Low dose rituximab in adult patients with idiopathic autoimmune hemolytic anemia: clinical efficacy and biological studies

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Abstract

This prospective study investigated the efficacy, safety and the duration of the response of low dose (LD)-rituximab (100 mg fixed-dose x 4 weekly infusions) together with a short course of steroids, as first or second line therapy in 23 patients with primary autoimmune hemolytic anemia (AIHA). Overall response (OR) was 82.6% at month +2, and subsequently stabilized ~90% at month +6 and +12; response was better in warm AIHA (WAIHA) (OR 100% at all time-points) than in cold hemagglutinin disease (CHD) (on average 60%); the relapse free survival (RFS) was 100% for WAIHA at +6 and +12 months versus 89% and 59% in CHD, and the estimated RFS at 2 years 81% and 40% for warm and cold forms, respectively. The risk of relapse was higher in CHD and in patients with longer interval between diagnosis and enrolment. Steroid administration was reduced both as cumulative dose (~50%) and duration compared with the patient’s past history. Treatment was well tolerated and no adverse events or infections were recorded; re-treatment was also effective. The clinical response correlated with amelioration biological markers, such as cytokine production (IFN-γ, IL-12, TNF-α, and IL-17), suggesting that LD-rituximab exerts an immunomodulating activity. This study is registered at http://clinicaltrials.gov as NCT01345708.
Introduction

Autoimmune hemolytic anemias (AIHA) are acquired autoimmune diseases characterized by the production of antibodies directed against autologous red blood cells (RBC). AIHA are classified into warm- and cold-reactive antibody types, and can be either primary or secondary to lymphoproliferative disease, infections, immunodeficiency, and tumors. The degree of hemolysis, from fully compensated to fulminating, depends on the characteristics of the autoantibody (class, quantity, specificity, thermal amplitude, ability to fix complement and to react with tissue macrophages), on the activity of the reticulo-endothelial system, and on the efficacy of the erythroblastic response. Conventional therapy of warm AIHA (WAIHA) include administration of corticosteroids as first line therapy, which is effective in roughly 2/3 of cases; patients who are refractory or relapse after initial response (approximately 1/3) need additional second-line therapies which include splenectomy and immunosuppressive agents, which are reported to provide a 60-75% and 40-60% response rate, respectively. Conventional treatment with corticosteroids in cold hemagglutinin disease (CHD) induced responses in 14% of cases only, and additional treatments, including alkylating agents, interferon-α, and low-dose cladribine, have little efficacy.

Rituximab is a chimeric monoclonal antibody directed against CD20 first developed for the treatment of lymphoproliferative malignancies. The drug was shown effective also in rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and several hematologic autoimmune diseases, such as primary immune thrombocytopenia (previously referred to as idiopathic thrombocytopenic purpura, ITP), acquired hemophilia, thrombotic thrombocytopenic purpura (TTP), and AIHA. In WAIHA rituximab is effective in about 60% of patients, suggesting that it may represent an alternative to splenectomy and/or immunosuppressive/cytotoxic therapy; furthermore, it induces durable responses in CHD, offering an effective treatment to a group of patients with limited therapeutic options. Moreover, rituximab and orally fludarabine combination therapy was proven very effective in CHD, resulting in 75% response rate, complete remissions in about 20%, and more than 66 months estimated response duration.

In these hematologic autoimmune diseases the drug was generally administered at the dose scheduled for the treatment of lymphomas (375 mg/mq/weekly for 4 weeks). In attempt to minimize side effects and reduce costs, and considering that the lymphocyte burden in autoimmune hematologic conditions is lower than the tumor mass in lymphoproliferative diseases, low dose (LD)-rituximab (100 mg fixed dose) has been successfully used in autoimmune cytopenias. In ITP a prospective study demonstrated that it is effective in roughly 60% of patients but has moderate long-term effect; furthermore, a randomized trial reported more pronounced sustained responses in ITP patients treated with LD-rituximab and steroids compared with steroids alone, but with similar response rates. Finally, a prospective study in patients with steroid-refractory AIHA and ITP showed that LD-rituximab plus alemtuzumab subcutaneously (10 mg on days 1 to 3) induced an overall response rate of 100%, with complete responses in 58% of cases. The aim of this prospective study was to evaluate the efficacy, safety and the duration of the response of LD-rituximab associated with a short course of prednisone (PDN) as first line therapy in newly diagnosed WAIHA and CHD, and as second line therapy in WAIHA relapsed after standard oral PDN. Further aim was to correlate the clinical response with cytokine production in cultures.
Materials and Methods

