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RESEARCH ARTICLE

Luspatercept stimulates erythropoiesis, increases iron utilization, and redistributes body iron in transfusion-dependent thalassemia

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Abstract

Luspatercept, a ligand-trapping fusion protein, binds select TGF-β superfamily ligands implicated in thalassemic erythropoiesis, promoting late-stage erythroid maturation. Luspatercept reduced transfusion burden in the BELIEVE trial (NCT02604433) of 336 adults with transfusion-dependent thalassemia (TDT). Analysis of biomarkers in BELIEVE offers novel physiological and clinical insights into benefits offered by luspatercept. Transfusion iron loading rates decreased 20% by 1.4 g (~7 blood units; median iron loading rate difference: −0.05 ± 0.07 mg Fe/kg/day, $p < .0001$) and serum ferritin (s-ferritin) decreased 19.2% by 269.3 ± 963.7 μg/L ($p < .0001$), indicating reduced macrophage iron. However, liver iron content (LIC) did not decrease but showed statistically nonsignificant increases from 5.3 to 6.7 mg/g dw. Erythropoietin, growth differentiation factor 15, soluble transferrin receptor 1 (sTfR1), and reticulocytes rose by 93%, 59%, 66%, and 112%, respectively; accordingly, erythroferrone increased by 51% and hepcidin decreased by 53% (all $p < .0001$). Decreased transfusion with luspatercept in patients with TDT was associated with increased erythropoietic markers and decreasing hepcidin. Furthermore, s-ferritin reduction associated with increased erythroid iron incorporation (marked by sTfR1) allowed increased erythrocyte marrow output, consequently reducing transfusion needs and enhancing rerouting of hemolysis (heme) iron and non-transferrin-bound iron to the liver. LIC increased in patients with intact spleens, consistent with iron redistribution given the hepcidin reduction. Thus, erythropoietic and hepcidin changes with luspatercept in TDT lower transfusion dependency and may redistribute iron from macrophages to hepatocytes, necessitating the use of concomitant chelator cover for effective iron management.

Maciej W. Garbowski and Manuel U. Guerrero contributed equally to this study.

1 | INTRODUCTION

Ineffective erythropoiesis is a hallmark of β-thalassemia and is characterized by anemia, red bone marrow hyperplasia, expansion of erythroid progenitors and pre-polychromatophilic erythroblasts, maturation arrest, and subsequent apoptosis. Several TGF-β family members (e.g., growth differentiation factor [GDF] 11) contribute to erythroid progenitor expansion.[1–3] Expansion occurs as erythroblasts fail to proceed through polychromatophilic-to-orthochromatophilic maturation to reticulocytes and red blood cells (RBCs). This is due to β-globin gene mutation-dependent imbalance of α-globin and β-globin chains, cytosolic retention of Heat Shock Protein 70, and GATA-1 cleavage by caspases.[4,5] Transfusion-dependent thalassemia (TDT) occurs when insufficient RBC output from bone marrow, or insufficient circulating RBC survival, causes anemia requiring regular transfusions. Each transfusion transiently suppresses erythropoiesis by reducing erythropoietin[6] and erythroferrone,[7] and increasing hepcidin,[6,8] while transitory loading of macrophages with erythrophagocytotic iron increases serum ferritin (s-ferritin).[6] Conversely, peripheral hypoxia increases erythropoietin synthesis, resulting in hyperplasia of early erythroblasts and inducing iron overload through increased production of erythroid factors (erythroferrone[7,9] and GDF15), which lower liver hepcidin synthesis. This reduction in hepcidin increases duodenal iron absorption and, more importantly in transfused patients, increases macrophage iron release. Inefficient iron utilization by the thalassemic erythron increases iron storage due to hepcidin suppression in non-transfused patients, a relationship more pronounced in severely anemic patients. In sporadically transfused non-transfusion-dependent thalassemia (NTDT) patients, this inverse hemoglobin (Hb)–liver iron content (LIC) relationship is perturbed,[10] and completely abolished in TDT patients.

Luspatercept is a ligand-trapping fusion protein that binds select TGF-β superfamily ligands, promoting late-stage erythroid maturation,[11] and is approved[12,13] for treatment of anemia in adult patients with TDT and NTDT. Luspatercept binds certain signal-transduction-activating ligands for Smad2/3 (GDF11, GDF8, and activin B), and Smad1/5/8 (bone morphogenetic protein [BMP] 6) leading to the reduction of phosphorylated Smad2/3 proteins (pSmad2/3), thereby modulating their regulatory effect on erythropoiesis.[11,14] Here, we report physiological and clinical insights from biomarker analysis of the BELIEVE trial, confirming hepcidin changes in TDT that lead to iron redistribution from spleen to hepatocytes and the erythropoietic compartment.

2 | METHODS

2.1 | Patients

BELIEVE (NCT02604433) is a randomized, double-blind, placebo-controlled phase 3 trial of TDT patients aged ≥18 years who were randomly assigned (2:1) to subcutaneous luspatercept or placebo injections every 21 days for ≤48 weeks; the primary outcome measures and study design have been published.[12] The study was approved by an institutional review board or ethics committee at each participating site and was conducted in accordance with the Declaration of Helsinki.

2.2 | Transfusion burden estimate

Transfusion burden was encoded as a longitudinal variable using a 12-week sliding window and was calculated as the sum of the RBC units transfused during the previous 12 weeks at each visit. Iron loading rate in mg/kg/day[13] was derived from transfusion data (see Supplemental Methods).

2.3 | Chelation relative efficacy calculation

Three chelators, deferasirox (DFX), deferiprone (DFN), and deferoxamine (DFO), were used during the study according to local preferences. The percentage of patients receiving single-agent or combination treatment was as follows: DFX: 49.2%, DFN: 14.9%, DFO: 5.8%, DFN + DFO: 16.4%, DFX + DFO: 7.2%, DFX + DFN: 4.6%, and DFX + DFN + DFO: 1.9%. In order to compare across all chelation modalities, we calculated the efficient iron-binding equivalents of each chelation regime for each patient as follows. The doses (in mg/kg) of each chelator ([DFO, molecular weight (mw) 656.8 g/mol], [DFX, mw 373.4], and [DFN, mw 139.15]) were recalculated into μmol/kg (dose[mg/kg]/1000/mw[g/mol]/10^6). Next, the molar dose was adjusted divided by the denticity constant (1 for DFO, 2 for DFX, 3 for DFN) to obtain the chelator iron-binding equivalents, in addition, DFO molar iron-binding equivalent dose was corrected for DFO posology (number of days per week; if 5 days out of 7, the correction factor was 0.714). Chelation efficiency adjustment was 0.135 for DFO, 0.27 for DFX, and 0.04 for DFN. The adjusted iron-binding equivalent dose was multiplied by the number of days between each study visit (typically 21, unless otherwise captured, and including recorded missed doses or compliance adjustment) to represent exposure to iron-binding equivalent of chelators in μmol*-days/kg. There was no evidence that average chelation exposure changed in the first 48 weeks.

