Novel insights into the treatment of complement-mediated hemolytic anemias

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***Abstract*:** Complement-mediated hemolytic anemias can either be caused by deficiencies in regulatory complement components or by autoimmune pathogenesis that triggers inappropriate complement activation. In paroxysmal nocturnal hemoglobinuria (PNH) hemolysis is entirely complement-driven. Hemolysis is also thought to be complement-dependent in cold agglutinin disease (CAD) and in paroxysmal cold hemoglobinuria (PCH), whereas warm antibody autoimmune hemolytic anemia (wAIHA) is a partially complement-mediated disorder, depending on the subtype of wAIHA and the extent of complement activation. The pathophysiology, clinical presentation, and current therapies for these diseases are reviewed in this article. Novel, complement-directed therapies are being rapidly developed. Therapeutic terminal complement inhibition using eculizumab has revolutionized the therapy and prognosis in PNH but has proved less efficacious in CAD. Upstream complement modulation is currently being investigated and appears to be a highly promising therapy, and two such agents have entered phase II and III trials. Of these, the anti-C1s monoclonal antibody sutimlimab has shown favorable activity in CAD, while the anti-C3 cyclic peptide pegcetacoplan appears to be promising in PNH as well as CAD, and may also have a therapeutic potential in wAIHA.

Keywords: autoimmune hemolytic anemia, cold agglutinin disease, complement, complement inhibitors, paroxysmal nocturnal hemoglobinuria, therapy

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Introduction

In several immune or primarily nonimmune hemolytic anemias, the breakdown of red blood cells (RBCs) is mediated by the activation of complement.[1–4] The complement system requires strict regulation in order to direct its response against pathogens and, at the same time, prevent damage to host cells and tissues. Complement-dependent hemolytic diseases can either be caused by deficiencies in regulatory complement components or by inappropriate autoantibody-driven complement activation. The hemolytic process in these disorders may be completely or partially complement-mediated.

The role and mechanisms of complement activation in hemolytic anemias has become particularly important with the introduction of complement-directed therapies, which are being rapidly developed.[4] In this review, we discuss warm and cold reactive antibody-mediated autoimmune hemolytic anemias (AIHAs) as examples of immune-initiated hematologic diseases in which complement is involved,[5] and we will also discuss paroxysmal nocturnal hemoglobinuria (PNH) as an example of nonimmune complement-driven hemolytic anemia.[6,7] Complement also plays an important role in some thrombotic microangiopathies, including the *Shiga* toxin associated and atypical hemolytic uremic syndromes,[4] these multifactorial disorders are not addressed in this review. Table 1 provides an overview of the relevant diseases.[1,4,8,9] Therapeutic considerations are focused on current and future possibilities for complement modulation, while immunosuppressive and other noncomplement therapies are only briefly discussed.

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Table 1. Complement-driven hemolytic anemias.

Primary complement disorders	
Nonimmune hemolytic anemias	Paroxysmal nocturnal hemoglobinuria (PNH)
	Congenital CD59 deficiency
Thrombotic microangiopathies (TMA)	Atypical hemolytic uremic syndrome (aHUS)
Secondary complement involvement	
Autoimmune hemolytic anemias (AIHA)	Cold agglutinin disease (CAD)
	Secondary cold agglutinin syndrome (CAS)
	Paroxysmal cold hemoglobinuria (PCH)
	A proportion (~50%?) of warm antibody AIHA
Thrombotic microangiopathies (TMA)	Hemolytic uremic syndrome (HUS)
	transplant-associated TMA

Based on data from Baines and Brodsky,[1] Risitano,[4] Dacie,[8] Berentsen and Tjønnfjord.[9]
aHUS, atypical hemolytic uremic syndrome; AIHI, autoimmune hemolytic anemias; CAD, cold agglutinin disease; CAS, secondary cold agglutinin syndrome; HUS, hemolytic uremic syndrome; PCH paroxysmal cold hemoglobinuria; PNH, paroxysmal nocturnal hemoglobinuria; TMA, thrombotic microangiopathies.

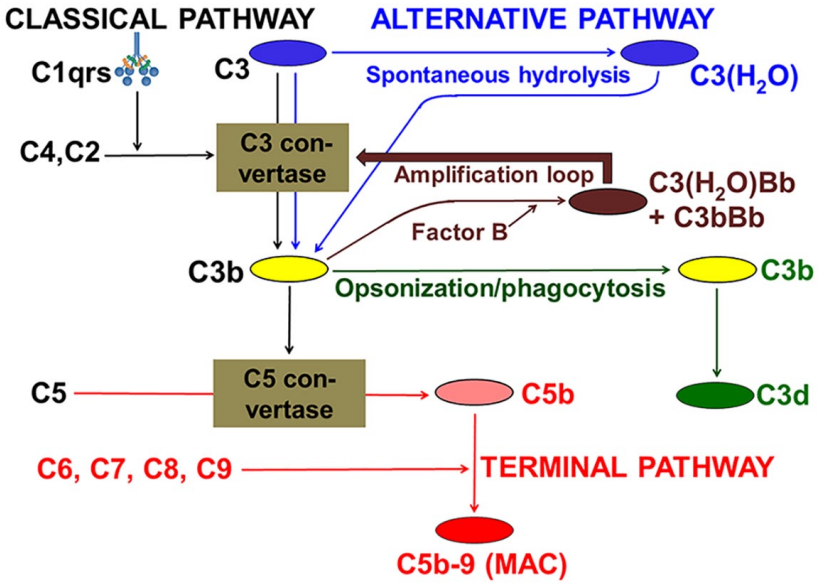

Figure 1. The complement system simplified. Only the relevant pathways and components are shown.
C, complement protein; MAC, membrane attack complex.

Hemolysis and the complement system

A simplified model of the complement system is shown in Figure 1. Complement is an important component of the innate immune response and is composed of more than 50 proteins that are widely distributed throughout body fluids and tissues.[3] Many of these components act as proteases that are themselves activated by proteolytic cleavage. Splitting of a precursor protein generates an active enzyme, which then cleaves its substrate, another

complement protein, into its activated enzymatic form. This, in turn, triggers the next step in the cascade. If activated on cell surfaces, the system can initiate successive steps of proteolysis and inflammatory and lytic processes.

