haematologica
Journal of the Ferrata Storti Foundation

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Received: November 29, 2024.
Accepted: June 3, 2025.

Citation: Chen Mei, Gaixiang Xu, Cuiping Zheng, Yaping Xie, Minming Li, Yanping Shao, Rongxin Yao, Shi Tao, Wei Jiang, Jun Guo, Zhiyin Zheng, Wei Wang, Xinping Zhou, Liya Ma, Li Ye, Yingwan Luo, Chunmei Yang, Wenjuan Yu, Wanzhuo Xie, Jie Jin and Hongyan Tong. Recombinant human erythropoietin plus all-trans retinoic acid and testosterone undecanoate for the treatment of anemia in patients with lower-risk myelodysplastic syndromes: a multicenter, single-arm, prospective trial.
Haematologica. 2025 June 12. doi: 10.3324/haematol.2024.287055 [Epub ahead of print]

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Running Title: A triple therapy for LR-MDS patients with anemia

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Clinical Trial Registration

This study was registered with the Chinese Clinical Trial Registry (http://www.chictr.org.cn, identifier ChiCTR2000032845) on 13 May 2020, prior to participant enrollment.

Author contributions

HT and CM designed the study; CM, GX, CZ, and YX analyzed the date; CM and GX wrote the manuscript. CM, GX, WW, YL, and LY performed the research; CZ, YX, ML, YS, RY, ST, WJ, JG, ZZ, XZ, LM, CY, WY, and WX collected the data; JJ and HT guided the project design and provided administrative support; all authors reviewed and approved the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Funding

This work was supported by the Key R&D Program of Zhejiang province (2024C03164). The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report.

Acknowledgments

The authors thank the patients, their families, and medical staff who participated in this study. We acknowledge Maryam Maleki Goli and Parnia Ghanad (both from Zhejiang University, School of Medicine, Hanagzhou, China) for their assistance with language editing.

Abstract

Erythropoiesis-stimulating agents (ESAs) achieve hematological improvement-erythroid (HI-E) in only 30% of ESA-naïve lower risk myelodysplastic syndrome (LR-MDS) patients with anemia, highlighting the need for developing novel drugs or new treatment strategies to improve the outcome of these patients. We conducted this multicenter, single-arm trial to investigate the efficacy and safety of a triple regimen consisting of recombinant human erythropoietin (rhEPO), all-trans retinoic acid (ATRA) and testosterone undecanoate in patients with anemia due to lower-risk MDS based on Revised International Prognostic Scoring System. Eligible patients received rhEPO 10000 IU/day, oral ATRA 25 mg/m^2/day and oral testosterone undecanoate 80 mg twice daily for 12 weeks. The primary endpoint was the proportion of patients achieving HI-E during 12 weeks of treatment. Of 52 eligible patients, 32 (61.5%, 95%CI 48.0%-73.5%) achieved HI-E, meeting the primary endpoint. Fifteen patients (65.2% [15/23]) with baseline serum erythropoietin ≤500 IU/L had HI-E *versus* 58.6% of those (17/29) with baseline serum erythropoietin >500 IU/L. More patients with very low or low risk had HI-E than those with intermediate risk (73.3% *vs.* 45.5%, P = 0.041) and fewer patients with mutated *ASXL1* had HI-E than those with wildtype *ASXL1* (33.3% *vs.* 70.0%, P = 0.040). The regimen had an acceptable safety profile compatible with individual agents. In conclusion, the triple regimen of rhEPO combined with ATRA and testosterone undecanoate attained HI-E in approximately 61.5% of patients regardless of baseline serum EPO levels, supporting further development of this regimen for LR-MDS patients with anemia. This study was registered at CHICTR.ORG.CN as ChiCTR2000032845.

Introduction

Myelodysplastic syndromes (MDS) are a group of clonal myeloid disorders that are characterized by ineffective hematopoiesis, persistent peripheral cytopenias, and a risk of progression to acute myeloid leukemia (AML). Symptomatic anemia is the most common cytopenia in lower-risk MDS (low or intermediate-1 risk per the International Prognostic Scoring System [IPSS] or very low, low, or intermediate-risk per the revised IPSS [IPSS-R])[1] and is associated with red blood cell (RBC) transfusion requirement, poor health-related quality of life (HRQoL) and multiple comorbidities such as cardiovascular diseases[2]. Worsening anemia could render lower-risk MDS patients eventually dependent on RBC transfusions and negatively impacts on HRQoL and overall survival[3, 4].

Treatment goals for patients with lower-risk MDS include transfusion independence, improvement in cytopenia and hemoglobin levels, and amelioration of HRQoL[5]. Though the landscape of treatment for lower-risk MDS is rapidly evolving with the advent of transforming growth factor-β (TGF-β) inhibitor luspatercept, telomerase inhibitor imetelstat, lenalidomide, and hypomethylating agents[6-8], they are not broadly applicable to lower-risk MDS patients with anemia. For instance, lenalidomide is approved for lower-risk MDS patients with chromosome 5q deletion (del[5q]) while luspatercept is approved for MDS with ring sideroblasts (MDS-RS). Currently, erythropoiesis-stimulating agents (ESAs) including recombinant human erythropoietin (rhEPO) are the first-line treatment for non-del(5q) lower-risk MDS patients with anemia. They target early erythropoiesis by stimulating erythropoietin-responsive erythroid precursor proliferation. However, ESAs sustain a meaningful improvement in hemoglobin levels in only a small proportion of lower-risk MDS patients[5, 6, 9, 10]. Higher serum erythropoietin levels, especially when higher than 500 IU/L, are associated with lower response rates. Presently, ESA monotherapy is not recommended for patients with serum erythropoietin levels above 500 IU/L[5, 6]. Given limited treatment options,

new treatment strategies are needed for anemia in lower-risk MDS patients.

An early interest in all-trans retinoic acid (ATRA) for the treatment of MDS emerged following the exemplary success of ATRA as a differentiation-induction therapy for acute promyelocytic leukemia, due to its role in regulating normal hematopoiesis by promoting hematopoietic cell differentiation and enhancing erythroid colony formation[11-15]. However, a flurry of trials revealed that ATRA only yielded modest activities in improving anemia in MDS[16]. It is of note that ATRA, when added to rhEPO, led to clinically significant erythroid responses in nearly half (48.0%, 13/27) of the patients with lower-risk MDS, which were sustained in over half of the responders (53.8%, 7/13) at 13 months of follow-up[17]. Testosterone undecanoate, a type of androgen, is known to be a potent stimulator of erythropoiesis by acting directly on bipotential hematopoietic precursor cells and potentially increasing the sensitivity of erythroid progenitor cells to erythropoietin[18-20]. The combination therapy with ATRA and androgens has also been attempted for the treatment of anemia in patients with MDS[21-23]. Prompted by these findings and given the fact that the currently available treatment options fail to elicit a hematological improvement-erythroid (HI-E) response in a sizable proportion of lower-risk MDS patients with anemia, we hypothesized that a triple regimen consisting of rhEPO, ATRA and testosterone undecanoate could potentially improve the proportion of lower-risk MDS patients with anemia to achieve an HI-E response. This trial was conducted as a part of a prospective study to evaluate the efficacy and safety of rhEPO plus ATRA and testosterone undecanoate for lower-risk MDS patients with anemia.

