Efficacy of recombinant erythropoietin in autoimmune hemolytic anemia: a multicenter international study

Autoimmune hemolytic anemia (AIHA) is due to autoantibody mediated hemolysis with or without complement (C) activation.[1,2] Increasing attention has been paid to the role of bone marrow compensation,[3,4] since inadequate reticulocytosis is observed in 20-40% of cases and correlates with poor prognosis.[3,5] Moreover, features resembling bone marrow failure syndromes have been described in patients with chronic and relapsed/refractory AIHA, including dyserythropoiesis and fibrosis.[6,7] Recombinant erythropoietin (rEPO) is an established treatment of myelodysplastic syndromes, which has been used in a limited number of AIHA cases.[8,9]

In this study, we systematically evaluated the safety and efficacy of EPO treatment in a multicenter, international European cohort of AIHA patients. The protocol was approved by the Ethics Committees of Human Experimentation, and patients gave informed consent in accordance with the Declaration of Helsinki. We included 51 patients with primary or secondary AIHA treated with recombinant EPO, either alone or concomitantly with other therapies. Patients were treated from June 2007 until October 2019 at nine centers in Italy, France, Norway, Austria, UK, Denmark, and the Netherlands. Efficacy of EPO was evaluated at 15 and 30 days, and then at 3, 6 and 12 months. Response was defined as complete response (CR) (hemoglobin [Hb]>12 g/dL and normalization of all hemolytic markers), partial response (PR) (Hb>10 g/dL or at least 2 g/dL increase in Hb, and no transfusion requirement). Finally, retrospective data on AIHA diagnosis and course were obtained from clinical charts. Statistical analysis was performed using Student's *t*-test for continuous variables and χ^2 or Fisher's exact tests for categorical ones.

Table 1 shows the patients' characteristics at diagnosis: 66% of cases were aged >60 years and the male to female ratio was 1. Main AIHA types (warm, cold, mixed, and direct antiglobulin test [DAT] negative) were represented. Five cases were secondary to a lymphoproliferative disorder (two chronic lymphocytic leukemia, two Waldenstrom macroglobulinemia, and one marginal zone lymphoma), without active disease at the time of commencement of EPO. At commencement of EPO, the majority of patients (90%) had been pretreated with at least one therapy line, and the median time from diagnosis to EPO was 24 months (range: 0.03-187 months). Sixty-seven % of cases received EPO treatment because of non-response to a concomitant therapy, including steroids (n=24), rituximab (n=9), cytotoxic immune-suppressor (n=8), or sutimlimab (n=1). Patients who had been treated with rituximab received this drug within a median of 1 month (range: 0-5 months) before EPO. The hematologic parameters were similar to those observed at diagnosis, with 37% of cases displaying severe anemia and 53% lactate dehydrogenase levels >1.5xU/L. Inadequate reticulocytosis was observed in the majority of patients (31of 42). Regarding bone marrow characteristics, erythroid hyperplasia was present in the majority (hypercellularity of all lineages in 53%), with dyserythropoietic features in 40% and reticulin fibrosis in 9% of patients. A lymphoid infiltrate was demonstrated in 24 cases, being greater than 10% only in patients with underlying lymphoproliferative disease. In the primary forms, lymphoid infiltrate was polyclonal (T-cells in seven cases, B-cells in two, and mixed in six), but for five CAD patients who showed typical monoclonal CAD-associated lymphoid cells. The median endogenous EPO was 32 U/L (9.3-1,328) greater than the normal range, but inadequate in 88% of AIHA subjects considering Hb levels. Renal function was normal in all patients but two

Table 1. Patients' characteristics at diagnosis.