The classical complement pathway (CCP) forms a link between the adaptive immune system and the complement system.[3,10] This pathway is initiated by fixation of complement protein 1q (C1q) to an antigen–antibody (ag-ab) complex at a pathogen or host cell surface. Subsequent activation of C1r and C1s generates a serine protease that, in turn, cleaves C4 and C2. The products, C4b and C2a, combine to form C3 convertase, which then cleaves C3 into C3a, an anaphylatoxin, and C3b, which bind covalently to the cell and act as part of a proteolytic enzyme (C5 convertase) as well as an opsonin.[5,10,11]

Two other complement activation pathways are also known, the lectin pathway (LCP) which is triggered by terminal mannose groups on microbe surfaces, and the alternative complement pathway (ACP) which is initiated by continuous spontaneous hydrolytic activation (tick-over) of C3.[6,12] Similar to the CCP, these pathways lead to the formation of C3 convertase, deposition of C3b on cell surfaces, and, under some conditions, activation of the terminal pathway. C3 activation, therefore, is the point of convergence between the three initial pathways and is essential for further complement activation.

Receptors on phagocytes can bind C3b-opsonized cells, which results in phagocytosis by the mononuclear phagocytic system.[10,11,13–15] When this process affects RBCs it is known as extravascular hemolysis. Alternatively or concomitantly, surface-bound C3b can be degraded into its more or less inactive constituent products iC3b, C3c, and C3d.[3] It should be noted that the surface protein CD46, which has cofactor activity for inactivation of C3b and C4b by factor I, is not present on RBCs.[16]

Surface-bound C3b can also bind the C4bC2a complex, which generates C5 convertase. This step initiates the terminal (lytic) complement pathway, where C5 convertase cleaves C5 into C5a, a potent anaphylatoxin, and C5b, a surface bound protein that in turn binds C6, C7, C8, and C9. The resulting C5b–C9 complex, also known as the membrane attack complex (MAC), is capable of inducing cell lysis.[3,6]

If the terminal pathway is activated, the amplifying nature of the cascade and positive feedback loops can result in accelerated, and sometimes fatal, cell lysis and inflammation.[3] Endogenous complement inhibitors and negative feedback loops, however, prevent this from occurring under normal physiological conditions.[5,6] Cell bound regulators particularly relevant for complement-driven hemolytic anemias are: CD55, which has an inhibitory function at the C4–C3 level in the CCP and ACP, and CD59, which blocks assembly of the C5b–C9 complex at the C8–C9 stage.[3,16]

There is a close interaction at multiple points (cross-talk) between the complement system and the coagulation cascade.[17,18] In some complement-driven hemolytic anemias, this probably contributes to the increased risk of thrombosis.[18,19]

Autoimmune and nonimmune hemolytic anemias

AIHA is a heterogeneous group of diseases characterized by the reduced lifespan of RBCs because of autoimmune-mediated cell destruction.[20] The AIHAs are classified into warm reactive antibody types (wAIHA), which are further divided into primary and secondary forms, and cold reactive antibody types (cAIHA), further classified as primary cold agglutinin disease (CAD), secondary cold agglutinin syndrome (CAS), and paroxysmal cold hemoglobinuria (PCH).[9,20–22] The nonimmune hemolytic anemias comprise a larger and even more heterogeneous group of acquired and hereditary disorders. Of these, PNH is a rare and severe, acquired clonal stem cell disease in which hemolysis is entirely complement-driven.[1,6]

wAIHA

Diagnosis and basic features

In wAIHA, polyclonal autoantibodies with a temperature optimum at 37°C bind to the RBC surface. The involved antibody class is most often immunoglobulin (Ig) G (mostly IgG1 or IgG3) but can be IgA or warm-reactive IgM combined with IgG, or, infrequently, IgM or IgA alone.[22–24] Approximately 50% of wAIHA cases are

secondary to (i.e. associated with or caused by) other diseases, including lymphoproliferative disorders (LPDs), autoimmune diseases, or other immune dysregulation including common variable immunodeficiency.[18–20, 22] The remaining 50% are designated as primary. Chronic lymphocytic leukemia is the most commonly associated LPD.[8,20]

The direct antiglobulin test (DAT) is used to demonstrate the presence of Ig, complement, or both on the RBC surface, and the monospecific DAT in the majority of cases can identify the Ig class and type of complement protein.[20,22] wAIHA is diagnosed in patients with biochemically confirmed hemolytic anemia and a DAT positive for IgG alone, IgG and C3 (more specifically C3 fragments, typically C3d), C3 alone, or, rarely IgA. If C3 is found, the absence of cold agglutinin in serum at a significant titer is an additional requirement for wAIHA diagnosis.[9,21] Surface bound IgM is a potent CCP activator, but any RBC-bound IgM will usually detach from the cell surface before it can be detected by DAT.[24] In patients with wAIHA, therefore, finding only C3 by monospecific DAT should be considered a 'footprint' of warm reactive IgM involvement, and IgM can sometimes be involved even if C3 positivity is found in combination with IgG or IgA.[24,25] More sensitive or quantitative methods for detecting complement or Ig (including IgM) on RBCs are available at specialized laboratories.[26]

Complement involvement in wAIHA

The immune-initiated RBC breakdown in wAIHA is not entirely complement-mediated (Figure 2). Based on the DAT pattern, complement is involved in 28–65% of wAIHA.[22] Major noncomplement mechanisms are macrophage-inflicted membrane damage with subsequent formation of spherocytes, which are prone to destruction in the red pulp of the spleen and, concomitantly or alternatively, phagocytosis of Ig-opsonized RBCs by the mononuclear phagocytic system, which mainly occurs in the spleen.[5,27,28]

On RBCs opsonized with IgM or heavily coated with IgG, the ag-ab complex will initiate the CCP.[10,11] IgG is a weaker complement activator than IgM. Of the IgG subclasses, it is mainly IgG3, and to a lesser extent IgG1, that is able to activate complement, while IgG2 is an even weaker activator.[29,30] IgG4 and IgA do not trigger the complement system. However, IgA-mediated wAIHA can be fulminant, possibly because of concomitant IgM involvement.[31,32] CCP activation will leave the RBCs opsonized with C3b and, therefore, susceptible to extravascular hemolysis by the mononuclear phagocytic system, primarily by Kupfer cells in the liver, while intravascular hemolysis mediated by the terminal pathway is prominent only in severe cases.[5] The explanation is probably the protective effect of the CD55 and CD59 which, unlike in PNH, are intact in AIHA.