Patients
This phase II, single-arm prospective multicenter study started in June 2008 and concluded in May 2011, and involved 3 Italian academic centers or hospitals. The study protocol was approved by the Ethical Committee of Human Experimentation of all participating institutions, registered at http://clinicaltrials.gov, and patients gave informed consent in accordance with the Declaration of Helsinki (EudraCT number 2008-006713-25, NCT01345708). Patients were aged >18 years and affected by WAIHA or CHD either newly diagnosed or relapsed after first line treatment with oral prednisone; AIHA was defined by symptomatic anemia and positive direct antiglobulin test (DAT), in the absence of underlying lymphoproliferative, infectious or neoplastic disease (according to the single Center diagnostic criteria). Exclusion criteria were active bacterial, viral (HIV, HCV, HBV), or fungal infection requiring systemic therapy, immunologic deficit (congenital or acquired), history of malignancies within 3 years prior to study entry, concomitant immunosuppressive or cytotoxic treatment, pregnancy, or any condition that would preclude ability to give informed consent.

Treatment
Rituximab was administered at the fixed dose of 100 mg as an intravenous (iv) infusion on days +7, +14, +21, +28 along with oral prednisone 1 mg/kg/day p.o. from day +1 to +30, followed by tapering according to the following schedule: 10 mg/week until 0.5/mg/kg/day, then 5 mg/week until stop. Patients received oral acetaminophen 500 mg and IV chlorphenamine 10 mg as pre-medication therapy.

Assessments and outcome measures
Complete clinical examination, complete blood counts, hemolytic markers (reticulocytes, total and unconjugated bilirubin, LDH, and haptoglobin), were performed at enrolment, and at day +7, +14, +21, +28, +35, monthly until month +6, then every 3 months until the end of follow-up; DAT, hepatic and renal function tests were performed at enrolment, and at day +35, at month +3, and then every 3 months until the end of follow-up.

The primary objective of the study was to evaluate the efficacy in terms of overall response (OR), complete response (CR, defined as Hb>12 g/dL, and normalization of all hemolytic markers) and partial response (PR, defined as Hb=10-12 g/dL or at least 2 g/dL increase in hemoglobin, and no transfusion requirement). Further objectives were to evaluate the time to response, the duration of the response (sustained response, SR, defined as Hb>10 g/dL in the absence of any treatment), and the relapse free survival (RFS). Moreover, we assessed the safety profile, as adverse event incidence up to 12 months from the beginning of therapy, according to NCI-CTC version 3.0. Finally, to verify a possible steroid-sparing effect of LD-rituximab, we compared in previously relapsed patients the amount of past steroid therapy with that administered in association with LD-rituximab, at equal time intervals.

Immunological investigations
To evaluate cytokine production (TNF-α, IFN-γ, interleukin (IL)-12, IL-4, and IL-17), heparinized blood samples from patients (N=12) and controls (N=16) were diluted 1:6 with RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) and stimulated with 2μg/mL phytohemagglutinin (PHA) (Sigma, St. Louis, MO, USA) in 24-well plates for 48 hour. Cytokines production was measured using commercially available ELISA kits (R&D systems, Minneapolis, MN, USA). DAT for the detection of IgG and complement bound to RBCs was performed using the tube technique employing the standard method with polyspecific anti-human globulin (anti-IgG+C) and monospecific anti-IgG and anti-C3 antisera (Ortho Clinicals Diagnostics, Raritan, NJ, USA).
**Statistical analysis**

