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Daratumumab monotherapy in refractory warm autoimmune hemolytic anemia and cold agglutinin disease.

Tracking no: ADV-2024-012585R1

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Abstract:
Autoimmune hemolytic anemia (AIHA) is a rare autoantibody-mediated disease. For steroid and/or rituximab-refractory AIHA, there is no consensus on optimal treatment. Daratumumab, a monoclonal antibody targeting CD38, could be beneficial by suppression of CD38+ plasmacells and thus autoantibody secretion. In addition, since CD38 is also expressed by activated T-cells, daratumumab may also act via immunomodulatory effects. We evaluated efficacy and safety of daratumumab monotherapy in an international retrospective study including 19 adult patients with heavily pretreated refractory AIHA. In warm AIHA (wAIHA, n=12), overall response was 50% with a median response duration of 5.5 months (range, 2-12 months) including ongoing response in 2 patients after 6 and 12 months. Of 6 non-responders, 4 had Evans syndrome. In cold AIHA (cAIHA, n=7) overall hemoglobin (Hb) response was 57%, with ongoing response in 3/7 patients. One additional non-anemic cAIHA patient was treated for severe acrocyanosis and reached a clinical acrocyanosis response as well as a Hb increase. Of 6 cAIHA patients with acrocyanosis, 4 had improved symptoms after daratumumab treatment. In two patients with wAIHA treated with daratumumab in whom we prospectively collected blood samples, we found complete CD38+ T cells depletion after daratumumab, as well as altered T-cell subset differentiation and a severely diminished capacity for cell activation and proliferation. Reappearance of CD38+ T-cells coincided with disease relapse in one patient. In conclusion, our data show that daratumumab therapy may be a treatment option for refractory AIHA. The observed immunomodulatory effects that may contribute to the clinical response deserve further exploration.

Conflict of interest: COI declared - see note

COI notes: Bruno Fattizzo received consultancy honoraria from Alexion, Novartis, Janssen and Sobi. Marc Michel received consultancy fees and/or speakers fees received from Novartis, Alexion, Sanofi, UCB, Argenx and Sobi. Etienne Crickx received honoraria (advisory boards, speaker's fees) from Novartis, UCB, Amgen and Sanofi. Quentin A. Hill received speaker honoraria from Grifols and Novartis; consultancy: Amgen, Argenx, Gliknik, Incyte, Immunovant, Janssen, Novartis, Sanofi and Sobi. Ulrich Jaeger received honoraria from Sanofi, Roche, Novartis, Incyte, Janssen and BMS; Advisory Role: Sanofi, Roche and Novartis. Arnon Kater received research funding from AbbVie, AstraZeneca, BMS, Janssen, and Roche/Genentech; received patent royalties from Janssen and LAVA; and served on the board of directors or advisory committees for AstraZeneca, BMS, Roche/Genentech, Janssen, AbbVie, and LAVA; and received speaker's fees from AbbVie, AstraZeneca, and Janssen. Shirley D' Sa received research funding from BeiGene and Janssen; advisory boards: BeiGene, Sanofi, Janssen and Cellectar; speakers bureau: Janssen and BeiGene. Josephine Vos received consultancy and advisory board honoraria from Sanofi and Janssen, research support from Beigene and Abbvie/Genmab, and participating in the speakers' bureau for BMS, Sanofi, and Amgen. All honoraria received are directed to the institute.

Preprint server: No;

Author contributions and disclosures: All authors were involved in data collection and the development of the manuscript. M.J and J.M.I.V designed the study and analyzed the data. All authors critically reviewed the manuscript.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: The authors confirm that the data supporting the findings of this study are available within the article and its supplementary figure. Additional raw data supporting the findings of this study are on request from the corresponding author.

Clinical trial registration information (if any):

Title page

Title: Daratumumab monotherapy in refractory warm autoimmune hemolytic anemia and cold agglutinin disease.

Short title: Daratumumab monotherapy in refractory AIHA.

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Data-sharing statement:
The authors confirm that the data supporting the findings of this study are available within the article and its supplementary figure. Additional raw data supporting the findings of this study are on request from the corresponding author.

Word count abstract: 250

Word count main text: 3925

Reference count: 39

Table count: 3 tables

Figure count: 3 figures main text, 1 figure in supplementals

Keypoints:

1. Daratumumab monotherapy may be an effective and well tolerated treatment in more than half of refractory AIHA and CAD patients.
2. Daratumumab can be effective on both the hemolysis as well as cold agglutinin induced circulatory symptoms.

Abstract

Autoimmune hemolytic anemia (AIHA) is a rare autoantibody-mediated disease. For steroid and/or rituximab-refractory AIHA, there is no consensus on optimal treatment. Daratumumab, a monoclonal antibody targeting CD38, could be beneficial by suppression of CD38+ plasmacells and thus autoantibody secretion. In addition, since CD38 is also expressed by activated T-cells, daratumumab may also act via immunomodulatory effects. We evaluated efficacy and safety of daratumumab monotherapy in an international retrospective study including 19 adult patients with heavily pretreated refractory AIHA. In warm AIHA (wAIHA, n=12), overall response was 50% with a median response duration of 5.5 months (range, 2-12 months) including ongoing response in 2 patients after 6 and 12 months. Of 6 non-responders, 4 had Evans syndrome. In cold AIHA (cAIHA, n=7) overall hemoglobin (Hb) response was 57%, with ongoing response in 3/7 patients. One additional non-anemic cAIHA patient was treated for severe acrocyanosis and reached a clinical acrocyanosis response as well as a Hb increase. Of 6 cAIHA patients with acrocyanosis, 4 had improved symptoms after daratumumab treatment. In two patients with wAIHA treated with daratumumab in whom we prospectively collected blood samples, we found complete $CD38^{+}$ T cells depletion after daratumumab, as well as altered T-cell subset differentiation and a severely diminished capacity for cell activation and proliferation. Reappearance of CD38+ T-cells coincided with disease relapse in one patient. In conclusion, our data show that daratumumab therapy may be a treatment option for refractory AIHA. The observed immunomodulatory effects that may contribute to the clinical response deserve further exploration.