2.4 | Biomarkers

Iron, hematological, and erythropoietic biomarkers were profiled at each visit for ≥48 weeks (see Supplemental Methods); time points at which biomarkers were measured are listed in Table S1.

2.5 | Liver iron content measurements

LIC measurements were performed on 1.5 T magnetic resonance imaging (MRI) scanners, using spin-echo sequence R2-FerriScan LIC[15] or gradient-echo sequence T2* LIC methods; the latter used various LIC calibrations[16–19] to report hepatic T2* values in mg Fe/g dry weight (dw) (see Supplemental Methods for details). Total body iron stores were

estimated based on the Angelucci formula[20] and iron balance was calculated from changes in total body iron stores and transfused iron.[21] The Angelucci formula predicts that the amount of total body iron stores (in mg/kg body weight) is equivalent to 10.6 times the LIC (in mg/g dw),[20] for example, LIC of 5 mg/g dw predicts 53 mg/kg of total body iron stores, which in a 70-kg man equals 3.71 g.

2.6 | Statistical analysis

Biomarkers comparisons at different time points were performed using non-parametric, pairwise Wilcoxon tests. The relationship between s-ferritin slope and other biomarkers was evaluated by linear regression. Fisher's exact test was used to assess the association between luspatercept and hematological response (negative iron loading rate difference), and to test the arm-wise association between hematological response and s-ferritin change over time. Longitudinal models were constructed to analyze long-term trends in s-ferritin and other biomarkers. A mixed-effects linear regression approach allowed for identification of the patient-nested (within-subjects) effects and the population-level (between-subjects) effects of different explanatory variables (see Supplemental Methods).

3 | RESULTS

3.1 | Patients

All 336 patients received ≥1 dose of luspatercept or placebo. Patients were well balanced between arms for baseline parameters ($p < .05$; Table 1 and Table S2). Treatment started with luspatercept (starting dose: 1 mg/kg) or placebo received over 48 weeks (345 days, range: 294–518) with up-titration allowed to 1.25 mg/kg per protocol.

TABLE 1 Baseline parameters overall and in placebo and luspatercept arms.

Baseline variables	Luspatercept ($N = 224$)	Placebo ($N = 112$)	Overall ($N = 336$)
Female sex, *n* (%)	132 (58.9%)	63 (56.3%)	195 (58.0%)
Age, median (range), years	30 (18–66)	30 (18–59)	30 (18–66)
β^0/β^0 genotype, *n* (%)	68 (30.4%)	35 (31.3%)	103 (30.7%)
Prior splenectomy, *n* (%)	129 (57.6%)	65 (58.0%)	194 (57.7%)
Pre-treatment transfusion burden, *n* (%)			
Low (≤10 units/24 weeks)	33 (14.7%)	14 (12.5%)	47 (14.0%)
Medium (>10–≤15 units/24 weeks)	96 (42.9%)	47 (42.0%)	143 (42.6%)
High (>15 units/24 weeks)	95 (42.4%)	51 (45.5%)	146 (43.5%)
Pre-treatment iron loading rate[a], median (range), mg Fe/kg/day	0.309 (0.0992–0.644)	0.315 (0.103–0.558)	0.313 (0.0992–0.644)
Pre-treatment mean pre-transfusion Hb, median (range), g/L	93.1 (45.1–114.0)	91.5 (58.0–117.0)	92.7 (45.1–117.0)
R2 LIC, mg/g dry weight (FerriScan)			
n	53	20	73
Median (range)	4.6 (0.9–42.0)	3.8 (1.0–43.0)	4.35 (0.9–43.0)
T2*1 LIC, mg/g dry weight (T2*)			
n	171	92	263
Median (range)	5.67 (0.73–40.60)	5.02 (0.18–31.94)	5.3 (0.18–40.60)
s-ferritin, median (range), µg/L	1510 (70.0–6400.0)	1150 (111.0–6400.0)	1400 (70.0–6400.0)
GDF11, median (range), pg/mL	109 (53.0–197.0)	108 (49.0–239.0)	109 (49.0–239.0)
EPO, median (range), IU/L	60.6 (2.40–972.00)	45.3 (4.65–422.00)	56.5 (2.40–97.200)
ERFE, median (range), ng/mL	20.0 (6.49–81.90)	18.2 (6.49–78.00)	19.4 (6.49–81.90)
Hepcidin, median (range), ng/mL	24.8 (2.5–249.0)	25.3 (2.5–220.0)	25.1 (2.5–249.0)
GDF15, median (range), pg/mL	8920 (733–48 000)	8430 (657–48 000)	8840 (657–48 000)
sTfR1, median (range), nM	69.4 (10.4–242.0)	64.4 (11.0–167.0)	67.2 (10.4–242.0)
Fetal Hb, median (range)	8.5 (0.5–130.0)	13.2 (0.5–75.3)	8.0 (0.5–130.0)
Reticulocytes, median (range), $\times 10^9$/L	53.8 (0.73–822.00)	50.4 (3.90–605.00)	50.4 (0.73–822.00)
LDH, median (range), median (range), U/L	168 (75–739)	159 (96–1250)	164 (75–1250)
Indirect bilirubin, median (range), µmol/L	23 (5–188)	23 (6–182)	23 (5–188)

Abbreviations: ERFE, erythroferrone; EPO, erythropoietin; GDF, growth differentiation factor; Hb, hemoglobin; LDH, lactate dehydrogenase; LIC, liver iron content; s-ferritin, serum ferritin; sTfR1, soluble transferrin receptor 1.

[a]Pre-treatment period of 48 weeks.

