REGULAR ARTICLE

blood advances

C1-inhibitor treatment in patients with severe complement-mediated autoimmune hemolytic anemia

Esther C. W. de Boer,[1,2,*] Marit Jalink,[3-5,*] Laura Delvasto-Nuñez,[1] Elisabeth M. Meulenbroek,[1] Inge Baas,[1] Susanne R. Janssen,[4] Claudia C. Folman,[6] Kyra A. Gelderman,[7] Diana Wouters,[8] Marije D. Engel,[4] Masja de Haas,[3,6,9] Marie José Kersten,[10] Ilse Jongerius,[1,2,*] Sacha Zeerleder,[11,12,*] and Josephine M. I. Vos[6,10,*]

[1]Department of Immunopathology, Sanquin Research and Landsteiner Laboratory, Amsterdam University Medical Centre, Amsterdam Infection and Immunity Institute, Amsterdam, The Netherlands; [2]Department of Pediatric Immunology, Rheumatology, and Infectious Diseases, Emma Children's Hospital, Amsterdam University Medical Center, Amsterdam, The Netherlands; [3]Department of Clinical Transfusion Research, Sanquin Research, Amsterdam, The Netherlands; [4]Department of Hematology, Amsterdam University Medical Center, Amsterdam, The Netherlands; [5]Department of Transfusion Medicine, Sanquin Blood Supply, Amsterdam, The Netherlands; [6]Department of Immunohematology Diagnostics, Sanquin, Amsterdam, The Netherlands; [7]Sanquin Diagnostic Services, Amsterdam, The Netherlands; [8]Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, The Netherlands; [9]Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands; [10]Department of Hematology, Amsterdam University Medical Centers, Location University of Amsterdam, Cancer Center Amsterdam and Lymphoma and Myeloma Center Amsterdam, Amsterdam, The Netherlands; [11]Department of Hematology, Luzerner Kantonsspital, Luzern, Switzerland; and [12]Department for BioMedical Research, University of Bern, Bern, Switzerland

Key Points

- Human plasma–derived C1-INH temporarily reduced complement deposition on RBCs but not systemic complement activation.
- C1-INH did not halt hemolytic activity in patients with severe CM-AIHA.

Complement-mediated (CM) autoimmune hemolytic anemia (AIHA) is characterized by the destruction of red blood cells (RBCs) by autoantibodies that activate the classical complement pathway. These antibodies also reduce transfusion efficacy via the lysis of donor RBCs. Because C1-inhibitor (C1-INH) is an endogenous regulator of the classical complement pathway, we hypothesized that peritransfusional C1-INH in patients with severe CM-AIHA reduces complement activation and hemolysis, and thus enhances RBC transfusion efficacy. We conducted a prospective, single-center, phase 2, open-label trial (EudraCT2012-003710-13). Patients with confirmed CM-AIHA and indication for the transfusion of 2 RBC units were eligible for inclusion. Four IV C1-INH doses (6000, 3000, 2000, and 1000 U) were administered with 12-hour intervals around RBC transfusion. Serial blood samples were analyzed for hemolytic activity, RBC opsonization, complement activation, and inflammation markers. Ten patients were included in the study. C1-INH administration increased plasma C1-INH antigen and activity, peaking at 48 hours after the first dose and accompanied by a significant reduction of RBC C3d deposition. Hemoglobin levels increased briefly after transfusion but returned to baseline within 48 hours. Overall, markers of hemolysis, inflammation, and complement activation remained unchanged. Five grade 3 and 1 grade 4 adverse event occurred but were considered unrelated to the study medication. In conclusion, peritransfusional C1-INH temporarily reduced complement activation. However, C1-INH failed to halt hemolytic activity in severe transfusion-dependent–CM-AIHA. We cannot exclude that posttransfusional hemolytic activity would have been even higher without C1-INH. The potential of complement inhibition on transfusion efficacy in severe CM-AIHA remains to be determined.

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*E.C.W.d.B. and M.J. contributed equally to this study, I.J., S.Z., and J.M.I.V. contributed equally to this study.

Deidentified individual participant data underlying the reported results as well as the protocol will be made available 3 months after the publication for 5 years via the authors. Data are available on request from author, Josephine M. I. Vos (j.m.i.vos@amsterdamumc.nl).

The full-text version of this article contains a data supplement.

Introduction

Complement-mediated (CM) autoimmune hemolytic anemia (AIHA) is caused by the destruction of red blood cells (RBCs) owing to autoantibodies that trigger the classical pathway (CP) of the complement system.[1,2] The antibodies involved in CM-AIHA are mostly cold agglutinins (CAs).[1] CAs are typically immunoglobulin M (IgM) autoantibodies against RBCs that bind optimally from 0 to 4°C but also from 16 to 30°C.[1,2] CAs are found in primary CA disease (CAD) and secondary CA syndrome.[1] CM-AIHA is most frequently based on cold-reactive IgM antibodies but also on warm-reactive IgM antibodies, complement-activating IgG, or a combination thereof.[3]

CM-AIHA can lead to severe hemolytic episodes which can be life-threatening, prompting hospitalization and RBC transfusion. Transfusion may be ineffective because in up to 30% of patients with AIHA, donor RBCs undergo rapid destruction by their autoantibodies, which can lead to decreased RBC transfusion efficacy and a state of refractory anemia.[4] Severe AIHA refers to a subset of patients with AIHA defined by the presence of an unsupported hemoglobin (Hb) level below 8.0 g/dL and repeated transfusion requirement with an interval ≤7 days, characterized by severe anemia symptoms and Hb instability.[5] This is a rare and life-threatening condition for which, to the best of our knowledge, prospective studies are lacking so far.

The treatment of CM-AIHA depends on the type of anti-RBC antibodies involved and the underlying disease. In CAD, treatment with anti-CD20 antibodies with(out) chemotherapy is directed against underlying B-cell lymphoproliferation to attenuate autoantibody production.[1,2] In IgG^+ CM-AIHA, steroids and other immunosuppressants are the mainstay of treatment. However, the time to response can be weeks to months, and responses are often only partial.[6,7] Consequently, in severe CM-AIHA, a rapid-acting agent that leads to an effective halt of the hemolytic activity and better RBC transfusion efficacy is an unmet need.