Current management of wAIHA

The majority of patients with wAIHA present with a decompensated hemolytic anemia and require immediate therapy. Transfusions can be administered in severe symptomatic anemia, but it is nearly always impossible to find serologically compatible donor erythrocytes. To avoid transfusion reactions and induction of alloantibodies following transfusions with 'incompatible blood', extended phenotyping of the donor RBCs is advisable in the majority of cases and an '*in vivo* biological compatibility test' is recommended in many countries.[23,33] In this test, approximately 20 ml of blood is given as a rapid infusion, the infusion is stopped for 20–30 min and, if no reaction occurs, the remaining infusion is administered at a normal rate.[23]

Therapy with predniso(lo)ne at high initial doses appears to result in approximately 80% initial response rate and remains the preferred first-line treatment. However, only one-third of patients are able to successfully discontinue corticosteroids and maintain long-term remission.[34] Based on two randomized trials, it has recently been suggested that predniso(lo)ne plus rituximab, which leads to an overall 75% response rate at 12 months, should be the preferred first-line treatment, at least in selected cases.[34,35] The preferred second-line option is rituximab, if not given as the first-line therapy.[21,23,36] A high number of third-line, or subsequent, treatments, including splenectomy and immunosuppressive agents, have been used, often based on case reports or small retrospective series.[21,37] In secondary wAIHA, treatment of the underlying or associated disease should be given in selected cases.[37,38] Comprehensive reviews on non-complement

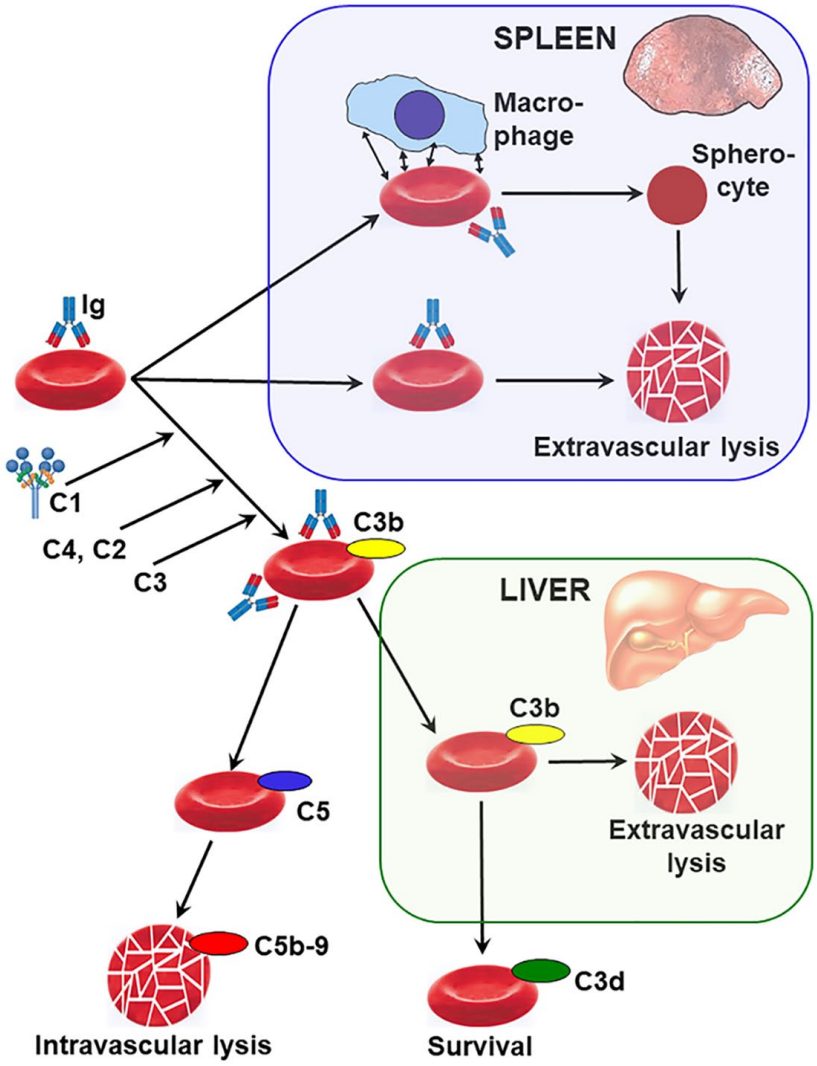

Figure 2. Mechanisms of hemolysis in warm antibody autoimmune hemolytic anemia.
C, complement protein; Ig, immunoglobulin.

therapies for wAIHA can be found elsewhere in the literature.[21,37,38]

CAD

Diagnosis and basic features

Definition, and diagnostic tests. Cold agglutinins (CAs) are autoantibodies, in most cases of the IgM class, that agglutinate RBCs upon binding to the cell surface at an optimum temperature of 3–4°C.[39] Most CAs in CAD are specific for the surface carbohydrate antigen termed I. Rare specificities include anti-Pr or anti-i.[39,40] Primary CAD is defined by chronic hemolysis, a significant CA titer (usually defined as ⩾64) at 4°C, typical findings by the DAT, and the absence of an underlying specific infection or overt (i.e. clinically or radiologically detectable) malignancy.[9,21,41] Typically, monospecific DAT is strongly positive for C3d only, but additional weak positivity for IgG can be seen in up to 20% of patients.[41,42] There may be a small number of cases with a CA titer <64. The thermal amplitude (TA) is the highest temperature at which the CA will react with its antigen.[43] TA determination is useful in selected patients to exclude low-titer, low-TA CAs as a cause of false-positive results.[21,44] Such naturally occurring CAs can be detected in a minority of healthy people with the

absence of hemolysis and with negative DAT. A frequency of 0.3% has been suggested based on a cohort of patients with unrelated diseases.[45] Clinical and histological assessment, supplemented by radiological examinations if needed, will rule out CAS that is secondary to a malignant disease.[9]

Clinical presentation. Anemia in CAD is often mild to moderate, and in some cases fully compensated hemolysis occurs. A large number of patients, however, suffer from severe anemia.[41,42] In a descriptive study of 86 unselected patients, the median hemoglobin level was 8.9 g/dl (range, 4.5–15.6 g/dl; lower tertile, 8.0 g/dl).[41] Up to 90% of the patients according to a Norwegian study (possibly less in warmer climates) experience cold-induced circulatory symptoms affecting acral parts of the body. Acrocyanosis is the most common circulatory symptom, but Raynaud-like phenomena can also occur and in some patients, this can be disabling.[41,42] The presence and severity of acrocyanosis does not correlate with the severity of anemia.[41] Estimates on transfusion requirements show large variations, probably due to patient selection and variable transfusion criteria. In unselected cohorts, approximately half of the patients received transfusions.[41,42]