Student’s t and chi-square test were used to analyze continuous and categorical variables, respectively. RFS (%) was evaluated by using the Kaplan-Meier method. Cox regression models were fitted to calculate the relapse hazard ratios (HR) and 95% confidence intervals (95% CI) according to select demographic and clinical variables. Multiple random intercept models were used to evaluate trend of continuous variables (prednisone use, hematologic variables) in the periods before and after LD-rituximab taking into account the positive correlations within subjects at follow-up visits. The average daily prednisone doses before and after LD-rituximab were compared considering each day of therapy prescription and also the days between follow-up visits. The gender and an indicator variable for the period (after/before start of therapy) were included in the random-effect models. The modifying effect of AIHA on prednisone administration was evaluated by including a product-term in the regression model and by performing two separate analyses for WAIHA and CHD. Multiple random-effect models were used to assess cytokine levels over time, including as covariates the AIHA and the time since diagnosis to start of rituximab therapy. All the analyses were performed with the software Stata, version 11.
Results

Clinical characteristics of patients at enrolment
Table 1 shows the clinical and serological characteristics of patients at enrolment. Warm forms of AIHA were more frequent than CHD. The latter were almost all females, relapsed after steroid treatment, and had a longer median interval from diagnosis to rituximab treatment. None of the patients was splenectomized. The median Hb value at enrolment was lower in WAIHA than CHD (median 8.65 g/dL, range 4.4-13.4 for the former and 9.4 g/dL, range 7.1-12.2 for the latter), and 6/14 (43%) WAIHA and 2/9 (22%) CHD patients showed Hb values lower than 8 g/dL. Hemolytic markers were abnormally elevated in almost all patients with no differences between WAIHA and CHD (see also baseline values, Table 3).

Response to therapy
All patients included in the study completed the therapeutic program receiving the four infusions of LD-rituximab as scheduled, and were subsequently followed up for a median of 15 months (range 6-35); the follow-up was at least 12 months in 18/23 (78%), 18 months in 11/23 (48%), and 24 months in 7/23 (30%). On the whole OR at month +2, +6 and +12 were 82.6%, 91.3% and 84.2%, respectively. Considering separately warm and cold forms (Table 2), all cases of WAIHA responded at month +2 and maintained the response until month +6 and +12; response rates were lower in CHD, with an OR at month +2 in 55.6% of cases, and a SR at month +6 and +12 in 77.7% and 66.7%, respectively; relapse rates were 11.1% and 33.3% at month +6 and +12, respectively. The median time to OR was 16 days (range 6-62) for WAIHA and 19 days (range 6-166) for CHD. In univariate analysis response was significantly associated with WAIHA (P=0.023, P=0.047, and P=0.013 at month +2, +6, and +12, respectively), and the probability of CR at month +2 was associated with younger age (P=0.023, \( \chi^2 \)), higher weight (P=0.013, \( \chi^2 \)), and shorter interval between diagnosis and rituximab therapy (P=0.077, \( \chi^2 \)). In multivariate analysis the thermal characteristic of hemolytic anemia emerged among age, weight and interval between diagnosis and rituximab therapy as the only variable associated with a sustained response at month +6 (OR 9.6, 95% CI 0.9-105.1, P=0.064), and +12 (OR 21.9, 95% CI 0.9-484, P=0.05). The cumulative RFS for all cases of AIHA at +6 and +12 months was 96% and 86%, respectively, and the estimated RFS at 2 years 68%; considering separately WAIHA and CHD, the cumulative RFS at +6 and +12 months were significantly reduced in the latter (89% and 59% for CHD versus 100% for WAIHA at both time points, P=0.015) (Figure 1); the estimated RFS at +24 months was 40% and 81% for cold and warm cases, respectively. In addition, univariate Cox regression models showed that risk of relapse was higher in CHD (HR 6.1, 95% CI 1.1-33.8; P=0.04) and in patients with longer interval between diagnosis and rituximab therapy (HR 1.01, 95% CI 1.00-1.02; P=0.03); age, gender, and weight showed no relationship with relapse risk. Among 6 relapsed patients (4 CHD, 2 WAIHA, 3 in the first year after LD-rituximab and 3 subsequently) it is noteworthy that one CHD patient who relapsed at month +7 was retreated with a second LD-rituximab (achieving PR and SR at +9 and +13, respectively), and with a third cycle achieving an ongoing SR at month +20; the patient was therefore able to stop steroids for several months between cycles. Another patient with WAIHA who had a severe relapse (Hb 6.5 g/dL) at month +16 was retreated with LD-rituximab achieving a PR until month +24.

