Luspatercept lowers hepcidin and ferritin but redistributes body iron in transfusion-dependent thalassemia

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SUPPORTING INFORMATION

SUPPLEMENTAL METHODS

Transfusion methods

Pre-treatment and end-of-study (EOS) iron loading rate (ILR) was calculated from a 48-week time window. The formula used is showed below:

$$ILR\ (mgFe\ kg^{-1}day^{-1}) = \frac{\frac{RBC\ units\ transfused\ for\ 48\ weeks\ x\ 200\ (mgFe)}{mean\ weight\ (kg)}}{48\ weeks\ x\ 7\ (days)}$$

where RBC is red blood cell. In this analysis, hematological response criteria were defined by ILR difference, with those patients being considered responders if $ILR_{EOS} < ILR_{BASELINE}$, indicating a transfusion burden reduction during treatment compared to the pre-treatment period. This method of analysis differs from the prior publication,[1] but allows closer examination of the relationship of transfusion response to expected reductions in body iron as measured by serum ferritin (s-ferritin) and liver iron content (LIC).

Biomarkers

In this analysis we considered the following measurements: hepcidin (Intertek, San Diego, CA, USA), erythroferrone (ERFE), erythropoietin (EPO), growth differentiation factor 15 (GDF15), soluble transferrin receptor 1 (sTfR1) with a validated ELISA (Intertek, San Diego,

CA, USA), GDF11 was measured by QHSP-GDF-11 Simoa™ ultra-high sensitive assay (Myriad RBM, Austin, TX, USA), fetal hemoglobin, reticulocytes, bilirubin, lactate dehydrogenase (LDH), s-ferritin, pretransfusion hemoglobin, and liver function tests (alanine aminotransferase [ALAT] and aspartate aminotransferase [ASPAT] by standard methods, assessed at study sites), thalassemia genotype (beta globin only, coded as β^0/β^0, or non-β^0/β^0) by chart review, splenectomy flag, and spleen size (measured by ultrasonography or MRI at study sites per standard of care) (Table S1).

LIC measurements

LIC measurements were performed on 1.5 T scanners by various methods, including spin-echo sequence R2-FerriScan LIC,[2] and gradient-echo sequence T2*-LIC methods with the latter utilizing various LIC calibrations[3–6] to report hepatic T2* values in mg Fe/g dry weight (dw). While this reflects real-life practices in the management of iron overload, it unfortunately prevents a meaningful across-site comparison of LIC or derivation of study-wide average statistics. We have therefore reported LIC separately for FerriScan- and T2*-based methods, since these 2 methods remain non-interchangeable[6] for absolute LIC values. We pooled all T2* LIC results into one category by deriving LIC values (mg/g dw) from T2* values (ms) according to one calibration formula:[6] $LIC\ [mg/g\ dw] = 31.94 \times (T2^*[ms])^{-1.014}$. If R2* ($s^{-1}$) values were provided, they were first back-transformed into T2* values ($R2^*[s^{-1}] = 1000/(T2^*[ms])$ before LIC calculation using the calibration formula. The T2* MRI acquisition parameters from study sites were compared for echo time structure (first echo time, number and spacing of echo times) and other parameters[7] in order to ensure that the derived T2* LIC values can be validly pooled. Total body iron stores were estimated based on the Angelucci formula,[8] and the total body iron balance was derived from total body iron stores and transfusion iron.[9]

Statistics

Box-and-whisker plots, bar plots and scatter plots represented in this article were generated using the ggplot2 package (v.3.3.3) in the R programming language. Other R packages were

used for adding statistics to plots: rstatix (v.0.7.0), ggpubr (v.0.4.0), gridGraphics (v.0.5.0), and gridExtra (v.2.3).

We constructed the so-called benchmark model, which aimed to explain s-ferritin trend (absolute s-ferritin) in patients with transfusion-dependent thalassemia (TDT) on standard-of-care treatment (transfusion) and study arm (luspatercept or placebo). This was intended to control the effect of luspatercept treatment on s-ferritin with effects and variables that are known to influence s-ferritin behavior during standard-of-care treatment of TDT. This model was tested using the following potential explanatory variables: time on study, baseline s-ferritin, arm allocation (luspatercept or placebo), transfusion effect, genotype, baseline LIC, LIC change, chelator exposure, and their interaction elements.

Linear mixed-effect models (lmer4 R package v.0.1.23) were used to investigate the impact of each biomarker on s-ferritin over time. Patient ID was considered as the random grouping factor, and time (in days) plus transfusion burden (RBC units) were considered as random effects. Time, baseline s-ferritin, arm, transfusion burden, and LIC15 (categorical variable derived from LIC describing whether baseline LIC is below or above 15 mg/g dw) were used to formulate the benchmark model:

$$Serum\ ferritin \sim ARM * time + baseline\ SF + transfusion\ burden + time{:}\ baseline\ SF + ARM{:}\ time{:}\ LIC\ 15 + (time + transfusion\ burden \mid patient\ ID)$$

The model was tested using time on study, baseline s-ferritin, arm allocation (luspatercept or placebo), transfusion effect, genotype, baseline LIC, LIC change, chelator exposure, and their interaction elements as potential explanatory variables. Table S4 shows the final benchmark model, with successfully retained predictor variables of time, baseline s-ferritin, transfusion, arm allocation, their interaction elements, and an interaction element with LIC. Once the benchmark model was optimized, the remaining biomarkers were added to the model one by one, considering single and interaction effects. Non-longitudinal variables

such as hepcidin were encoded into 2 different derivative variables: baseline and delta, representing initial value and difference between baseline and EOS, respectively. Only significant variables were retained in the benchmark model. Next, we evaluated interactions between the biomarkers in the model. As most of the biomarkers refer to body iron metabolism, collinearity issues may impair the robustness and interpretability of linear models. Thus, we performed a randomization test for each biomarker included in the model, i.e. to permutate each biomarker to assess its impact in the treatment coefficient. A variable included in the model is considered truly significant if it strongly affects the treatment coefficient while having a minimum effect after permutations.