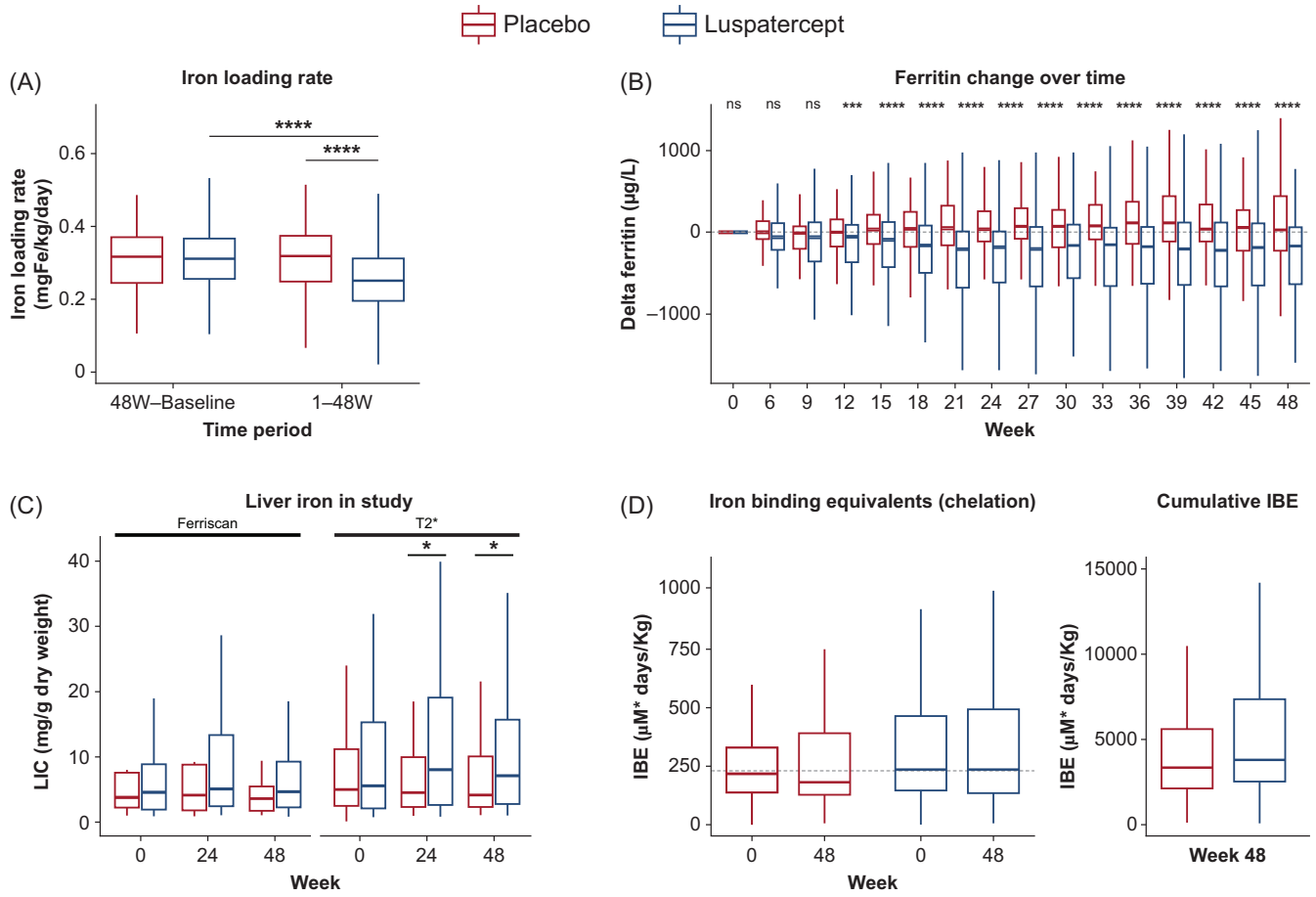

FIGURE 1 Iron parameter responses. (A) Transfusion dependence shown as iron loading rate in mg Fe/kg/day at baseline (i.e., over the total pre-baseline period of 1 year) and during treatment (Weeks 1–48) for placebo and luspatercept. Box-and-whisker plot and paired and unpaired Wilcoxon tests shown; $n = 333$. (B) s-ferritin response shown as s-ferritin change with baseline level subtracted at each visit, levels across 16 visits shown with box-and-whisker plots in placebo and luspatercept groups; $n = 334$. (C) Unpaired Wilcoxon test p values shown, comparing placebo and luspatercept at each visit. LIC levels at baseline, Week 24 and Week 48, for placebo and luspatercept groups shown separately for FerriScan R2 LIC and T2* LIC methods, box-and-whisker plots; $n = 321$, Wilcoxon test for assessing significance. (D) Efficient chelator IBEs (see Methods) at baseline and during treatment for placebo and luspatercept; $n = 334$. Dashed line represents a theoretical IBE calculation considering a deferasirox exposure of 15.4 mg/kg/day (left); cumulative exposure of chelator IBE, Wilcoxon test applied (right). * $p \leq .05$; **** $p \leq .0001$. IBE, iron-binding equivalent; LIC, liver iron content; s-ferritin, serum ferritin.

3.2 | Transfusion dependence, transfusion iron loading rate, s-ferritin, LIC response, and changes in chelation exposure

Transfusion dependence was similar between arms in the year before treatment, with a mean ± SD transfusion iron loading rate of 0.31 ± 0.09 mg Fe/kg/day for both arms (Table 1; Figure 1A). Luspatercept reduced mean transfusion iron loading rate by 20% during the 48-week treatment period (placebo vs. luspatercept: 0.31 vs. 0.25 mg Fe/kg/day, $p < .0001$; Figure 1A), amounting to 1.4 g of iron (approximately 7 RBC units/year). Luspatercept decreased s-ferritin after four doses (12 weeks) reaching −103.6 ± 690.3 µg/L ($p < .0001$), or − 4.9 ± 33%, and this reduction continued further up to Week 48, reaching −269.3 ± 963.7 µg/L ($p < .0001$), or −12.8 ± 45.9%. s-ferritin levels remained stable in the placebo arm (Figure 1B).

Surprisingly, luspatercept treatment led to a decrease in transfusion burden without a concomitant decrease in LIC. The Angelucci formula predicts an LIC decrease of 1.9 mg/g dw for a 70-kg patient for comparative reduction in transfusion burden. Paradoxically, a nonsignificant trend toward increased mean LIC was observed in the first year, from approximately 5.0 to 6.5 mg/g dw by FerriScan, and from approximately 10.0 to 11.5 mg/g dw by the T2* method (Figure 1C).

Daily exposure to chelator iron-binding equivalents adjusted for chelation efficiency was analyzed at baseline and at Week 48. A nonsignificant trend toward higher chelation exposure with luspatercept versus placebo was seen at baseline and Week 48 (Figure 1D; Figure S1). The contrasting LIC findings, decreased s-ferritin, and reduced iron loading rate, in combination with unchanged chelation rates, suggest iron redistribution from macrophages to hepatocytes.