Hematologic evaluation
The main laboratory data of WAIHA and CHD are shown in Table 3. In WAIHA, hematologic parameters at month +2 were normalized in most cases: Hb levels significantly increased (P<0.0001 versus enrolment), and hemolytic markers decreased (P=0.005, P=0.03, P=0.002, for absolute reticulocyte, LDH and unconjugated bilirubin, respectively); at month +6 and +12 hematologic parameters were stable. At variance, in CHD hemoglobin levels increased to a lesser extent at month +2 (P=0.02 versus enrolment) and hemolytic markers decreased albeit still above normal
ranges; at month +6 hemoglobin levels further increased and hemolytic parameters were almost normalized; hematologic data at month +12 were stable in the 4 evaluable CHD cases. The increase of hemoglobin and the reduction of hemolytic markers were confirmed by analyzing all hematologic data before and after LD-rituximab with multiple random effect models: Hb +0.6 mg/dL, P<0.001, unconjugated bilirubin -0.5 mg/dL, P<0.001, reticulocytes -36.3x10^9/L, P<0.001. Interestingly, this statistical analysis allowed the comparison of prednisone administrations (491 cumulative doses-period) before and after LD-rituximab in the 15 patients with an adequate follow-up pre-enrolment (795±170 days, mean±SE): the average cumulative prednisone doses were reduced (roughly 50%) after LD-rituximab compared with the pre-study treatment (3,995 versus 7,810 mg×days, P=0.08, Figure 2); the average daily dosage was reduced as well (11.7 versus 17.2 mg/day, P=0.10), however the reduction was less evident in the 6 CHD (-0.6 mg/day, P=0.05) than in the 9 WAIHA (-4.1 mg/day, P<0.001). As regards the duration of steroid therapy, it is noteworthy that 9/14 (64%) WAIHA patients and 5/9 (56%) CHD patients had completely stopped steroids at +89 days (median values, range 58-150); moreover, the overall median duration was significantly reduced after LD-rituximab compared with pre-study treatment (150 days, range 58-548, 56% of time, versus 548 days, range 30-1459, 87% of time, P=0.027).

Safety
Overall rituximab therapy was well tolerated and no patients experienced the most frequently described infusion-related reactions. No infectious, hematologic or extra-hematologic complications were documented during follow up. As a consequence of steroid therapy, 5 patients experienced increase of body weight (on average 5 Kg), and one patient worsening of pre-existing hypertension that required temporary adjustment of therapy. An acute ischemic heart attack followed by fatal ischemic stroke occurred in one patient (who was in a CR/SR) 7 months after the end of rituximab therapy. No significant changes were observed for leukocyte and lymphocyte counts, as well as liver and renal function tests (data not shown).