Introduction

Autoimmune hemolytic anemia (AIHA) is a rare and heterogeneous antibody mediated disease in which red blood cell (RBC) autoantibodies lead to the accelerated destruction of RBCs. Depending on the optimal binding temperature of the autoantibodies in-vitro, AIHA can be classified as warm, cold or mixed (both) AIHA.[1] In warm AIHA (wAIHA) polyclonal autoantibodies, IgG and/or IgA and rarely IgM isotype, bind to the RBC antigens at an optimum temperature of 37 °C. Based on the presence of an underlying disorder (such as a malignancy or other autoimmune disorders like systemic lupus erythematosus (SLE)), wAIHA can be subdivided in primary or secondary wAIHA.(1) In cold AIHA (cAIHA), RBC autoantibodies lead to complement-mediated hemolysis mostly via extravascular hemolysis in the liver. cAIHA is often due to "CAD-LPD" a lymphoproliferative disorder with an IgM kappa paraproteinemia which is called cold agglutinin disease (CAD).[2, 3] Cold agglutinins secondary to other conditions such as overt malignancy, or infections are classified as cold agglutinin syndrome (CAS). At least 50 percent of patients with cAIHA have symptoms of acrocyanosis after exposure to low temperatures, causing skin discoloration with or without numbness and tingling. Cold associated symptoms adversely impact quality of life.[4-6] The cornerstone of treatment for wAIHA are corticosteroids followed by rituximab in case of steroid refractoriness or dependency. As with ITP, there is evidence suggesting that a lack of response to rituximab could be due to the expansion of auto-reactive long-lived plasma cells lacking CD20 expression.[7] For patients with CAD requiring therapy, rituximab alone or combined with bendamustine may be used, however long-term response to rituximab is much lower than in wAIHA and chemotherapy-based regimens may not be suitable for elderly patients with comorbidities. Additionally, the recently approved complement inhibitor sutimlimab is able to improve hemolysis and anemia in CAD, but has no effect on peripheral acrocyanosis symptoms.[8] For relapsed/refractory wAIHA/CAD there is little evidence on optimal treatment and this population represents a pressing unmet need.

Daratumumab is a humanized monoclonal antibody which targets the CD38 glycoprotein highly expressed on plasma cells and is widely used for its registered application in the treatment of patients with multiple myeloma (MM) in whom it shows a favorable toxicity profile.[9-11] We and others hypothesize that daratumumab might suppress the secretion of autoantibodies by long-lived plasma cells (and any remaining CD38+ B-cells) in AIHA patients.[12, 13] In addition, due to the expression of CD38 glycoproteins on other immune cells like natural killer cells, monocytes, B- and T-cells, daratumumab has an additional immunomodulatory effect.[9-11, 14, 15] Recent literature describes the successful off label use of daratumumab in patients with autoimmune mediated cytopenias.[12, 13, 16-21] These retrospective series mainly involve children and immune-mediated cytopenias in the post-hematopoietic stem cell transplantation setting. Only small numbers of successful AIHA treatments with daratumumab monotherapy in adult patients have been published.[12, 13, 17, 19-22] We aimed to evaluate the efficacy and safety of daratumumab monotherapy in a larger series of adult patients with both warm and cold antibody mediated refractory AIHA. Furthermore, the immunomodulatory effects of daratumumab treatment in AIHA patients with regards to the T cell compartment were of special interest. The pathophysiology of especially warm AIHA is complex and not fully elucidated, however it is clear that there is immune dysregulation including at the T-cell level.[23] The production of auto-antibodies by B cells from AIHA patients is thought to be mediated by $CD4^+$ T cells, previously shown to be in a hyperactive state in AIHA *in vitro*.[24] In addition, the presence of sufficient T_{reg} was critical for protecting against AIHA development in a murine model.[24-26] We therefore characterized immune cell composition, specifically T cell subsets, numbers and function in longitudinally collected samples in two wAIHA patients before, during and after daratumumab therapy.

Patients and methods

We undertook a multinational retrospective observational study of patients with warm and/or cold AIHA treated with daratumumab monotherapy. Inclusion criteria were AIHA with or without immune thrombocytopenia (Evans syndrome),[27] diagnosed as a combination of anemia, hemolysis (defined by increased

reticulocytes, lactate dehydrogenase [LDH], and bilirubin with a decreased haptoglobin) and a positive direct antiglobulin test (DAT) for IgG and/or IgA and/or IgM and/or strongly positive for complement deposition. AIHA was classified according to recent international consensus.[1] Patients with AIHA after hematopoietic stem cell transplantation were excluded. To ensure the presence of an adequate bone marrow response, patients with reticulocytopenia were excluded. Each investigator was asked to report all consecutive patients with AIHA that received at least one dose of daratumumab therapy. Laboratory and clinical data were retrospectively collected regarding underlying disease, bone marrow pathology, hemolytic parameters, and grading of patient-reported acrocyanosis (scored as stable, better, or resolved) at diagnosis, 2 weeks and 1, 3, 6, and 12 months,[34] and last date of follow-up. Patients were included in 10 different centers in 5 countries (Austria, Italy, France, The Netherlands, United Kingdom). Hemoglobin (Hb) response was considered none, partial (PR; Hb 10-12 g/dL or >2 g/dL increase), or complete (CR, Hb > 12 g/dL), in the absence of recent RBC transfusion.. Patients who were not anemic at baseline (Hb>12) were not evaluated for Hb response. Distribution of data regarding continuous variables was described in terms of a median and ranges. The initial overall response (OR) is expressed as a percentage of patients with complete and partial response out of the total number of patients. All patients were followed until last available data or loss of response. Adverse events were graded according to the Common Terminology Criteria, version 5.0 (2017). All patients gave informed consent based on local legislation; patients providing material for translational studies signed an additional informed consent. The data of two deceased patients were used according to local legislation.

Immune cell phenotyping

Peripheral blood mononuclear cells (PBMCs) were isolated from prospectively collected blood samples from 2 wAIHA patients (patient 1 and 3) taken at several time points; pre-treatment, pre cycle 5, 1 week after cycle 8 and 3 months after end of treatment and cryopreserved as described previously[2]. PBMCs were stained with the multifluorochrome antibody panels (details in supplementary methods, supplementary Table 2). T cells were analyzed either directly ex vivo, or stimulated using CD3 (clone1XE) and CD28 (clone15E8) for 2 or 5 days. For assessment of proliferation, PBMCs were stained using CellTrace™ Violet (C34557; ThermoFisher Scientific) according to manufacturer's description prior to stimulation. Cells were acquired on a LSR Fortessa (BD Biosciences), and data were analyzed using FlowJo 10.5.3. Analysis of proliferation was performed using the FlowJo proliferation tool.

All patients gave written informed consent for the retrospective data analysis concerning their treatment of auto-immune hemolytic anemia. Retrospective data analysis was approved by the Ethics committee of the Amsterdam University Medical center. Patients providing material for translational studies signed an additional informed consent and were included in the DRAIHA study, an observational cohort study which is approved by the Medical Ethics Committee Leiden The Hague Delft.