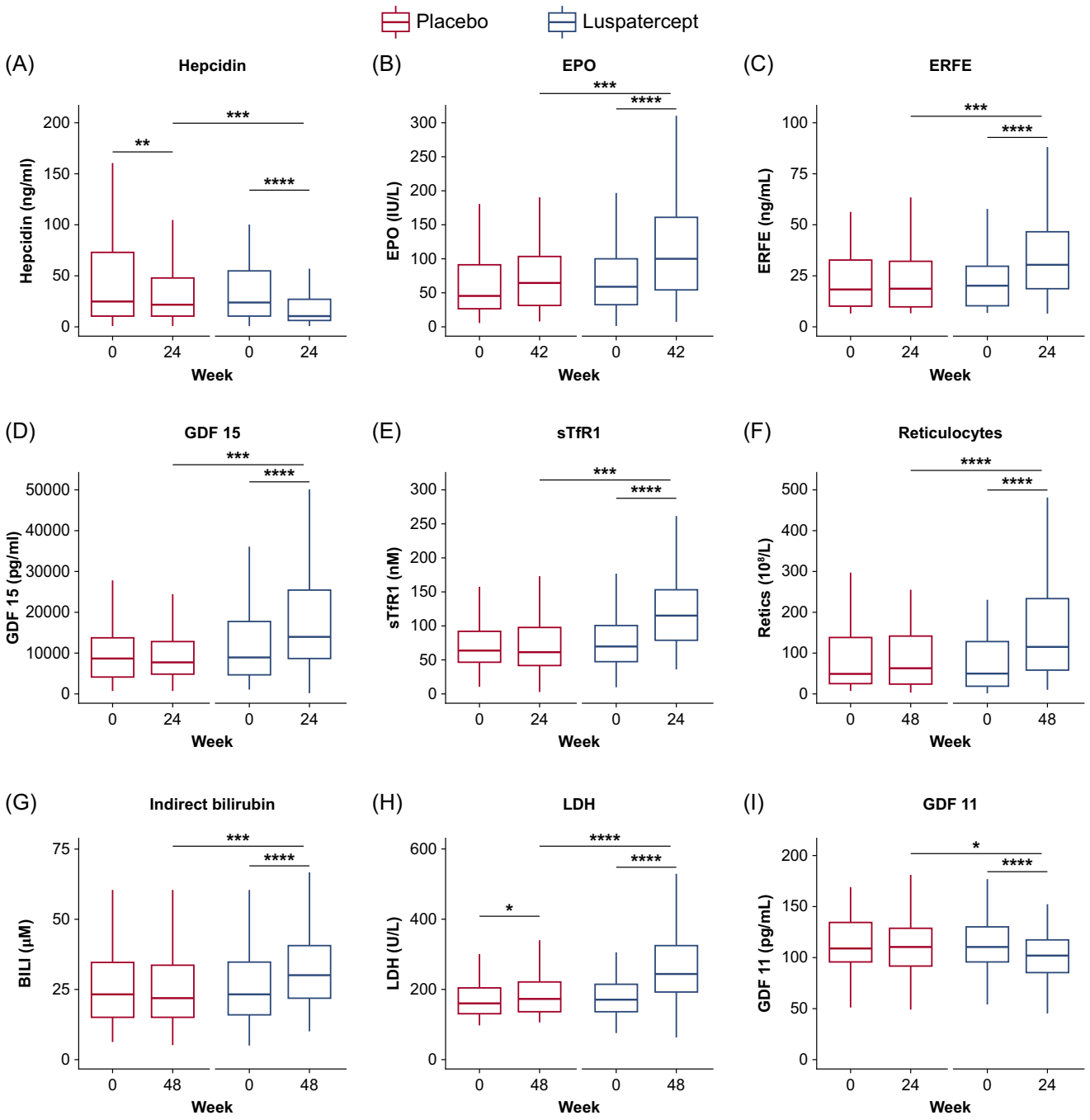

FIGURE 2 Biomarker response on study. Biomarker levels are shown for placebo (red) and luspatercept (blue) at baseline and at the indicated post-baseline time points. Box-and-whisker plots and *p* values computed using Wilcoxon paired or unpaired tests are shown. (A) Hepcidin; $n = 327$. (B) EPO; $n = 330$. (C) ERFE; $n = 323$. (D) GDF15; $n = 324$. (E) sTfR1; $n = 325$. (F) Reticulocytes; $n = 258$. (G) Indirect bilirubin; $n = 330$. (H) LDH; $n = 329$. (I) GDF11; $n = 327$. * $p \leq .05$; ** $p \leq .01$; *** $p \leq .001$; **** $p \leq .0001$. BILI, bilirubin; EPO, erythropoietin; ERFE, erythroferrone; GDF, growth differentiation factor; LDH, lactate dehydrogenase; retics, reticulocytes; sTfR1, soluble transferrin receptor 1.

3.3 | Iron and erythropoietic response biomarkers

With luspatercept treatment, the levels of all biomarkers changed significantly (median [interquartile range (IQR)] at baseline and Week 24, unless otherwise specified): hepcidin decreased by 53% from 24.8 (44.8) ng/mL to 11.6 (20.1) ng/mL; erythropoietin increased by 93% from 60.6 (68.7) IU/L to 116.7 (156.1) IU/L at Week 42, erythroferrone increased by 51% from 20 (19.4) ng/mL to 30.1 (28) ng/mL, GDF15 increased by 59% from 8916.1 (12 835.5) pg/mL to 14 146 (17 095.3) pg/mL, soluble transferrin receptor 1 (sTfR1) increased by 66% from 69.4 (52.8) nM to 115.3 (73.8) nM, reticulocytes increased by 112% from 53.8 (117.95) $\times$ 10^9/L to 114 (157) $\times$ 10^9/L, and

GDF11 decreased by 8% from 109 (35) pg/mL to 100 (33) pg/mL ($p < .0001$ for all; Figure 2A-I). Timing of samples within the transfusion cycle accounted for <6% of biomarker variability, hence changes in these biomarkers can be attributed to luspatercept treatment (Table S3).