Data at diagnosis	All patients (N=51)
Age years, median(range)	68 (25-92)
M/F	24/27
Secondary AIHA, N(%)	5 (9)
Type of AIHA	
CAD, N(%)	21 (41)
WAIHA IgG, N(%)	11 (22)
WAIHA IgG+C, N(%)	15 (29)
MIXED, N(%)	3 (6)
DAT neg, N(%)	1 (2)
Hematologic parameters at diagnosis	
Hb g/L, median(range)	72 (40-118)
LDH U/L, median (range)	477 (174-6,000)
Ret x10^9/L, median(range)	137 (10-310)
BMRI, median(range)	77 (4-193)
Previous therapy lines	
Treated , N(%)	46 (90)
N of lines, mean+SD	2.3+1.4
steroids, N(%)	41 (80)
rituximab, N(%)	35 (68)
splenectomy, N(%)	5 (9)
immunosuppressor, N(%)	23 (45)
Time from diagnosis to EPO, months, median (range)	24 (0.03-187)
Bone marrow features	**N=32**
Cellularity %, median (range)	50 (20-95)
Hypercellularity, N(%)	17 (53)
Dyserythropoiesis, N(%)	13 (40)
Lymphoid infiltrate %, median (range)	5 (0-90)
Reticulinic fibrosis MF1, N(%)	3 (9)
Data at EPO start	**N=51**
Hematologic parameters	
Hb g/L, median(range)	85 (37-109)
LDH U/L, median (range)	344 (193-1,030)
Ret x10^9/L, median(range)	117 (11-310)
BMRI, median(range)	85 (5-222)
Concomitant therapy, N(%)	34 (67)
Time on EPO days, median (range)	188 (11-1,550)

Primary autoimmune hemolytic anemia (AIHA) was defined by hemolytic anemia and positive direct antiglobulin test (DAT), in the absence of associated overt lymphoproliferative, infectious, autoimmune, or neoplastic diseases. None of the patients had a drug-induced AIHA. Patients were classified as warm (wAIHA; DAT positive for IgG or IgG+C), cold agglutinin disease (CAD; DAT positive for C only, with high titer cold agglutinins), mixed (DAT positive for IgG+C with high titer cold agglutinins) and atypical (DAT negative, DAT positive for IgA only, warm IgM, mitogen stimulated -DAT positive only). The efficacy of the compensatory erythroblastic response was expressed as absolute reticulocyte count as well as bone marrow responsiveness index (BMRI: absolute reticulocyte count x patient's Hb/normal Hb) [Russo R, Gambale A, Langella C, *et al*. Am J Hematol. 2014;89(10):e169–e175]. M: male; F: female. Hb: hemoglobin; LDH: lacatae dehydrogenase; Ret: reticulocytes; EPO: erythropoietin.

(for whom endogenous EPO levels were not available). As shown in Figure 1A, an expected negative correlation was present between EPO and Hb levels (r=-0.64, *P*=0.0004). However, EPO levels in AIHA were reduced compared with expected values of controls with other types of anemia.[10] Moreover, EPO levels were significantly reduced in transfusion dependent patients *versus* transfusion independent ones (30 U/L, range: 8-91, *vs.* 44 U/L, range: 10-1,328; *P*=0.05). Finally, bone marrow compensatory response (BMRI) negatively correlated with EPO levels (r=-0.42, *P*=0.03), and positively with Hb (r=0.28, *P*=0.05). Regarding the efficacy of rEPO treatment (Figure 1B), the overall response rate (ORR) was 55% at 15 days, 71% at month+1, 73% at month+3, 76% at month+6, and 78% at month+12 with a progressive increase of the CR rate. The median Hb and reticulocyte increase from baseline was 24 (range: 2-83) g/L (*P*<0.001) and 25 (range: 0-220) $x10^9$/L at month+1; 30 (range: 0-94) g/L (*P*<0.001) and 33 (range: 0-352) $x10^9$/L at month+3; and 42 (9-94) g/L (*P*=0.01) and 21 (range: 0-218) $x10^9$/L at month+6. Hb increased independently of the AIHA type and number of previous therapy lines (see the *Online Supplementary Figure S1*). Response rates were greater in patients who started rEPO within the first year from AIHA diagnosis compared with patients with a longer AIHA history (85% *vs.* 64%, *P*=0.06). Likewise, a better response was observed in primary *versus* secondary AIHA patients (77% *vs.* 44%), although the difference was not statistically significant. Concerning rEPO discontinuation and outcome, 23 patients were still on EPO and 28 had discontinued treatment at the last follow-up. Reasons for discontinuation were long standing CR (n=14), suboptimal response (n=12, Hb increase <20 g/L or Hb<100 g/L), and AIHA relapse (n=2). During treatment, two patients experienced a thrombotic event (one thrombosis of a peripheral inserted central vein catheter and one pulmonary embolism concomitant to AIHA relapse in a splenectomized patient). The occurrence of thrombotic episodes is a concern in AIHA, being observed in up to 20% of patients,[1-4,11] and associated with intravascular hemolysis, complement activation, and previous splenectomy.[1-4] On the other hand, rEPO treatment has been associated with thrombotic diathesis although in our patients, other risk factors were also present.