Results

Nineteen patients with warm and cold AIHA were included in our study. Of these patients, 4 were previously published,[12, 13, 22] and we here update the clinical response data of 2. Patient baseline characteristics are summarized in table 1. Nine patients were diagnosed with primary wAIHA, of whom 5 patients with Evans syndrome, and 3 patients with secondary wAIHA (1x myasthenia gravis, 2x SLE). Of 7 patients with cAIHA, 2 were diagnosed with lymphoplasmacytic lymphoma (LPL) and 5 were primary CAD patients. Patients had received a median number of treatments of 5 (range, 2-10). Most of the patients received ongoing immune suppressive treatments at start of daratumumab therapy (13/19). Median Hb at start of daratumumab was 8.6 g/dL (range 4-12.5). At baseline, 9/19 patients were transfusion dependent and received a median of 10 RBC units (range, 1-36) during the month before starting daratumumab. In 1 CAD patient, treatment indication was refractory acrocyanosis only, without significant hemolytic anemia. Six out of 7 patients with

cAIHA reported symptoms of acrocyanosis. Of 12 patients with wAIHA, 9 received a fixed duration of daratumumab therapy with either 4 or 8 weekly doses, 3 patients received a median of 11 doses of daratumumab (range, 11-13). Of the 7 patients with cAIHA, 3 patients received a fixed duration of 8 weekly doses of daratumumab, while 4 patients received daratumumab maintenance treatment for a median duration of months (range, 11-40). For all AIHA patients, the median duration of follow up was 5 months (range, 2-40). Two patients (1 wAIHA and 1 cAIHA) died 3 months after the start of daratumumab, due to uncontrolled severe hemolytic anemia. No patients were lost to follow up.

Treatment response in warm AIHA

Median Hb at start of daratumumab treatment was 8.5 g/dL (range, 4-11.2). Daratumumab treatment resulted in an overall complete Hb response in 6/12 (50%) of the wAIHA patients, with a median time to overall response of 2 weeks (range, 2-8). Complete Hb response was reached after a median of 4 weeks after the start of daratumumab (range, 2-9 weeks), with a median Hb increase of 2.3 g/dL (range, 1.3-8.1 g/dL). Median duration of response was 5.5 months (range, 2-12 months) after the start of daratumumab. Median duration of therapy was 2 months (range, 1-6 months) with a median duration of follow up of 4 months (range, 2-12 months). At 4 months after the start of daratumumab, one wAIHA patient with a CR from 2 months of daratumumab therapy onwards started belimumab, a human monoclonal antibody that is thought to inhibit B-cell survival and thereby production of autoantibodies, [28] for underlying SLE. This patient is still in CR after 2 years, however. Data from this patient were censored from the time of start of belimumab.

At the time of data collection 2 heavily pretreated wAIHA patients with a median number of previous therapies of 6 (range, 5-7) showed an ongoing complete Hb response at 6 and 12 months after fixed duration daratumumab treatment (4 and 8 doses respectively). Three additional patients relapsed 2, 5 and 9 months after the start of daratumumab. Six of 12 (50%) wAIHA patients did not show an improvement of hemolysis after a median of duration of therapy of 2.5 months (range, 1-6 months) and a median duration of follow up of 3 months (range, 2-6 months). These were all patients with a median of 7 previous lines of therapies (range, 3-10) of whom 4/6 with underlying Evans syndrome.

Five wAIHA patients were heavily RBC transfusion-dependent at the start of daratumumab treatment with a median of 20 RBC transfusions per month before start daratumumab (range 1-36 units). Four patients had an ongoing need for transfusion after a median duration of therapy of 3 months (range, 1-6 months). One patient who received 36 units of RBC transfusions in the month before starting daratumumab treatment, became transfusion independent after two weeks. However, due to the severity of the disease, this patient received more lines of therapy in the month before starting daratumumab treatment, so we cannot exclude the contribution of co-medication (see table 1).

Treatment response in cold AIHA

Median Hb at start of daratumumab treatment was 8.6 g/dL (range, 5-12.5). Daratumumab treatment resulted in an overall Hb response in 4/7 (57%) patients with cAIHA with a median time to overall response of 3 weeks (range, 2-12). Complete Hb response was reached in 3 patients, with a median Hb increase of 3.2 g/dL (range, 1.5-4.2 g/dL) 3 months after the start of daratumumab (range, 0.5-4 months). One cAIHA patient reached a long lasting PR with an increase of hemoglobin of 2.1 g/dL after 12 weeks. One patient started daratumumab treatment because of acrocyanosis without significant hemolytic anemia (Hb at start 12.5g/L), yet showed a Hb increase of 4.1 g/dL after 2 months, with an ongoing Hb response at 40 months. However, because of our definition of CR of Hb at start daratumumab, this patient was excluded from assessment of Hb response.

Median duration of therapy was 11 months (range, 2-40 months) with a median duration of follow up of 11 months (range, 2-40 months). At the time of data collection 2/3 cAIHA patients on daratumumab monthly maintenance therapy had an ongoing Hb response after a median of 11.5months (range, 11-12). One patient showed progression of AIHA after 19 months of maintenance therapy. Of the 3 patients with a fixed duration of two months daratumumab therapy, 1 patient showed an ongoing CR of Hb after 8 months. The two other patients with a fixed duration of two months daratumumab therapy did not have a response. Four patients were transfusion dependent before the start of daratumumab therapy with a median of 3 RBC transfusions per month (range, 1-20). Two patients became transfusion independent after 3 and 6 months respectively.
Of the 6 patients with acrocyanosis, 4 patients reported clinical improvement, of whom 2 within 14 days and the other 2 patients after 3 and 6 months. Complete resolution of acrocyanosis was reached in 2 patients after 3 and 12 months respectively.

Safety

Three patients had a grade 2 infusion reaction after the first dose of intravenous daratumumab. One patient suffered from a grade 3 chronic COVID infection requiring hospitalization. There was one varicella zoster virus infection grade 1 reported. One patient reported a grade 3 febrile neutropenia and presumed pneumonia requiring hospitalization. One patient had a grade 3 bacterial sepsis and an anal abscess requiring surgery. This patient was heavily pretreated with immunosuppressive drugs and was still on prednisolone and sirolimus therapy when daratumumab treatment was initiated.