CAD is a clonal, lymphoproliferative bone marrow disorder. There is an increasing body of evidence for considering primary or 'idiopathic' CAD as a specific clonal LPD of the bone marrow in all cases. The occurrence of monoclonal IgMκ in most CAD patients has been recognized for many decades, and IgMκ has been shown to carry the relevant autoantibody properties and to be identical with the CA.[39,46,47] The first monoclonal protein ever described was a CA from a patient with primary CAD.[46] Selective usage of heavy and light chain gene segments has also been demonstrated.[48,49] Flow cytometric and histological evidence for a clonal bone marrow disorder was documented during the 1990s.[41,50,51] In 2014, Randen and colleagues published a study of histomorphological, immune histochemical, flow cytometric, and molecular findings in bone marrow samples from 54 patients with well-documented primary CAD.[52] Nodular B-cell aggregates were found in biopsy specimens from 40 out of 54 patients, while the remaining 14 patients showed only scattered clonal B cells. The median extent of lymphoid infiltration was 10%. Morphological, immune histochemical and flow cytometric assessment showed marked differences from lymphoplasmacytic lymphoma, marginal zone lymphoma, and other previously recognized lymphoma entities, and the *MYD88* L265P mutation could not be detected. The authors proposed the term 'CA-associated LPD' for this distinct, homogeneous bone marrow disorder.[52] Later studies have confirmed many of these findings and have added new data on the molecular genetic characteristics.[53–56] In an Austrian study of 20 patients with cAIHA, four had no signs of clonal bone marrow disease by electrophoresis, immune fixation, histology, or flow cytometry. In three of the patients, however, it was still possible to show a clonal rearrangement of their Ig heavy and light chain genes.[56]

Complement involvement in CAD

CAs bind to the RBC surface at temperatures below the central body temperature. These temperatures are normally found in acral parts of the body. This results in RBC agglutination and, frequently, ischemic symptoms from the capillary circulation as described previously. These symptoms are not complement-mediated.

In more than 90% of patients with CAD, the CA is an IgMκ and therefore a powerful activator of the CCP.[11,13,41,57] CAs of the IgG class are rare but descriptions that they result in hemolytic anemia have been convincing.[41,58] Some differences from typical, IgM-mediated CAD may indicate a different mechanism of hemolysis in the IgG-mediated cases, for example, some of the cases have been reported to respond to splenectomy, which is not effective in CAD of the IgM type.[58] Infrequently, CAs of the IgA class have also been observed, although IgA is not expected to activate complement.[31,41,59] A careful study of the literature reveals that some patients reported as having monoclonal IgA with CA activity did not have hemolysis.[31] Another patient had clinical CAD together with monoclonal IgA but turned out to have two independent clonal disorders, indicating that the CA was not identical to the clonal IgA.[60] This indicates that IgA-mediated CAD does not exist.

Upon the binding of IgM-CA to its antigen, activation of the CCP leaves the RBCs opsonized with C3b and, therefore, susceptible to extravascular hemolysis by the mononuclear phagocytic

system; this predominantly occurs in the liver.[11,13,57] On the surviving RBCs, C3b is metabolized to C3d, which most likely protects the cell against further phagocytic attack. The terminal pathway is probably not significantly activated in mild and steady-state CAD,[11,13,57] but has been shown to be active in severe disease and acute exacerbations.[61–64] Part of the explanation for the limited role of the terminal pathway is probably the protective effect of the membrane-bound physiologic inhibitors CD55 and CD59, which are intact in CAD.

A unique phenomenon in CAD is an exacerbation of hemolytic anemia during febrile infections and other conditions with acute-phase reaction, originally described as 'paradoxical' hemolysis[65] and later found to occur in at least 70% of the patients.[41] The constant complement consumption during steady-state disease results in low serum levels of C3 and, in particular, C4, which appears to be rate-limiting for CCP-dependent hemolysis. Acute-phase reaction has been shown to increase the production of these components. The serum levels are replete, CCP activity is enhanced, and exacerbation of hemolysis ensues.[66] Thus, CAD patients should probably not receive transfusion with complement-rich blood products, for example, plasma, even though this has not been systematically investigated.

Current management of CAD

Patients with mild disease can be managed by thermal protection without drug therapy. These precautions have been described elsewhere in the literature.[21,44]

Several therapies active in wAIHA, including corticosteroids, other untargeted immune suppressive agents, and splenectomy, have little or no effect in CAD.[23,41,42] Until now, documented therapies have been directed at the pathogenic B-cell clone.[44] Rituximab monotherapy has been shown to be effective in approximately 50% of the patients.[67,68] Responses are almost always partial (PR) and the median time to response (TTR) has been found to be 1.5 months, median response duration is approximately 1 year, but toxicity is low and repeat treatment is often effective in relapsed patients. At the time of clinical response, a major reduction in the monoclonal antibodies (as assessed by the IgM levels) has been documented. They are, however, not completely eliminated, in contrast with what is seen in the complete responders (CRs) following combination chemoimmunotherapy.[67,69]

Rituximab and bendamustine combination therapy is more efficacious and has been found to produce responses in 76% of the patients, with a 40% CR rate and long response duration (<10% relapses at 32 months).[69] Toxicity is acceptable and manageable but should be taken into consideration when treating less-fit patients. Median TTR was 1.9 months (range, 0.25–7 months), often with an even longer time to optimal response.

Several treatment options are available as second-line therapies, including rituximab plus bendamustine, if not given as first-line therapy,[69] rituximab plus fludarabine (approximately as efficacious as rituximab-bendamustine but more toxic),[70] and bortezomib-based therapies (which has a reported overall response rate of 6/19 if administered as one cycle of monotherapy).[71] The possibility of using Bruton tyrosine kinase inhibitors, or other novel B-cell-directed therapies, is attractive but has not been systematically studied.

The prospective studies of B-cell-directed therapies have used strict response definitions, for example, most studies have required the absence of any detectable bone marrow LPD in order to qualify for CR.[67,69,70] Further data analysis has supported these criteria, as response duration has been shown to correlate with the depth of response of the underlying LPD. The same criteria, however, will not be applicable to therapeutic complement inhibition in CAD, as this treatment is not directed against the underlying clonal disorder.[61,64,72]

Secondary CAS

Diagnosis and basic features

CAS is a clinical syndrome similar to CAD, occurring secondary to another well-defined disease, and consists of CA-induced hemolysis and, in some cases, cold-induced acral circulatory symptoms.[9] In an adult population, CAS occurs much less frequently than CAD.

CAS can be associated with aggressive non-Hodgkin B-cell lymphoma.[9] In these cases, the CAs are monoclonal IgM and have anti-I specificity as in CAD, but the light chain restriction can be λ as

well as κ.[73] Association with several nonlymphatic malignancies has also been described, the majority in case reports. Some of these associations are very rare, and others can be questioned because coexistence of CAD and malignancy will sometimes occur.