In CM-AIHA, C1q binds autoantibodies on RBCs and triggers the CP via the activation of serine proteases C1r and C1s, ultimately leading to C3b deposition. C3b-opsonized RBCs can be phagocytosed, mainly in the liver, leading to extravascular hemolysis, which is usually predominant in RBC destruction in CM-AIHA.[8,9] Ultimately, terminal complement activation induces membrane attack complex formation, resulting in intravascular hemolysis. This can be involved in fulminant cases but is usually not predominant.[9] Complement activation contributes to a proinflammatory response via anaphylatoxins C3a and C5a, but this response has not been thoroughly investigated in CM-AIHA.[2,10-12]

C1-inhibitor (C1-INH) is a serine protease inhibitor regulating vascular permeability and inflammation and a CP and lectin pathway regulator by inhibiting C1r, C1s, and mannan-binding lectin serine proteases 1 and 2.[13,14] C1-INH has been used for >30 years in patients with hereditary angioedema (heterozygous deficiency of C1-INH).[15] Based on its anti-inflammatory properties, C1-INH has also been used in clinical trials for inflammatory conditions, such as sepsis and ischemic reperfusion injury.[16-21] Inflammation is also present in CM-AIHA, induced among others by complement activation, neutrophil extracellular trap formation, and cell-free heme, which also contribute to the prothrombotic state in patients with AIHA.[22,23] Furthermore, the inflammatory state possibly contributes to fatigue, an often-reported symptom in CM-AIHA[12,24] and in other CM anemic diseases such as paroxysmal nocturnal hemoglobinuria.[25,26] This illustrates the relevance of investigating (the effect of C1-INH on) inflammation in CM-AIHA. In vitro, C1-INH abrogated autoantibody-mediated complement deposition and lysis of RBC after incubation with AIHA sera.[27,28] We reported a case of CM-AIHA in which peritransfusional administration of C1-INH led to an attenuation of complement deposition on RBCs and improved the efficacy of RBC transfusion.[28] Other studies reported 4 patients with severe AIHA and direct antiglobulin test (DAT) positive for C3d who responded to C1-INH, which was administered for 6 to 20 days.[29]

We hypothesized that temporary treatment with C1-INH will suppress CM hemolytic activity and thus improve the RBC transfusion efficacy in patients with severe CM-AIHA. C1-INH treatment might thus overcome an acute and severe hemolytic episode while awaiting the effect of additional therapies.

In addition, we hypothesized that C1-INH could attenuate the systemic inflammatory state in CM-AIHA. Therefore, we designed a "proof-of-principle" study to assess the effect of peritransfusional C1-INH administration on RBC transfusion efficacy as well as on hemolytic activity and complement and inflammatory markers in patients with severe CM-AIHA in need of a transfusion.

Methods

Patients and healthy controls (HCs)

We designed a prospective single-center phase 2 open-label trial. Adult patients with CM-AIHA, Hb <8 g/dL, and a current indication for transfusion of at least 2 RBC units were eligible for inclusion. CM-AIHA was defined by clinical signs of hemolysis (non-detectable haptoglobin) and increased lactate dehydrogenase [LDH]), in combination with a positive (≥1+) monospecific DAT for C3b or C3d with/without positivity for IgM or strongly positive (≥3+) monospecific DAT for C3b and/or C3d with positivity for IgG. Concurrent IgG deposition was not excluded in the presence of clear complement deposition because IgG, mainly subclass 1 and 3, is also able to activate the CP.

Transfusion indication was based on clinical assessment. Key exclusion criteria were a history of arterial and/or venous thromboembolic events in the absence of current treatment with vitamin K antagonists or concomitant use of therapeutic doses of heparin and the presence of active infections or any other severe and/or uncontrolled medical conditions. The full list of in- and exclusion criteria can be found in supplemental Table 1. As controls for the biomarker studies and flow cytometry, plasma and RBCs were obtained from anonymous healthy volunteers (HCs) after their informed consent.

Treatment and study design

The study medication was human plasma–derived C1-INH (Cinryze, Takeda). Subjects received the following 4 administrations of IV C1-INH: 6000, 3000, 2000, and 1000 U, with the first dose just before RBC transfusion and the subsequent doses after 12, 24, and 36 hours respectively (Figure 1). Data to guide optimal dosing of C1-INH to inhibit complement activation in vivo are scarce. In a clinical trial in patients with sepsis, the aforementioned protocol was

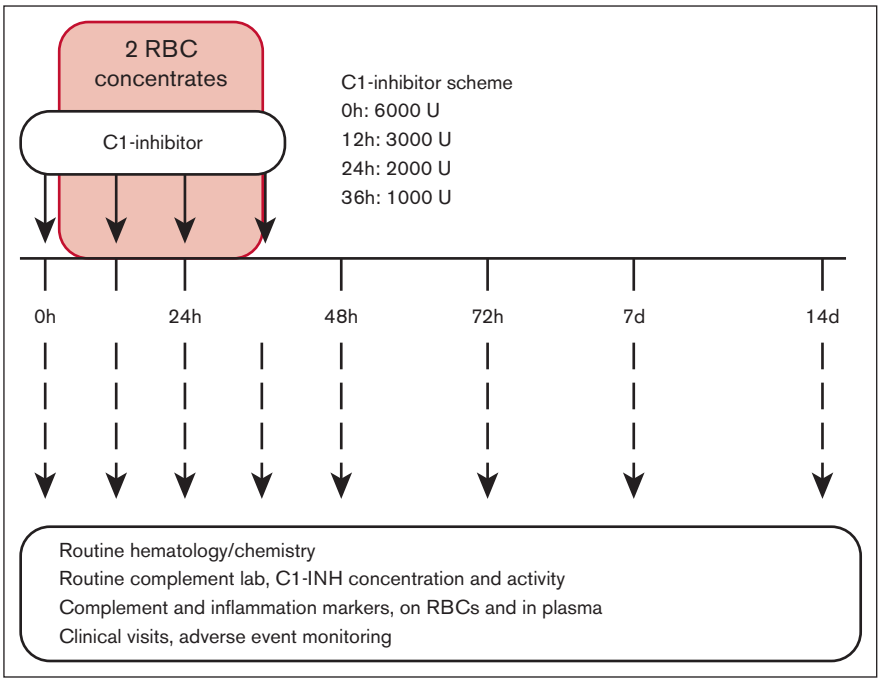

Figure 1. Study design.

safe and efficiently blocked the activation of the CP as evidenced by a decrease in C4b/c;[16] this was the rationale for the dose used in this trial. Blood samples to determine Hb levels, markers of hemolytic activity (LDH and bilirubin), complement activation and inflammation, and immunohematological diagnostics (serology and flow cytometry) were collected at baseline and 12 hours, 24 hours, 48 hours, 72 hours, 7 days, and 14 days. As per national policy, all RBC units were leucodepleted with hematocrit between 0.50 and 0.65 L/L and an average volume of 280 mL. Transfused units were Rh and K-antigen matched and, if applicable, antigen-negative for preexisting allo-antibodies.

Study oversight

The trial protocol was approved by the Medical Ethics Committee of the Amsterdam University Medical Center (AUMC). The trial was registered in The Netherlands Trial Register (#NL8164) and conducted in accordance with the Declaration of Helsinki and Dutch regulations. All patients and HCs provided written informed consent. Data were treated according to the EU General Data Protection Regulation. E.C.W.d.B., M.J., M.D.E., J.M.I.V., I.J., S.Z., and L.D.-N. analyzed the data, with access to data for all authors. AUMC, location AMC was the sponsor of the study. Takeda (previously Viropharma and Shire) gave financial support and supplied study medication. Takeda did not contribute to data acquisition and reviewed the manuscript after its development. Takeda had no other role in the conduct of the trial.