In summary, EPO therapy was able to increase Hb levels (median Hb increase greater than 2 g/dL) in more than 70% of patients, both frontline and in relapsed/refractory chronic disease. Most responses were complete and long-lasting, allowing EPO discontinuation in about one third of responders. Almost all responses were observed between month+1 and +3, with more than half as soon as day+15. EPO efficacy was higher in patients treated within the first year from diagnosis, suggesting that the duration of the immune-suppressive therapy is particularly detrimental for bone marrow responsiveness. Accordingly, a worse response was observed in secondary forms, where both underlying condition and cytotoxic therapy may have affected bone marrow function. Additional possible confounders of EPO efficacy may be concomitant treatments or recent therapy with rituximab. However, EPO was started because of persistent non-response to these agents and an early response promptly observed, suggesting a primary role of EPO stimulation.

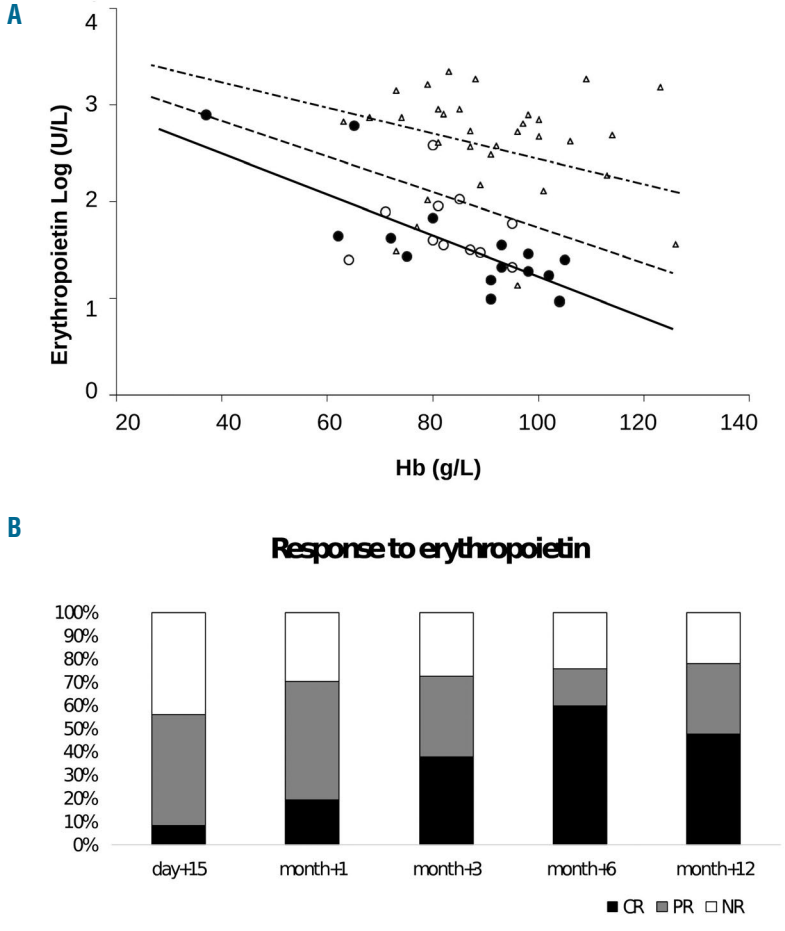

Figure 1. Relationship between endogenous erythropoietin and hemoglobin levels and response evaluation after recombinant erythropoietin treatment. (A) Continuous line shows the relationship in patients with autoimmune hemolytic anemia. Black circles represent patients responding to recombinant erythropoietin (rEPO) and white circles non responders; no significant differences were observed between these two groups. As controls, 49 aplastic anemia patients (white triangles) are shown, with the corresponding correlation (dotted-dashed line); dashed line represent patients with other types of anemia, including iron and vitamin deficiency [log(Epo)=4.478-(0.284xHb)] [Bergamaschi *et al.*, Haematologica 2008 Dec;93(12):1785-91]. (B) Overall response rate to rEPO at different time points. Patients were treated for a median of 7 months and received mainly epoetin α 40,000 U/week (n=17, 33%) or darbepoetin α 20-300 mcg/week (n=20, 39%); a minority of cases received epoetin ζ 30,000 U/week (n=6, 12%) or β 30,000 U/week (N=1, 2%). Two patients received epoetin α 4,000 U/week because of co-existent chronic kidney disease. CR: complete response: PR: partial response; NR: no response).