Total body iron stores

The mean amount of iron transfused on luspatercept was approximately 1.4 g lower versus placebo (6.1 versus 4.7 g; $P < .0001$; Figure S11A). This difference was not borne out in the between-arm comparison of total body iron stores (TBIS) (Figure S11B). A non-significant trend toward increased TBIS in patients receiving luspatercept was observed. However, TBIS based on T2* LIC were higher for luspatercept versus placebo at 6 and 12 months ($P < .05$; Figure S11B). The mean of 1.4 g of iron saved on luspatercept was approximately 35% of the median baseline TBIS in a 70-kg patient (median baseline LIC: 5.4 mg/g dw). This suggests that comparable amounts of iron were released from spleen (or from the macrophage compartment after splenectomy), or (much less likely) a significant amount of intestinal iron absorption occurred secondary to hepcidin reduction, or both. Iron redistribution phenomena may alter the validity of Angelucci formula, but this particular issue has not been raised before or studied.

Supplemental Discussion

Why we think that intestinal iron loading in TDT on luspatercept is trivial.

In non-transfusion-dependent thalassemia (NTDT) patients followed up in the deferasirox THALASSA study,[10] the annual s-ferritin increase was 115 µg/L/48 weeks and LIC increase

was 0.38 mg/g dw per year. This is equivalent, in non-transfused patients, to a total body iron stores increase of 0.011 mg/kg/day[10] based on the Angelucci formula[8] (0.38 × 10.6 = 4.03 mg Fe/kg; further divided by 365 days equals 0.011 mg/kg/day). The total body iron stores change in the THALASSA study would therefore be 281 mg in a 70-kg person (4.03 mg Fe/kg × 70 kg). In our present study, the median change of total body iron stores on luspatercept was 500 mg increase from baseline (from 3.6 to 4.1 g, not statistically significant vs baseline). This increase would still amount to a 1.78 times higher gut iron loading than in NTDT.

Let us consider a significant increase in LIC: if the median 1.19 mg/g dry weight LIC change from baseline over 48 weeks on luspatercept in patients with spleens (*P* = .006, Figure S8A) would be dependent on the increase of the duodenal iron uptake rate, this would amount to a rate increase of 3.13 times that of NTDT iron loading rate from the gut in the THALASSA study (1.19 × 10.6 = 12.614 mg Fe/kg; further divided by 365 days equals 0.034 mg/kg/day). To repeat, this would assume that the duodenal iron loading rate in TDT would increase to a more than 3 times greater rate than the NTDT rate. This is highly unlikely in our opinion. The TDT versus NTDT intestinal iron loading rate has been previously compared by Pippard et al. (1977) and estimated as being approximately 8 times lower in TDT than in NTDT.[11] Furthermore, when hepcidin is reduced in TDT by luspatercept, it is highly likely that the duodenal iron uptake rate would increase in parallel to macrophage iron release rate as both are regulated by hepcidin in a similar fashion. As macrophage iron release is 25 times greater than duodenal iron flux, we still think that the gut iron loading rate changes are negligible for our purposes in this analysis.

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Supplemental Tables and Figures

TABLE S1 Time points at which biomarkers were measured

Biomarker	VISIT															
	Baseline parameter	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Hepcidin		x							x								
Erythroferrone		x							x								
Erythropoietin		x	x				x				x				x		
GDF11		x					x		x								x
GDF15		x							x								
sTfR1		x							x								
HbF		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Reticulocytes		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Indirect bilirubin		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
LDH		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Serum ferritin		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Spleen size		x							x								x
LIC		x							x								x
MyoIC		x															x
ALAT		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
ASPAT		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Genotype	x																
Splenectomy flag	x																

Abbreviations: ALAT, alanine aminotransferase; ASPAT, aspartate aminotransferase; HbF, fetal hemoglobin; GDF, growth differentiation factor; LDH, lactate dehydrogenase; LIC, liver iron content; MyoIC, myosin IC; sTfR1, soluble transferrin receptor 1.

TABLE S2 Baseline parameters overall and in placebo and luspatercept arms

Baseline variables	Luspatercept (N = 224)	Placebo (N = 112)	Overall (N = 336)
Genotype–n (%)			
β^0/β^0	68 (30.4%)	35 (31.3%)	103 (30.7%)
Non-β^0/β^0	155 (69.2%)	77 (67.0%)	232 (69.0%)
Missing	1 (0.4%)	0	1 (0.3%)
Iron loading rate–pre-treatment period (48 weeks)–mg Fe/kg/day			
Missing–n (%)	1 (0.4%)	0 (0%)	1 (0.3%)
R2 LIC–mg/g dry weight (FerriScan)			
Missing–n (%)	4 (7.5%) (n = 53)	1 (5%) (n = 20)	5 (6.8%) (n = 73)
T2*1 LIC–mg/g dry weight (T2*)			
Missing–n (%)	18 (10.5%) (n = 171)	8 (8.7%) (n = 92)	26 (9.9%) (n = 263)
Serum ferritin–µg/L			
Mean (SD)	2100 (1760)	1890 (1770)	2030 (1760)
Missing–n (%)	14 (6.3%)	12 (10.7%)	26 (7.7%)
GDF11–pg/mL			
Missing–n (%)	7 (3.1%)	6 (5.4%)	13 (3.9%)
EPO–IU/L			
Missing–n (%)	8 (3.6%)	12 (10.7%)	20 (6.0%)
EFRE–ng/mL			
Missing–n (%)	23 (10.3%)	13 (11.6%)	36 (10.7%)
Hepcidin–ng/mL			
Missing–n (%)	23 (10.3%)	15 (13.4%)	38 (11.3%)
GDF15–pg/mL			
Mean (SD)	12300 (9910)	10700 (9440)	11800 (9780)
sTfR1–nM			
Missing–n (%)	15 (6.7%)	10 (8.9%)	25 (7.4%)
Fetal Hb–%			
Missing–n (%)	11 (4.9%)	10 (8.9%)	21 (6.3%)
Reticulocytes–10^9/L			
Missing	114 (50.9%)	53 (47.3%)	167 (49.7%)
LDH–U/L			
Missing–n (%)	12 (5.4%)	6 (5.4%)	18 (5.4%)
Indirect bilirubin–µmol/L			
Missing–n (%)	5 (2.2%)	6 (5.4%)	11 (3.3%)

Abbreviations: EFRE, erythroferrone; EPO, erythropoietin; GDF, growth differentiation factor; Hb, hemoglobin; LDH, lactate dehydrogenase; LIC, liver iron content; SD, standard deviation; sTfR1, soluble transferrin receptor 1.