Main study end points

The predefined primary end point of the study was improved RBC transfusion efficacy, defined as a posttransfusion Hb decrease of <1 g/dL per 24 hours. In normovolemic, nonbleeding patients, the Hb level measured just after RBC transfusion remains stable after 24 hours.[29,30] However, in the setting of fulminant CM-AIHA, donor RBCs have a severely shortened survival and ongoing hemolytic activity typically results in a lower or no effect on Hb levels, despite transfusion.[28] Because normal values for transfusion efficacy in the setting of severe CM-AIHA are lacking, this predefined end point was not based on published clinical data, nor is an existing end point for clinical studies. Rather, the chosen cut-off was an educated guess to substantiate the primary outcome.

For the primary end point analysis, we compared the peak Hb level after the transfusion of 2 RBCs with the Hb level 24 hours later. To gain insight into the validity of the used cut-off for the primary end point, we analyzed the RBC transfusion efficacy in transfusion episodes 30 days before the study enrollment. For this post hoc analysis, we included only those episodes that had sufficient data available.

Predefined secondary end points were the inhibition of complement activation and complement deposition on RBCs upon C1-INH treatment and changes in their levels and inflammatory markers. As inflammatory markers, we measured nucleosomes and human neutrophil elastase α1-antitrypsin (EA) complexes, which are released upon neutrophil extracellular trap formation or cell damage[30,31] and neutrophil activation, respectively. Adverse events (AEs) were tracked from the time of administration of the first dose of the study drug through 14 days after the start of the C1-INH treatment. Toxicity and AEs were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 5.0.

Laboratory techniques

Parameters of AIHA (Hb, hemolytic markers, and DAT) were determined at the AUMC and total complement protein levels were determined at Sanquin Diagnostic Services, both according to standard diagnostic practice.

C1-INH antigen concentration was determined using nephelometric assay on the Siemens Dimension Vista, using Siemens reagents. C1-INH activity was determined with the Berichrom

Table 1. Baseline characteristics and posttransfusion Hb decrease

Case	Sex	Age, y	Diagnosis	DAT results	Type of AIHA*	RBC units transfused†	Hb, g/dL ‡	LDH, U/L ‡	Bilirubin total, μmol/L ‡	Posttransfusion Hb decrease <1 g/dL per 24 hr	
										Before enrollment (without C1-INH) §	On trial (with C1-INH) ‖
1	F	45	CAS	IgG: 4+	Mixed	2	5.6	975	65	Yes	Yes
			APS	IgM: 2+							
				IgA: 2+							
				C3d: 3+							
2	F	37	CAS	IgG:4+, IgM: 2+,	Mixed	3	3.7	1118	152	No	No
			IBD	IgA: neg							
			T-cell NHL, allo-SCT	C3d: 3+							
3	F	38	CAS	IgG: 4+	Mixed	7	3.5	911	55	No data	No
			SLE	IgM: 4+,							
			RA	IgA: 3+							
			IBD	C3d: 4+							
			ITP								
4	M	69	CAS	IgG: 4+	Mixed	2	5.3	559	36	No data	Yes
			B-cell NHL	IgM: neg							
				IgA: neg							
				C3d: 3+							
5	M	59	CAD	IgG: neg IgM: neg IgA: neg C3d: 2+	Cold	1	4.8	605	30	Yes	Yes
6	F	46	CAS, allo-SCT, MF	IgG: 2+ IgM: neg IgA: neg C3d: 3+	Mixed	23	5.3	270	53	N/A	Yes
7	M	65	CAS, CLL	IgG: neg¶ IgM: 1+ IgA: neg C3d: 3+	Cold	8	5.5	526	96	No	No
8	M	72	CAS,	IgG: neg	Cold	2	6.2	476	13	Yes	Yes
			CLL	IgM: neg							
				IgA: neg							
				C3d: 4+							
9	F	77	CAD	IgG: neg	Cold	4	6.9	570	26	Yes	Yes
				IgA: neg							
				IgM: 2+							
				C3d: 4+							
10	M	75	CAD	IgG: neg	Cold	1	6.9	495	55	Yes	Yes
				IgM: neg							
				IgA: neg							
				C3d: 3+							
Median		62				2.5	5.4	564.5	54		
Response/no. of patients										5/7	7/10

Eight out of 10 patients received either a prophylactic or therapeutic dose of anticoagulation.

allo-SCT, allogeneic stem cell transplantation; APS, antiphospholipid syndrome; CAS, cold agglutinin syndrome; CLL, chronic lymphocytic lymphoma; F, female; IBD, inflammatory bowel disease; ITP, immune thrombocytopenia; neg, negative; NHL, non-Hodgkin lymphoma; M, male; MF, myelofibrosis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

*AIHA classified as warm AIHA: DAT positive for IgG and/or IgA ± C3d when a clinically significant cold-reactive antibody has been excluded; cold AIHA: monospecific DAT strongly positive for C3d (and negative or weakly positive with IgG) and CAs are present; mixed AIHA: diagnosed in patients with a DAT positive for C3d and IgG DAT, a CA with a thermal amplitude ≥30 °C, and evidence of a warm IgG antibody by eluate.

†Total in the 30 days before inclusion.

‡Normal values: Hb male, 13.2 to 16.6 g/dL; Hb female, 11.6 to 15 g/dL; LDH, 0 to 247 U/L; total bilirubin, 0 to 17 μmol.

§Post hoc analysis (see "Methods").

‖Per protocol analysis.

¶IgG weakly positive in DAT, No evidence for IgG reacting autoantibodies in the eluate.