Immunologic assessment
All patients displayed a positive DAT at enrolment, 14 with anti-IgG (WAIHA) and 9 with anti-C antisera (CHD); at month +6 it was still positive in all cases, while at month +12 2/18 patients became DAT negative (12.5%); titers and scores were available in 8 patients still DAT+: 2 showed a clear decrease of both parameters, 2 a little decrease, and 4 cases displayed no significant changes. Concerning Th-1 cytokine production, at enrolment TNF-α was lower in patients compared with controls although not significantly (946±208 versus 1574±139 pg/mL, mean±SE), and increased at month +3 and +6, reaching normal values (1289±152 and 1591±189 pg/mL, respectively); likewise, IL-12 production was lower than normal values (697±253 versus 1022±38 pg/mL), and increased at month +3 and +6 (1130±140 and 1295±214 pg/mL, respectively); IFN-γ was significantly reduced as well (1245±412 in patients versus 2897±55.3 pg/mL in controls, P=0.04), and increased after therapy without reaching normal values. These findings were confirmed by multiple random-effect models (data not shown). Regarding Th-2 cytokines, at enrolment IL-4 was higher in patients compared with controls (24.6±8.6 versus 12.6±1.2 pg/mL), diminished at month +3 reaching normal values (11.0±3.4 pg/mL) but returned to baseline levels at month +6 (22.6±11.6 pg/mL); likewise, at enrolment IL-17 was significantly increased compared with controls (37.8±7.4 versus 5.6±0.1 pg/mL, P=0.03), and showed a slight reduction at month +6 (30.2±6.2 pg/mL, P=0.04 versus controls). Although the number of patients evaluated is too small to draw definite conclusions, IL-17 at month +6 was lower in responding (n=7) than in non responding patients (n=4) (19.6±5.0 versus 43.9±13.0 pg/mL, P=0.068), whereas there was no difference in the other cytokines investigated considering separately the two populations.
B-cell depletion was evaluated in 3 patients (2 responders and 1 non responder): median baseline value of CD20 positive cells was 0.18 x 10^9/L, (range 0.09-0.49) versus 0.03 x 10^9/L (range 0.01-
0.14) at month +6, without any difference between responding and non responding cases. Serum IgG, IgA and IgM levels were comparable at baseline and at month +6 (data not shown).
Discussion

First line therapy of autoimmune hemolytic anemias is based on corticosteroids, which are reported to provide a response in 70-85% of WAIHA; however, only one third of patients remain in long term remission once the drug is discontinued, and a further 50% require maintenance doses³. This long-term steroid therapy even at low doses is known to have potentially harmful side effects. Second line therapy with cytotoxic and immunosuppressive drugs (such as azathioprine, cyclophosphamide, cyclosporine) is reported to provide a 40-60% response rate but their use may be associated with serious side effects, and the effectiveness of other options, such as IVIg, plasmapheresis and danazol, is controversial³-⁵. In CHD corticosteroids are effective in 1/6 of cases only, and other treatments (alkylating agents, interferon-α, and low-dose cladribine) have little efficacy.²,⁴,⁶ Therefore, we pursued the idea to develop a “potentially curative” therapy for AIHA, combining two well-demonstrated effective treatments, i.e steroids and rituximab, at doses lower than those usually employed; the aim was to minimize side-effects and costs, and synergize their curative actions. Furthermore, the reduction of rituximab doses may decrease the risk of complications, such as the reactivation of viral infections and progressive multifocal encephalopathy, although a specific study is needed to address this issue; of note, progressive multifocal encephalopathy, the most harmful of these complications, has been reported earlier after rituximab treatment (median time from last rituximab 5.5 months), and generally in heavily immunosuppressed lymphoma patients²⁰-²⁴.

The results of this prospective study show that low doses of rituximab, i.e. nearly one seventh of the standard dose, together with a short course of prednisone is effective in AIHA patients with a sustained response at one year in 90% of cases, and an estimated relapse free survival in roughly 2/3 of patients at two years. These responses are greater than those reported in the literature for conventional steroids alone, and undoubtedly superior in comparison with those of second line therapy with cytotoxic and immunosuppressive drugs. The response observed in this study was associated with a significant increase of hemoglobin levels and a reduction of hemolytic markers, even if only two patients became DAT negative. Treatment was well tolerated and no rituximab-related adverse events were recorded, including leucopenia/neutropenia and infections. Our results compare favorably with those reported with conventional doses of rituximab (effective in roughly 60% of cases, with a great variability, range 40-100%)⁷,¹¹, and are similar to the more recent studies showing response rates of 80-90%, and a disease free survival at two years in one half of cases²⁵-²⁸. Despite the small numbers, results clearly show that response rates and relapse free survival, as well as amelioration of hematological parameters, were more evident in warm than cold cases, with virtually all cases of the former responding. Steroid therapy certainly may contribute to improve and accelerate the response of WAIHAs, which are known to be more sensitive to steroids than CHD. Cold forms respond to a lesser extent to low doses of rituximab; likewise, the time to response was particularly prolonged in some cases of CHD, as already observed with standard doses⁷,¹¹,²⁸-³¹ supporting the idea that CHD is a different clinical entity from classic WAIHA³,⁶. Consistently, the presence of a clonal B-cell lymphoproliferation was found by re-examining bone marrow biopsies in more than 2/3 of otherwise classified primary CHD, suggesting the existence of a continuous spectrum between CHD and lymphoproliferative diseases such as lymphoplasmacytic lymphoma, marginal zone lymphoma, and Waldenstrom macroglobulinemia⁶. In line with the “clonality” of CHD, more aggressive therapy with rituximab and orally fludarabine was proven more effective than rituximab alone at standard doses (3/4 compared with half of cases)¹². Finally, we found that the response to LD-rituximab was associated with a shorter disease duration, suggesting that it may be more appropriate as earlier treatment; however, this finding may also reflect the longer disease duration of CHD, that do worst, compared with WAIHA.