Methods

Study design and patients

This investigator-initiated, prospective, single-arm trial enrolled adult patients (18-80 years)

with primary MDS according to the World Health Organization (WHO) 2016 criteria[24] at 10 Chinese centers. The key inclusion criteria were: IPSS-R-defined very low, low, or intermediate-risk MDS[25]; bone marrow blasts < 5%; baseline hemoglobin < 100 g/L, regardless of erythropoietin levels and RBC transfusion support; Eastern Cooperative Oncology Group (ECOG) performance status of 0–2; and adequate renal and hepatic function. The main exclusion criteria included del(5q) cytogenetic abnormality, prior stem cell transplant, and recent treatment with ESA, immunosuppressants, lenalidomide, or hypomethylating agents prior to enrolment. The full eligibility criteria are available in Supplementary Methods.

The study protocol was approved by the Clinical Research Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine (Reference number: 197). The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines. All patients provided written informed consent before any trial-related activities. This trial is registered with Chinese Clinical Trial Registry (ChiCTR2000032845).

Procedures

Patients received rhEPO 10,000 IU/day subcutaneously, oral ATRA 25 mg/m^2/day and oral testosterone undecanoate 80 mg twice daily for 12 weeks. Dose modification based on biweekly measurement of hemoglobin values was allowed according to a prespecified algorithm. After 12 weeks of treatment, patients with clinical response (HI-E response or transfusion independence) could continue triple treatment until disease progression or unacceptable toxicities. Patients who completed or discontinued treatment were followed up for 8 weeks.

Assessments and Endpoints

Baseline assessments included bone marrow morphology, cytogenetics, flow cytometry, and

next-generation sequencing of 20 MDS-related genes (**Supplementary table 1**). Hemoglobin levels, transfusion requirements, and safety were assessed biweekly throughout the study period. Adverse Events (AEs) were monitored using the National Cancer Institute Common Terminology Criteria of Adverse Events version 4.0 (NCI CTCAE v4.0).

The primary efficacy endpoint was the proportion of patients who had achieved an HI-E response, defined as a hemoglobin increase ≥ 15 g/L from baseline lasting for ≥ 8 consecutive weeks in the absence of RBC transfusions during 12 weeks of treatment according to the modified 2006 International Working Group (IWG) response criteria[26]. Secondary endpoints included the median time to HI-E response and progression to higher-risk disease. Exploratory endpoints included achieving RBC transfusion independence for ≥ 8 weeks and progression to AML.

Statistical analysis

The sample size was calculated using the Simon two-stage optimal design[27], aiming to differentiate an erythroid response rate of 60% from a minimal erythroid response rate of 40%, with 80% power, a one-sided α of 0.05, and a 10% dropout rate, resulting in approximately 52 evaluable patients. In this intention-to-treat study, efficacy analyses used the full analysis set (FAS), supported by the per-protocol set (PPS). Summary statistics were employed to describe patients' demographics and laboratory measurements, and a logistic regression model was utilized to identify risk factors for HI-E response. Post hoc subgroup efficacy analyses included age, hemoglobin, IPSS-R risk, and molecular profiles. Methodological details are provided in Supplementary Methods.

Results

Patient baseline and treatment characteristics

Between July 26, 2020 and August 4, 2023, of 55 patients assessed for eligibility, two did not

meet the eligibility criteria, and one withdrew before the start of treatment. Finally, 52 patients who received study medications were included in the FAS. The PPS included 50 patients after exclusion of one patient who withdrew from the study due to lack of efficacy and one who discontinued the study treatment due to severe AEs (**Figure 1**). The median age of the patients in the FAS was 65 years (range 20-78) and 57.7% (30/52) of the patients were male. The median hemoglobin (Hb) was 62 g/L (range 40-91), with 30 patients (57.7%) having Hb ≥ 60 g/L and 22 patients (42.3%) having Hb <60 g/L. Baseline serum erythropoietin (EPO) levels were distributed as follows: 55.8% (29/52) of patients had levels > 500 IU/L, 15.4% (8/52) had levels between > 200 and ≤ 500 IU/L, and 28.8% (15/52) had levels ≤ 200 IU/L. With regard to the IPSS-R categories, 57.7% (30/52) and 42.3% (22/52) of the patients were categorized in the very low or low risk group and the intermediate risk group, respectively. Twenty-four patients (46.2%) had MDS-RS, 5 (9.6%) had MDS-SLD, and 23 (44.2%) had MDS-MLD. Twenty-five patients (48.1%) had no transfusion burden (NTB), defined as receiving no RBC transfusion in 8 weeks before treatment, and 27 (51.9%) had a transfusion burden (TB), defined as receiving 1 or more RBC units within 8 weeks before treatment. Most patients (47/52, 90.4%) had no prior ESA therapy, and no patient previously received lenalidomide, luspatercept, imetelstat or hypomethylating agents (HMAs). Next generation sequencing showed that *SF3B1* was mutated in 40.4% (21/52) of the patients. Other mutated genes included *ASXL1* (12/52, 23.1%), *TET2* (10/52, 19.2%), *U2AF1* (7/52, 13.5%), *DNMT3A* (7/52, 13.5%), and *SRSF2* (5/52, 9.6%) (**Table 1**).

Efficacy

In the FAS, 32 of 52 patients (61.5%, 95% CI 48.0%-73.5%) achieved an HI-E response lasting for ≥ 8 consecutive weeks during 12 weeks of treatment, thus rejecting the null hypothesis. The median follow-up time was 20 weeks (rang 4-20 weeks) after treatment initiation. The median time to achieve an HI-E response was 40 days (95% CI 34.4-45.5

days). In the PPS, 64.0% of the patients (32/50, 95% CI 50.1%-75.9%) achieved an HI-E response lasting for ≥ 8 consecutive weeks during 12 weeks of treatment. Among responders achieving HI-E, the post-treatment median Hb was 95 g/L (range: 68-152) compared to 73 g/L (range 46-91) in non-responders. Patients with baseline Hb ≥ 60 g/L achieved a post-treatment median Hb of 92 g/L (range 69-152), whereas those with baseline Hb <60 g/L had a lower post-treatment median Hb of 73 g/L (range 46-106). Fifteen of 23 patients (65.2%) with baseline serum erythropoietin ≤ 500 IU/L had an HI-E response as compared to 58.6% of those (17/29) with baseline serum erythropoietin > 500 IU/L (P = 0.627). They had a shorter median time to an HI-E response as compared with those with baseline serum erythropoietin > 500 IU/L (27 days, 95% CI 16.9-37.1 days *vs.* 44 days, 95% CI 34.9-53.1 days, P = 0.051). When evaluated according to the baseline transfusion burden, 63% (17/27) of the patients with transfusion burden achieved RBC transfusion independence (RBC-TI) lasting for ≥ 8 consecutive weeks. Furthermore, 55.6% (15/27) of the patients were transfusion-free for ≥ 8 weeks, and 48.1% (13/27) maintained RBC-TI lasting for ≥ 12 weeks. The maximum percentage change from baseline over 8 weeks in patients with RBC transfusion burden is shown in **Figure 2**. Among NTB patients at baseline, the median hemoglobin level was 72 g/L (range: 60-91), which increased to a post-treatment median hemoglobin of 92 g/L (range: 69-152).