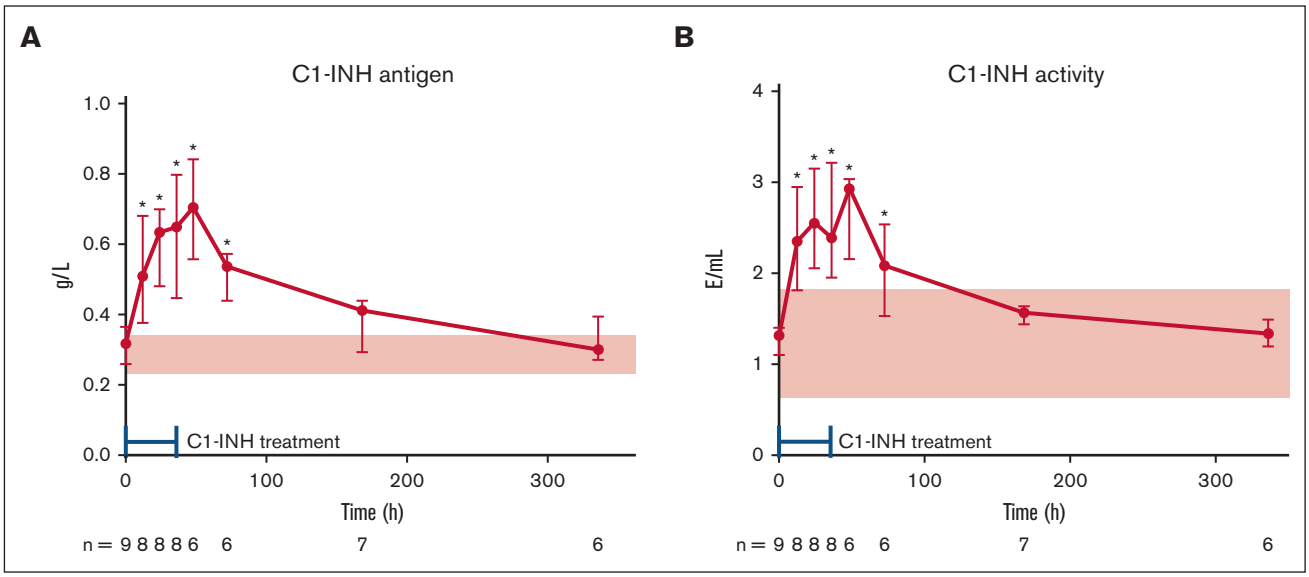

Figure 2. C1-INH antigen and activity levels increase upon Cinryze administration. Both C1-INH antigen (A) and activity (B) levels increase upon administration of four C1-INH doses in the first 36 h, reaching maximum levels at 48 h after first administration respectively. Red area indicates normal healthy levels of C1-INH antigen and activity. Data expressed as median + IQR, significance tested using Wilcoxon matched-pairs signed rank test. *$P \leq 0.05$.

Chromogenic Assay (Siemens Healthineers). An in-house plasma standard was used, targeted on NIBSC08/256, results are expressed in U/mL.

IgG, IgM, and IgA binding and C3 deposition were determined using flow cytometry on patients' RBCs isolated from EDTA blood samples using rabbit antihuman IgG, rabbit antihuman IgM, rabbit antihuman IgA, and antihuman C3.19 (Sanquin, The Netherlands), as described previously.[27] To measure systemic complement activation, C4b/c and C3b/c levels were determined in EDTA plasma using a previously described enzyme-linked immunosorbent assay (ELISA) in which neoepitopes on C4b/bi/c (C4b/c) or C3b/bi/c (C3b/c) are specifically recognized.[32,33] Nucleosome levels were assessed using ELISA as previously described[34] using monoclonal antibody ANA-60 (Sanquin) against histone 3 and $F(ab)_2$ fragments ANA-58 (Sanquin), recognizing an epitope on histone 2A, 2B, and double-stranded DNA complexes. EA complexes were measured using ELISA as previously described[31] using a polyclonal antihuman neutrophil elastase antibody (Sanquin) and a monoclonal anti-α1 antitrypsin antibody (Sanquin) in combination.

Statistics

Statistical analysis was performed using GraphPad Prism version 9.1.1. This trial was designed as a "proof-of-principle" study and therefore descriptive statistics are used. Data were analyzed using paired tests and the D'Agostino-Pearson normality test for normal distribution. C1-INH antigen and activity, C3b/c, C4b/c, nucleosomes, and EA complexes were not normally distributed and compared with t = 0 using Wilcoxon matched-pairs signed rank tests. Hb, bilirubin, LDH, C2, C4, and C3 levels were normally distributed and tested using mixed-effect analysis with Geisser-Greenhouse correction and Holm-Šídák multiple comparisons test comparing each timepoint with t = 0. Deposition of C3 and binding IgG, IgM, and IgA on RBCs were not normally distributed and compared with HCs using a Mann-Whitney test. C3 deposition was normalized to t = 0 hours for each patient, to which each time point was tested using a 1-sample Wilcoxon test to compare with t = 0.

Results

Patient characteristics

From 2014 to 2021, 10 patients were included. Five patients were classified as cold AIHA and the other 5 as mixed AIHA. Baseline characteristics are summarized in Table 1. Median Hb at inclusion was 5.4 g/dL. All patients had received at least 1 previous line of AIHA treatment (median, 4 [range, 1-5] in the 2 months before inclusion) (supplemental Table 2) and a median of 2.5 RBC units (range, 1-23) in the 30-day period before inclusion, consistent with severe AIHA. All but 2 patients received C1-INH according to the study protocol. Patient 4 received a second round of 4 infusions of C1-INH after completing the first round because of fulminant transfusion–refractory AIHA, thus all timepoints from 72 hours were excluded from the analysis. Patient 10 missed the second dose of C1-INH because of a technical error and was therefore only analyzed up to that timepoint.

Hb and hemolytic parameters

Hb levels showed a significant increase after RBC transfusion and C1-INH infusion at t = 12 hours, 24 hours, and 14 days (Figure 2A). LDH and bilirubin remained stable during the study, indicating no change in hemolytic activity (Figure 2B-C). After 2 initial RBC transfusions in the study protocol, 6 of 10 patients remained transfusion-dependent during the trial period (median, 3 [range, 1-9] additional RBC transfusions).

For the predefined primary end point, we compared the peak Hb level measured after transfusion with 2 RBC units to the Hb level 24 hours later. In 7 patients, Hb levels were stable or decreased <1 g/dL after 24 hours, thus meeting the predefined end point. Despite C1-INH administration, 3 patients showed a decrease in