3.4 | Biomarkers and hematological response assessed by transfusion iron loading rate

The correlations between changes in hematological response and changes in biomarker levels were assessed (Figure S2A-I). Hematological response was expressed as the change in transfusion iron loading rate on treatment versus baseline (negative delta iron loading rate indicates that hematological response was present). Erythropoietin increase correlated with hematological response as assessed by study change of transfusion iron loading rate in luspatercept-treated patients ($p = .02$; Figure S2A), despite mean Hb remaining unchanged (Figure S3). Hematological response also correlated with decreased hepcidin concentration ($p = .02$; Figure S2C) and increase in sTfR1 ($p = .002$; Figure S2D). However, no correlation was observed between hematological response and erythroferrone or reticulocytes ($p = .15$) (Figure S2B and S2F), or GDF15, or GDF11 (Figure S2E, S2I). Finally, changes in bilirubin inversely correlated with hematological response (Figure S2G) but not those of LDH (Figure S2H). Considering iron fluxes, hepcidin changes are mechanistically consistent with a relationship between increased sTfR1 and transfusion reductions. Given that iron overload—not iron deficiency—is present, this increase in sTfR1 is indicative of more of differentiating and hemoglobinizing cells in the erythron, and hence a greater iron sink.[22,23] The inverse correlation of hematological response with bilirubin ($p = .04$; Figure S2G) suggests that the iron incorporated into erythron (marked by sTfR1) undergoes hemolytic recycling following increased endogenous erythropoietic output.

3.5 | Baseline predictors of s-ferritin reduction

In the following sections, we present the response of s-ferritin to study treatment and analyze single variables affecting that response before we move on to the multiple predictor model of s-ferritin response to treatment. As s-ferritin is a fundamental marker of iron metabolism and was measured at each visit, it was the most information-rich biomarker of all in the study and selecting it for modeling allowed us to investigate substantive predictors of that response in a controlled model, thereby explaining luspatercept mechanism of action in terms of relevant biomarker changes.

3.5.1 | Hepcidin

Among luspatercept-treated patients, including s-ferritin responders and non-responders, 70% experienced a significant reduction in hepcidin before hematological response; this effect was not observed in the placebo arm. Baseline hepcidin levels were higher in s-ferritin responders versus non-responders ($p < .05$; Figure S4A), which was not confounded by differences in the time between baseline blood samples being taken and transfusion being received (Figure S5), the effect of that difference having been previously studied.[6] With respect to ferritin response, baseline hepcidin levels correlated with the s-ferritin slope gradient (Figure S6A), suggesting that patients starting treatment with higher hepcidin at the start of treatment have a sharper absolute s-ferritin decline.

3.5.2 | Erythropoietic markers

Baseline erythroferrone levels were lower in s-ferritin responders versus non-responders receiving luspatercept ($p < .05$; Figure S4B). Values <40 ng/mL corresponded to steeper future s-ferritin reduction slopes (Figure S6B). Since erythroferrone increased in patients on luspatercept, and hepcidin changes inversely correlated with erythroferrone changes ($p < .001$; Figure S7), increases in erythroferrone may have different s-ferritin consequences depending on the baseline erythroferrone value. Baseline erythropoietin, GDF15, GDF11, sTfR1, and lactate dehydrogenase (LDH) levels were comparable between luspatercept-treated s-ferritin responders and non-responders (Figure S4C-G). However, baseline erythropoietin values <250 IU/L corresponded to a steeper s-ferritin reduction slope (Figure S6C), consistent with increased erythropoietin being necessary for erythropoietic response.

3.5.3 | Hemolytic markers

Indirect bilirubin increased by 35% at 48 weeks in patients receiving luspatercept, from median (IQR) 23 (19) μM to 31 (21) μM in s-ferritin responders, who had baseline levels approximately 25% lower than non-responders ($p < .05$; Figure S4H). Furthermore, baseline bilirubin <50 μM corresponded to steeper s-ferritin reduction slopes in patients on luspatercept (Figure S6D). LDH increased by 67% in s-ferritin responders receiving luspatercept (166 [89.5] IU/L to 276 [160.5] IU/L; $p < .0001$; Figure S4G). The finding that lower baseline bilirubin allows a greater reduction in s-ferritin after luspatercept is consistent with responder patients having lower endogenous erythropoiesis. Decreasing s-ferritin correlated with increasing bilirubin and LDH, indicating both more peripheral hemolysis and ineffective erythropoiesis—and more heme breakdown from endogenous erythropoiesis against the background of catabolized transfused cells (i.e., export of iron from erythrocyte heme turnover to plasma rather than retention by macrophages).

3.5.4 | Transfusion response as iron loading rate

Baseline transfusion dependence expressed as transfusion iron loading rate (mg/kg/day) did not predict s-ferritin response (Figure S4I). However, 131 of 184 (71%) patients with transfusion response had negative s-ferritin slopes (including 75 of 93 [81%] patients with

significant slopes), whereas 13 of 30 (43%) patients without erythropoietic response had negative s-ferritin slopes (including 4 of 16 [25%] patients with significant slopes) ($p < .0001$; Figure S8C).

3.5.5 | Spleen status

Contrary to animal data,[3,24] spleen volume in humans did not change overall in the BELIEVE luspatercept study.[12] At baseline, however, s-ferritin responders to luspatercept had significantly lower spleen volume than non-responders (Figure S4J). Previous splenectomy did not affect the proportion of s-ferritin responders and non-responders (Figure S4K). Baseline spleen volume did not correlate with the steepness of s-ferritin slopes (Figure S6E). As shown above, the effect of luspatercept increasing macrophage iron release is relevant to the redistribution of iron from spleen to liver: luspatercept patients with intact spleens had significantly greater increases in median LIC than splenectomy patients (median [IQR] 1.19 [5.6] vs. 0 [3.5]; $p = .006$; Figure S8A) and experienced a greater s-ferritin reduction (Figure S4K and S6F).

3.5.6 | Genotype

A comparable reduction in s-ferritin occurs in both β0/β0 and non-β0/β0 genotypes (Figure S6G) in the first 48 weeks of treatment, whereas hematological response mostly occurs in the non-β^0/β^0 genotype as judged by subgroup analyses.[12] This implies a partial disconnect between erythropoiesis and iron metabolism on luspatercept. Since a proportion of patients reduced their ferritin without any hematological response, iron metabolism changes are either independent from hematological response or precede it. It is also possible that the disconnect between changes in s-ferritin and changes in hematological response might relate to a different amount of time required for the latter response.

3.5.7 | Relationship between liver iron content, s-ferritin levels, and hematological response

Five-fold more patients receiving luspatercept versus placebo had a reduction in iron loading rate ($p < .0001$; Figure S8D). Patients with decreased s-ferritin and reduced transfusion dependence were more likely to experience increased LIC. In reticulocyte responders who reduced ferritin, LIC generally reduced on placebo, but on luspatercept their LIC increases were slightly more often observed than LIC reductions (Figure S9). On placebo, regardless of the LIC measurement method used, the changes in s-ferritin and LIC were correlated positively. In contrast, among luspatercept-treated patients, LIC methods were important in that the FerriScan LIC trend did not show the expected correlation with the s-ferritin trend (Figure S8B, upper panel), whereas the T2* LIC trend did show a correlation with s-ferritin that had a steeper slope with luspatercept than with placebo. In summary, there are luspatercept-treated patients (unlike placebo) in whom s-ferritin decreased while LIC increased, consistent with iron redistribution from macrophages to the hepatocyte compartment.