'Less infrequent' underlying diseases are specific infections, in particular, *Mycoplasma pneumoniae* pneumonia and Epstein–Barr virus (EBV) infection.[8,9,38] In CAS complicating *Mycoplasma* pneumonia, the CA is a polyclonal IgM with anti-I specificity, while anti-i polyclonal CAs are usually found in EBV-induced CAS, either of the IgM or IgG class.[9] CAS may also, infrequently, complicate infections with cytomegalovirus, other viruses, and *Chlamydia* species.[9] Occasional cases have been reported following stem cell transplantation and, recently, during therapy with the immune checkpoint-inhibitor nivolumab.[9,74]

Complement involvement in CAS

Although specific data are sparse, immune hemolysis in CAS is thought to be entirely complement-driven and mediated by the CCP as in CAD.[57,75]

Current management of CAS

Infection-associated CAS will resolve after recovery from the causative infection, but anemia can be profound, transfusion-dependent, and the resolution can be slow, especially in *Mycoplasma*-associated CAS.[9] There is no documented therapy apart from treatment of the underlying infection, if relevant, and transfusions if required. It has not been demonstrated whether a short course of predniso(lo)ne is able to shorten the duration of hemolysis.

PCH

Diagnosis, basic features, and complement involvement

Complement-mediated pathogenesis. The pathogenic autoantibody in PCH is an anti-P specific, strongly complement-activating biphasic polyclonal IgG hemolysin, termed Donath-Landsteiner's antibody after those who first described the disease in 1904.[76,77] The ag-ab fixation takes place at temperatures below normal central body temperature, while CCP activation beyond the C4–C2 stage occurs after warming to 37°C in the central circulation, followed by terminal pathway activation and intravascular hemolysis.[77–79]

Epidemiology and clinical presentation. PCH is a rare AIHA, that is mostly seen as an acute, transient complication during or following virus infection in small children.[77–79] The epidemiology and clinical presentation have changed dramatically over the last 40–60 years. Although always considered rare, PCH was previously reported as a chronic hemolytic anemia in adults with tertiary syphilis, hematologic malignancies, or without any identifiable underlying disease.[8,77,80,81] The syphilitic form is now rarely seen, and adult PCH is extremely rare.

PCH is currently most frequently reported as a complication of a viral infection in children, usually aged ⩽5 years.[77,78] The onset of post-viral childhood PCH is typically sudden with pallor, hemoglobinuria, chills, and myalgia.[77,79]

Post-viral childhood PCH is thought to be very rare but the current incidence has not been established. All types of AIHA are uncommon in children, with PCH patients making up 6–32% of all AIHA cases in two series.[78,82] The reported incidence may have been influenced by the awareness of the clinician and the sensitivity of diagnostic tests.[77,79] In addition, few laboratories are able to perform the Donath-Landsteiner test and the DAT can be negative,[82,83] as a result PCH may be underdiagnosed as a cause for adult and childhood hemolytic anemia.

Diagnosis. Laboratory tests show anemia and signs of intravascular hemolysis, including markedly elevated lactate dehydrogenase (LDH) levels, low haptoglobin, indirect hyperbilirubinemia, hemoglobinuria, and hemosiderinuiria.[77] Reticulocytosis may be absent in the initial phase. Monospecific DAT is usually negative for IgG but frequently positive for C3 fragments.[77,82] The diagnosis is confirmed by the Donath-Landsteiner's test.[76,83] In this assay, patient serum is incubated with RBCs at 4°C to fix complement to the IgG-P complex at the cell surface, and then incubated at 37°C. A parallel patient sample is incubated at 37°C without previous cooling. The test is positive if hemolysis is observed in the sample exposed to cold incubation, but not in the control sample. This original version of Donath-Landsteiner's test is highly specific but has limited sensitivity because the patient serum is often

complement-depleted.[77,79] More sensitive versions of the test have been developed by using papain-treated donor RBCs, or by adding complement, and the test should be carried out in a specialized laboratory.

Current management of PCH

Management consists of close monitoring, thermal protection, and transfusions if required.[77] Although transient, hemolysis can be severe with profound anemia requiring transfusions, and there is an obvious unmet need for efficient treatment. Anecdotal reports on corticosteroid therapy do not provide any reliable evidence, as childhood PCH is self-remitting. Convincing improvement following rituximab therapy has been reported in an adult patient with chronic PCH.[84]

PNH

Basic features

PNH is a rare, clonal, nonneoplastic, acquired hematopoietic stem cell disorder, characterized by nonimmune hemolytic anemia driven by uncontrolled complement activation. PNH is frequently accompanied by bone marrow failure and is associated with a high risk of thrombosis and a severely reduced survival rate and quality of life, if not treated successfully.[6,85]

Cellular pathogenesis. The causative acquired mutation in clonal PNH stem cells is a defect in the *PIGA* gene on the p arm of chromosome X, which encodes for the glycosyl-phosphatidylinositol (GPI) anchor expressed on cell surfaces.[86–88] The GPI anchor is required for the binding of several surface proteins to the cell. If this structure is absent, or nonfunctional, the cell surface cannot bind the physiological complement inhibitors CD55 and CD59. GPI-deficient cells (including RBCs, granulocytes, monocytes, and platelets that originate from the PNH stem cells) therefore lack these proteins on their surface.[88,89]

In patients with PNH, normal stem cells coexist alongside the PNH clone.[88,90] The development of clinical disease is believed to be a two- or multistep process that starts with the somatic mutation. The resulting lack of a functional GPI anchor is probably not enough to promote the expansion of the pathologic clone and, therefore, a 'second hit' is required.[90] This will occur if the normal stem cells are affected by, for example, an immune attack that may result in aplastic anemia or other permanent or temporary bone marrow suppression. Suppression of the normal stem cells confers a proliferative advantage to the PNH clone, and clonal expansion ensues, in some cases with development of clinical PNH.

Complement involvement in PNH

The ACP is subject to a low-grade, continuous activation by spontaneous hydrolysis (tick-over) of C3 (Figure 1), starting with the formation of $C3(H_2O)$, a hydrolytic and conformationally rearranged C3 derivative.[91,92] $C3(H_2O)$ and C3b, respectively, can form complexes with factor B.[2] Factor D, a serine protease, then cleaves factor B into the components Ba and Bb, resulting in the formation of $C3(H_2O)Bb$ and C3bBb, both of which act as ACP C3 convertases.[12,91,92] Subsequent generation of more C3b feeds a positive feedback loop. ACP C3 convertase, stabilized after binding of factor P (properdin), can bind another molecule of C3b, forming the ACP C5 convertase (C3bBbC3b).[2,12,91,93] This proteolytic enzyme has the potential to cleave C5, thus generating the powerful anaphylatoxin C5a in addition to C5b, which triggers the terminal complement cascade with the formation of the MAC and intravascular hemolysis.