In post hoc subgroup analysis, when evaluated according to the IPSS-R risk category, the percentages of patients with an HI-E response were significantly higher in patients with very low or low risk than those with intermediate risk (73.3% [22/30, 95% CI 55.6%-85.8% *vs.* 45.5% [10/22, 95% CI 25.1%-67.3%], P = 0.041). Additionally, the HI-E response rates were comparable between patients with and without ring sideroblasts (RS) (61.5% [16/26] vs. 61.5% [16/26]; P =1.000). No statistical difference was observed in the HI-E response rate when the patients were evaluated according to prior use of ESAs (yes *vs.* no), age (< 60 years *vs.* ≥ 60

years), serum hemoglobin level (< 60 g/L *vs*. ≥ 60 g/L), serum ferritin level (< 500 ng/mL *vs.* ≥ 500 ng/mL), bone marrow fibrosis grade (0 *vs.* 1-3), bone marrow blast percentage (≤ 2% vs. > 2 - < 5%), MDS subtypes (RS *vs.* SLD/MLD), IPSS-R karyotype (very good/good *vs.* intermediate), and baseline transfusion burden (no *vs.* yes) (**Figure 3**).

The patients were also evaluated according to gene mutational status. Patients with mutated *ASXL1* had a significantly lower HI-E response rate than those with wildtype *ASXL1* (33.3% [4/12, 95% CI 11.3%-64.6%] *vs.* 70.0% [28/40, 95% CI 54.6%-81.9%], $P = 0.040$). The percentages of patients with an HI-E response were higher in patients harboring *SF3B1* mutation than those with wildtype *SF3B1*, but without statistical difference (71.4% [15/21, 95% CI 50.0%-86.2%] *vs.* 54.8% [17/31, 95% CI 37.8%-70.8%], $P = 0.228$). No statistical difference was observed in the percentages of patients with an HI-E response with wildtype *TET2*, *U2AF1*, *DNMT3A,* and *SRSF2* as compared to those with mutated *TET2*, *U2AF1*, *DNMT3A,* and *SRSF2*.

Safety

All 52 patients received at least one dose of the study medications and were included in the safety set. Fifty patients completed 12 weeks of treatment; two patients withdrew after receiving 4 and 8 weeks of treatment, respectively.

Primary toxicity data were systematically collected throughout the entire 20-week study period, including the initial 12-week treatment phase and the subsequent 8-week safety follow-up. Treatment-related AEs (TRAEs) of any grade occurred in all 52 patients (100%). Fatigue was the most frequently reported TRAE (38.4%), followed by dry skin (36.5%), headache (15.4%), edema (13.4%) and hypertriglyceridemia (11.5%). Grade 3 TRAEs occurred in 4 patients (7.7%). Grade 3 infections (pneumonia or skin and soft tissue infections) occurred in 3 patients (5.8%) and deep vein thrombosis was reported in 1 patient (1.9%) (**Table 2**). Three patients (5.8%) had dose reduction, and one (1.9%) discontinued

treatment due to grade 3 deep vein thrombosis. No new or worsening toxicities attributable to the triple regimen were observed during the 20-week study period. No progression to higher-risk MDS or AML, and no death was reported throughout the study period.

Discussion

Current treatment options for lower-risk MDS patients with anemia remain limited. In this trial, the triple regimen of rhEPO plus ATRA and testosterone undecanoate achieved an HI-E response lasting for ≥ 8 consecutive weeks during 12 weeks of treatment in 61.5% of the patients with non-del (5q) lower-risk MDS with anemia, meeting the primary study endpoint. Besides, the triple regimen was well-tolerated, and the toxicity profiles were consistent with those of individual components in the regimen. No unexpected toxicities emerged during the trial. The findings indicated that the study regimen could offer an effective and safe therapeutic option for lower-risk MDS patients with anemia, supporting further exploration of the regimen in advanced trials.

Currently, ESA monotherapy as the first-line treatment for non-del(5q) lower-risk MDS patients with anemia remains unsatisfactory. In a randomized trial, epoetin-alpha led to an HI-E response in 31.8% of the patients with lower-risk MDS patients with no or moderate transfusion burden[28]. In a separate trial, 14.7% of lower-risk MDS patients with no or moderate transfusion burden who were treated with darbepoetin alfa had an HI-E response[10]. These findings highlight the inadequacy of ESA monotherapy for lower-risk MDS patients with anemia. In a study on dual agent therapy for lower-risk MDS patients with anemia, ATRA plus rhEPO led to clinically significant erythroid responses, defined as increases of hemoglobin levels of ≥ 1 g/dL or reduction of transfusion needs, in nearly half (48.0%, 13/27) of the patients with lower-risk MDS, which were sustained in over half of the responders (53.8%, 7/13) at 13 months of follow-up[17], suggesting that combination therapy might lead to

improved treatment response among patients with lower-risk MDS patients with anemia. In our study, 61.5% (32/52) of the patients achieved an HI-E response lasting for ≥ 8 consecutive weeks during 12 weeks of treatment, and the response rates were 68% (17/25) in patients with no transfusion burden at baseline and 55.6% (15/27) in those with transfusion burden. While these response rates numerically exceed historical ESA monotherapy data[10, 28], our regimen yielded a lower post-treatment median hemoglobin level (95 g/L) compared to previous studies (106 g/L). Cross trial comparisons are inherently limited by differences in trial design and patient populations. For instance, our study utilized higher ESA dosing (70,000 IU/week vs ≤ 40,000 IU/week in ESA monotherapy trials) and shorter response evaluation period (12 weeks vs 24-48 weeks in ESA trials). Key population differences included a lower baseline median Hb level in our study (62 g/L vs 90 g/L in ESA trials), the absence of EPO level restrictions (55.8% > 500 IU/L vs 0% in ESA trials), distinct genetic profiles (0% del[5q] MDS vs 3.8-8.9% in ESA trials), prior ESA exposure in 9.6% of patients (vs 0% in ESA trials), and a more varied IPSS-R risk distribution (42.3% intermediate-risk vs 8.9-54.6% in ESA trials). Additionally, differing definitions of transfusion dependence across studies further limit direct efficacy comparisons. Rigorous clinical trials are required to determine whether the triple regimen is more effective than ESA monotherapy or ESA-based dual agent therapy for lower risk MDS patients with anemia.