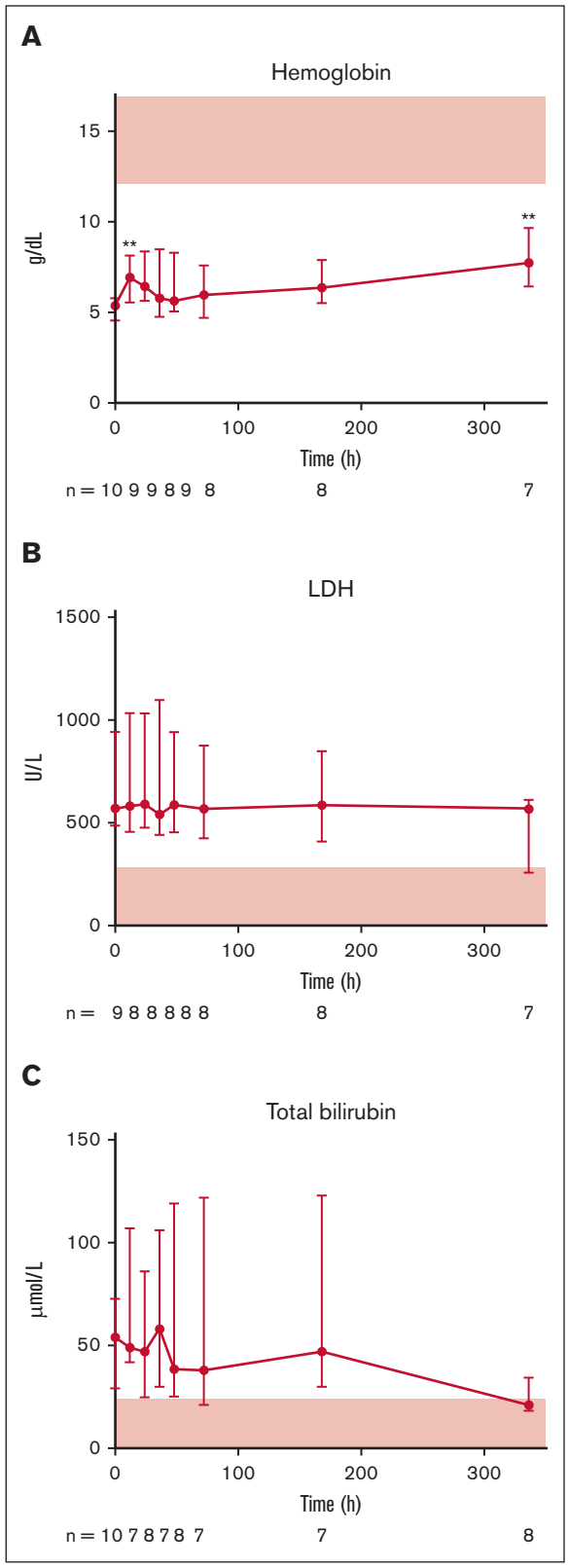

Figure 3. Hemolytic parameters remain unaffected by C1-INH treatment. (A) Hb significantly increased after 12 and 24 hours as well as after 7 and 14 days. (B,C) No significant change in LDH and total bilirubin levels was found. (A-C) Red area indicates normal values. Data shown as median with interquartile range (IQR),

Hb level of >1 g/dL within 24 hours (Table 1). For comparison, we then performed a post hoc analysis of the 24-hour course of Hb levels after each transfusion episode that occurred in the 30 days before study enrollment (Table 1). In 6 of 10 patients, the Hb level decreased <1 g/dL in <24 hours in most of pre-enrollment transfusion episodes, meaning that most patients already met the predefined primary end point before entering the study. Pre-enrollment analysis showed a decrease in Hb level of >1 g/dL within 24 hours in 2 patients. The remaining 2 patients were not evaluable for this analysis.

Patient C1-INH levels increase upon C1-INH administration

At the beginning of the study, all patients had normal levels of both C1-INH antigen and activity (Figure 3). C1-INH antigen levels increased 12 hours after the administration of the first dose compared with t = 0, peaking at 48 hours and decreasing to baseline after 7 days. C1-INH activity levels, indicating the ability of C1-INH to bind C1s, showed a similar pattern. In an exploratory analysis, the C1-INH dose per kg body weight for each patient was calculated. There was no difference in complement activation in patients who received higher doses of C1-INH per kg, shown by C1-INH antigen and C3b/c in plasma (supplemental Figure 1).

Complement and inflammatory parameters

At baseline, patient RBCs showed deposition of C3 and binding of IgG and IgM but not IgA (Figure 4A-D). C3 deposition was the most distinct and found on the RBCs of all participants. This is expected based on our inclusion criteria (positive C3 antiglobulin test). After C1-INH treatment, C3 deposition on RBCs reduced significantly when normalized to baseline for each patient at t = 12 and 48 hours (Figure 4E).

Plasma C4b/c, a marker for systemic activation of the CP and lectin pathway, was not significantly different in patients with AIHA compared with HCs (Figure 5A) and remained in the healthy range throughout the trial (Figure 5B). Even though we did not observe increased C4b/c levels, total C4 and C2 levels were below the normal range at t = 0, which indicates consumption of these CP complement proteins (Figure 5F-G). We observed no increase in C4 and C2 levels upon C1-INH administration. An exploratory analysis of the ratio between C4b/c and C4 showed a slight but nonsignificant reduction after C1-INH administration (supplemental Figure 2). Plasma C3b/c was significantly increased in patients compared with HCs, indicating complement activation at the level of C3 (Figure 5C). A trend toward reduction was observed after C1-INH treatment (Figure. 5D). C3 levels were close to normal throughout the trial and did not change during C1-INH treatment (Figure 5E).

At baseline, patients showed increased levels of inflammation and neutrophil activation markers compared with HCs, as indicated by plasma nucleosome (Figure 6A) and EA complexes levels (Figure 6B). Nucleosomes and EA complexes remained unchanged upon C1-INH administration.

Figure 3 (continued) significance tested using mixed-effect analysis with Geisser-Greenhouse correction and Holm-Šídák multiple comparisons test comparing each timepoint with t = 0.

