset was 9.3 g/dL (range 4.5–15.3) with 27% of patients having Hb <8 g/dL. Thus, the severity of anemia is clearly inferior to that of wAIHA. Considering the main symptoms, about 70% of patients had hemolytic anemia with no or mild peripheral circulatory symptoms, 21% hemolytic anemia with severe circulatory symptoms, and 9% circulatory symptoms with compensated hemolysis. Around half of patients required transfusions during the chronic course/acute exacerbations of the disease [12]. Importantly, fatigue is a hallmark of chronic CAD, greater than expected for a given hemoglobin value, possibly related to continuous complement activation and production of pro-inflammatory mediators. Finally, an increased risk of thrombosis has been reported in this disease [23], as in other complement-mediated hemolytic conditions [33].

Treatment of CAD is based on supportive measures (cold avoidance, folic acid, transfusions) while standard treatments of wAIHA are poorly effective: steroids may be useful in the acute setting but only at high doses, tapering is usually accompanied by recurrence of hemolysis, and chronic administration discouraged. Historical immunosuppressants/cytotoxic agents (azathioprine, cyclophosphamide, cyclosporine, chlorambucil) had little effect and splenectomy is clearly discouraged as chronic hemolysis occurs mainly in the liver. In mild cases, a watch-and-wait approach may be indicated, while treatment should be considered for symptomatic anemia, marked fatigue and/or important circulatory symptoms. In the acute/severe setting plasma-exchange may be an option, although temporary and not easy to organize [34]. As already stated for wAIHA transfusions should be guided by symptomatic anemia, administered with a blood warmer and ensuring a warm environment, although the evidence of benefit is limited [3, 12, 35]. Thromboprophylaxis is indicated as well, and the use of recombinant erythropoietin is advised particularly in case of reticulocytopenia. For secondary forms, treatment of underlying condition is essential [1, 20, 21].

Standard therapy has two main targets: inhibition of autoantibody production by targeting B-cells or plasma cells and blocking complement activation, the main pathogenetic mechanisms of the disease. Regarding the former, the anti-CD20 monoclonal antibody rituximab has been used since more than 20 years at the dose of 375 mg/sqm/week for 4 weeks with a response in about 50% of patients. Nevertheless, complete responses are rare (less than 5%) and the median duration of response is less than 1 year [12]. Addition of fludarabine increased

Table 2. Novel drugs for cold agglutinin disease (CAD)

Drug	Phase/status	Target	Comments
Plasma cell targeting agents			
Bortezomib	Phase 2/case reports	Proteasome inhibitor	30% response after one course; some patients repeated treatment with efficacy
Daratumumab/ Isatuximab	Case reports	Anti-CD38 MoAb	Case reports of daratumumab efficacy in severe multi-refractory CAD. Efficacy on cold induced symptoms reported
B-cell targeting agents			
Ibrutinib	Retrospective study	BTK inhibitor	Effective in a recent case series of 15 patients, 4 CAD and 11 CAS. 13 responded, 2 died due to underlying disease. Benefit on peripheral symptoms too
Parsaclisib	Phase 2/3	PI3K inhibitor	64% response rates in a phase 2 study of rel/ref wAIHA and CAD. Phase 3 study withhold
Complement inhibitors			
Eculizumab	Phase 2 study	Anti-C5 MoAb	Improved hemolysis and transfusion need in about 1/3 of CAD patients. No effect on peripheral symptoms
Sutimlimab	FDA and EMA approved	Anti-C1s MoAb	>80% anemia response in two phase 3 studies open label and placebo controlled. No effect on peripheral symptoms
Riliprubart	Phase 1b	Anti-active C1s MoAb	Rapid and sustained improvement of hemoglobin and bilirubin
Pegcetacoplan	Phase 2/3	C3 inhibitor	About 2/3 of CAD patients responded in the phase 2 study. A phase 3 study was withheld
Iptacopan	Phase 1	Factor B inhibitor	/