An interesting finding of our study is that steroid administration during LD-rituximab was reduced, particularly in WAIHA, both in terms of cumulative dose (roughly 50%) and duration compared with the patient’s past history, suggesting a steroid-sparing activity of this treatment. Moreover, it is
of particular interest the sustained response that leaves the patient free of therapy for longer periods in comparison to the pre-treatment history, and the possibility to obtain again a response with a retreatment, as already reported with conventional doses of rituximab.\textsuperscript{7,11}

It is well established that the response to rituximab is related to B-cell depletion, with the consequent inhibition of several B-cell pathologic activities, such as the production of autoantibodies, cytokines secretion, and antigen-presenting cell function.\textsuperscript{9} B-cell depletion has been documented at low-doses in ITP\textsuperscript{14,15} and, although in limited cases, also in our series. However, it has recently been proposed that the drug exerts also an immunomodulating activity, i.e. in ITP it normalizes the abnormal autoreactive T-cell response and rituximab-opsonized B cells block the macrophage Fc-receptor function consequently reducing the sequestration of platelets in the spleen.\textsuperscript{32,33} To investigate this issue, we tested in vitro production of several cytokines: we focused on Th1 cytokines (IFN-\(\gamma\), IL-12, and TNF-\(\alpha\)), which are reported to be reduced in autoimmune diseases including AIHA\textsuperscript{34-39}; in addition we studied IL-17 which is produced by a novel Th cell subset distinct from Th1 and Th2 cells, critical in inflammation and autoimmunity.\textsuperscript{38} IL-17 expression was reported increased at the site of inflammation in patients with rheumatoid arthritis, psoriasis, multiple sclerosis, and uveitis\textsuperscript{40-43}; furthermore, in animal models, mice genetically deficient in IL-17A were less susceptible to collagen-induced arthritis and experimental autoimmune encephalitis and neutralization of IL-17 by treatment with anti-IL-17 antibodies in vivo protects mice from experimental autoimmune uveitis\textsuperscript{40,44}. On the other side, Th1 cytokines like IFN-\(\gamma\) and IL-12, inhibit Th17 differentiation and thus exert a protective role against IL-17-mediated autoimmune inflammation.\textsuperscript{45} We found that AIHA patients displayed reduced IFN-\(\gamma\), IL-12, and TNF-\(\alpha\), compared with healthy controls; at variance, levels of IL-17 were greatly increased, as already found in other autoimmune diseases.\textsuperscript{40-43} Treatment with LD-rituximab has minor effects on IL-17, with only a slight reduction at month +6, which was more evident in responding patients; however, IFN-\(\gamma\), IL-12, and TNF-\(\alpha\), which exert a protective role against IL-17-mediated autoimmune damage, increased over time, sometimes reaching normal values. This data suggest that LD-rituximab treatment, in addition to its B-cell depleting activity, may translate in a favorable immunomodulating effect, which persists after steroid discontinuation.