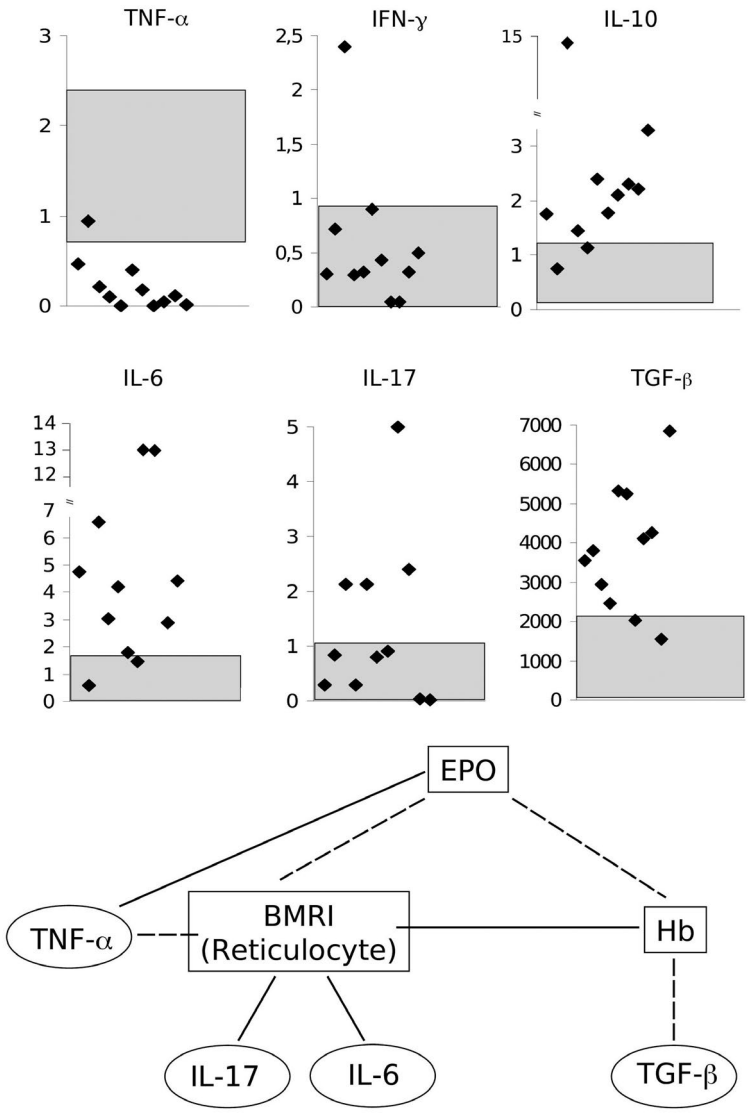

Figure 2. Cytokine serum levels in patients with autoimmune hemolytic anemia and relationship with hematologic parameters and endogenous erythropoietin levels. Upper panel: serum cytokine individual values of autoimmune hemolytic anemia (AIHA) patients before therapy with recombinant erythropoietin (rEPO). Grey areas indicate mean+1 standard deviation of 40 age and sex matched healthy controls. TNF-α level was lower in AIHA than in controls (P<0.001), whereas IL10, IL6, IL17, and TGF-β levels were all higher (P<0.001, P<0.001, P=0.014, and P=0.002, respectively); IFN-γ level was comparable between patients and controls. Cytokines were evaluated using commercialeEnzyme-linked immunosorbent assay kits. Lower panel: correlation between endogenous EPO, bone marrow reticulocytes index (BMRI), hemoglobin (Hb) and cytokines. TNF-α positively correlated with endogenous EPO (r=0.77, P=0.005), and negatively with BMRI (r=-0.052, P=0.05). Moreover, IL6 and IL17 positively correlated with BMRI (r=0.45, not significant; r=0.53, P=0.04, respectively). Similar correlations were observed for reticulocytes (TNF-α: r=-0.051, P=0.05; IL6: r=0.53, P=0.04; IL17: r=0.61, P=0.02). Finally, a negative correlation was observed between TGF-β and Hb values (r=-0.63, P=0.04). Dashed line: negative correlation; continuous line: positive correlation.

For the first time, we showed that EPO levels were inappropriately low compared to hemoglobin levels in the majority of patients. Even if reduced, endogenous EPO maintained a negative correlation with Hb values, reticulocyte counts and bone marrow responsiveness index. As expected, patients who were transfused showed lower levels of endogenous EPO, possibly indicating that the feed-back loop (anemia- hypoxia – EPO production) is intact in AIHA. Interestingly, patients with AIHA showed significantly reduced EPO values compared to other types of anemia. These findings are similar to what has been described for immune thrombocytopenia, where low or inappropriately normal levels of endogenous thrombopoietin have been demonstrated[12] and have led the way to TPO-receptor agonists use. The question is why AIHA patients show reduced EPO levels compared to other anemias. It may be speculated that reticulocyte response to hemolysis, by reversing the relative hypoxia (of the anemic state), may give a negative feed-back to the kidney. This may result in inhibition of EPO production, similar to what has been observed in transfused patients. Another possible mechanism may be due to the quick instauration of anemia in AIHA where massive erythrocyte destruction may occur in a few hours, whilst bone marrow compensation requires more time.