The effectiveness of treatment for non-del(5q) MDS patients with anemia is impacted by serum erythropoietin levels, with a strong inverse correlation with hemoglobin levels[29-31] and a lower response rate for those with > 500 IU/L[6]. Given that only 10-20% of patients with serum erythropoietin levels above 500 IU/L would likely respond to ESAs[32, 33], the National Comprehensive Cancer Network guidelines do not recommend ESA monotherapy for this patient subpopulation[34]. Nevertheless, it is interesting to note that in our study, patients were included regardless of baseline serum erythropoietin levels, and there was no statistical

difference in the HI-E response rates and the median time to achieve an HI-E response between patients with serum erythropoietin levels > 500 IU/L and those ≤ 500 IU/L. The two randomized trials of epoetin-alpha and darbepoetin alfa excluded patients with baseline serum erythropoietin ≥ 500 □IU/L[10, 28] while our study included patients regardless of baseline serum erythropoietin levels. In a prospective study, ATRA plus rhEPO led to an HI-E response in 19% of the patients with baseline serum erythropoietin > 500□ IU/L[35]. In our study, 58.6% (17/29) of the patients with baseline serum erythropoietin > 500□ IU/L showed an HI-E response to the triple regimen, suggesting that the study regimen could also benefit this subgroup of lower-risk MDS patients. Our analysis further revealed that HI-E rates were not significantly dependent on Nordic Score prognostic factors[36]. However, the response rates observed in our cohort differed from those reported by Fenaux et al. (e.g., High Nordic Score: 68.8% vs. 44.7%), which may be due to variations in study design and patient populations. We speculated that the triple regimen may improve or restore the response to ESAs in lower risk MDS patients with anemia with baseline serum erythropoietin > 500□ IU/L, possibly due to synergy among the individual agents or enhancement of erythroid colony formation by ATRA and increment of sensitivity to erythropoietin by testosterone undecanoate[12, 37]. The mechanisms underlying these clinical observations require further in-depth studies.

Our post hoc subgroup analysis also showed that the triple regimen conferred benefits across diverse subgroups of patients with regards to prior use of ESAs, age, baseline serum hemoglobin level, baseline serum ferritin level, baseline serum erythropoietin levels, bone marrow fibrosis, bone marrow blast percentage, MDS subtypes, IPSS-R karyotype, and baseline transfusion burden. One notable exception is the IPSS-R risk category. Paitents with intermediate risk had a significantly lower HI-E response rate than those with very low or low risk (45.5% *vs.* 73.3%, P = 0.041). The finding is consistent with a previous study showing higher IPSS-R risk may be associated with a lower response rate to ESAs[38], and

such patients are recommended to receive alternative treatments. Patients with lower-risk MDS-RS have limited treatment options, except luspatercept[39, 40], if they do not respond to ESAs. In the current trial, two thirds (66.7% [16/24] of the MDS-RS patients responded to the triple regimen, suggesting that the triple regimen could be an effective treatment option for lower-risk MDS-RS patients with anemia.

SF3B1 was the most frequently mutated gene in the study cohort, occurring in 40.4% of the patients. Despite a higher HI-E response rate in patients with mutated *SF3B1*, no statistical difference was observed from that of patients with wildtype *SF3B1* (71.4% *vs.* 54.8%). This trend is similar to the differential efficacy of luspatercept, approved for lower-risk MDS with *SF3B1* mutation[41], which demonstrated superior erythroid response in *SF3B1*-mutated versus wildtype patients (70% vs. 42%)[40]. In contrast, epoetin alfa monotherapy in the COMMANDS trial obtained similar erythroid response rates in patients with and without *SF3B1* mutation (31% *vs.* 32%)[40]. Cross-trial comparisons must be interpreted with caution. The COMMANDS trial exclusively enrolled transfusion-dependent patients requiring 2-6 RBC units every 8 weeks pre-treatment and utilized stringent endpoints defined as RBC transfusion independence for at least 12 weeks with a concurrent mean hemoglobin increase of at least 1.5 g/dL (weeks 1-24)[40]. In contrast, our study included both transfusion-independent and dependent patients, and applied IWG 2006 criteria (≥ 8-week HI-E within 12 weeks). The non-randomized design of our study limits definitive attribution of efficacy differences to the triple regimen, rather than to inherent population biases. Although the higher HI-E response rate with the triple regimen for *SF3B1*-mutated patients suggests that it could be a promising treatment option, similar to luspatercept, this finding remains exploratory. Randomized controlled trials directly comparing EPO-based combinations with luspatercept in molecularly stratified cohorts are essential to clarify their therapeutic roles.

ASXL1 is mutated in approximately 20% of patients with MDS and a predictor of an

adverse prognostic outcome[42]. In the current trial, *ASXL1* was mutated in 23.1% of the patients and was associated with a significantly lower HI-E response rate as compared with wildtype *ASXL1* (33.3% *vs.* 70.0%, $P = 0.040$). In the W-JHS MDS01 trial, *ASXL1* mutation was associated with a worse response rate to ESA monotherapy in patients with baseline serum erythropoietin levels ≥ 100 IU/L (mutated *ASXL1* 0% *vs.* wildtype *ASXL1* 60.0%)[43]. *ASXL1* loss was shown to hinder erythroid development and differentiation, indicating that ineffective erythropoiesis of MDS may occur as a result of *ASXL1* mutation[44]. However, the precise mechanism with regards to poor response to ESAs could not be identified for *ASXL1*-mutated subjects and needs to be explored in future studies. Therefore, when lower-risk MDS patients have a predictor of poor response such as *ASXL1* mutation, especially when concurrent with high baseline serum erythropoietin levels, it would be advisable to initiate treatment with other therapeutic options.

The study has several limitations. The trial design was a single-arm study with no comparator. The study findings await confirmation in randomized controlled trials with a larger patient population. Most patients had a relatively short diagnostic duration (≤6 months) and received no prior treatment, which may limit the conclusions of the study. Sustaining an HI-E response remains an important goal of treatment for lower-risk MDS with anemia and impacts on the HRQoL of the patients. Given the short duration of the study, the durability of the treatment response observed in the patients and the long-term safety of this combination therapy remained unknown. While the IWG 2006 criteria were rigorously applied, there has been limited clinically meaningful improvement in some patients, highlighting the need for future trials to adopt more comprehensive response criteria to better evaluate therapeutic impact and clinical benefit.

In conclusion, the triple regimen of rhEPO, ATRA and testosterone undecanoate led to an HI-E response in 61.5% of the patients with lower-risk MDS with anemia regardless of

baseline serum erythropoietin levels and demonstrated broad activity across diverse patient subgroups except those in the IPSS-R intermediate-risk category or with mutated *ASXL1*. The triple regimen could offer a meaningful treatment option for lower-risk MDS patients with anemia. These findings should be taken into consideration for the design of randomized, controlled trials aimed at evaluating the efficacy and safety of the triple regimen for lower-risk MDS.

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Table 1. Patient demographic and baseline characteristics-FAS