In conclusion, LD-rituximab associated with a short course of steroid therapy is a safe and effective treatment, particularly in WAIHA and in patients with newly diagnosed disease, with response rates and sustained responses comparable to standard doses of rituximab; treatment leaves patients free of therapy for longer periods and results in a steroid-sparing effect, although specific controlled studies should be designed to address all these issues; re-treatment with LD-rituximab is also effective. LD-rituximab seems more appropriate in WAIHA than CHD, raising the question of whether a higher dose is required for latter disease, which often shows bone marrow clonal lymphoproliferation. Finally, LD-rituximab has immunomodulating effects, confirming that B-cell depletion is not the unique mechanism of action of the drug.
Acknowledgements

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Author Contributions

Barcellini Wilma designed research, collected, analyzed and interpreted data, and wrote the manuscript.
Zaja Francesco collected data and critically revised the manuscript.
Zaninoni Anna performed research and biological investigation, partially wrote the manuscript, collected data for statistical analysis.
Imperiali Francesca G performed research and biological investigation, partially wrote the manuscript, collected data for statistical analysis.
Battista Marta Lisa collected data.
Di Bona Eros collected data and critically revised the manuscript.
Fattizzo Bruno collected data and partially wrote the manuscript.
Consonni Dario performed statistical analysis, contributed to the interpretation of data, and partially wrote the manuscript.
Cortelezzi Agostino revised the manuscript.
Renato Fanin revised the manuscript.
Zanella Alberto contributed to study design, interpreted data and wrote the manuscript.
Conflict of Interests Disclosures
Dr Zaja is on Roche advisory board. The other Authors disclose any commercial affiliation as well as consultancies, stock or equity interests and patent licensing that could be considered to pose a conflict of interest regarding the submitted article.
References


Table 1. Clinical and serological characteristics of patients at enrolment.

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<tr>
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<th>All patients (23)</th>
<th>WAIHA patients (14)</th>
<th>CHD patients (9)</th>
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<tr>
<td>Male (%)</td>
<td>7 (30.4%)</td>
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<td>Female (%)</td>
<td>16 (69.6%)</td>
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<td>Median age (range)</td>
<td>56 (27-75)</td>
<td>46.5 (27-75)</td>
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<td>Newly-diagnosed patients</td>
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<td>Relapsed after steroid treatment</td>
<td>16 (69.6%)</td>
<td>8 (57%)</td>
<td>8 (89%)</td>
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<tr>
<td>Median interval from diagnosis to rituximab, months (range)</td>
<td>23 (0-276)</td>
<td>8 (0-122)</td>
<td>46 (7-276)</td>
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Table 2. Response rate and outcome after rituximab therapy.

<table>
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<th>Month +2</th>
<th>Month +6</th>
<th>Month +12</th>
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<tr>
<td></td>
<td>OR 14/14 (100%)</td>
<td>SR 14/14 (100%)</td>
<td>SR 13/13 (100%)</td>
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<tr>
<td>WAIHA</td>
<td>CR 11 (78.6%)</td>
<td>CR 10 (71.4%)</td>
<td>CR 9 (69.2%)</td>
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<td>PR 3 (21.4%)</td>
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<td>PR 4 (30.8%)</td>
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<td>NR 0 (0%)</td>
<td>NR 0 (0%)</td>
<td>NR 0 (0%)</td>
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<td></td>
<td>Relapse rate 0 (0%)</td>
<td>Relapse rate 0 (0%)</td>
<td>Relapse rate 0 (0%)</td>
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<td></td>
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<tr>
<td>CHD</td>
<td>OR 5/9 (55.6%)</td>
<td>SR 7/9 (77.7%)</td>
<td>SR 3/6 (50%)</td>
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<td></td>
<td>CR 4 (44.4%)</td>
<td>CR 4 (44.4%)</td>
<td>CR 1 (16.6%)</td>
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<td>PR 3 (33.3%)</td>
<td>PR 2 (33.3%)</td>
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<tr>
<td></td>
<td>NR 4 (44.4%)</td>
<td>NR 1 (11.15%)</td>
<td>NR 0 (0%)</td>
</tr>
<tr>
<td></td>
<td>Relapse rate 0 (0%)</td>
<td>Relapse rate 1 (11.15%)</td>
<td>Relapse rate 2 (33.3%)</td>
</tr>
</tbody>
</table>

WAIHA, warm AIHA; CHD, cold hemagglutinin disease; OR, overall response; CR, complete response (i.e. Hb ≥ 12 g/dL); PR, partial response (i.e. Hb ≥ 10 g/dL); SR, sustained response (i.e. Hb ≥ 10 g/dL); NR, no response.
Table 3. Hematologic parameters of patients at enrolment and after rituximab treatment.