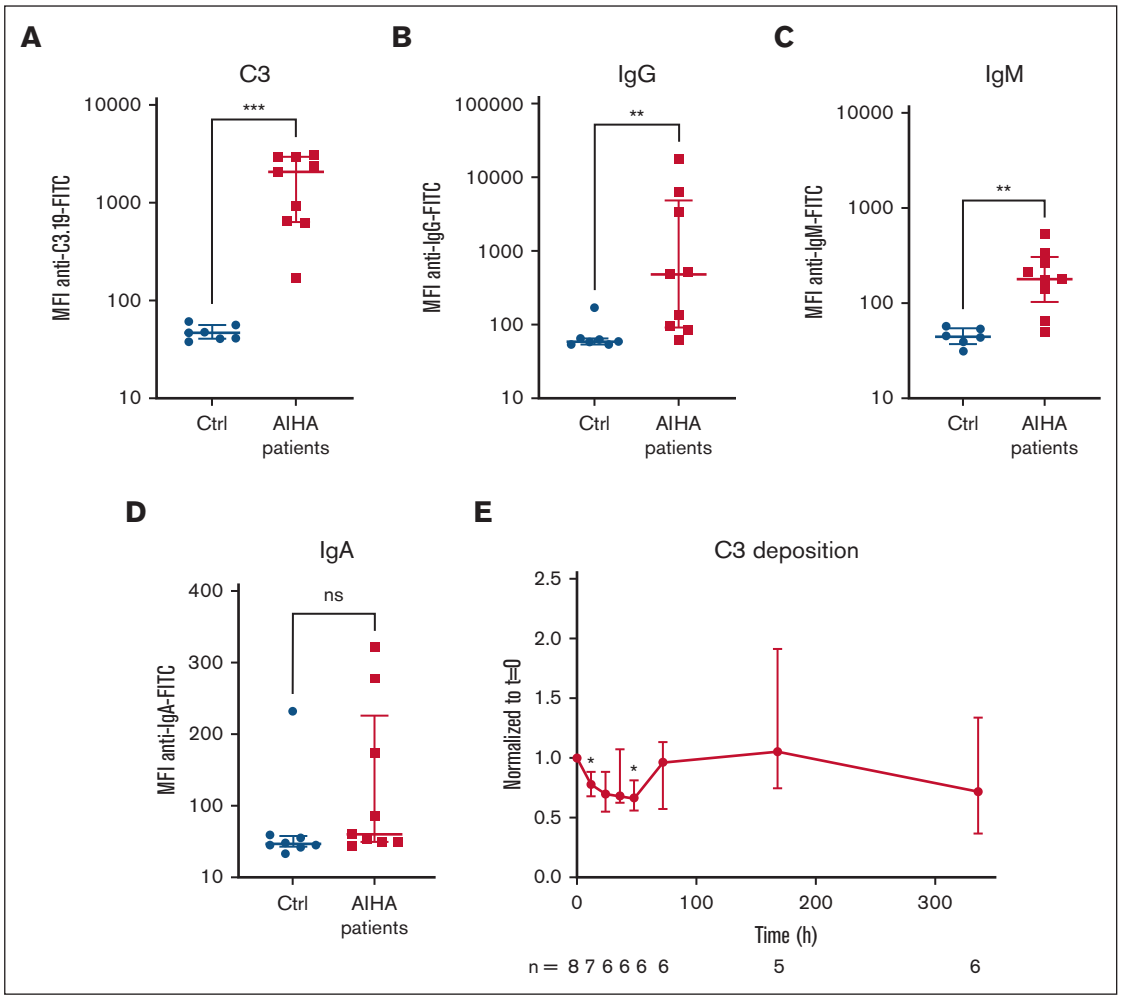

Figure 4. Complement deposition and antibody binding on RBCs at baseline and after treatment. (A-D) Baseline deposition of C3, IgG, IgM, and IgA on RBCs as analyzed by flow cytometry. (A-C) C3 deposition and IgG and IgM binding is significantly increased in patients with AIHA compared with HCs. (D) IgA binding is not increased compared with the HCs, which were comparable to background geometric mean fluorescence intensity (MFI) except for 1 individual. (E) C3 deposition on RBCs is significantly reduced at 12, 24, and 48 hours compared with baseline in the trial participants. All data are expressed as a scatter plot with median + IQR. Significance was tested using the Mann-Whitney test (A-D) and 1-sample Wilcoxon test (E). *$P \leq .05$, **$P \leq .01$, ***$P \leq .001$. Ctrl, control; ns, not significant.

Safety

A total of 40 AEs occurred in 7 out of 10 patients (70%). The full list is provided in supplemental Table 3. There were a total of 34 grade 1 or 2 AEs in 7 patients, of which 16 were probably or possibly related to the study medication. There were a total of 5 grade 3 AEs in 2 patients (constipation, hypertension, hyponatremia, and dyspnea), none of which were determined to be related to the study medication. Hyponatremia and hypertension occurred in a patient with renal failure (already present before enrollment) and the dyspnea was due to a hypersensitivity reaction to pentamidine inhalation. There was 1 grade 4 AE, worsening of AIHA with a need for plasmapheresis and blood transfusions. The investigator determined this to be unrelated to the study medication but attributed it to ongoing underlying AIHA. No thrombotic events or serious AEs were observed.

Discussion

In this prospective phase 2 study, we investigated the efficacy and safety of peritransfusional C1-INH administration in patients who were deeply anemic with severe CM-AIHA. To our knowledge, this is the first prospective study in this population. Although peritransfusional C1-INH temporarily reduced C3d deposition on RBCs, it did not significantly reduce hemolytic activity, systemic complement activation, or inflammation in the study population.

The predefined primary study end point, defined as a post-transfusion decrease of Hb <1 g/dL per 24 hours, was met in 7 of 10 patients. However, we found that at least 6 of 10 patients already met the primary end point before study enrollment. We therefore conclude that, in hindsight, this is not a suitable end point. For future interventional studies in this population, we would