Characteristics	N=52
Median age in years (range)	65(20-78)
≥ 60, n (%)	35(67.3)
< 60, n (%)	17(32.7)
Sex, n (%)	
Male	30(57.7)
Female	22(42.3)
Time since MDS diagnosis, n (%)	
> 6 months	4 (7.7)
≤ 6 months	48 (92.3)
Prior use of ESAs, n (%)	
Yes	5 (9.6)
No	47 (90.4)
ECOG performance status, n (%)	
0	25 (48.1)
1	21 (40.4)
2	6 (11.5)
White blood cell count ($\times 10^9$/L), median (range)	3.0(1.8-9.9)
Neutrophil count ($\times 10^9$/L), median (range)	1.51(0.8-5.57)
Platelet count ($\times 10^9$/L), median (range)	133(39-399)
Hemoglobin level (g/L), median (range)	62(40-91)
≥ 60, n (%)	30(57.7)
< 60, n (%)	22(42.3)
LDH, IU/L, n (%)	
≤ 250	40(76.9)
> 250	12(23.1)
Serum ferritin concentration, ng/mL, n (%)	
≥ 500	35(67.3)
< 500	17(32.7)
Baseline serum erythropoietin level, IU/L, n (%)	
> 500	29(55.8)
> 200 - ≤ 500	8(15.4)
≤ 200	15(28.8)
Bone marrow fibrosis, n (%)	
Grade 0	39(75.0)
Grade 1-3	13(25.0)
Bone marrow blast, n (%)	
≤ 2%	40(76.9)
> 2 - < 5%	12(23.1)
WHO (2016) classification of MDS, n (%)	
MDS-SLD	5(9.6)
MDS-RS	24(46.2)
MDS-MLD	23(44.2)
Ring sideroblasts (RS), n (%)	
RS-positive*	26 (50.0)
RS-negative□	26 (50.0)
IPSS-R karyotype, n (%)	
Very good/good	40(76.9)
Intermediate	12(23.1)
IPSS-R risk category, n (%)	
Very low/low	30(57.7)
Intermediate	22(42.3)
RBC transfusion burden, n (%)	
No transfusion burden†	25(48.1)
Transfusion burden‡	27(51.9)
Selected gene mutations, n (%)	
SF3B1	21(40.4)
ASXL1	12(23.1)
TET2	10(19.2)
U2AF1	7(13.5)
DNMT3A	7(13.5)
SRSF2	5(9.6)

Abbreviations: FAS, Full Analysis Set; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; MDS-MLD, myelodysplastic syndrome (MDS) with multilineage dysplasia; MDS-RS, MDS with ring sideroblasts; MDS-SLD, MDS with single lineage dysplasia;

RBC, red blood cells; WHO, World Health Organization; RS, Ring sideroblasts; IPSS-R, International Prognostic Scoring System-Revised;

*RS-positive is defined as the presence of ring sideroblasts in patients, including those diagnosed with MDS-RS and patients exhibiting RS without fulfilling the diagnostic criteria for MDS-RS.

RS-negative is defined as the absence of ring sideroblasts in patients.

†No transfusion burden is defined as no RBC transfusion within 8 weeks before study treatment.

‡Transfusion burden is defined as requiring 1 or more RBC units within 8 weeks before study treatment.

Table 2 Treatment-related adverse events in the safety set

AEs	Grade 1-2	Grade 3
Fatigue	20(38.5)	0
Dry skin	19(36.5)	0
Headache	8(15.4)	0
Edema peripheral	7(13.4)	0
Hypertriglyceridemia	6(11.5)	0
Mucositis	5(9.6)	0
Nausea	5(9.6)	0
Liver enzymes increased	4(7.7)	0
Infection	0	3(5.8)
Rash	1(1.9)	0
Myalgias	1(1.9)	0
Hypertension	1(1.9)	0
Creatinine increased	1(1.9)	0
Deep vein thrombosis	0	1(1.9)

No grade 4 or 5 treatment-related adverse events.
Abbreviations: AEs, adverse events

Figures legend

Figure 1 Patient disposition chart.

Figure 2 Maximum percentage change in red blood cell (RBC) transfusion burden in previously transfused patients (n = 27). Of these 27 patients, 13 (48.1%) had a low transfusion burden (LTB), defined as receiving <4 RBC units in 8 weeks, and 14 (51.9%) had a high transfusion burden (HTB), defined as receiving ≥ 4 RBC units in 8 weeks. RBC, red blood cell; TD, transfusion dependence; TI, transfusion independence.

Figure 3 Forest plots of HI-E responses in subgroups of patients. HI-E, hematological improvement-erythroid; ESAs, erythropoiesis-stimulating agents; WHO, World Health Organization; MDS-RS, MDS with ringed sideroblasts; MDS-SLD/MLD, MDS with single lineage dysplasia/multilineage dysplasia; IPSS-R, Revised International Prognostic Scoring System; RBC, red blood cell; mut, mutation; wt, wildtype.

Figure 1

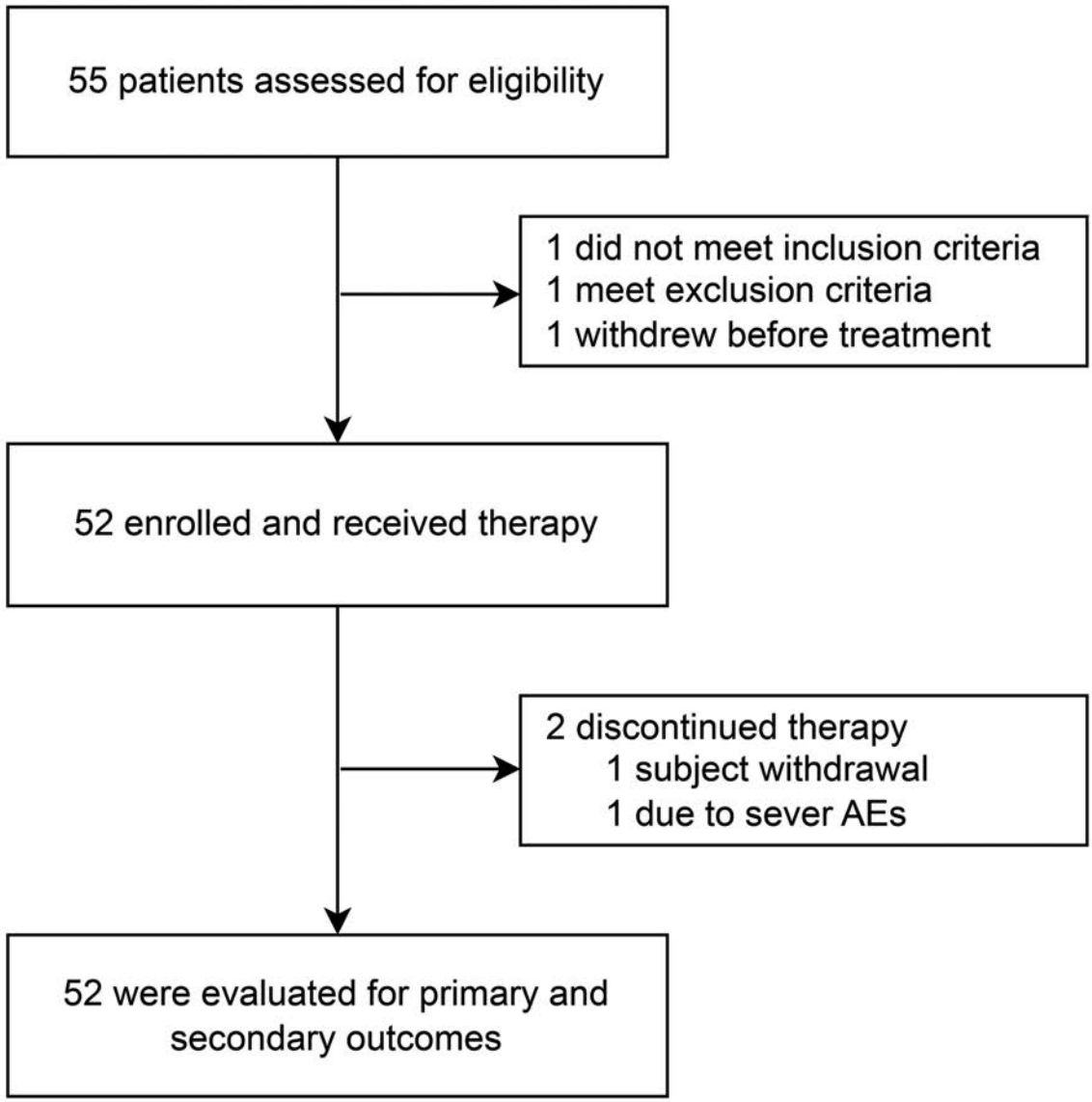

55 patients assessed for eligibility
1 did not meet inclusion criteria
1 meet exclusion criteria
1 withdrew before treatment
52 enrolled and received therapy
2 discontinued therapy
1 subject withdrawal
1 due to sever AEs
52 were evaluated for primary and secondary outcomes