Immunomodulatory effects of daratumumab treatment

To characterize the immune cell composition and potential immunomodulatory effects of daratumumab in patients with AIHA, response evaluable PBMC samples prospectively collected from 2 wAIHA (patient 1 and 3, see Table 1) patients were analyzed at baseline (Pre), during treatment (Mid), immediately after treatment cessation (EOT) and 3 months after end of treatment (Post). In addition, for patient 3, a sample taken 6 weeks after the last cycle (6 weeks) was also included. The percentage of B cells, which usually constitutes about 10% of lymphocytes, was relatively low at baseline in the PBMC pool of these patients and declined further over the course of treatment (Figure **2**A).[29] The pre-existing low B cell abundance could be related to the heavily pre-treated status of the studied patients, including immunosuppressive agents as described in Table 1. In the six months before the initiation of daratumumab, patient 1 was treated with steroids in combination with first sirolimus followed by cyclosporin because progression of disease. Due to cyclosporin intolerance, steroid monotherapy was continued in the last three months before the start of daratumumab. Patient 3 was only treated with steroids in the six months before start of daratumumab. Last dose of rituximab was 15 and 2 years before start daratumumab for patient 1 and 3 respectively.
The composition of the B-cell subsets, characterized using CD27 and IgD, did not change during the treatment (Figure 2A, Figure S1A). We also observed patient specific changes in the percentage of T_{reg}, and in the $CD4^+$: $CD8^+$ ratio, but not in the total abundance of T cells at baseline(Figure 2B, Figure S1B). When characterizing T-cell subsets using CD27 and CD45RA, we identified a reduction in central memory (CM; $CD27^+CD45RA^-$) and an increase in effector memory (EM; $CD27^-CD45RA^-$) in $CD4^+$ (but not in $CD8^+$) T cells at the EOT time point (Figure 2B). This shift partially normalized at 3 months after end of treatment (Figure 2B, Figure S1C). As expected, the population of T cells expressing CD38 disappeared during daratumumab treatment. In one patient, the $CD38^+$ subset reappeared after treatment cessation which coincided with a swift disease relapse, shown by corresponding changes in Hb levels (Figure 2B, Figure 2C, Figure S1C). Next, the unsupervised clustering algorithm FlowSOM [30] was applied on these samples. The eight metaclusters were named, based on the combination of markers expressed (Figure 3A, Figure S1D). The tSNE maps of the different time points clearly visualized the changes in subset

distribution during treatment, which were mostly consistent in these two patients. After treatment, the aforementioned reappearance of the $CD38^+$ subset was visible in patient 1 (Figure **X**A). To investigate functional responses of T cells at the different time points, we stimulated the patient samples using CD3/CD28 antibodies (Figure S1E). Following stimulation, expression of the activation marker CD25 was reduced on T cells from samples taken at later time points during treatment (Figure 3B, Figure S1F). However, CD38 is also an activation marker on healthy T cells and daratumumab treatment caused depletion of CD38 expressing T cells (Figure 3B, Figure S1F). Following *in vitro* T-cell activation, no effects of daratumumab treatment on T-cell frequency or $CD4^+$: $CD8^+$ ratio were seen (Figure S1F). An important functionality of T cells is proliferation, which was measured after five days of stimulation. Our data showed that proliferation capacity in both $CD4^+$ and $CD8^+$ T cells was severely diminished (Figure 3C, Figure S1G). Altogether, these data indicate that in addition to altering immune composition, daratumumab also disrupts normal T cell function.

Discussion

To our knowledge, this is the largest study on daratumumab monotherapy in AIHA, with a total of 19 adult patients with refractory AIHA and a particularly high burden of disease , of whom 9 were transfusion dependent and 5 had Evans syndrome. The effect of daratumumab in wAIHA in our series (50%) is lower compared previously published retrospective data in which 83% (5/6) patients showed a Hb response with a median duration of response of 5 months (range, 2-20 months) after a median of 6 doses of daratumumab (range, 4-12), see Table 3.[13, 17, 19, 20, 31] This may be related to positive reporting bias in previously published case reports and smaller case series.

Still, all wAIHA patients in our study had severe refractory and heavily pretreated AIHA, with median previous lines of therapy of 6, where little treatment options are available. Also, the time to response (median of 2 weeks) is short, which is very relevant in the setting of severe hemolytic disease. Interestingly, most non-responders with wAIHA had Evans syndrome, which may suggest that anti-CD38 therapy is less promising in this setting. Indeed Evans syndrome is associated with a more aggressive course and is more treatment resistant suggesting another mechanism of disease compared to normal wAIHA[27].

In cAIHA, daratumumab therapy was effective in more than half of patients with cAIHA, with an overall Hb response of 57% and a clinical improvement of acrocyanosis in 67% of the patients. One non-responding cAIHA patient had no detectible M-protein and no evidence of a lymphoproliferative disorder in the bone marrow. It is thought that in classic CAD-LPD and LPL, monoclonal autoantibodies are produced by B-cells and long-lived plasma cells which may explain the sensitivity to daratumumab treatment. Consequently, the mechanism of disease might have been different from this non-responding patient without underlying LDP and thus less sensitive to daratumumab.[32, 33]

All cAIHA patients receiving maintenance daratumumab treatment showed long-lasting responses, with one patient showing a relapse of AIHA after 19 months of daratumumab treatment. These results suggest that there might be a role for maintenance daratumumab therapy in a responsive patients. This is in line with previously published retrospective data on 4 primary cAIHA patients, of whom we could update the response data of 2 patients in our study, treated with daratumumab maintenance therapy. All 4 cAIHA patients had an ongoing Hb response after a median of 12.5 months maintenance therapy (range, 8-16 months) (table 3) .

Prospective studies should confirm the efficacy of antiCD38 therapy in both wAIHA as well as cAIHA, and explore if maintenance therapy with daratumumab might be beneficial in patients that show an initial response. A phase 1b/2 trial with the anti-CD38 antibody isatuximab in patients with wAIHA was initiated, but terminated prematurely due to strategic sponsor decisions [NCT04661033]. We are not aware of any other ongoing clinical studies.

Importantly, after the initiation of daratumumab therapy, acrocyanosis symptoms improved in 4/6 cAIHA patients, with complete resolution in two. This is an notable finding, since acrocyanosis can significantly affect quality of life in a large proportion of cAIHA patients. In patients with disabling acrocyanosis, daratumumab therapy or other clone directed therapy might therefore be preferred over the novel complement inhibitors now being approved for CAD, since cold autoantibody induced circulatory symptoms are not complement mediated and symptoms might even worsen after the start of complement inhibitors.[5, 34, 35]

In our cohort, infections were reported in 4/19 (21%) patients, including three of grade 3 and one of grade 1. Due to the retrospective nature of this study and missing data on immunoglobulin titers, we could not assess to what extent these infections were causally related to the initiation of daratumumab in addition to the patients' heavily pretreatments with other immunosuppressive drugs. By comparison, in heavily pretreated myeloma patients, infection-related serious adverse events occurred in 10-17% of the patients treated with daratumumab monotherapy. [36] The potential for hypogammaglobulinemia and infections should be kept in mind when considering daratumumab treatment.

Whether daratumumab treatment has additional immunomodulatory effects on the active T-cell involvement in AIHA has not been investigated. In 2 wAIHA patients (patient 1 and 3), we showed alterations in T-cell skewing towards an effector memory phenotype and a severely dampened capacity for T cell activation and proliferation during exposure to daratumumab, all indicators of immunomodulation. We did not however observe the reduced abundance of T_{reg} that was described earlier in daratumumab-treated myeloma patients[2] and thus found no evidence that daratumumab provides additional control of AIHA by modulating T_{reg} presence.