3.6 | Modeling the effect of luspatercept on s-ferritin

The predictor variables show increasing s-ferritin trend on placebo (reference population) with lower increases at higher baseline s-ferritin levels (Table S4). Baseline s-ferritin also had a reducing effect on the s-ferritin trend with luspatercept. Transfusion increased s-ferritin levels by 40 μg/L for every cumulative RBC unit in the 12 weeks preceding any time point on study in both treatment arms. The treatment effect on s-ferritin was only observed in patients with baseline LIC <15 mg/g dw; these patients may constitute a potential s-ferritin responder population among patients receiving luspatercept.

The benchmark model included the luspatercept treatment effect (−390 μg/L at 48 weeks [−1.16 μg/L/day]; $p < .01$), controlled for transfusion and other standard-of-care parameters. The model was then interrogated with substantive variables, including hepcidin, markers of erythropoiesis and hemolysis, spleen status, and genotype. Hepcidin (model B) and erythropoietin (model C) jointly explained 23% of the treatment effect of luspatercept on s-ferritin (model E), while making the model more parsimonious. Bilirubin (in model F) was the only other biomarker entered into the joint hepcidin and erythropoietin model (model E) that provided additional information beyond that provided by hepcidin and erythropoietin, jointly explaining 67% of the treatment effect. Therefore, s-ferritin reductions in patients receiving luspatercept can be explained by taking erythropoietin recovery, hepcidin reduction, and increased heme turnover into account. This suggests that elevated erythropoietin leads to erythroferrone-dependent hepcidin reduction, enabling iron exit from macrophages to the erythron, with subsequent release of thalassemic erythrocytes hemolyzed at a higher rate versus pre-treatment. Erythroferrone, sTfR1, GDF15, and fetal Hb (HbF) reduce the luspatercept treatment effect on s-ferritin when entered singly into the benchmark model, but do not provide information beyond the erythropoietin–hepcidin–bilirubin combination.

4 | DISCUSSION

Luspatercept-treated patients with TDT experienced decreased transfusion iron loading and, expectedly, a s-ferritin decrease. However, while hepcidin levels decreased LIC remained stable (or increased in patients with spleens) despite stable chelation. Erythropoietin, markers of erythropoiesis (sTfR1, GDF15), and hemolytic markers increased. Luspatercept-induced decreases in hepcidin explain decreased s-ferritin and partial iron redistribution from spleen to liver in patients with TDT (Figure 3). Macrophage iron, as estimated by s-ferritin, decreases with reduced iron recovery from fewer transfusions (and higher macrophage iron release via ferroportin at lower hepcidin levels). Hb levels are unchanged, indicating that transfusion reduction