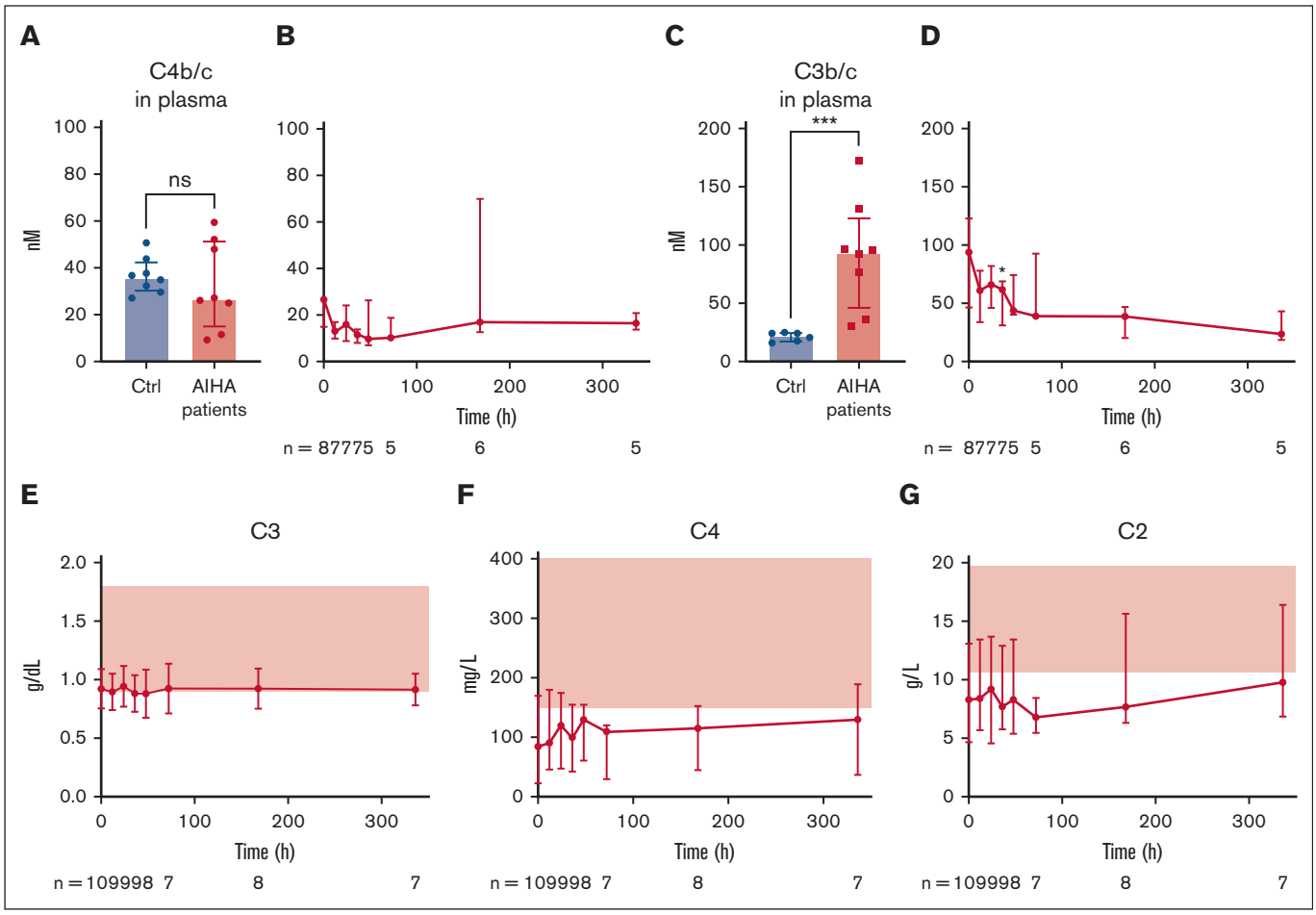

Figure 5. Complement activity in plasma is hardly affected by C1-INH treatment. (A-D) Complement activation as measured by C4b/c and C3b/c detection in EDTA plasma by ELISA. (A) C4b/c levels at baseline do not differ between patients with AIHA and HCs. (B) C4b/c is at low levels, similar to HCs, throughout the trial. (C-D) C3b/c levels at baseline are all significantly increased compared with HCs and are reduced significantly at t = 36 hours only. (E) C3 levels are at the lower end of normal values (red area). (F-G) C4 and C2 levels are below normal values (red area) at most timepoints, indicating high consumption of C2 and C4. Data expressed as median + IQR. Significance was tested using the Mann-Whitney test (A,C), Wilcoxon matched-pairs signed rank test (B,D), and mixed-effects analysis with Geisser-Greenhouse correction (E-G). $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$.

suggest the normalization of hemolysis and stabilization of Hb/ becoming transfusion-independent as potential suitable primary end points. We also conclude that C1-INH in the studied dose cannot normalize transfusion efficacy in CM-AIHA. Whether this short intervention still had a more subtle dampening effect on disease activity remains unknown. The observed long-term improvement (at 7 and 14 days after treatment) in Hb and hemolytic parameters, may very well be related to additional transfusions and treatments, and we can therefore not draw any conclusions on the effect of C1-INH at these later timepoints.

We also focused on the effect of C1-INH on the markers of CM-AIHA disease activity in more detail. Shortly after the RBC transfusion, Hb levels significantly increased, but at 48 hours (the peak of C1-INH activity), they had already returned to near baseline. Because RBC transfusion in patients without AIHA leads to sustained, stable elevation of Hb levels, these results are compatible with accelerated clearance of donor RBCs and/or ongoing lysis of autologous RBCs.[35,36] Indeed, in the first 48 hours after transfusion, LDH and bilirubin levels remained high. However, we cannot exclude that hemolytic activity after RBC transfusion might have been even higher without C1-INH because transfusion may also trigger additional lysis of donor RBCs.[28]

All patients showed strong complement deposition on the RBCs (as defined in the inclusion criteria) and the presence of cold-reactive antibodies. In addition, 5 of 10 patients showed IgG ± IgA binding, confirmed by the eluate showing IgG or IgA antibodies, which classifies these patients as having mixed type AIHA. Thus, ongoing hemolysis via the complement-independent IgG–mediated route (Fc-γ receptors in the spleen) could have contributed to ongoing hemolysis and lack of therapeutic effect in these 5 patients. Regardless, complement activation was not normalized upon C1-INH administration in these patients either, and the patients without IgG deposition did not show a better response to C1-INH.

Patients reached supraphysiological levels of C1-INH similar to the levels reached in the study of patients with sepsis using the same C1-INH dosing scheme.[16] We cannot exclude that higher dosing

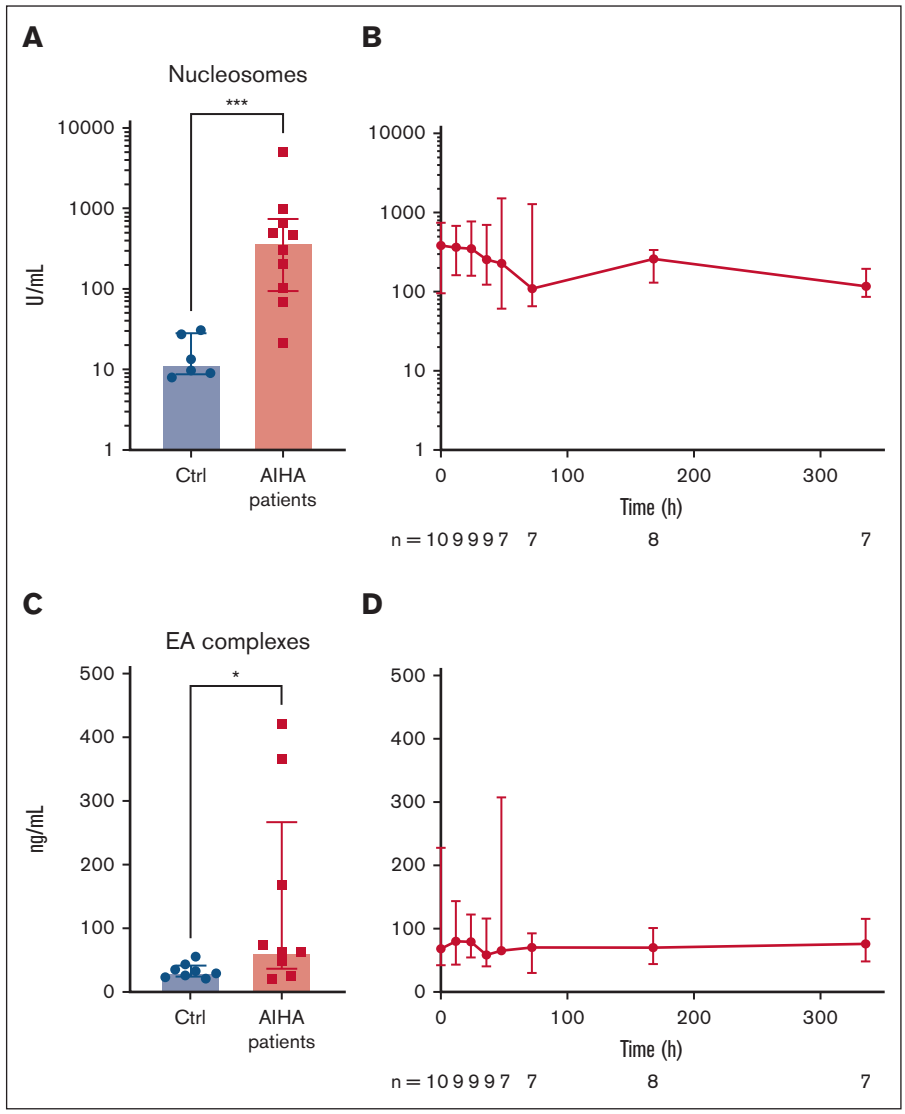

Figure 6. Inflammatory parameters are increased in patients with AIHA and are unaffected by C1-INH treatment. (A,C) Nucleosome and EA complex levels are increased in patients with AIHA compared with HCs, indicating inflammation and neutrophil activation. (B,D) Nucleosome and EA complexes are unaffected by C1-INH treatment. Data expressed as median + IQR, significance tested using Mann-Whitney test (A,C) and Wilcoxon matched-pairs signed rank test (B,D). *$P \leq .05$, **$P \leq .01$, ***$P \leq .001$.