Figure 2

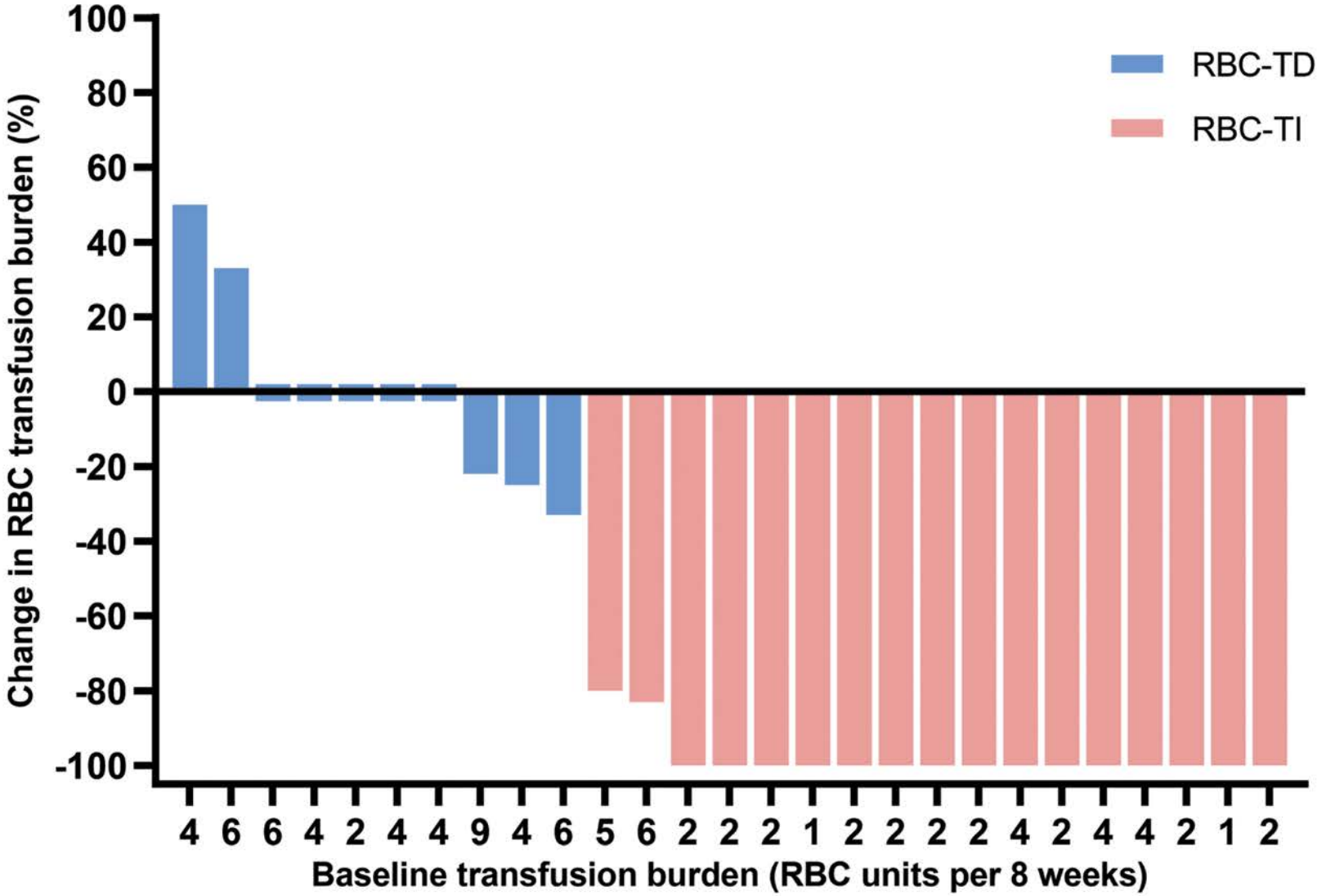
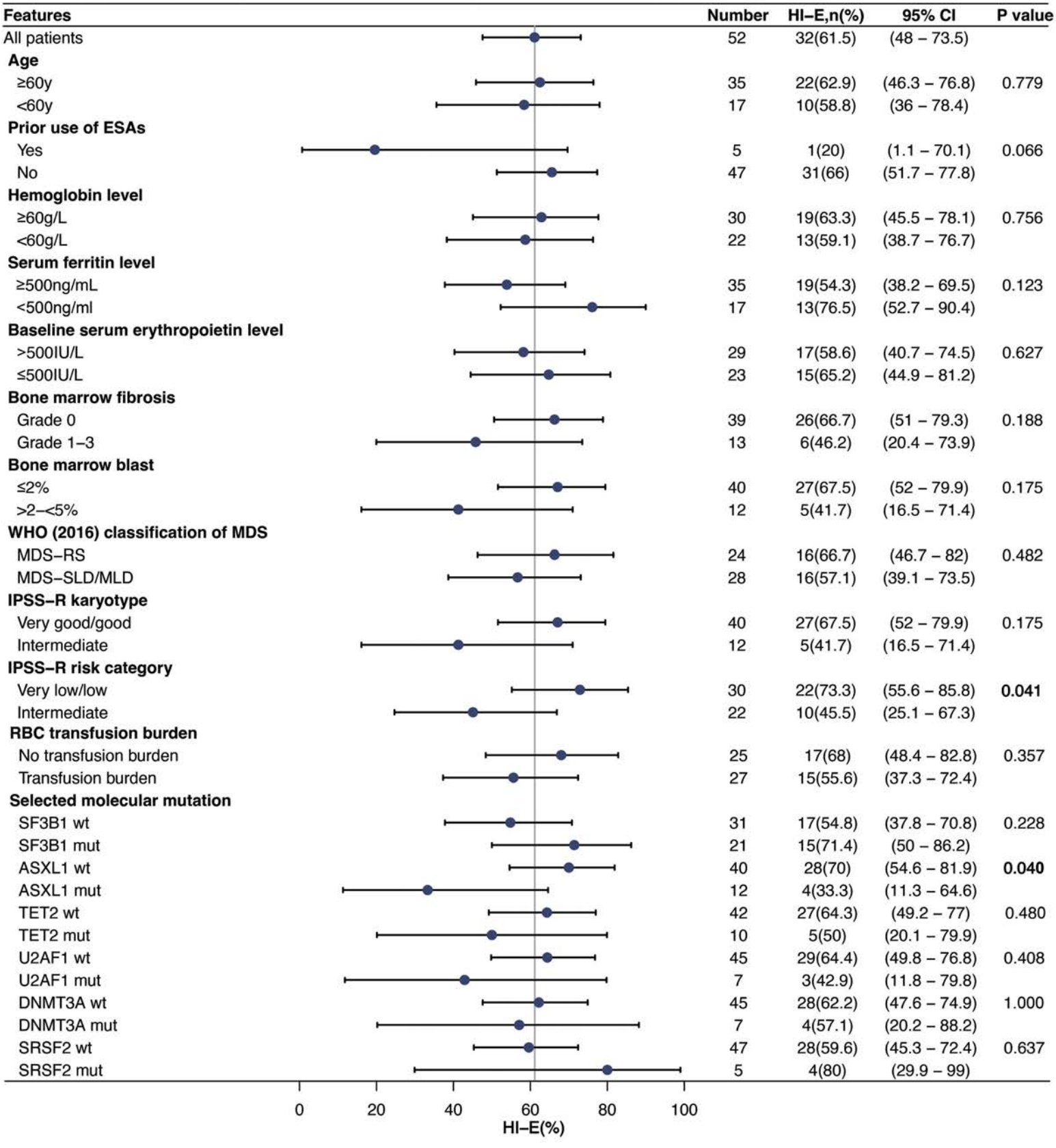