Strikingly, we observed a clear difference in the restoration of a CD38 expressing T-cell population between the 2 patients. In particular, the rapid recurrence in patient 1 at the same time of AIHA relapse shortly after treatment cessation warrants further attention. CD38 is a T-cell surface marker which is upregulated upon activation. It is involved in susceptibility to (viral) infections and the activation of cytotoxic T cells upon exposure to a pathogen by promoting intracellular calcium release.[37] The depletion of $CD38^+$ T cells likely leads to a broad reduction of activated T cells, which usually co-express markers such as CD25 (IL-2 receptor), CD69 and/or CD44.[38] The disappearance of these (activated) T cells could therefore hamper T-cell support of auto-antibody producing B cells. Additionally, the early recurrence of $CD38^+$ T-cells and AIHA disease relapse following shortly after in one patient, suggests that CD38 expression on T cells could be an early biomarker of (auto)immune reactivation and relapse after daratumumab treatment. These preliminary findings deserve further study in a larger cohort. Daratumumab has been used off label at a small scale in patients suffering from auto-immune disease such as SLE and (refractory) immune thrombocytopenia[13]. We hypothesize that the immunomodulatory effect of daratumumab on CD38 expressing T cells contributes to the responses achieved in these patients, as has been described in SLE patients who have been shown to portray altered CD38 expression in their T cell compartments.[39] Also, this may have relevance for the potential of anti-CD38 antibodies in other autoimmune diseases characterized by increased T cell activation, such as inflammatory bowel diseases and multiple sclerosis.[39]

Limitations of this study are related to its retrospective design and the low incidence of refractory AIHA that both leads to the potential for selection bias as well as incompleteness of data i.e. regarding adverse events, the small number of cases, the variable dosing schedules and the lack of a control group. Also, we cannot exclude late responses to the co-medication given before initiation of daratumumab treatment.

In conclusion, daratumumab monotherapy may be an effective and well tolerated treatment option associated with rapid responses for a proportion of refractory AIHA patients, with an effect on both the hemolytic anemia as well as the cold induced circulatory symptoms. Prospective studies should confirm these findings including the role of daratumumab maintenance therapy in patients showing an initial response. Also, it would be relevant to understand better which subpopulations of AIHA patients might be more likely to respond. The immunomodulatory effects that we observed may provide additional mechanisms via which daratumumab leads to response in refractory wAIHA patients.

Contribution:
All authors were involved in data collection and the development of the manuscript. M.J and J.M.I.V designed the study and analyzed the data. All authors critically reviewed the manuscript.

Conflicts-of-interest disclosure:
Bruno Fattizzo received consultancy honoraria from Alexion, Novartis, Janssen and Sobi.
Marc Michel received consultancy fees and/or speakers fees received from Novartis, Alexion, Sanofi, UCB, Argenx and Sobi.
Etienne Crickx received honoraria (advisory boards, speaker's fees) from Novartis, UCB, Amgen and Sanofi.
Quentin A. Hill received speaker honoraria from Grifols and Novartis; consultancy: Amgen, Argenx, Gliknik, Incyte, Immunovant, Janssen, Novartis, Sanofi and Sobi.
Ulrich Jaeger received honoraria from Sanofi, Roche, Novartis, Incyte, Janssen and BMS; Advisory Role: Sanofi, Roche and Novartis.
Arnon Kater received research funding from AbbVie, AstraZeneca, BMS, Janssen, and Roche/Genentech; received patent royalties from Janssen and LAVA; and served on the board of directors or advisory committees for AstraZeneca, BMS, Roche/Genentech, Janssen, AbbVie, and LAVA; and received speaker's fees from AbbVie, AstraZeneca, and Janssen.
Shirley D' Sa received research funding from BeiGene and Janssen; advisory boards: BeiGene, Sanofi, Janssen and Cellectar; speakers bureau: Janssen and BeiGene.
Josephine Vos received consultancy and advisory board honoraria from Sanofi and Janssen, research support from Beigene and Abbvie/Genmab, and participating in the speakers' bureau for BMS, Sanofi, and Amgen. All honoraria received are directed to the institute.

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Table 1. Baseline characteristics

	Age*/ Sex	AIHA, DAT results	Associated condition	Acrocy anosis	Previous AIHA treatments	Ongoing immune suppressive treatments at start daratumumab	Hb, g/dL, at start daratumumab	Bilirubin total, µmmol/L, at start daratumumab	LDH, U/L, at start daratumumab	RBC transfusion in month before start daratumumab
1.	37/F	wAIHA DAT >2+ IgG	-	-	Steroids, rituximab splenectomy, azathioprine, cyclophosphamide bortezomib, mycophenolate, sirolimus, cyclosporin	Steroids	11.2	53	425	-
2.	54/F	wAIHA, DAT IgG>2+ and IgA>2+	-		Steroids, rituximab EPO, mycophenolate, cyclosporin	Steroids	9.7	32	584	-
3	53/M	wAIHA, DAT IgG>2+	Myasthenia Gravis	-	Steroids, rituximab, EPO, azathioprine, mycophenolate, cyclosporin, bortezomib	Steroids	10	74	914	-
4	56/F	wAIHA DAT>2 + IgG	SLE	-	Steroids, mycophenolate, rituximab, IVIG EPO, cyclophosphamide,bortezomib	Steroids, hydroxychloro-quine EPO	10.5	11	288	-
5	53/F	wAIHA DAT IgG>2+ and C>2+	-	-	Steroids, rituximab splenectomy IVIG, EPO, azathioprine, bortezomib, sirolimus, cyclosporin, tacrolimus	Steroids, IVIG maintenance (monthly)	8.8	86	242	-
6 ¶	55/F	wAIHA DAT IgG>2+ and C<2+	Evans syndrome	-	Steroids, rituximab, splenectomy, azathioprine, bortezomib, everolimus, cyclosporin	-	8.2	31	1100	10
7 ¶	55/F	wAIHA DAT IgG>2+ and C<2+	Evans syndrome	-	Steroids, rituximab	Steroids	10.7	24	335	-