Under normal circumstances, this process is strictly controlled due to the suppression of the ACP by CD55 and, in particular, by the inhibitory effect on the terminal pathway of CD59.[3] PNH cells, being deficient in these surface bound regulatory proteins are, therefore, particularly prone to lysis by terminal pathway complement attack, which explains why patients with a significant proportion of PNH cells have chronic, severe intravascular hemolysis.

The intravascular hemolysis results in free hemoglobin in the serum, which acts as a nephrotoxic substance. In addition, free hemoglobin increases the nitric oxide (NO) consumption by a factor of 8–10.[94] The NO depletion results in dysregulation of smooth muscle cell tonus, which explains many of the symptoms and complications in PNH including gastrointestinal spasms, abdominal pain, dysphagia, vasoconstriction, pulmonary and systemic hypertension, and erectile dysfunction.[94,95] Depletion of NO has also been shown to

result in platelet activation and aggregation and, therefore, is thought to contribute to the high risk of thrombosis.[18,95,96] As mentioned previously, there are multiple points of interaction (cross-talk) between complement and the coagulation system.[17,18] Thus, the massive complement activation on its own contributes to the risk of thrombosis.

As expected, untreated patients with this nonimmune hemolytic disease usually have a negative DAT. If the terminal complement pathway is blocked by a therapeutic inhibitor, however, there will be more C3b-opsonized PNH cells available for extravascular hemolysis by the mononuclear phagocytic system. A proportion of the C3b-labeled RBCs will also survive, and C3b is metabolized to C3d.[14,15] Therefore, PNH patients on treatment with downstream complement inhibitors will have some residual hemolysis, and most of them will develop DAT positivity for C3d.[15]

Clinical presentation and diagnosis

Patients with PNH may present with anemia due to intravascular hemolysis, usually with highly elevated LDH levels and sometimes resulting in hemoglobinuria.[85] A large number of patients also suffer from arterial and venous thrombosis. These are commonly described in atypical localizations, such as the splanchnic or cerebral circulation, but can also occur in more common sites.[18,97] Hepatic and other splanchnic venous thrombosis have been shown to account for more than 35% of the thrombotic events described.[97] Thromboembolic complications are the major, direct cause of death in patients with PNH, accounting for 40–67% of mortality.[18,97] The prophylactic effect of warfarin is insufficient, and thrombotic events have been shown to occur approximately 10 times per 100 patient-years in PNH patients on anticoagulants.[97]

Fatigue is a typical symptom, and smooth muscle phenomena including gastrointestinal spasms, dysphagia, hypertension, and erectile dysfunction occur in the majority of cases.[85,94] Episodes of abdominal pain may be due to the smooth muscle dysfunction or ischemia, as well as thrombosis. Development of pulmonary hypertension is frequent, and some patients develop renal failure.[94,98]

In patients with DAT-negative hemolytic anemia, flow cytometry testing of peripheral blood for the absence of specific GPI-linked proteins, or the GPI anchor itself, will establish the diagnosis[6,99] Owing to the high mortality rate in PNH patients with thromboembolic events, flow cytometry should also be performed in patients with unexplained thrombosis, in particular, if they have signs of hemolysis.

Management and prognosis of PNH in the pre-eculizumab era

Before the event of effective anticomplement therapies, management of PNH was mainly supportive and included transfusions and anticoagulation. Some younger, severely affected patients received allogeneic stem cell transplantation, which could be curative, however, at a risk of treatment-related mortality and morbidity.[100] Prognosis can be poor in patients with hemolytic PNH who do not receive effective treatment (Figure 3),[85,101] and any thrombotic event further increases the risk of early death.[18,97]

Complement modulation as therapy for complement-driven hemolytic anemias

Targeting the terminal complement cascade

The humanized monoclonal anti-C5 antibody eculizumab was the first complement inhibitor shown to be effective in any complement-mediated hemolytic anemia and is today the standard treatment for patients with PNH requiring therapy. The drug is usually given at a loading dose of 600 mg weekly for 4 weeks, followed by 900 mg every other week for maintenance. In a phase II trial, this was shown to substantially improve hemolytic anemia and reduce transfusion requirements.[102] The effect was found to be sustained in an extension study, and the findings were confirmed by a large, randomized controlled trial followed by another extension study.[7,103] Further studies have also demonstrated improvement in fatigue score and quality of life, as well as dramatically reduced risk of developing thrombotic complications, pulmonary hypertension, and renal insufficiency. A highly promising effect has been documented on survival, which has approached normal life expectancy in an age-matched population (Figure 3).[101] Even in pregnant women with PNH, eculizumab therapy has been shown to be safe and substantially improve the outcome in these ultra-high-risk pregnancies.[104]

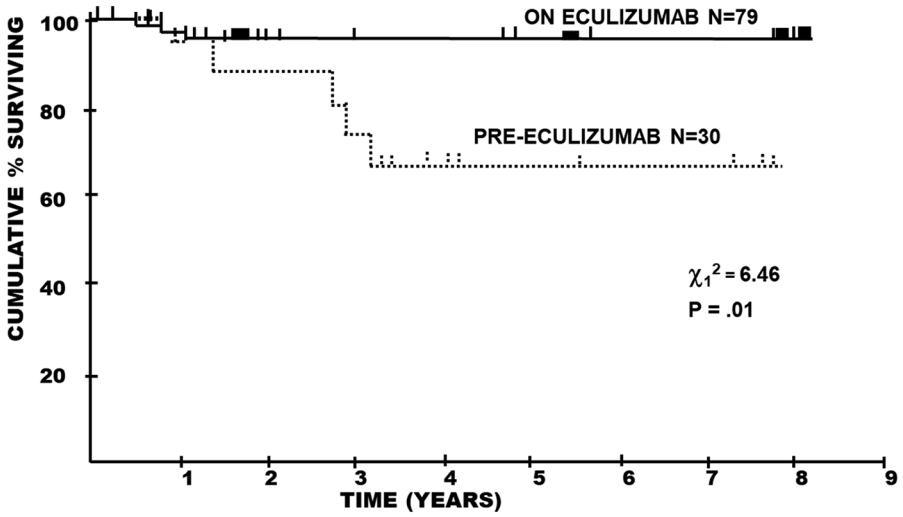

Figure 3. Survival among patients with paroxysmal nocturnal hemoglobinuria (PNH) on eculizumab therapy compared with survival before the eculizumab era.
First published in *Blood* by Kelly and colleagues,[101] reused under general permission. Copyright: *Blood*, the Journal of the American Society of Hematology.