TABLE S3 Data showing the bias effect on biomarker levels of the blood sample timing within a transfusion cycle, as transfusion cycle sample bias (TCSB), with percentage of explained variance attributable to that bias.

Biomarker	TCSB effect	*P*	R^2	Exp Var (%)	
Hepcidin	−0.0689266	4.04E-01	0.00354281	0.3543	Luspatercept
Liver iron	−0.0138892	0.29211374	0.00525857	0.5259	
EPO	1.76E+00	6.48E-05	7.17E-02	7.1691	
Indirect bilirubin	−1.67E-03	9.47E-01	2.19E-05	0.0022	
EFRE	0.03604608	2.77E-01	0.00602101	0.6021	
GDF15	−33.752753	9.43E-02	0.01393012	1.393	
GDF11	−0.0148924	0.75443495	0.00044975	0.045	
sTfR1	−0.058015	0.49755426	0.00224758	0.2248	
Reticulocytes	5.31E-02	9.03E-01	9.62E-05	0.0096	
Fetal Hb	0.0048366	0.77472251	0.00041257	0.0413	
Ferritin	0.12307092	0.94352715	2.47E-05	0.0025	
LDH	−0.5036299	0.10699696	0.01274773	1.2748	
Hepcidin	−0.27035	2.41E-03	0.08592	8.5921	Placebo
Liver iron	0.014969	0.284	0.01033	1.0331	
EPO	0.2952	0.147	0.01969	1.9691	
Indirect bilirubin	0..2881	0.0316	0.001743	0.833	
EFRE	0.13442	7.41E-04	0.1061	10.6082	
GDF15	70.9	5.50E-03	0.07174	7.1744	
GDF11	−0.01311	0.858	0.0002853	0.0285	
sTfR1	0.05149	0.523	0.003857	0.3857	
Reticulocytes	−0.2088	0.47	0.0002125	0.6396	
Fetal Hb	−0.02129	0.0508	0.03797	3.7975	
Ferritin	0.4729	0.815	0.0005313	0.0531	
LDH	0.1272	0.532	0.00381	0.381	

Abbreviations: EFRE, erythroferrone; EPO, erythropoietin; GDF, growth differentiation factor; Hb, hemoglobin; LDH, lactate dehydrogenase; sTfR1, soluble transferrin receptor 1.

TABLE S4 Multilevel models for change (mixed linear regression) explaining serum ferritin change on study.

Predictors	Benchmark (Placebo: LIC < 15 as ref.)			Model B (Hepcidin)			Model C (EPO)			Model D (Bilirubin)			Model E (B+C)			Model F (E+D)		
Fixed effects	**Coefficient**	**SE**	***P***	**Coefficient**	**SE**	***P***	**Coefficient**	**SE**	***P***	**Coefficient**	**SE**	***P***	**Coefficient**	**SE**	***P***	**Coefficient**	**SE**	***P***
Time (days)	0.8391	0.3313	< .05	0.6872	0.3638	.	0.6655	0.3063	< .05	0.238	0.4211		0.5194	0.3286		-0.0119	0.4449	
Serum ferritin at baseline	1007	0.01241	< .001	1.01	0.01316	< .001	1	0.01164	< .001	1009	0.01278	< .001	1009	0.01285	< .001	1016	0.01325	< .001
Arm (Placebo)	-270.5	66.68	< .001	-278.4	64.42	< .001	-285.1	58.98	< .001	-291.6	83.33	< .001	-335.3	66.36	< .001	-329.5	84.8	< .001
Arm (Luspatercept)	-309.6	59.52	< .001	-291.7	54.19	< .001	-294.4	49.88	< .001	-152	72.43	< .05	-297.4	56.04	< .001	-211.5	74.33	< .01
Transfusion	40.32	6252	< .001	40.39	5393	< .001	43.91	6012	< .001	39.59	6509	< .001	47.06	6484	< .001	41.86	6631	< .001
Time: Serum ferritin at baseline	-0.0004524	0.0001081	< .001	-0.0003387	0.0001157	< .01	-0.0002607	0.0000974	< .01	-0.0004261	0.0001074	< .001	-0.0001027	0.0001015		-0.0003027	0.0001134	< .01
Time: Arm (Luspatercept): LIC < 15	-1.157	0.3606	< .01	-0.8322	0.4056	< .05	-1151	0.3326	< .001	-0.7801	0.4882		-0.8957	0.3636	< .05	-0.3845	0.5323	
Time: Arm (Luspatercept): LIC > 15	0.2009	0.5118		0.193	0.5503		-0.5987	0.4556		0.4569	0.6097		-0.8484	0.4746	.	0.4095	0.65	
Time: Arm (Placebo): LIC > 15	0.7031	0.641		0.487	0.6763		-0.3604	0.5708		0.6058	0.6371		-0.7139	0.5765		0.3255	0.6669	
Arm (Placebo): hepcidin delta				0.2643	1296								-0.159	1277		-0.07122	1297	
Arm (Luspatercept): hepcidin delta				1106	0.7487								1309	0.7155	.	1412	0.7523	.
Time: Arm (Placebo): hepcidin delta				-0.001867	0.009007								0.003844	0.0082		-0.00587	0.00885	
Time: Arm (Luspatercept): hepcidin delta				0.01408	0.005014	< .01							0.01398	0.004597	< .01	0.01355	0.004903	< .01
Arm (Placebo): EPO delta							-0.4025	0.5578					-0.253	0.5841		-0.3323	0.5877	
Arm (Luspatercept): EPO delta							-0.4391	0.1209	< .001				-0.4541	0.1219	< .001	-0.4011	0.1211	< .01
Time: Arm (Placebo): EPO delta							-0.005392	0.003482					-0.00491	0.003451		-0.00492	0.003914	
Time: Arm (Luspatercept): EPO delta							0.00211	0.0007294	< .01				0.002217	0.0007016	< .01	0.002252	0.0008643	< .01