8	42/M	wAIHA DAT IgG>2+ and C<2+	Evans syndrome, MGUS IgGkappa Non clonal CD8 T-cell proliferation , T-LGL suspect	-	Steroids, rituximab , splenectomy, Plasmapheresis, Cyclophosphamide, sirolimus, cyclosporin, danazole, methotrexate	Steroids Sirolimus Plasmapheresis	4	140	4800	30
9	25/F	wAIHA DAT IgG>2+	Evans syndrome	-	Steroids, rituximab IVIG, bortezomib, sirolimus, danazol, EPO	Danazol Sirolimus	6.3	19	421	-
10	59/M	wAIHA DAT>2 + IgG	IgG MGUS	-	Steroids, rituximab IVIG, EPO, splenectomy	Steroids Rituximab, IVIG, EPO, splenectomy	6.1	80	1609	36
11	36/F	wAIHA DAT<2 + IgG	SLE	-	Steroids, rituximab, Obinutuzumab, plasmapheresis, azathioprine, ibrutinib	Steroids IVIG	7.8	41	282	10
12	64/F	wAIHA DAT IgG <2+	Evans syndrome, IgG kappa and lambda MGUS	-	Steroid, rituximab, IVIG	Steroids	7.3	61.2	671	1
13 ¶	59/M	cAIHA, DAT negative Low titer coldagglutinin present	CAD IgG kappa MGUS, HBV	yes	Steroids, rituximab EPO, bortezomib	-	7	67	518	2
14	62/M	cAIHA DAT C>2+	CAD	-	Steroids Rituximab IVIG Plasmapheresis Eculizumab	Steroids Eculizumab	8.8	73	480	20

15 ¶	56/M	cAIHA DAT IgG<2+, IgM>2+	LPL	Yes	Steroids, rituximab, cyclophosphamide, bortezomib, lenalidomide	-	12.5 †	10	351	-
16	74/M	cAIHA DAT C<2+	CAD	Yes	Rituximab, plasmapheresis	-	11.2	12	-	-
17	80/F	cAIHA DAT C>2+	LPL	Yes	Steroids, rituximab, cyclophosphamide, mycophenolate, ibrutinib	Steroids	8.5	46	972	-
18	75/F	cAIHA	CAD	yes	Steroids, rituximab, mycophenolate, azathioprine	-	8.6 ‡	57	809	1
19	70/M	cAIHA	CAD, type 1 cryoglobuli-naemia	yes	Steroids, plasmapheresis 9 doses of Pegcetacoplan/ placebo § 1 dose of BIVV020‖	-	5	30	1330	4
Median (range)	**56 (25-80)**				**5 (2-10)**		**8.6 (4-12.5)**	**46 (10-140)**	**584 (242-4800)**	**10 (1-36)**

AIHA indicates autoimmune hemolytic anemia; anti-HCVCAD, cold agglutinin disease; cAIHA, cold autoimmune hemolytic anemia; DAT, direct antiglobulin test; EPO, erythropoietin; Hb, hemoglobin; HBV, hepatitis B virus; HCV, hepatitis C virus; IVIG, intravenous immune globulin; LDH, lactate dehydrogenase; LPL, lymphoproliferative lymphoma; MGUS, monoclonal gammopathy of unknown significance; RBC, red blood cell; SC, subcutaneous; SLE, systemic lupus erythematosus.

*Age in years at start daratumumab,

† Treatment indication was severe acrocyanosis,

‡ After RBC transfusion,

§ Participated in the randomized, placebo-controlled phase 3 trial trial (ClinicalTrials.gov, NCT05096403),

‖ Participated in the PDY16370 study, received one dose of BIVV020 (anti-C1s Humanized IgG4 Monoclonal Antibody), but could not continue further doses due to neutropenia. (ClinicalTrials.gov, NCT04269551),

¶ Previously published.

Table 2. Individual response data

Case, AIHA type	Daratumumab treatment schedule	Time to partial Hb response in weeks* (Hb, g/dL, Δ)	Time to complete Hb response in weeks* (Hb, g/dL, Δ)	Best Hb response in weeks from start daratumumab (Hb, g/dL, Δ)	Transfusion dependence (if transfusion dependent at baseline)	Acrocyanosis improved/ resolved (for patients with acrocyanosis at baseline)	Duration of response after the start of daratumumab
1 wAIHA	8x weekly sc 1800 mg	-	4 (12.5, +1.3)	4 (12.5, +1.3)	-	-	2 months
2 wAIHA	8 x weekly sc 1800 mg	2 (11.9, +2.2)	4 (13.4, +3.7)	4 (13.4, +3.7)	-	-	5 months
3 wAIHA	8 x weekly sc 1800 mg	2 (11.0, +1.0)	9 (12.4, +2.4)	24 (13.4, +3.4)	-	-	Ongoing at 12 months
4 wAIHA	4x weekly sc 1800 mg	-	8 (12.7, +2.2)	12 (13.5, +2)	-	-	Ongoing at 4 months ‡
5 wAIHA	8x weekly sc 1800 mg, then 2x 2-weekly sc 1800mg.	-	-	-	-	-	No response after 4 months
6 wAIHA	8 x weekly 16 mg/kg iv, 3 x 2-weekly 16 mg/kg iv	-	-	-	Ongoing	-	No response after 3,5 months
7 wAIHA	4 x weekly 16mg/kg iv		2 (12,1, +1,4)	12 (14.6, +3.9)	-	-	9 months
8 wAIHA	8 x weekly 16 mg/kg iv	-	-	-	Ongoing	-	No response after 2,5 months
9 wAIHA †	4x weekly 16 mg/kg iv	-	-	-	-	-	No response after 2,5 months
10 wAIHA	4x weekly sc 1800 mg	2 (10.1, + 4.0)	4 (14.2, +8.1)	24 (15.3, +9.2)	Transfusion independent after 14 days	-	Ongoing at 6 months
11 wAIHA	4x weekly sc 1800 mg	-	-	-	Ongoing	-	No response after 2 months
12 wAIHA	2x weekly 16 mg/kg iv 11x 2-weekly sc 1800 mg	-	-	-	Ongoing	-	No response after 6 months
13 cAIHA	8x weekly 16 mg/kg iv, 16x 2-weekly 16 mg/kg iv, 9x monthly 16 mg/kg iv	12 (9.1, +2.1)	-	40 (10.2, +3.2)	Transfusion independent after 6 months	Improvement after 3 months	19 months §
14 cAIHA	8 x weekly sc 1800 mg	2 (10.1, +1.3)	16 (12.0, +3.2)	24 (14.5, +5.7)	Transfusion independent after 3 months	-	Ongoing at 8 months
15 cAIHA	First dose 16 mg/kg iv followed by 8x weekly sc 1800 mg, 8x 2-weekly sc 1800 mg, followed by maintenance 1800 mg sc monthly.	-	‖	8 (16.6, +4.1)	-	Improved after 2 weeks Resolved after 3 months	Ongoing at 40 months §
16 cAIHA	First dose 16mg/kg iv followed by 8x weekly sc 1800 mg, 8x 2-weekly sc 1800 mg, followed by maintenance 1800 mg sc monthly.	-	2 (12.7, +1.5)	24 (14.8, +3.6)	-	Improved after 2 weeks Resolved after 12 months	Ongoing at 12 months §
17 cAIHA	First dose 16mg/kg iv followed by 8x weekly sc 1800 mg, 8x 2-weekly sc	4 (11.2, +2.7)	12 (12.7, +4.2)	12 (12.7, +4.2)	-	Improved after 6 months	Ongoing at 11 months §

	1800 mg, followed by maintenance 1800 mg sc monthly.						
18 cAIHA	8 x weekly sc 1800 mg	-	-	-	Ongoing	No response	No response after 2 months
19 cAIHA †	8 x weekly sc 1800 mg	-	-	-	Ongoing	No response	No response after 3 months
Median (range)		**2 (2-12 weeks) Δ + 2.2 g/dL (1.0-4.0)**	**2 (2-16 weeks) Δ + 2.3 g/dL (1.3-8.1)**	**12 (2-40 weeks) Δ + 3.7 g/dL (1.3-9.2)**			

AIHA indicates autoimmune hemolytic anemia; Hb, hemoglobin; IV, intravenously; NA, not applicable; sc, subcutaneous; Δ, delta.