or a longer duration of C1-INH administration might have been more effective in overcoming the high hemolytic activity and normalization of complement levels, as was suggested in vitro.[28]

When our study was designed, there were concerns about the safety (increased thrombotic risk) of high dosage C1-INH which precluded using a higher dose,[37] although the possible thrombogenicity of C1-INH was later contradicted.[38] In an exploratory analysis, the C1-INH dose per kg body weight for each patient was calculated and we found no signs of a dose-dependent effect. A previous retrospective analysis[39] of 4 patients and a case report[29] suggested a beneficial effect of C1-INH (Berinert) administered from the day of admission for a minimum of 6 to 20 days in combination with prednisone and rituximab on Hb and hemolysis. The setup of these studies does not allow for direct comparison to our study because the treatment duration was longer and Berinert was given instead of Cinryze. It is however possible that similar beneficial effects of C1-INH were not confirmed in our prospective study owing to shorter treatment duration.

As expected in CM-AIHA, complement deposition on RBCs and most plasma complement parameters were increased in patients at baseline compared with HCs. Surprisingly, C4b/c levels were not increased, although complement activation at baseline was indicated by increased C3b/c levels and reduced total C4 and C2 levels. Possibly, high ongoing consumption of C4 has reduced its total levels, a known phenomenon in other autoimmune diseases such as systemic lupus erythematosus.[40] Our C4b/c assay might not be sufficiently sensitive to pick up the slight increase in C4b/c that can still be produced upon the activation of thee reduced amounts of C4. The exploratory analysis of C4b/c to C4 ratio showed a relatively high level of C4b/c to C4 at baseline, which has been suggested as a more sensitive marker of in vivo activation.[41]

To our knowledge, this is the first prospective study to look at the markers of systemic inflammation in severe CM-AIHA. We observed significantly elevated nucleosomes and neutrophil activation as evidenced by EA complexes at baseline. The nucleosome levels found in patients with CM-AIHA are in between levels of patients with deep venous thrombosis,[31] fever, or systemic inflammatory response syndrome on the lower end and sepsis and septic shock on the higher end.[20] A previous study found that nucleosomes, but not EA complexes, were increased in patients with paroxysmal nocturnal hemoglobinuria if they had a history of thrombosis.[42] In our study, we saw no change in inflammatory markers after C1-INH administration. This might be related to the lack of durable effect of C1-INH treatment on systemic complement activation or hemolytic activity[22,30] because their products are considered main activators of neutrophil activation.[30,43] Taken together, nucleosome and EA complexes are potential biomarkers in CM-AIHA to assess systemic inflammation and possibly prothrombotic state. It would be interesting to prospectively study these in a larger cohort in which correlations with thrombotic markers and disease activity can be made.

Because proximal complement activation is the main driver of hemolysis in CM-AIHA, complement inhibition is a logical approach. Indeed, the field of proximal complement inhibition has evolved in recent years, and many novel agents are under investigation, also in AIHA.[9] The first clinical study of complement inhibition in AIHA was a phase 2 prospective trial with eculizumab (DECADE),[11] which inhibits C5 and thus terminal complement. Eculizumab significantly reduced intravascular hemolysis and transfusion requirements, but the effect on the anemia was modest, probably because eculizumab does not target the usually predominant extravascular hemolysis. Sutimlimab, an anti-C1s monoclonal antibody, was studied in 2 clinical trials (CARDINAL and CADENZA) in patients with CAD, which were recently published and led to Food and Drug Administration approval in February 2022.[44,45] Sutimlimab treatment resulted in quick and almost complete inhibition of complement activation and hemolytic activity and a clinically significant increase in Hb levels, even in transfusion-dependent patients with CAD. Patients in these trials were not as acutely ill as those in the current trial (median baseline Hb, 5.4 g/dL; and 8.7 and 9.3 g/dL in the CARDINAL and CADENZA trials, respectively). The effects of sutimlimab and other novel proximal complement inhibitors in patients with CM-AIHA with severe presentation as in our cohort and on transfusion efficacy remain to be determined. We recommend future investigation of these novel complement inhibitors in the population of severe CM-AIHA specifically because this subpopulation was not included in the current studies.

Overall, our trial demonstrates that peritransfusional C1-INH did not significantly suppress hemolytic activity or systemic complement activation in severe CM-AIHA, although there was a decrease in complement deposition on circulating RBCs. An interesting novel finding was the strong increase in inflammatory markers in our cohort compared with HCs, which may be relevant for clinical correlations to the thrombotic risk and deserve further study. In conclusion, these data do not support the clinical use of C1-INH in the tested dose in patients with severe CM-AIHA but add to the increasing experience with proximal complement inhibitors in this patient population.

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Authorship

Contribution: E.M.M., K.A.G., D.W., I.J., J.M.I.V., M.J.K., M.d.H., L.D.-N., C.C.F., and S.Z. provided trial design, input, and data interpretation; M.J., M.J.K., J.M.I.V., and S.Z. enrolled patients; E.C.W.d.B., L.D.-N., E.M.M., I.B., and K.A.G. performed experiments; S.R.J. and M.D.E. collected samples and performed data and trial management; E.C.W.d.B., M.J., M.D.E., J.M.I.V., I.J., S.Z., and L.D.-N. analyzed the data; E.C.W.d.B. performed statistical analysis; E.C.W.d.B., M.J., and J.M.I.V. wrote the first version of the manuscript; and all authors commented on and approved the final version of the manuscript.

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The current affiliation for E.M.M. is Janssen Vaccines & Prevention B.V., Leiden, The Netherlands.

ORCID profiles: C.C.F., 0000-0003-1346-4202; K.A.G., 0000-0002-7815-032X.

Correspondence: Esther C. W. de Boer, Department of Immunopathology, Sanquin Research and Landsteiner Laboratory, Amsterdam University Medical Centre, Amsterdam Infection and Immunity Institute, Plesmanlaan 125, 1066CX Amsterdam, The Netherlands; email: e.deboer@sanquin.nl.

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