Arm (Placebo): bilirubin delta										0.6952	1225					1057	1233	
Arm (Luspatercept): bilirubin delta										-4028	0.91	< .001				-1795	0.9831	.
Time: Arm (Placebo): bilirubin delta										0.01591	0.007337	< .05				0.01726	0.007491	< .05
Time: Arm (Luspatercept): bilirubin delta										0.005761	0.00532					0.003073	0.005781	
Random effects	**Variance**	**L-hood ratio**	***P***	**Variance**	**L-hood ratio**	***P***	**Variance**	**L-hood ratio**	***P***	**Variance**	**L-hood ratio**	***P***	**Variance**	**L-hood ratio**	***P***	**Variance**	**L-hood ratio**	***P***
Intercept	366900	-	-	150800	-	-	142000	-	-	438500	-	-	212800	-	-	318600	-	-
Time (days)	5155	953.5	< .001	4967	840.82	< .001	4351	787.8	< .001	5094	918.42	< .001	4333	671.93	< .001	4792	824.05	< .001
Transfusion	3071	24.68	< .001	185.2	21.8	< .001	2359	-98.89		3783	51.37	< .001	2964	-128.55		3170	39.95	< .001
Residual	141200	-	-	134600	-	-	150700	-	-	140000	-	-	140500	-	-	131300	-	-
Observations	4656			4133			4619			4607			4111			4068		
Groups	297			251			291			297			249			249		
AIC	69863.3			61747.8			69455.3			69139			61557.6			60763.2		
BIC	69966.4			61874.4			69584			69267.7			61709.3			60939.9		
Deviance	69831.3			61707.8			69415.3			69099			61509.6			60707.2		
Df.resid	4640			4113			4599			4587			4087			4040		

AIC, Akaike information criterion; BIC, Bayesian information criterion; Df.resid, residual degrees of freedom; EPO, erythropoietin; LIC, liver iron content; SE, standard error.

TABLE S5 Mixed model of EPO as predicted by treatment, time, HbF.

Predictors	**EPO predicted by HbF**				
	Estimate	SE	df	Pr(>|t|)	
Intercept	80.15737	9.61275	480.99831	7.97E-16	***
Days	0.03308	0.08058	401.36605	0.681653	
Luspatercept:Days	0.33508	0.09833	411.24954	7.19E-04	***
HBF	0.76051	0.72556	517.67737	0.29505	
Luspatercept:HbF	1.36176	0.76407	506.24437	0.075309	.
Random effects	Variance	L-hood ratio	Significance		
Intercept	8.66E+03	-	-		
Time (days)	1.99E-01	113.43	***		
Residual	1.97E+04	-	-		
Observations	1.32E+03				
Groups	3.26E+02				
AIC	17394.7				
BIC	17441.38				
Deviance	17376.7				

Erythropoietin is increasing on treatment with luspatercept from 80.15 ± 9.6 IU/L at baseline by 0.33 ± 0.1 IU/L per day on luspatercept arm (but not placebo). HbF as a predictor is not significant, has no additional effect on erythropoietin above the effect of luspatercept, and interaction of HbF with luspatercept is not statistically significant. AIC, Akaike Information Criterion; BIC, Bayesian Information Criterion; df, degrees of freedom; HbF, fetal hemoglobin: EPO, erythropoietin; SE, standard error.

FIGURE S1 Cumulative chelation exposure as efficient chelator iron binding equivalents (IBE). Cumulative IBE at each visit during 48 weeks of treatment on (A) placebo and (B) luspatercept. IBE, iron binding equivalent.