response rate (76%, with complete response in 21% of patients), and extend response duration to more than 5 years, but was associated with a significant toxicity, mainly infective. Similarly, the combination of rituximab plus bendamustine resulted in increased response rate (78%, with complete responses in 53%), and long response duration (more than 7 years), with a moderate and manageable toxicity [36, 37]. Table 2 summarizes novel drugs for CAD. The proteosome inhibitor bortezomib has been shown effective in some case reports and in a pilot study of 21 patients [26, 38] (response in 32% of patients, 16% complete) with a median duration of response of 16 months and an acceptable toxicity. The anti-CD38 monoclonal antibody daratumumab has been reported effective in some relapsed/refractory cases, with the rationale of targeting long-lived plasma cells in the spleen and bone marrow [39]. Finally, the Bruton tyrosine kinase (BTK) inhibitor ibrutinib has been investigated in 15 patients with CAD primary or secondary CAD (all heavily pretreated, mostly transfusion dependent and with circulatory symptoms) showing improvement of both hemolytic anemia and acrocyanosis [40]. A last mention deserves the oral PI3K inhibitor parsaclisib induced a response in 64% of patients in a trial including CAD subjects but was further developed in wAIHA only. Regarding complement-directed therapies, the anti-C5 complement inhibitor eculizumab has been reported effective in reducing hemolysis and obtaining transfusion independence in an open-label trial involving 13 patients [41]. The monoclonal antibody anti-C1s sutimlimab has been more extensively studied in an open label study of 24 CAD patients with a history of recent transfusion [42], and in a placebo-controlled trial involving 42 patients without a recent history of transfusion (in both studies inclusion criteria for Hb was <10 g/dL at baseline) [43]. In the first study, 54% of patients met the primary end point (Hb ≥12.0 g/dL or Hb increase ≥2.0 g/dL) and 83% achieved a stable increase in Hb by >1.0 g/dL. Importantly, all the disease parameters (increase of Hb, reduction of bilirubin and C4 levels, improvement of fatigue) occurred in the first weeks of treatment. Vaccination against *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* was required, and no meningococcal infections or other serious drug-related adverse events related were reported. In the second study, results were confirmed, with 73% of patients in sutimlimab arm that met the primary end point (Hb increase ≥1.5 g/dL and avoidance of transfusion), as compared with 10% in the placebo arm. The 2-years extension studies confirmed the results, with sustained hemoglobin responses and more comprehensive improvement of patients' reported outcomes, particularly on fatigue, an important issue impacting quality of life [44, 45]. However, the efficacy of the drug requires continuous administration every 2 weeks, as disease activity reoccurs after treatment interruption. All this evidence led to the approval of sutimlimab in CAD by regulatory agencies FDA and EMA. More recently, the second-generation active C1s inhibitor riliprubart

(formerly SAR445088, BIVV020), characterized by a prolonged half-life and thus a longer dosing interval than that of sutimlimab, is currently under investigation in CAD. The open label study showed that a single IV dose led to classical complement inhibition, control of hemolysis, and improvement in anemia, which was sustained for 15 weeks [46]. Additionally, the C3 inhibitor pegcetacoplan showed a rapid efficacy in about 2/3 of CAD patients in the open-label, phase II pilot trial (NCT03226678), and a phase 3 trial is ongoing (NCT05096403). Finally, the orally available factor B inhibitor iptacopan is currently being evaluated in a trial including CAD and immune thrombocytopenia (NCT05086744).

Clinical Vignette of a Multi-Refractory CAD Patient

A 76-year-old lady previously followed for 5 years by the immunologist for a scleroderma-like syndrome with anemia and Raynaud phenomenon and treated with steroids, azathioprine, and cyclophosphamide, was referred to the hematologist due to worsening hemolytic markers. At re-evaluation the DAT was markedly positive for C3d with a cold agglutinin titer of 1:256; an IgM/kappa MGUS was noted, and bone marrow evaluation showed a mature B-cell infiltrate, 10% of total cellularity, with negative MYD88. CT scan was negative but for a mild splenomegaly 13 cm. Due to severe anemia (Hb 7 g/dL) with altered hemolytic markers (LDH 2 × ULN and unconjugated bilirubin 3.1 mg/dL), the patient was transfused and treated with rituximab with a partial response lasting about 8 months. Thereafter, acrocyanosis, dark urine, and severe anemia reappeared, and re-evaluation showed an increase of total IgM (6 g/dL) and of bone marrow lympho-plasmocytic infiltrate (25%), MYD88 positive, with stable CT scan. The patient was re-classified as "cold agglutinin syndrome" (CAS) and treated with 6 cycles of rituximab in combination with bendamustine 70 mg/sqm (reduced dose due to age and frailty) obtaining a marked decreased of total IgM but no improvement of hemolytic anemia; 2 months later, the patient developed a severe fungal pneumonia and sadly passed away. This case highlights the pitfalls in the differential diagnosis between CAD and CAS and how disease phenotype may evolve over time. Additionally, it points at the infectious risk of prior and subsequent immunosuppressive treatments in elderly and frail CAD patients.