[1-4] Finally, the existence of a "stunned bone marrow" unable to build up a prompt response to anemia as observed in patients with septic state[13] may be hypothesized. In this setting, a temporary EPO stimulation may be preferred to additional immunosuppression.

In a fraction of cases, serum levels of TNF-α, interleukin 10 (IL10), IL6, IL17, TGF-β, and IFN-α were evaluated (Figure 2) and showed a different pattern in patients *versus* matched controls. As already reported, a shift towards T-helper 2 (Th2) and T-helper 17 phenotype was observed, with reduced TNF-α and increased levels of IL6, IL10, TGF-β, and IL17, consistently with a prevalent humoral autoimmune response. Despite the limited number of cases and the known variability of cytokine levels, we found interesting correlations with endogenous erythropoietin levels. In particular, TNF-α, a known negative regulator of erythropoiesis, positively correlated with EPO levels and negatively with reticulocyte response. The Th2 cytokines IL6 and IL17 were positively related to reticulocytosis mirroring the degree of autoimmune attack. Finally, TGF-β has a negative impact on Hb levels, confirming its well-known inhibitory and detrimental effect.[14] All these cytokine abnormalities may

play a role in the inflammatory milieu and bone marrow dysregulation of AIHA. An interesting hypothesis is that an autoimmune attack may also occur against bone marrow precursors, resulting in peripheral reticulocytopenia and accounting for the severity of AIHA.[6,15] In this context, therapy with recombinant EPO may interrupt the vicious circle, by priming the reticulocyte compensation, sustaining erythropoiesis and leaving time for the resolution of the autoimmune flare avoiding excessive immune-suppression.

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Contributions: B F and W B designed the study, followed patients, collected and analyzed data, wrote the paper and revised the paper for important intellectual content. MM, JG, SG, HF, JMIV, FRM, BJ, AP, FZ, AH, and SB followed patients, collected data, and revised the paper for important intellectual content. AZ performed cytokines studies and revised the paper for important intellectual content.

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