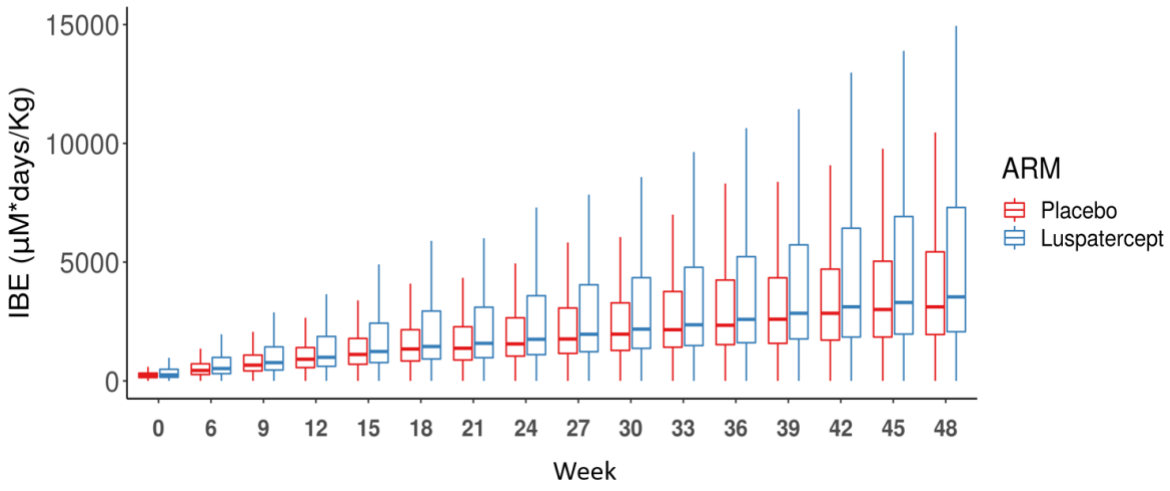


FIGURE S2 Relationship between biomarker changes (delta) on study and hematological response. Hematological response was assessed by ILR difference between baseline and EOS (see Methods). Linear regression models were used to compute the *P* values. (A) EPO, R^2 = .0003 (placebo) and .03 (luspatercept); n = 269. (B) ERFE, R^2 = .08 (placebo) and .01 (luspatercept); n = 279. (C) Hepcidin, R^2 = .02 (placebo) and .03 (luspatercept); n = 279. (D) sTfR1, R^2 = .06 (placebo) and .05 (luspatercept); n = 290. (E) GDF 15, R^2 = .006 (placebo) and .01 (luspatercept); n = 285. (F) Reticulocytes, R^2 = .002 (placebo) and .03 (luspatercept); n = 135. (G) Indirect bilirubin, R^2 = 7.64e−07 (placebo) and .02 (luspatercept); n = 276. (H) LDH, R^2 = .0009 (placebo) and .003 (luspatercept); n = 254. (I) GDF11, R^2 = .008 (placebo) and 1.56e-05 (luspatercept); n = 288. EOS, end of study; EPO, erythropoietin; ERFE, erythroferrone; GRD, growth differentiation factor; ILR, iron loading rate; LDH, lactate dehydrogenase sTfR1, soluble transferrin receptor 1.

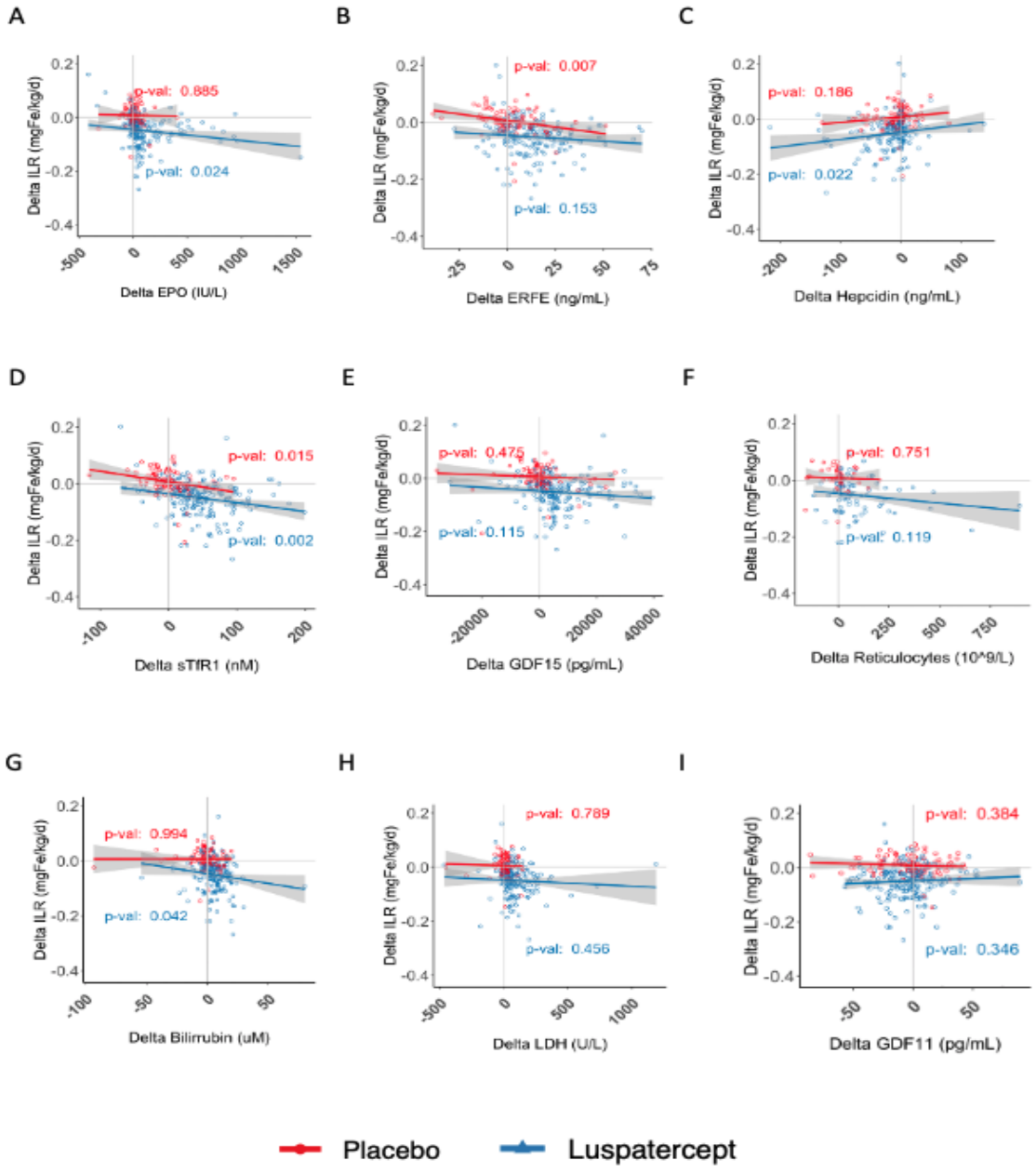

A
Delta ILR (mgFe/kg/d)
p-val: 0.885
p-val: 0.024
Delta EPO (IU/L)
B
p-val: 0.007
p-val: 0.153
Delta ERFE (ng/mL)
C
p-val: 0.186
p-val: 0.022
Delta Hepcidin (ng/mL)
D
p-val: 0.015
p-val: 0.002
Delta sTfR1 (nM)
E
p-val: 0.475
p-val: 0.115
Delta GDF15 (pg/mL)
F
p-val: 0.751
p-val: 0.119
Delta Reticulocytes (10^9/L)
G
p-val: 0.994
p-val: 0.042
Delta Bilirrubin (uM)
H
p-val: 0.789
p-val: 0.456
Delta LDH (U/L)
I
p-val: 0.384
p-val: 0.346
Delta GDF11 (pg/mL)
Placebo
Luspatercept

FIGURE S3 Total Hb quantification during 48 weeks of treatment. Arm-wise paired Wilcoxon tests were used. * *P* <.05. Hb, hemoglobin.