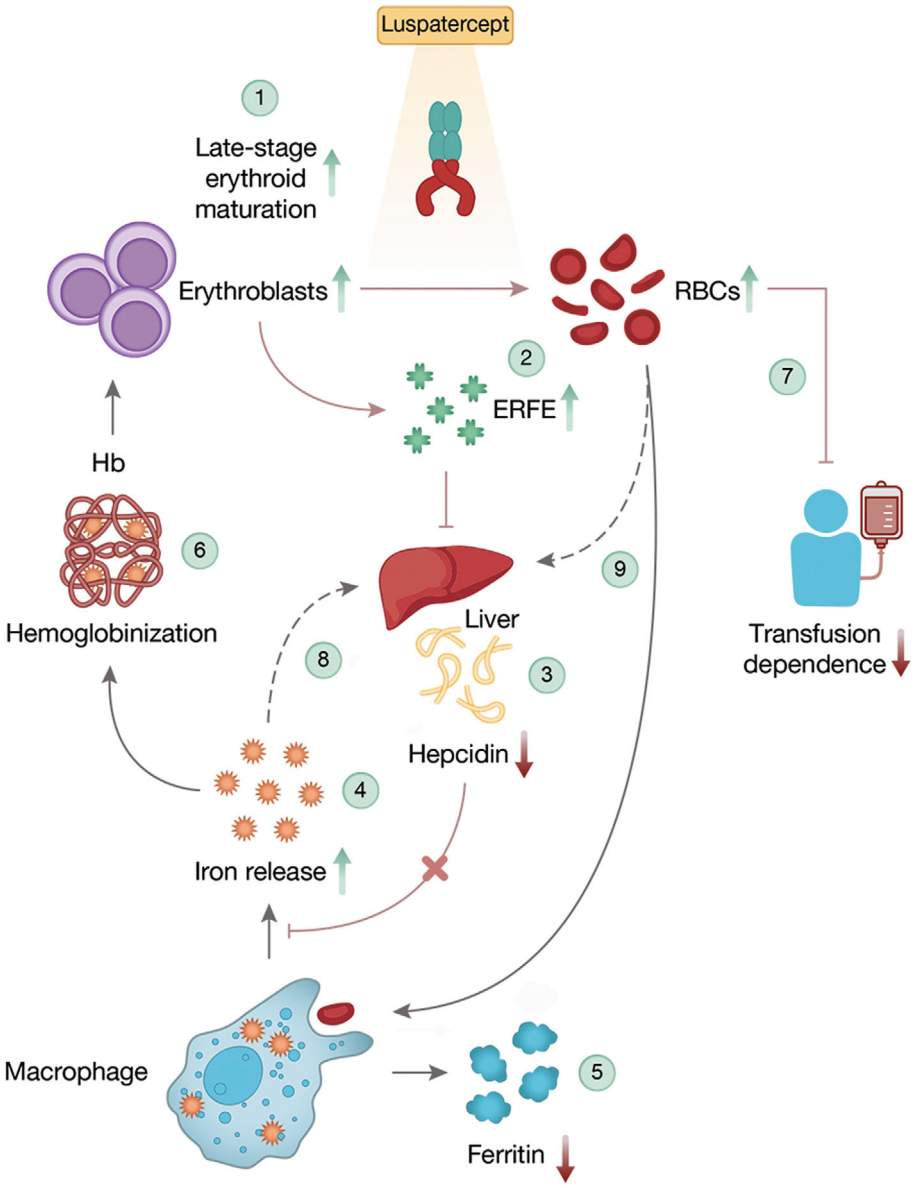

FIGURE 3 Mechanism of action of luspatercept in transfusion-dependent thalassemia. (1) As luspatercept increases late-stage maturation of RBC precursors, (2) more erythroblasts produce more ERFE. (3) ERFE suppresses the production of hepcidin by the liver, leading to (4) lower hepcidin levels, which allows more iron to be released from macrophage stores. This (5) increased iron release is consistent with the observed decrease in s-ferritin levels, and this (6) iron is now available to be incorporated into new Hb, which is used in the production of mature RBCs. (7) Increased numbers of thalassemic RBCs result in reduced RBC transfusion dependence. On the other hand, (8) macrophage-released iron is also transported to and taken up by hepatocytes in the liver (as both transferrin-bound and non-transferrin-bound iron), while (9) increased intravascular hemolysis of thalassemic RBCs releases heme iron that is transported to the liver, and increased extravascular hemolysis delivers iron to spleen macrophages or Kupffer cells in the liver. Taken together, this redistribution of iron results in iron availability for new Hb production but also increases LIC in some patients. ERFE, erythroferrone; Hb, hemoglobin; LIC, liver iron content; RBC, red blood cell; s-ferritin, serum ferritin.

is matched by increased endogenous RBC production, relying on iron recycled from RBCs, and released from stores due to lower hepcidin; released iron partly returns to the plasma and loads hepatocytes via a combination of heme, transferrin iron, and non-transferrin-bound iron (NTBI) uptake routes, the details of which are unclear. Hepcidin levels are reduced by increased erythroferrone due to increased survival of erythroferrone releasing erythroblasts. These surviving cells are a source of increased sTfR1 suggesting increased incorporation of iron into erythroid cells for hemoglobinization. Although these surviving cells remain thalassemic in phenotype and will be removed via hemolysis from circulation faster than transfused cells, they now represent an increased proportion of circulating RBCs, thus increasing hemolytic markers except where HbF increased in β^0/β^0 responders.

The events leading to increased erythroferrone, and increased erythropoietin, are less well understood. It is currently not certain if sequestration of single or several pSmad2/3-increasing ligands is required to achieve pSmad2/3 reduction in vivo. We have reported here the GDF11 reduction in humans receiving luspatercept treatment with decreases occurring both within and across dose cycles (Figure S8H). Given murine knockout experiments suggesting that GDF11 does not mediate luspatercept effects on murine erythropoiesis[25] and that therefore this mechanism might now be doubted[26]; the controversy around GDF11 emerged, even though murine thalassemia models differ substantially in biomarker response to luspatercept between models themselves (in hepcidin and erythropoietin, but also spleen size) and from our patient data. Nevertheless, pSmad2/3 reduction on luspatercept is still linked to reduction in the number of erythroid precursors regardless of preceding events. By increasing the differentiation rate of erythroid precursors without increasing their proliferation,[14] luspatercept will lead to global reduction of their numbers. These are mostly colony forming units-erythroid and early erythroblasts, which are the main erythropoietin receptor (EpoR)-bearing compartments.

The erythropoietic response to erythropoietin occurs among progenitor and early erythroblast cells bearing the EpoR.[27] Furthermore,

luspatercept reduces marrow cellularity and spleen size (and cellularity) in mice.[24,28] Contrary to thalassemic animal data where erythropoietin was reduced by 60% on RAP-536,[24] we observed a 93% increase in erythropoietin on luspatercept, but not on placebo. The mechanism of erythropoietin increase may relate to either decreased peripheral erythropoietin consumption[27] or increased tissue hypoxia. It could be argued that the HbF increase, observed on luspatercept as early as Week 9 (Figure S10, bottom panel), could account for increase in erythropoietin due to the leftward shift in the oxyhemoglobin dissociation curve of HbF; however, the HbF change on study did not predict erythropoietin changes on luspatercept, when corrected for relevant covariates (Table S5), so other causes need to be considered.

Luspatercept binding of pSmad2/3-increasing ligands therefore may reduce the cellularity of the erythropoietin-dependent, EpoR-bearing compartment, thus partially abrogating peripheral erythropoietin consumption.[27] Patients with RBC hypoplasia, for example, Diamond–Blackfan anemia, have 50- to 100-fold higher erythropoietin levels compared with TDT with similar Hb levels, with no sTfR1 present[29] since the erythropoietin-consuming, EpoR-expressing erythroid compartment is lacking. It is well established that JAK2-dependent lysosomal targeting of the ligand-receptor complexes constitutes the major mechanism of erythropoietin elimination.[30]

Importantly, the paradoxical erythropoietin increase in our study cannot be explained by total Hb changes, because the study was designed to maintain a constant Hb level (Figure S3) to prevent bias in transfusion requirements. In TDT, transfusion frequency reduction and/or the number of units transfused lowers the exposure to acute post-transfusional drops in erythropoietin,[6] further increasing erythropoietin levels. Erythropoietin increases were associated with transfusion response in TDT.

The late erythroblast pool (poly- and orthochromatophilic) is a significant source of erythroferrone,[9] so the expansion of this pool likely contributes to increased erythroferrone in thalassemia. Erythroferrone suppresses hepcidin production in the liver, leading to mobilization of stored iron and increased dietary iron absorption. The increased erythroferrone seen in approximately 90% of luspatercept-treated patients explains hepcidin reduction in approximately 70% of luspatercept-treated patients with TDT. Not all erythroferrone increases with luspatercept lead to hepcidin reductions or erythropoietic response; rather, high baseline LIC may interfere with effective downstream signaling through high baseline hepcidin (Figure S8E,F,G,H), explaining why some, but not all, erythroferrone increases translate into macrophage iron egress and increased erythrocyte output.

In contrast to animal data,[11] hepcidin was profoundly reduced in a large proportion of patients on luspatercept, and nearly all hepcidin reductions correlated with erythroferrone increases (Figure S7; $p < .0001$). Hepcidin reduction correlated with transfusion response (Figure S2C). Furthermore, a small proportion of patients with hematological response (<10%) had high baseline hepcidin; if hepcidin decrease occurred, it almost always resulted in reduction of transfusion requirements, and accounted for 90% of all hematological responses. It is evident, however, that some transfusion responses occur with increased hepcidin (Figure S2C).

Since the erythroferrone–hepcidin events precede hemoglobinization, hepcidin-dependent iron release from macrophages to plasma becomes a mechanism for iron redistribution. The increasing incorporation of iron into Hb (which feeds the hematological response) increases net hemolysis because proportionately more circulating RBCs will be derived from endogenous thalassemic erythropoiesis rather than from transfusion. With increased endogenous erythropoiesis, iron is rerouted via the NTBI, heme/hemopexin, and Hb/haptoglobin pathways to the liver, further explaining lack of LIC decreases in s-ferritin responders with hematological improvement (Figure S11C).