* Hb responses were defined as partial (PR, >2 g/dL Hb increase or >10g/dL) or complete (CR, >12g/dL).
‡ Pat 4 has started belimumab for underlying SLE 4 months after the start of daratumumab, Hb response data not analyzed after 4 months. Patient is after 2 years still in CR of AIHA.
† Patient died due to uncontrolled hemolytic anemia
§ Daratumumab maintenance therapy until progression
|| Treatment indication was severe acrocyanosis, therefore patient was excluded from assessment of Hb response.

Table 3.
Review of literature, daratumumab monotherapy in adult primary and secondary AIHA patients (non-stem cell transplantation setting).

age/sex	Disease	Previous treatments	Daratumumab schedule	Best response	Time to response Hb PR/CR ‡ and acrocyanosis	Duration of response	Reference
60, F	wAIHA	Steroids, Rituximab azathioprine	4x 16 mg/kg iv weekly	PR	10 weeks	5 months	[19]
44, F	wAIHA	Steroids, Rituximab, IVIG, HSA, splenectomy, cylcosporine, mycophenolate	6 x 16 mg/kg iv weekly + 6x 16 mg/kg iv maintenance	CR	na	5 months	[17]
55, F	wAIHA	Steroids, Rituximab	6x 16 mg/kg iv weekly	CR	na	2 months	[17]
55, F*	wAIHA	Steroids	6x 16 mg/kg iv weekly	CR	1 week	Relapse after 9 months	[13]
55, F*	wAIHA	Steroids, azathioprine, cyclosporine, everolimus, bortezomib	8 x 16 mg/kg iv weekly + 3 x 2-weekly 16mg/kg iv	No response	No response	-	[13]
64,F	wAIHA	Prednison, rituximab, splenectomy	8 x 16 mg/kg iv weekly	CR	na	Ongoing response after 20 months	[20]
56, M*	cAIHA	Steroids, rituximab, bortezomib, cyclophosfamide, lenalidomide	8x weekly 16mg/kg iv + 16x two weekly 16mg/kg iv + monthly 16mg/kg iv maintenance	CR Hb PR acrocyanosis	2 weeks 2 weeks improvement of acrocyanosis	Ongoing response after 10 months	[22]
59, M*	cAIHA	Steroids, rituximab, EPO bortezomib	8x weekly 16mg/kg iv + 16x two weekly 16mg/kg iv + monthly 16mg/kg iv maintenance	PR	12 weeks	Ongoing after 16 months	[12]
73, M	cAIHA	Rituximab, ibrutinib,	Na, maintenance therapy	PR	na	Ongoing	[20]

		bendamustine	ongoing at 15 months			response after 15 months	
64, F	cAIHA	Rituximab	8x weekly 16mg/kg iv + 8x two weekly 16mg/kg iv + monthly 16mg/kg iv maintenance Combination with HSA	CR	5 months PR 6 months CR	Ongoing response after 8 months	[21]

wAIHA indicates warm autoimmune hemolytic anemia; cAIHA, cold autoimmune hemolytic anemia; CR, complete response; EPO, erythropoietin; F, female; Hb, hemoglobin; HSA, haematopoiesis-stimulating agents

IVIG, intravenous immune globulin; M, male; PR, partial response; SC, subcutaneous; SLE, systemic lupus erythematosus.

*Case included and updated in our case series.

‡ Hemoglobin (Hb) response was considered partial (PR; Hb 10-12 g/dL or complete (CR, Hb > 12 g/dL).

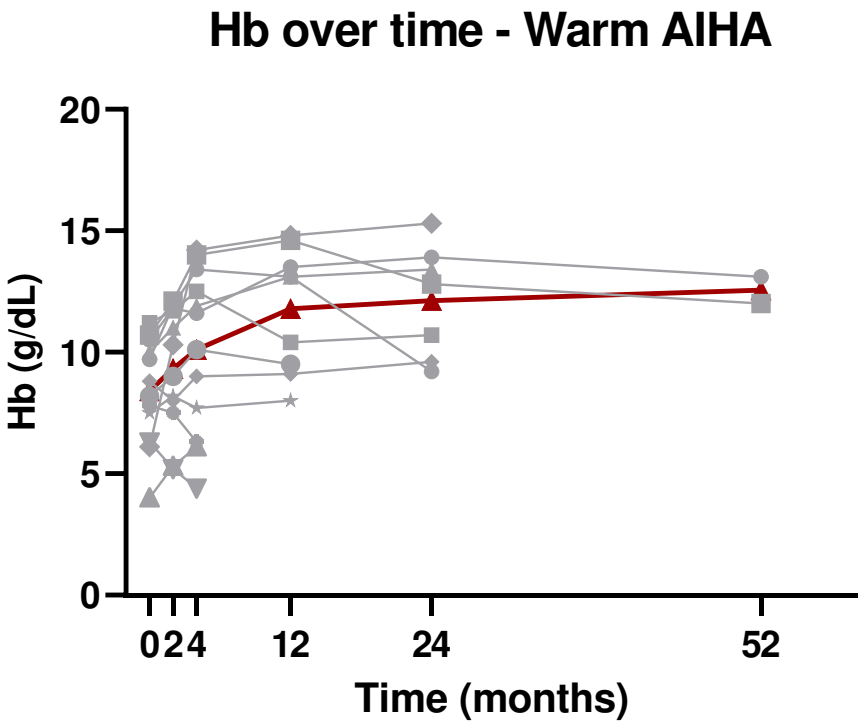
Figure 1. Hemoglobin (Hb) response after the initiation of daratumumab therapy.
A. Patients with warm autoimmune hemolytic anemia (AIHA). **B**. Patients with cold AIHA.
Gray lines represent individual data of Hb; bold line represents median Hb over time.