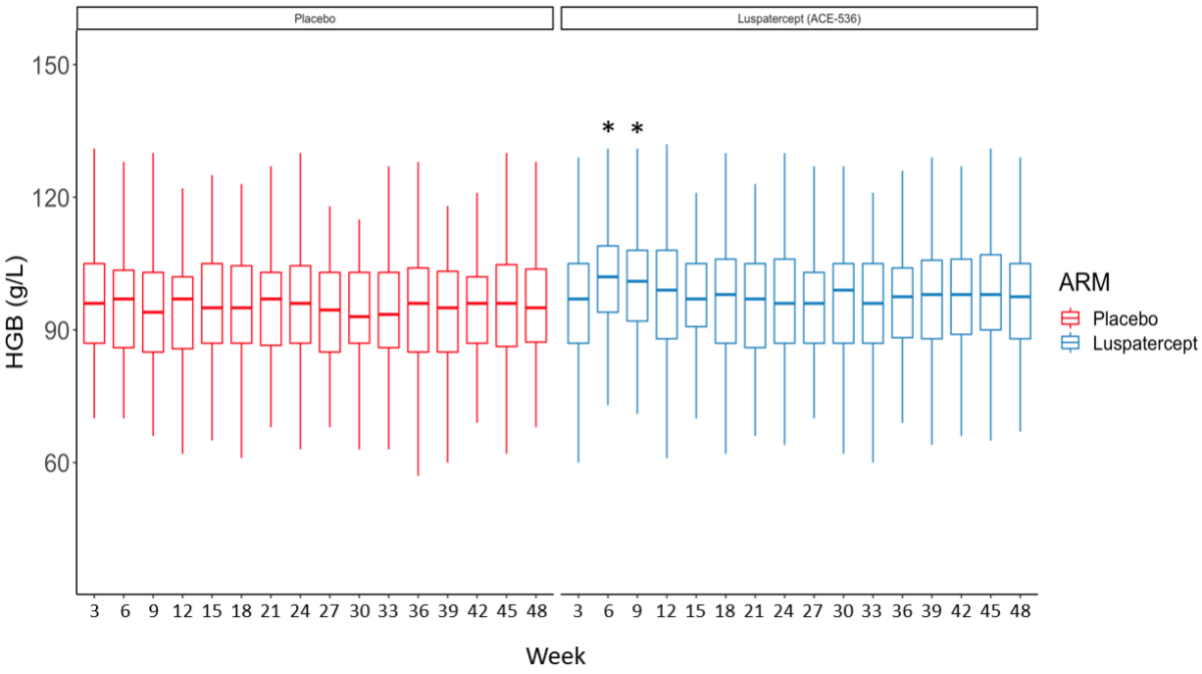


FIGURE S4 Association of biomarkers with s-ferritin response. s-ferritin response was assessed per patient by linear regression models, considering only significant time slopes as significant s-ferritin response (positive or negative). *P* values were computed using Wilcoxon test (A-J) and Fisher's exact test (K). Wilcoxon test *P* values were corrected, and box-and-whisker plots (A-J) and bar charts (K) were used. Baseline and last follow-up for each biomarker are represented for placebo and luspatercept. (A) Hepcidin; n = 156. (B) ERFE; n = 155. (C) GDF 15; n = 156. (D) sTfR1), n = 156. (E) EPO; n = 159. (F) GDF11; n = 156. (G) LDH; n = 160. (H) Indirect bilirubin; n = 159. (I) ILR at baseline, n = 160. (J) Spleen size; n = 64. (K) Association between spleen presence and s-ferritin response; n = 160. * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$; **** $P \leq .0001$. EPO, erythropoietin; ERFE, erythroferrone; GDF, growth differentiation factor; ILR, iron loading rate; LDH, lactate dehydrogenase; s-ferritin, serum ferritin; sTfR1, soluble transferrin receptor 1.