RBC-TD
RBC-TI
Change in RBC transfusion burden (%)
100
80
60
40
20
0
-20
-40
-60
-80
-100
4 6 6 4 2 4 4 9 4 6 5 6 2 2 2 1 2 2 2 2 4 2 4 4 2 1 2
Baseline transfusion burden (RBC units per 8 weeks)

Figure 3


Features
Number
HI–E,n(%)
95% CI
P value
All patients 52 32(61.5) (48 – 73.5)
Age
≥60y 35 22(62.9) (46.3 – 76.8) 0.779
<60y 17 10(58.8) (36 – 78.4)
Prior use of ESAs
Yes 5 1(20) (1.1 – 70.1) 0.066
No 47 31(66) (51.7 – 77.8)
Hemoglobin level
≥60g/L 30 19(63.3) (45.5 – 78.1) 0.756
<60g/L 22 13(59.1) (38.7 – 76.7)
Serum ferritin level
≥500ng/mL 35 19(54.3) (38.2 – 69.5) 0.123
<500ng/ml 17 13(76.5) (52.7 – 90.4)
Baseline serum erythropoietin level
>500IU/L 29 17(58.6) (40.7 – 74.5) 0.627
≤500IU/L 23 15(65.2) (44.9 – 81.2)
Bone marrow fibrosis
Grade 0 39 26(66.7) (51 – 79.3) 0.188
Grade 1–3 13 6(46.2) (20.4 – 73.9)
Bone marrow blast
≤2% 40 27(67.5) (52 – 79.9) 0.175
>2–<5% 12 5(41.7) (16.5 – 71.4)
WHO (2016) classification of MDS
MDS–RS 24 16(66.7) (46.7 – 82) 0.482
MDS–SLD/MLD 28 16(57.1) (39.1 – 73.5)
IPSS–R karyotype
Very good/good 40 27(67.5) (52 – 79.9) 0.175
Intermediate 12 5(41.7) (16.5 – 71.4)
IPSS–R risk category
Very low/low 30 22(73.3) (55.6 – 85.8) 0.041
Intermediate 22 10(45.5) (25.1 – 67.3)
RBC transfusion burden
No transfusion burden 25 17(68) (48.4 – 82.8) 0.357
Transfusion burden 27 15(55.6) (37.3 – 72.4)
Selected molecular mutation
SF3B1 wt 31 17(54.8) (37.8 – 70.8) 0.228
SF3B1 mut 21 15(71.4) (50 – 86.2)
ASXL1 wt 40 28(70) (54.6 – 81.9) 0.040
ASXL1 mut 12 4(33.3) (11.3 – 64.6)
TET2 wt 42 27(64.3) (49.2 – 77) 0.480
TET2 mut 10 5(50) (20.1 – 79.9)
U2AF1 wt 45 29(64.4) (49.8 – 76.8) 0.408
U2AF1 mut 7 3(42.9) (11.8 – 79.8)
DNMT3A wt 45 28(62.2) (47.6 – 74.9) 1.000
DNMT3A mut 7 4(57.1) (20.2 – 88.2)
SRSF2 wt 47 28(59.6) (45.3 – 72.4) 0.637
SRSF2 mut 5 4(80) (29.9 – 99)
0
20
40
60
80
100
HI–E(%)

Methods

Study design and patients

This investigator-initiated, prospective, single arm trial conducted at 10 sites across China enrolled adult patients between 18 and 80 years of age who had primary myelodysplastic syndrome (MDS) according to the World Health Organization (WHO) 2016 criteria[1] and were required to have baseline hemoglobin < 100 g/L regardless of the levels of endogenous serum erythropoietin and red blood cell (RBC) transfusion support. Patients were considered to have no transfusion burden if they had received no RBC transfusion in the 8 weeks before treatment and have transfusion burden if they required 1 or more RBC units in the 8 weeks before treatment.

Inclusion criteria:

Each potential subject must satisfy all of the following criteria to be entered in the study.

(1) Primary MDS classified as very low, low, or intermediate-risk by the Revised International Prognostic Scoring System (IPSS-R)[2] with <5% bone marrow blasts;

(2) Aged from 18 to 80 years, male or non-pregnant, non-lactating female;

(3) Eastern Cooperative Oncology Group (ECOG) performance status 0, 1, 2;

(4) Adequate renal and hepatic function was defined as serum creatinine ≤ 1.5 times of the upper limit of normal (× ULN), blood urea nitrogen (BUN) ≤ 1.5 × ULN, alanine aminotransferase (ALT) ≤ 2 × ULN, aspartate aminotransferase (AST) ≤ 2 × ULN, and total bilirubin ≤ 1.5 × ULN;

(5) Hemoglobin < 100 g/L, platelet ≥ 30×10^9/L and neutrophil absolute count ≥ 0.5 ×10^9/L;

(6) No erythropoiesis-stimulating agent (ESA), all-trans retinoic acid (ATRA), or

androgen treatment within 30 days prior to the first day;

(7) Ability to follow the study visit schedule and comply with all protocol requirements;

(8) Capacity to take oral medication and willingness to provide informed consent;

(9) Fertile individuals and their spouses must agree to use effective contraception during the trial and for at least 3 months post-last study drug administration.

Exclusion criteria:

Any potential subject who meets any of the following criteria will be excluded from participating in the study.

(1) Presence of del(5q) cytogenetic abnormality;

(2) Prior stem cell transplant;

(3) Treatment with granulocyte colony-stimulating factor (G-CSF), thrombopoietin, or thrombopoietin receptor agonists within 8 weeks prior to the first day;

(4) Treatment with anti-thymocyte globulin, azacytidine, decitabine, cyclosporine, thalidomide, lenalidomide within 12 weeks before the first day;

(5) Receipt of any trial drug within 28 days before the first day;

(6) Any active malignant tumor except for localized non-metastatic squamous cell or basal cell skin cancer or carcinoma in situ;

(7) Active infection requiring systemic antibiotic treatment;

(8) Life expectancy < 6 months;

(9) History of epilepsy;

(10) Thromboembolic events within six months prior to the first day;

(11) Currently receiving anticoagulant therapy;

(12) Known human immunodeficiency virus (HIV), hepatitis B, or hepatitis C infection;

(13) Severe heart disease, including New York Heart Association (NYHA) class III or IV congestive heart failure, uncontrolled hypertension or hypotension, or severe valve or endocardial disease;

(14) Clinically significant or uncontrolled persistent inflammatory/autoimmune diseases;

(15) Currently alcohol or drug abuse.