Figure 2. Daratumumab treatment reduced the abundance of B cells and affected subset distributions of T cells
PBMCs from 2 patients with warm autoimmune hemolytic anemia at different time points (baseline (Pre), during treatment (Mid), immediately after treatment cessation (EOT), 6 weeks after the last cycle (6 weeks) and 3 months after end of treatment (Post)) were analyzed by flow cytometry, either right after thawing or after a 2-5 day culture-cell stimulation by αCD3/αCD28 antibodies. A) Frequency and subset distribution of B cells over the course of daratumumab treatment. B) Analysis of $CD3^+$ and T_{reg} frequency, $CD4^+$: $CD8^+$ ratio, $CD4^+$subset distribution and CD38 expression. C) Hemoglobin (g/dL) levels of patients over course of treatment. CM= central memory, EM= effector memory, EMRA= effector memory RA+.

Figure 3. T cell function and populations altered by Daratumumab treatment
A) Pooled tSNE map, minimal spanning tree and heatmap of the 8 FlowSOM metaclusters based on marker intensity, followed by tSNE maps of metaclusters in individual patients over treatment course(baseline (Pre), during treatment (Mid), immediately after treatment cessation (EOT), 6 weeks after the last cycle (6 weeks) and 3 months after end of treatment (Post)). B) Expression of CD25, CD38 on $CD4^+$ T cells measured after a 48h T cell stimulation. C) Samples were stained with CTV and proliferation was assessed after 5 days of T cell stimulation. CM= central memory, EM= effector memory, EMRA= effector memory RA+. Treg= T-regulatory.

Figure 1

Figure 1.



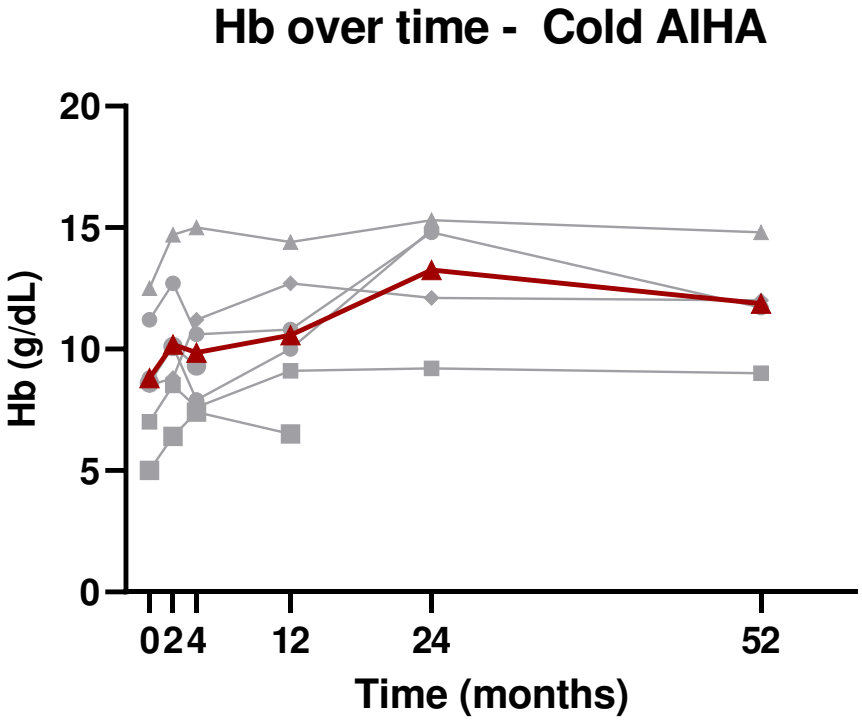


Figure 2

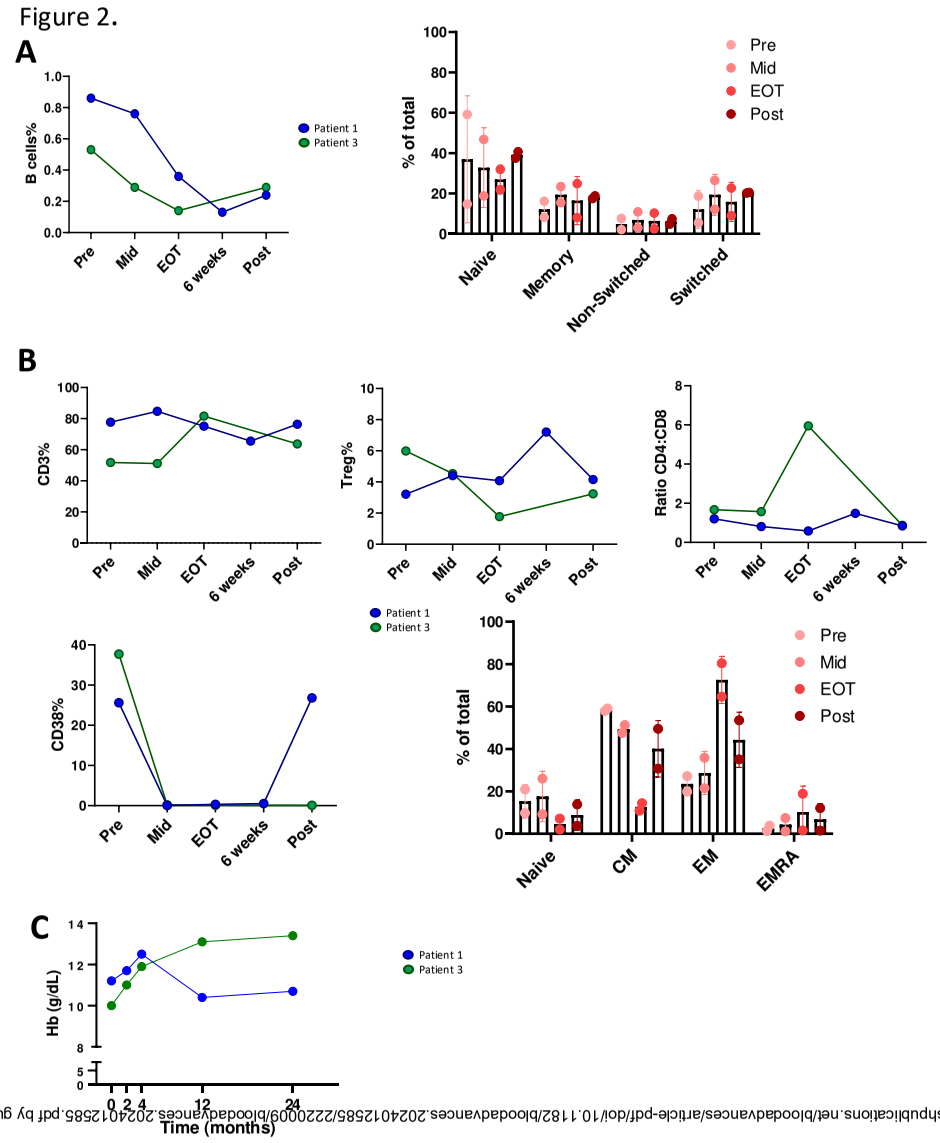


Figure 3