Mixed AIHA: Clinical Features and Therapy

Mixed AIHA is defined by the presence of both warm IgG autoantibodies and high-titer cold agglutinin (DAT titer >1:64). DAT is generally strongly positive for IgG and C3d [1]. Since DAT positivity for IgG and C3d may be observed even in wAIHA, the titer of the cold agglutinin and the presence of spontaneous agglutination at 20°C are useful tools. In large retrospective series, mixed AIHAs were characterized by a more severe onset and a relapsing course as compared to wAIHA [10, 11]. The mixed positivity of the DAT may imply uncertainty in the treatment algorithm that may be more similar to wAIHA or CAD depending on the predominant clinical features on a case-by-case basis. Corticosteroids should be given at high doses and early rituximab at standard dose may be necessary, whilst splenectomy seems less effective and is not generally advised [1]. Notably, most clinical trials for wAIHA and CAD exclude patients with mixed AIHAs.

Clinical Vignette of a Complex Case of Mixed AIHA

A 66-year-old man was referred to our center with AIHA diagnosed in a local hospital since about 6 months. He had been treated with steroids with partial response. At referral, he had Hb 9.6 g/dL, unconjugated bilirubin 2.1 mg/dL, LDH 1.9 x ULN, reticulocytes 180 × 10^9/L. The DAT with monospecific antisera was positive with anti-IgG, and C3 antisera and cold agglutinin titer was 1:128, leading to the diagnosis of mixed AIHA. Serum electrophoresis revealed a monoclonal IgMκ and bone marrow evaluation (morphology, cytometry, cytogenetics, and biopsy) was consistent with the diagnosis of CAD (MYD88 L265P negative). Total body CT, infectious and autoimmune investigation were negative, liver and kidney function, peripheral lymphocyte subpopulations, vitamins, and iron were normal as well. The patient was treated with rituximab 375 mg/sqm/week for 4 weeks (added to ongoing daily prednisone of 0.5 mg/kg/day), obtaining a complete response and allowing steroid discontinuation after 4 months. A relapse was observed after about 1 year, with Hb 7.2 g/dL, unconjugated bilirubin 2.9 mg/dL, LDH 3.2 x ULN, reticulocytes 70 × 10^9/L. Severe and disabling circulatory symptoms appeared (acrocyanosis and Raynaud). The DAT was positive with anti-C3 antisera and cold agglutinin titer increased to 1:256, whilst anti-IgG was no longer present. Most probably rituximab was effective on the "warm" IgG, at variance with the "cold" IgM. The patient was transfused because of symptomatic anemia but was ineligible for sutimlimab trials because of the original diagnosis of mixed AIHA. Given the inadequate reticulocyte compensation, he received recombinant erythropoietin (epoetin alpha 40,000 U/week for 3 weeks) and started bortezomib (1.3 mg/sqm IV on days 1, 4, 8, 11). A partial response was observed (Hb 9–10 g/dL), with amelioration of circulatory symptoms. However, after about 10 months a new relapse occurred (Hb 8 g/dL), and he received sutimlimab through a named program. A rapid and sustained increase of Hb was observed (12–12.5 g/dL) with normalization of hemolytic markers, but no further

improvement of circulatory symptoms. This case illustrates the difficult management of mixed AIHAs, as the disease is more severe and relapsing than wAIHA and CAD alone. More importantly, new therapeutic options are unavailable for these patients, as trials are generally performed in wAIHA or CAD, resulting in a true unmet need.