Eculizumab is generally well tolerated except for the potential risk of infection. The terminal complement cascade is an important part of the host defense against encapsulated bacteria including the *Neisseria* species. In the prospective eculizumab trials, as well as clinical practice, patients have been vaccinated against *Neisseria meningitidis* and in some countries, antibiotic prophylaxis (usually phenoxymethylpenicillin) is also recommended. Despite this, very rare cases of meningococcal infection have been reported.[105]

Other limitations of eculizumab therapy for PNH may also be significant. First, slightly variable *in vivo* half-life causes some patients to have breakthrough hemolysis in the days before the next dose is due. In patients who frequently experience this problem, it can usually be avoided by increasing the dose to 1200 mg or higher. Second, for some patients, the relatively frequent dosage every second week may be inconvenient. These two disadvantages are being addressed by the development of second-generation C5 inhibitors.[106] Third, hemolysis is not completely abrogated by eculizumab, resulting in residual hemolytic anemia in the majority of patients, but sufficient to require transfusion in a minority of patients. The explanation for this problem is the extravascular hemolysis by phagocytosis of C3b-opsonized RBCs (Figure 1), which is a minor pathway of hemolysis in C5-inhibitor naïve PNH patients but is accentuated by blocking the terminal complement pathway.[14,15] As described in the following, this problem may be resolved by complement inhibition at the ACP level. Fourth, eculizumab is not effective in patients with the p.Arg885His polymorphism in C5.[107]

Ravulizumab, a novel anti-C5 monoclonal antibody designed for dosage every 8 weeks, has recently been studied in two trials of PNH patients that were eculizumab-naïve or previously treated with eculizumab.[106,108] This C5 inhibitor has been found to be noninferior to eculizumab in effect on complement activation markers, parameters of hemolysis, clinical outcome, and frequency of breakthrough hemolysis. Tolerability and safety did not differ significantly from the eculizumab data.

An open, non-randomized prospective trial showed some effect of C5 inhibition with eculizumab in CAD.[61] However, although intravascular hemolysis was significantly reduced and most patients became transfusion independent, only a marginal increase in hemoglobin level was observed and there was no significant improvement in the quality of life scores. This could be explained by the predominance of C3b-opsonization mediated extravascular hemolysis, which does not involve the terminal pathway.[61,72] Therefore, C5 is obviously not the

optimal target of complement modulation in CAD, although a meaningful effect has been reported in severely affected patients[62,109] and as prophylaxis against exacerbation during heart surgery.[63]

A case of secondary CAS responding well to eculizumab has been reported as part of a CAD study.[61] In theory, the terminal pathway mediated pathogenesis in PCH should make this disease an ideal candidate indication for C5 inhibition. In the only case report published, no increase in hemoglobin level was observed although intravascular hemolysis was inhibited.[81] However, this patient was an adult with PCH secondary to a hematologic malignancy and thus not representative of typical PCH.

Case reports also document the use of eculizumab to rescue some patients with treatment-refractory, severe wAIHA. These cases were IgM-mediated secondary to Churg-Strauss syndrome,[110] IgG-mediated secondary to Waldenström macroglobulinemia,[111] and primary wAIHA with a DAT positive to IgG only.[112]

Proximal classical pathway modulation

The first available drug for modulating the CCP was plasma-derived C1 esterase inhibitor (C1-INH), used for the treatment of hereditary angioedema, which is not a complement-mediated disorder. High doses of C1-INH were found to abrogate complement activation and hemolysis and efficiently improve anemia when given as rescue therapy in a patient with a severe, IgM-mediated wAIHA,[113] and a similar effect was subsequently observed in a patient with acute, severe CAS.[114] However, there is no evidence of endogenous C1-INH deficiency in AIHA, and frequently repeated high doses are likely to be required. Thus, C1-INH is probably not suitable for long-term therapy, and no systematic clinical trial has been published.

Sutimlimab (also known as BIVV009 or TNT009) is a humanized monoclonal antibody inhibitor of C1s.[115] *In vitro* experiments with TNT003, the murine antibody from which sutimlimab is derived, demonstrated efficient and dose-dependent inhibition of complement activation, C3 deposition, and phagocytosis of RBCs in the presence of patient sera as a source of CA and normal human serum as a source of complement.[11] Sutimlimab therapy, administered as weekly intravenous infusions, increased hemoglobin levels by a median of 1.6 g/dl within the first week of treatment and by 3.9 g/dl within 6 weeks in a recent clinical phase Ib trial of 10 patients with CAD.[115] Extravascular hemolysis was abrogated in most patients, bilirubin levels often normalized within 24 h, and all of six previously transfusion-dependent patients became transfusion-free. Hemolytic anemia recurred 3-4 weeks after discontinuation, but re-administration of sutimlimab restored the remission.

Adverse events related to the study drug were not observed. Participants were vaccinated against *N. meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*, but received no other prophylaxis. Sutimlimab is now undergoing phase III trials in patients with CAD (ClinicalTrials.gov identifiers: NCT03347396 and NCT03347422).

In vitro experiments have shown prompt inhibition of CA-induced CCP activation and hemolysis by ANX005, a humanized monoclonal antibody to C1q.[116] A safety study in healthy volunteers has been completed but has not been published.

A class of peptide inhibitors of C1 (PIC1) has also been developed.[117] These are small molecules that target C1q, blocking the activation of associated serine proteases (C1s-C1r-C1r-C1s) and subsequent CCP activation. A recent experiment described in a case report has demonstrated *in vitro* reversal of CCP-mediated hemolysis initiated by serum from a patient with drug-induced AIHA.[118] No systematic human clinical study has been published.

In common with C5 inhibition, CCP blockade might carry a risk of severe infection. Clinical data so far indicate that C1 inhibition is also safe in this regard, provided the patients are vaccinated against meningococci, pneumococci, and *Haemophilus*.[115] This issue will need to be carefully addressed in future clinical trials. As hereditary deficiencies in proximal CCP components are associated with systemic lupus erythematosus (SLE), a risk of developing SLE might be suspected when C1 is blocked.[119] Until now, clinical data has not supported this concern.[115]

CCP inhibition in CAD will encounter some limitations. Unlike chemoimmunotherapy, treatment will probably have to continue indefinitely to maintain its effect. Furthermore, circulatory symptoms, such as acrocyanosis and Raynaud-like phenomena, are not complement-mediated and will not be relieved. In theory, C1 inhibition may be a suitable approach in secondary CAS and PCH but no clinical data have been published.