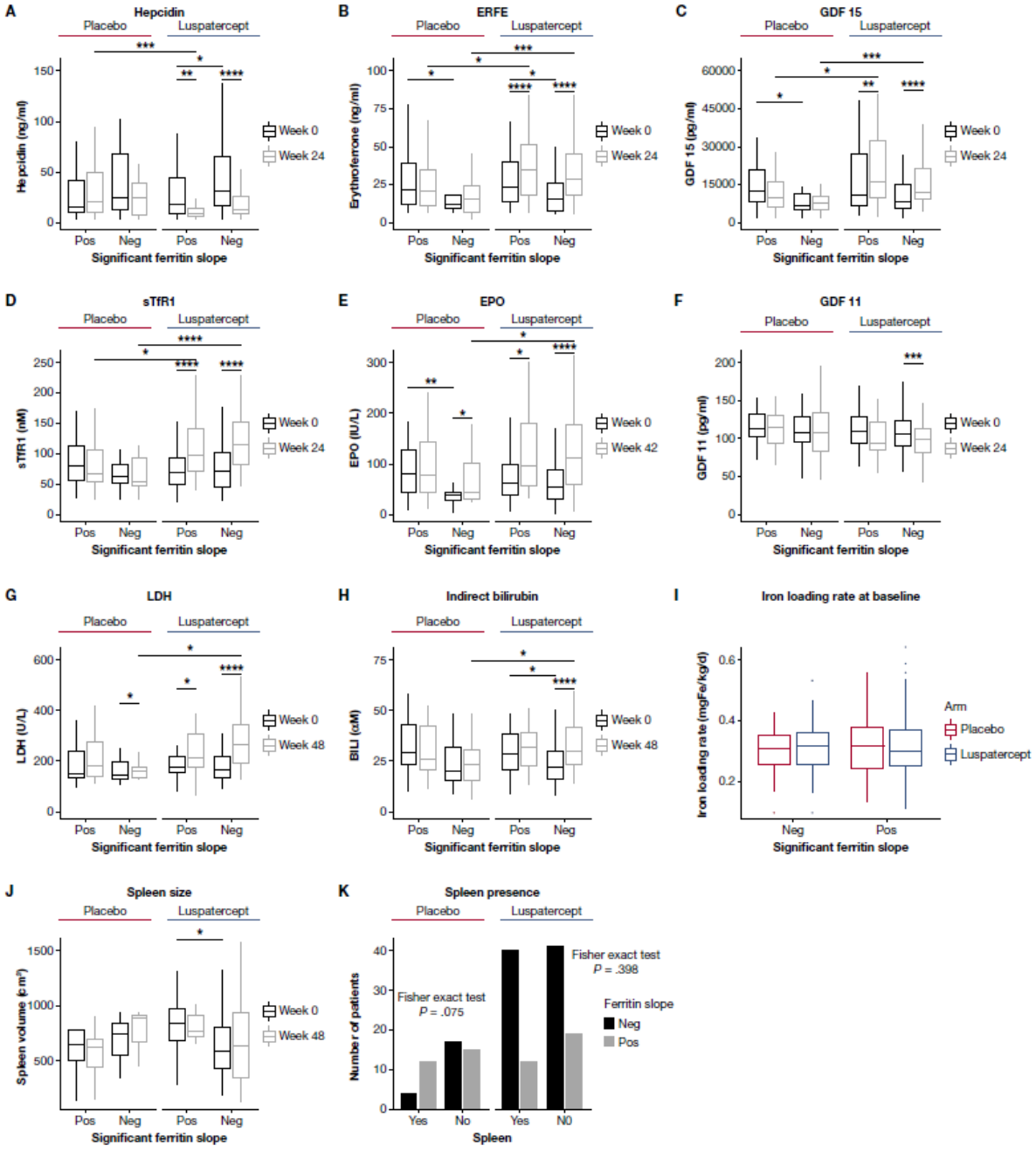
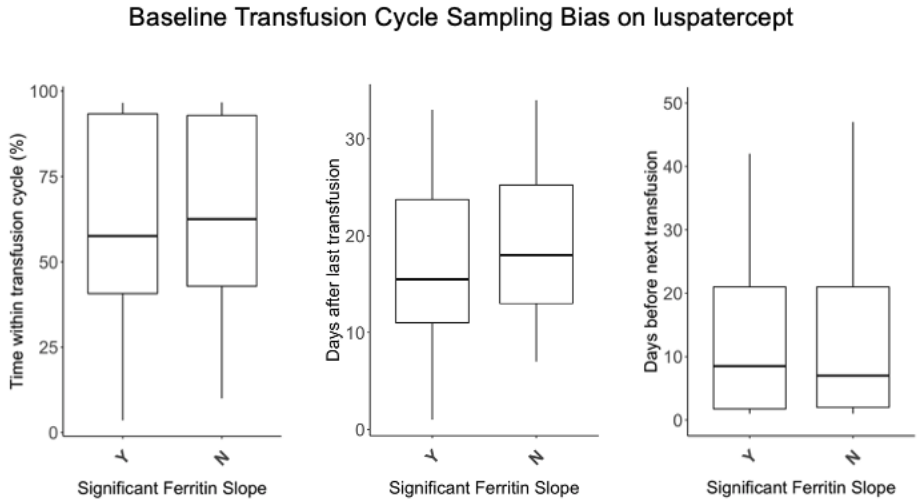

A
Hepcidin
Placebo
Luspatercept
Hepcidin (ng/ml)
Week 0
Week 24
Pos
Neg
Significant ferritin slope
B
ERFE
Erythroferrone (ng/ml)
Week 0
Week 24
C
GDF 15
GDF 15 (pg/ml)
Week 0
Week 24
D
sTfR1
sTfR1 (nM)
Week 0
Week 24
E
EPO
EPO (IU/L)
Week 0
Week 42
F
GDF 11
GDF 11 (pg/ml)
Week 0
Week 24
G
LDH
LDH (U/L)
Week 0
Week 48
H
Indirect bilirubin
BILI (uM)
Week 0
Week 48
I
Iron loading rate at baseline
Iron loading rate (mgFe/kg/d)
Arm
Placebo
Luspatercept
J
Spleen size
Spleen volume (cm³)
Week 0
Week 48
K
Spleen presence
Number of patients
Fisher exact test
P = .075
Fisher exact test
P = .398
Ferritin slope
Neg
Pos
Yes
No
N0
Spleen

FIGURE S5 Transfusion cycle sampling bias at baseline for s-ferritin responders and non-responders on luspatercept. (A) Time within transfusion cycle; (B) days after last transfusion; (C) days before next transfusion. Unpaired Wilcoxon and *t*-tests were used; no significant differences were found. s-ferritin, serum ferritin



* not significant differences

FIGURE S6 Relationships with s-ferritin response. Coefficients shown in tables are computed using multiple linear regressions (A-E). Significant difference between placebo and luspatercept slopes (A-E) was obtained using a separate regression model (not shown) with the interaction delta biomarker – arm. In every plot (A-F), regression lines are shown separately for placebo (red) and luspatercept (blue). Box-and-whisker plots and unpaired Wilcoxon tests were used for categorical variables (F-G). (A) Hepcidin; n = 286; (B) ERFE; n = 288; (C) EPO; n = 300. (D) Indirect bilirubin; n = 317. (E) Spleen size; n = 127. (F) Spleen presence; n = 317. (G) Binary genotype; n = 317. ERFE, erythroferrone: EPO, erythropoietin; NS, not significant; SE, standard error; s-ferritin, serum ferritin; sTfR1, soluble transferrin receptor 1.