Study approval and registration

The study protocol and subsequent amendments were approved by the Clinical Research Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine (Reference number: 197). The study was conducted in accordance with the principles of the Declaration of Helsinki and the Good Clinical Practice guidelines. All patients provided written informed consent before any trial-related activities. This trial is registered with Chinese Clinical Trial Registry (ChiCTR2000032845).

Procedures

Patients received recombinant human erythropoietin (rhEPO, 3SBio, Shenyang, China) 10000 IU/day subcutaneously, oral ATRA (Liangfu, Shandong, China) 25 mg/m^2/day and oral testosterone undecanoate (Catalent, Beinheim S.A, France) 80 mg twice daily for 12 weeks. Dose modification based on central or local biweekly measurement of hemoglobin values was allowed according to a prespecified algorithm. Treatment with rhEPO 10000 IU was adjusted to three times weekly if hemoglobin levels increased to ≥100 g/L and interrupted if hemoglobin levels increased to ≥ 120g/L. All patients who completed or discontinued treatment were followed up for 8 weeks, resulting in a total study duration of 20 weeks (12 weeks of treatment plus 8 weeks of follow-up). At the end of the 12-week treatment, patients who had attained clinical response

(hematological improvement-erythroid [HI-E] response or transfusion independence) were allowed to continue treatment with rhEPO, ATRA and testosterone undecanoate. These patients received continuous treatment with the prior regimen, with dosages adjusted based on hemoglobin levels, until disease progression or unacceptable toxicities occurred, rather than repeating the initial 12-week cycle. Beyond the 20-week timeframe, treatment continuation was at the discretion of patients and their treating physicians but was not protocol-mandated nor systematically tracked.

Best supportive care, including RBC transfusion and antimicrobial therapy, was permitted to ensure patient safety. According to the RBC transfusion guideline in China[3], the transfusion threshold for chronic anemia is hemoglobin level below 60 g/L. However, transfusion decisions should be individualized. For patients with clinically significant symptoms such as dyspnea, syncope, or impaired functional capacity, RBC transfusion can be considered even if their hemoglobin levels are ≥ 60 g/L.

Assessments

All patients were assessed for bone marrow morphology, cytogenetics, and flow cytometry at baseline. Furthermore, bone marrow mononuclear cells were obtained at baseline for mutational analyses by next-generation sequencing for a panel of 20 genes frequently mutated in MDS (**Supplementary table 1**). Additional baseline evaluations included comprehensive biochemistry panels, electrocardiograms (ECGs), echocardiography, and vascular ultrasound scans. Hemoglobin levels, blood transfusion requirements and safety were assessed biweekly until 8 weeks after the end of treatment. Other tests including pulmonary CT scans and cranial MRI scans were conducted at the discretion of the physician based on clinical need. Adverse Events (AEs) were monitored using the National Cancer Institute Common

Terminology Criteria of Adverse Events (NCI CTCAE), version 4.0 and described in MedDRA version 23.0 preferred terms and CTCAE grade. The occurrences, frequencies, and severities of AEs were summarized.

Endpoints

The primary efficacy endpoint was the proportion of patients who had achieved an HI-E response, defined as a hemoglobin increase ≥ 15 g/L from baseline lasting for ≥ 8 consecutive weeks in the absence of RBC transfusions during 12 weeks of treatment according to the modified 2006 International Working Group (IWG) response criteria[4]. Secondary endpoints included the median time to HI-E response and the percentage of progression to higher-risk disease. Exploratory endpoints included the percentage of patients achieving RBC transfusion independence for 8 consecutive weeks or longer, and the percentage of patients who progressed to AML.

Statistical analysis

A Simon two-stage optimal design was used to determine whether the triple regimen had sufficient activity to warrant further development[5]. An HI-E response rate ≤ 40% for the triple regimen was assumed unacceptable (null hypothesis) whereas an HI-E rate of ≥ 60% warranted further study (alternative hypothesis). Assuming a power (1-β) of 0.80 and one-sided $\alpha = 0.05$, target accrual was a minimum of 16 patients in Simon stage 1. If 7 patients achieved an HI-E response, 30 additional patients were recruited in Simon stage 2. Assuming a dropout rate of 10%, a population of 52 patients was required.

The study followed the intention-to-treat principle. The Full Analysis Set (FAS) included all patients who received at least one dose of the study medications and had a baseline assessment and at least one post-baseline assessment. The Per Protocol Set (PPS) included all patients who met the study eligibility criteria, had completed all

scheduled visits and laboratory studies, showed good compliance, and had no major study protocol violations. Good compliance was defined as ≥ 90% medication adherence, 100% completion of critical study visits, and ≤ 1 minor protocol deviation. These criteria were established to ensure the robustness of the dataset and to minimize potential biases that could arise from non-adherence, in accordance with Good Clinical Practice (GCP) guidelines. Efficacy analysis was mainly based on the FAS and supported by the PPS. Last observation carried forward (LOCF) was used for missing efficacy data.

Data were summarized by median (range or interquartile range [IQR]) for continuous variables and frequency (percentage) for categorical values. Risk factors for an HI-E response were performed using a logistic regression model. Time-to-event analyses were performed using the nonparametric tests. Post hoc subgroup efficacy analyses by prior use of ESAs (yes *vs*. no), age (< 60 years *vs.* ≥ 60 years), serum hemoglobin level (< 60 g/L *vs*. ≥ 60 g/L), serum ferritin level (< 500 ng/mL *vs.* ≥ 500 ng/mL), baseline serum erythropoietin levels (> 500 IU/L *vs.* ≤ 500 IU/L), bone marrow fibrosis (grade 0 *vs.* grade 1-3), bone marrow blast percentage (≤ 2% vs. > 2 - < 5%), MDS subtypes (MDS-RS *vs.* MDS with single lineage dysplasia/ multilineage dysplasia [SLD/MLD]), IPSS-R risk category (very low or low risk *vs.* intermediate risk), IPSS R karyotype (very good/good *vs.* intermediate), baseline transfusion burden (no *vs.* yes) and gene mutational status (yes *vs.* no). The safety set included all patients who had received at least one dose of the study medications and had at least one safety evaluation. AEs were analyzed using descriptive statistics. Statistical analyses were performed using SAS version 9.1.3 (The SAS Institute, Cary, NC, USA) and GraphPad Prism (version 8.0). A two-sided α level of 0.05 was considered significant.

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Supplementary Table 1. List of genes in the targeted next-generation sequencing panel

ASXL1	***RUNX1***	***U2AF1***	***TP53***	***TET2***
DNMT3A	*STAG2*	*SETBP1*	*BCOR*	*SRSF2*
IDH2	*EZH2*	*NRAS*	*ZRSR2*	*IDH1*
ETV6	*SF3B1*	*FLT3*	*CBL*	*JAK2*