Inhibition at the C3 level

Because cleavage of C3 by C3 convertase is a point of convergence between all three initial complement pathways and critical for activation of the terminal pathway, modulation at this level has the potential to block the entire complement system and thus provide an attractive option in a variety of complement-mediated disorders.[120,121] Experiments with a peptide inhibitor of C3 convertase formation, compstatin Cp40, found a dose-dependent inhibition of hemolysis and prevention of C3b deposition on PNH-RBCs in an *in vitro* system, and preclinical studies indicated favorable pharmacokinetics following subcutaneous injection.[122,123]

Pegcetacoplan (previously termed APL-2) is a pegylated compstatin analog designed for subcutaneous administration.[118] Systemic administration of pegcetacoplan was investigated in two phase I trials comprising 51 healthy patients.[124] Participants received vaccination as in the C1 inhibition trials. Monitoring showed high efficiency of pegcetacoplan in inhibiting the classical and alternative pathways, and the results of pharmacodynamic assessments were favorable. There were few adverse events and no serious adverse events, although the maximal duration of drug administration was only 28 days.[124]

Clinical phase II trials have found efficacy of pegcetacoplan in PNH as well as AIHA, including CAD (Figure 4).[125,126] A phase III study is ongoing in PNH (ClinicalTrials.gov identifier: NCT03500549), and further clinical studies in CAD and wAIHA are warranted. In theory, the drug may also be a promising approach in CAS and PCH.

When compared with C1 inhibition, which targets the CCP specifically and leaves the ACP and LCP intact, the potentially universal complement blockade brought about by C3 inhibition might pose a still greater risk of severe infection. Until now, clinical observation has not supported this concern,[124–126] but experience is limited and larger studies are ongoing. Appropriate vaccination as in the C1 inhibition trials and, possibly, antibiotic prophylaxis will be advisable.

Conclusion

Several hemolytic anemias are driven by complement activation, either completely as in PNH, PCH and probably CAD, or partially as in wAIHA. C5 inhibition has revolutionized the management and prognosis of PNH but appears to be less efficacious in CAD. Although complement modulation at the C1 or C3 level is still under investigation, C3 inhibition has the potential to further improve therapy in PNH by eliminating the residual extravascular hemolysis. If preliminary results are confirmed, C1 as well as C3 inhibition may provide efficacious treatment options in CAD and CAS, especially in patients who fail on chemoimmunotherapy for CAD or require rapidly acting therapeutic intervention. A potential role of CCP inhibition as the first potentially effective therapy in severe PCH should also be explored. Further clinical studies are warranted.

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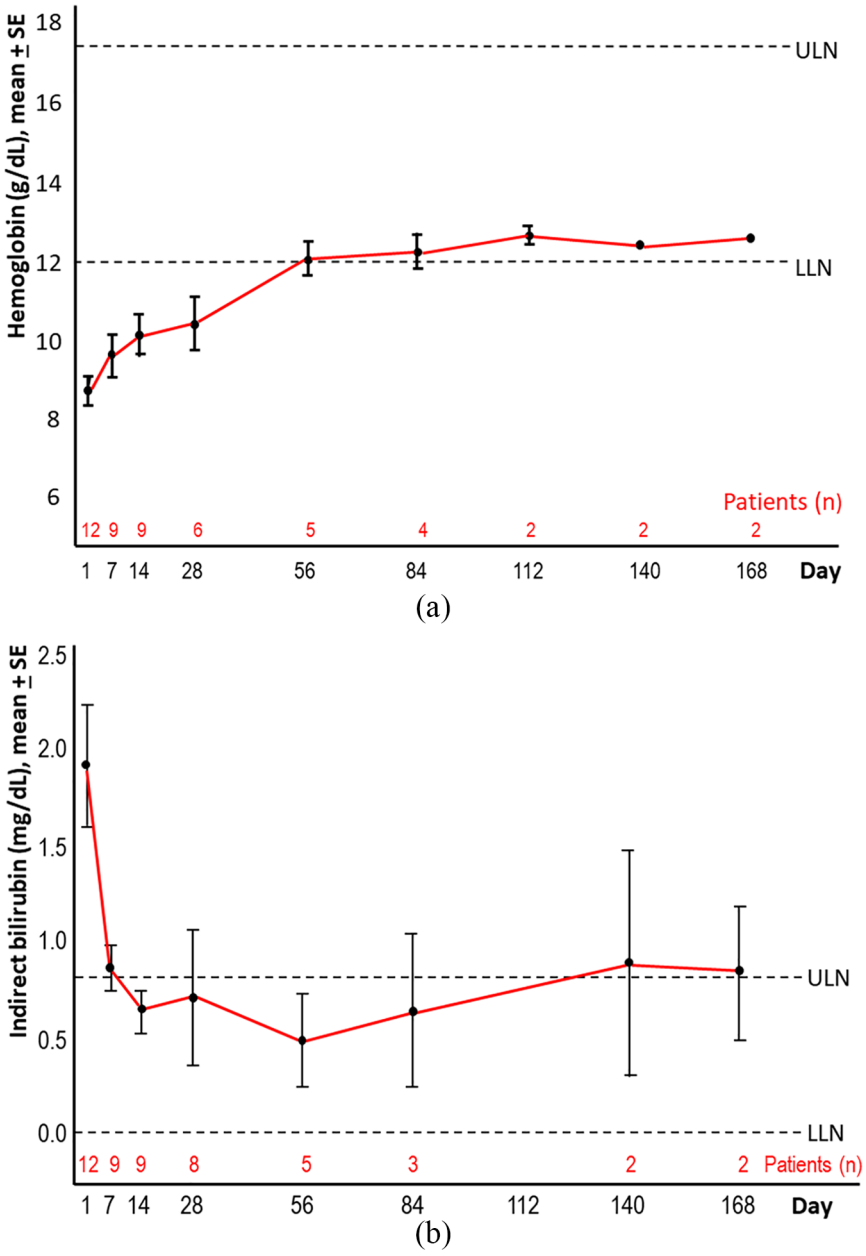

Figure 4. Effect of pegcetacoplan in cold agglutinin disease (CAD). Data from a phase II study, showing normalization of hemoglobin levels within 56 days of medication in the majority of patients and within 84 days in all patients (a). Normalization of indirect bilirubin levels within 1–2 weeks in all patients (b).
LLN, lower limit of normal; ULN, upper limit of normal. Originally presented by F. Grossi and colleagues.[126] at the 60. Annual Meeting of the American Society of Hematology, 2018, reproduced with permission. Courtesy of F. Stout and A. Shen. Copyright: Apellis Pharmaceuticals.

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