<table>
<thead>
<tr>
<th>WAIHA</th>
<th>Enrolment (N=14)</th>
<th>month 2 (N=14)</th>
<th>month 6 (N=14)</th>
<th>month 12 (N=14)</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td><strong>Hb (g/dL)</strong></td>
<td>8.7 (4.4-13.2)</td>
<td>13.1 (11.2-15.3)</td>
<td>13.5 (10.4-17.1)</td>
<td>13.3 (10.6-15.4)</td>
</tr>
<tr>
<td><strong>Reticulocyte (10⁹/L)</strong></td>
<td>205 (40-320)</td>
<td>90 (30-274)</td>
<td>76 (40-250)</td>
<td>83 (45-160)</td>
</tr>
<tr>
<td><strong>LDH (U/L)</strong></td>
<td>623 (314-1961)</td>
<td>479 (341-1031)</td>
<td>417 (276-605)</td>
<td>403 (281-816)</td>
</tr>
<tr>
<td><strong>Unconjugated bilirubin (mg/dL)</strong></td>
<td>1.66 (0.4-6.4)</td>
<td>0.5 (0.2-2.1)</td>
<td>0.6 (0.2-1.3)</td>
<td>0.63 (0.2-1.4)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHD</th>
<th>Enrolment (N=9)</th>
<th>month 2 (N=9)</th>
<th>month 6 (N=8)</th>
<th>month 12 (N=4)</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td><strong>Hb (g/dL)</strong></td>
<td>9.4 (7.1-12.2)</td>
<td>9.8 (9.1-14.9)</td>
<td>11.7 (9.2-14.5)</td>
<td>11.6 (11.1-12.6)</td>
</tr>
<tr>
<td><strong>Reticulocyte (10⁹/L)</strong></td>
<td>140 (52-269)</td>
<td>101 (40-210)</td>
<td>95 (72-130)</td>
<td>90 (36-148)</td>
</tr>
<tr>
<td><strong>LDH (U/L)</strong></td>
<td>629 (420-2173)</td>
<td>547 (257-1367)</td>
<td>457 (273-718)</td>
<td>414 (318-464)</td>
</tr>
<tr>
<td><strong>Unconjugated bilirubin (mg/dL)</strong></td>
<td>1.66 (0.6-3.6)</td>
<td>1.34 (0.4-2.7)</td>
<td>0.8 (0.7-1.3)</td>
<td>0.7 (0.5-0.8)</td>
</tr>
</tbody>
</table>

Values are expressed as median (range). Normal ranges: Hb: 13.6-16.7 g/dL; reticulocytes: 16-84 x 10⁹/L; LDH: 240-480 U/L; unconjugated bilirubin <0.75 mg/dL. CHD values at month +6 are shown for 8 cases (excluding 1 relapsed at month +4), and at month +12 for 4 cases (excluding 2 further relapses, 1 who has not yet reached the follow-up, and 1 who died at month +7).
Figure Legends

Figure 1 – Relapse free survival of 14 WAIHA (dotted line) and 9 CHD (continuous line) patients estimated by Kaplan-Meier method.

Figure 2 – Steroid treatment before and after LD-rituximab in AIHA patients: A) average locally weighted smoothed (lowess) of 15 AIHA patients relapsed after standard steroid treatment; B-G, typical examples of prednisone administration in six AIHA patients: Case B and C, WAIHA responders to LD-rituximab; case D and E, WAIHA responders, relapsed at month +13 and +16 after LD-rituximab; case F, CHD responder to LD-rituximab; case G, CHD non responder, relapsed at month +10 after LD-rituximab.
FIGURE 1
FIGURE 2
Low dose rituximab in adult patients with idiopathic autoimmune hemolytic anemia: clinical efficacy and biological studies

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