There is a well-established correlation between s-ferritin and LIC among patients with TDT receiving chelation, where s-ferritin responders are expected to reduce LIC in 1 year.[31] In luspatercept-treated patients who had an erythroid response, the relationship between s-ferritin and LIC was perturbed. LIC increased or stabilized with decreased transfusion dependence (Figure S11D) and tended to increase with increasing s-ferritin (Figure S11C). In the absence of hematological response, however, luspatercept did not perturb the relationship between LIC and s-ferritin: as s-ferritin dropped, so did LIC (Figure S11C). Moreover, while LIC decreased on placebo in reticulocyte responders who reduced s-ferritin, on luspatercept, in contrast, LIC either increased or decreased (Figure S9), net effect being no LIC reduction. However, reticulocyte change did not predict transfusion response (Figure S2F) even though absolute reticulocytes increased as early as Week 6 after treatment initiation (Figure S10, top panel). The likely consequence of thalassemic hematological response is greater hemolysis resulting in iron rerouting and NTBI uptake by the liver. These changes appear to be attenuated at high baseline LIC values (which increase hepcidin levels), presumably because higher hepatocyte iron signaling to the BMP receptor complex via BMP6 is no longer sufficiently antagonized by erythroferrone and luspatercept. Using linear regression modeling, we confirmed that the effect of luspatercept treatment on s-ferritin reduction over 48 weeks depends mechanistically on low baseline LIC (<15 mg/g dw) and increasing erythropoietin, causing erythroferrone increase, hepcidin reduction, and rising bilirubin. These observations jointly indicate that, unlike with high LIC which may increase hepcidin further, luspatercept allows for macrophage iron egress at relatively low LIC following erythroid events that increase erythropoietin; such events link greater iron flux via the plasma compartment (for incorporation into the erythron) with subsequently increased turnover of heme iron. Sustained reduction in s-ferritin, but without significant LIC reduction at Week 48 of luspatercept treatment, suggests a redistribution of iron from splenic macrophages to hepatocytes. Redistribution is consistent with the observed 25% reduction in transfusional iron loading (approximately 1.5–2 g of heme iron), together with chelator exposure overall remaining unchanged. Redistribution is also favored with luspatercept because >70% of patients have a significant reduction in plasma

hepcidin. As macrophage iron turnover is 25–50 times greater than intestinal iron uptake, the magnitude of iron redistribution from macrophages to plasma likely dominates over any potential increases in gut iron uptake. Here, therefore, we justifiably do not consider potential increase in gut iron uptake in TDT, which is relatively trivial in contrast to non-TDT (see Supplemental Discussion), although such intestinal iron uptake increase cannot be excluded. Interestingly, in this study, LIC increased with luspatercept treatment only in patients with intact spleens (and did not increase in the placebo group, Figure S8A), which is consistent with iron redistribution from intact spleens to hepatocytes.

Regarding study limitations, this study claims iron redistribution phenomenon from spleen to liver in the absence of liver biopsy. Direct proof of Kupfer cell iron reduction concomitant with hepatocyte iron increase is therefore lacking. Liver biopsy was not part of the study protocol and would be unethical at this point. We were also unable to obtain retrospectively spleen T2* as a proxy measurement of iron to conclude redistribution if T2* change showed reduction of spleen iron while liver iron is stable or increased. Further limitations consist in relying on serum biomarker signature of erythropoietic activity without direct erythropoietic assays from patient bone marrow samples. Bone marrow assays were not part of the protocol and would be unethical to obtain at this stage.

In conclusion, during luspatercept treatment, pSmad2/3 reduction increases the erythroblastic differentiation of thalassemic erythroid precursors thus depleting their number, which—likely through lower peripheral consumption of erythropoietin—raises erythropoietin levels, while improved differentiation expands the pool of erythroferrone-producing erythroblasts and consequently lowers plasma hepcidin. This favors greater macrophage and splenic iron release for hemoglobinization. Iron redistribution is mediated by marrow iron incorporation into Hb, with increased thalassemic erythrocyte production followed by hemolysis. Additionally, a hepcidin-driven, s-ferritin-lowering redistribution of iron from macrophage stores to hepatocytes occurs via NTBI, bypassing marrow iron incorporation. Together, these counter the iron-sparing reduction of transfusion and effectively increase LIC in a proportion of luspatercept-treated patients. The iron redistribution phenomenon during luspatercept treatment may require dosing and/or regimen adjustment of iron chelation therapy for more effective management of iron overload in TDT patients.

AUTHOR CONTRIBUTIONS

Maciej W. Garbowski jointly conceived the study, analyzed the data, planned the figures, and wrote the manuscript; Manuel Ugidos, Alberto Risueño, Anjan Thakurta, and Sadanand Vodala analyzed the data, prepared the figures, and co-wrote the manuscript; Jeevan K. Shetty reviewed the manuscript; Martin Schwickart jointly conceived the study and reviewed the manuscript; Olivier Hermine analyzed the data and reviewed the manuscript; John B. Porter jointly conceived the study, interpreted the data, and wrote the manuscript. All authors reviewed and approved the manuscript prior to submission.

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CONFLICT OF INTEREST STATEMENT

Maciej W. Garbowski reports consultancy agreement with Celgene BMS, Imara, and Vifor; and is a member of the advisory committee for Vifor. Manuel Ugidos reports current employment by BMS. Alberto Risueño reports holding a patent, current employment at and currently holding equity in BMS (a publicly traded company). Sadanand Vodala reports current employment at and currently holding equity in BMS (a publicly traded company). Martin Schwickart reports former employment at and currently holding equity in BMS (a publicly traded company). Jeevan K. Shetty reports former employment at BMS. Olivier Hermine reports receiving honoraria from BMS. Anjan Thakurta reports former employment at and currently holding equity in BMS (a publicly traded company). John B. Porter reports consultancy agreement with Agios, bluebird bio, and Celgene BMS; honoraria from bluebird bio, Celgene BMS, La Jolla Pharmaceuticals, Protagonist, Silence Therapeutics, and Vifor; and is a member of the advisory committee for bluebird bio, Celgene BMS, Silence Therapeutics, and Vifor.

DATA AVAILABILITY STATEMENT

Bristol Myers Squibb policy on data sharing may be found at https://www.bms.com/researchers-and-partners/independent-research/data-sharing-request-process.html.

CLINICAL TRIAL REGISTRATION

ClinicalTrials.gov (NCT02604433).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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