Other Rare and Complex Clinical Settings

Several underlying conditions may be associated with AIHA development or trigger hemolytic exacerbations. Some of these represent rare associations with heterogeneous presentation and diagnostic challenges. A typical example is drug induced AIHA [7], that may be due to: (1) drug-dependent antibodies that activate an immune response in the presence of the drug only and (2) drug-independent antibodies, which induce AIHA via adsorption, immune dysregulation or unknown mechanisms [1, 3]. Drug induced AIHA is rare, with an estimated incidence of 1 in 1 million of the population [47], difficult to diagnose without an experienced reference laboratory, and involves medications such as neuroleptics (i.e., historical alpha-methyldopa), antimicrobials (penicillin and cephalosporins), anti-inflammatory, and anti-neoplastic drugs. Novel anti-cancer immunotherapies may also be associated with AIHA, particularly checkpoint inhibitors and chimeric antigen T-cells that are aimed at restoring immune surveillance against neoplastic cells but may in turn provoke immune related adverse events [6, 48]. AIHA post-checkpoint inhibitors may occur after nivolumab, pembrolizumab, ipilimumab, and atezolizumab; it is mainly marked by severe hemolysis, transfusion requirement (80%), high prevalence of DAT negativity (38%), and carries a mortality as high as 17%, pinpointing the importance of clinician awareness and active monitoring of hemolytic markers in patients treated with checkpoint inhibitors. Finally, AIHA may complicate the course of solid and hematopoietic transplant: up to 5–10% of liver and intestinal transplantation and 2.5% of pancreas transplants may be associated with AIHA [1]. Even post-transplant AIHAs are often DAT negative with a possible diagnostic and treatment delay and significant mortality. In this context, "the passenger lymphocyte syndrome" has long been known to be due to immune competent lymphocytes, transferred with the donor organ, which produce antibodies against the recipient's erythrocytes. Onset is between 3- and 24-days posttransplant, the risk is proportional to the burden of transplanted lymphocytes, ranging from 9 to 70% of cases (kidney < liver < heart-lung transplants) [49], and hemolysis is generally transient.

Conclusions

AIHA, although rare, is the most frequent cause of acquired hemolytic anemia in adults and it is easy to diagnose in most cases by monospecific DAT. However, diagnosis may be challenging in some circumstances since the DAT is neither 100% sensitive nor specific so that a reference laboratory is essential. On one hand first level polyspecific DAT performed in all transfusion center may miss several cases, does not distinguish wAIHA from CAD, and may be confounded by previous treatments. On the other hand, concomitant hemolytic conditions may jeopardize AIHA diagnosis as described for hemolytic PNH, inborn errors of immunity, and bone marrow failures (clinical vignette 1). All in all, ex adjuvantibus steroid therapy may be attempted, but what about a second-line approach with rituximab in a DAT negative hemolytic anemia? Though there is no answer from available evidence, the importance of a comprehensive diagnostic approach to exclude associated conditions has to be underlined. Such associations do also impact on treatment choices and outcome (clinical vignette 2): IVIG may be beneficial in septic patients particularly in the presence of inborn errors of immunity, whilst cytotoxic immunosuppressants may be preferred for patients with associated connective tissue diseases, and chemo-immunotherapy for those with underlying lymphoproliferative disease. Additionally, Evans syndrome is characterized by a much higher risk of relapse and infectious and thrombotic complications. Notably, most of these "secondary AIHAs" are excluded from clinical trials thus limiting treatment approach. The result is an endless sequence of steroid courses and immunosuppressants that may eventually increase the risk of infectious complications and add on patient' morbidity, particularly in the elderly (clinical vignette 3). Other unmet needs are noncanonical AIHAs, such as mixed forms that may show phenotypical similarities to wAIHA or CAD and evolve over time (clinical vignette 4). For these patients, that tend to relapse more often, rituximab, anti-plasma cell agents and sutimlimab may show variable efficacy depending on DAT features and complement activation that should be re-evaluated at each relapse. Finally, several new drugs are increasingly used or are in trials for relapsed/refractory AIHAs, including B-cell (parsaclisib, ibrutinib, rilzabrutinib), and plasma cell target therapies (bortezomib, daratumumab), bispecific agents (ianalumab, obexelimab, povetacicept), neonatal Fc receptor blockers (nipocalimab), and complement inhibitors (sutimlimab, riliprubart, pegcetacoplan, iptacopan). Particularly, FcRn and complement inhibitors, by silencing the final step of hemolysis pathogenesis, may be beneficial for the acute and very severe setting, an unmet need with high mortality. In this

evolving scenario, further investigation of combination therapies and a patient-centered precision medicine approach will likely improve AIHA care in the upcoming years.

Conflict of Interest Statement

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Author Contributions

W.B. designed and wrote the article; B.F. wrote the article and revised it for important intellectual content. All authors approved the article.

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