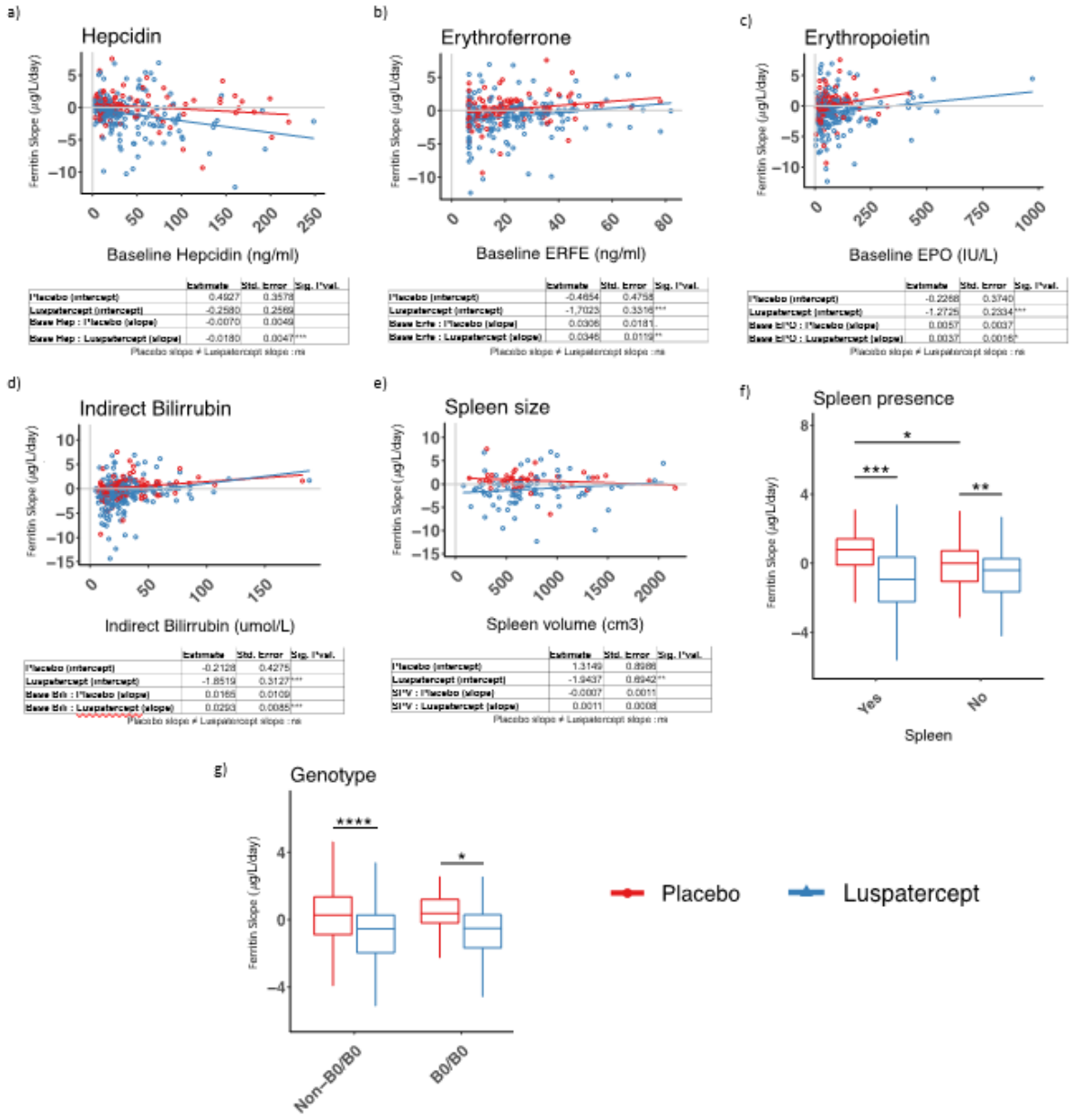
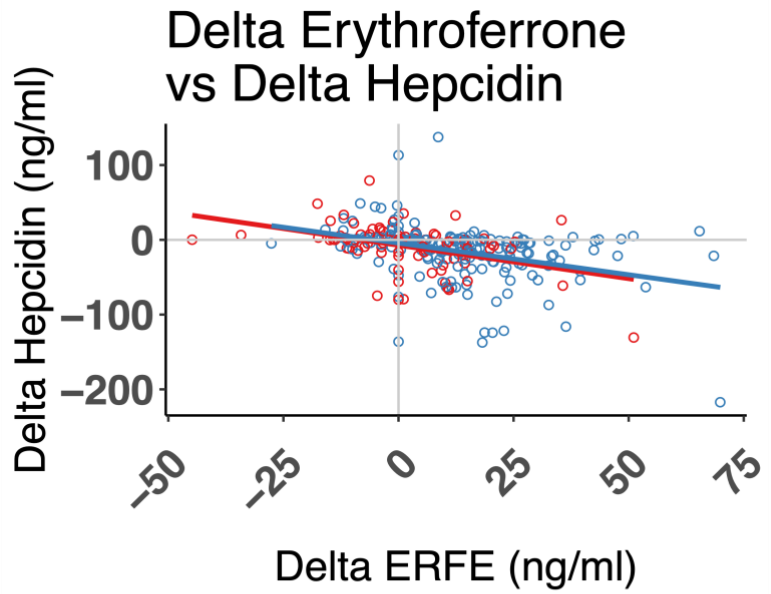

a)
Hepcidin
Ferritin Slope (µg/L/day)
Baseline Hepcidin (ng/ml)
b)
Erythroferrone
Ferritin Slope (µg/L/day)
Baseline ERFE (ng/ml)
c)
Erythropoietin
Ferritin Slope (µg/L/day)
Baseline EPO (IU/L)
d)
Indirect Bilirrubin
Ferritin Slope (µg/L/day)
Indirect Bilirrubin (umol/L)
e)
Spleen size
Ferritin Slope (µg/L/day)
Spleen volume (cm3)
f)
Spleen presence
Ferritin Slope (µg/L/day)
Yes
No
Spleen
g)
Genotype
Ferritin Slope (µg/L/day)
Non−B0/B0
B0/B0
Placebo
Luspatercept

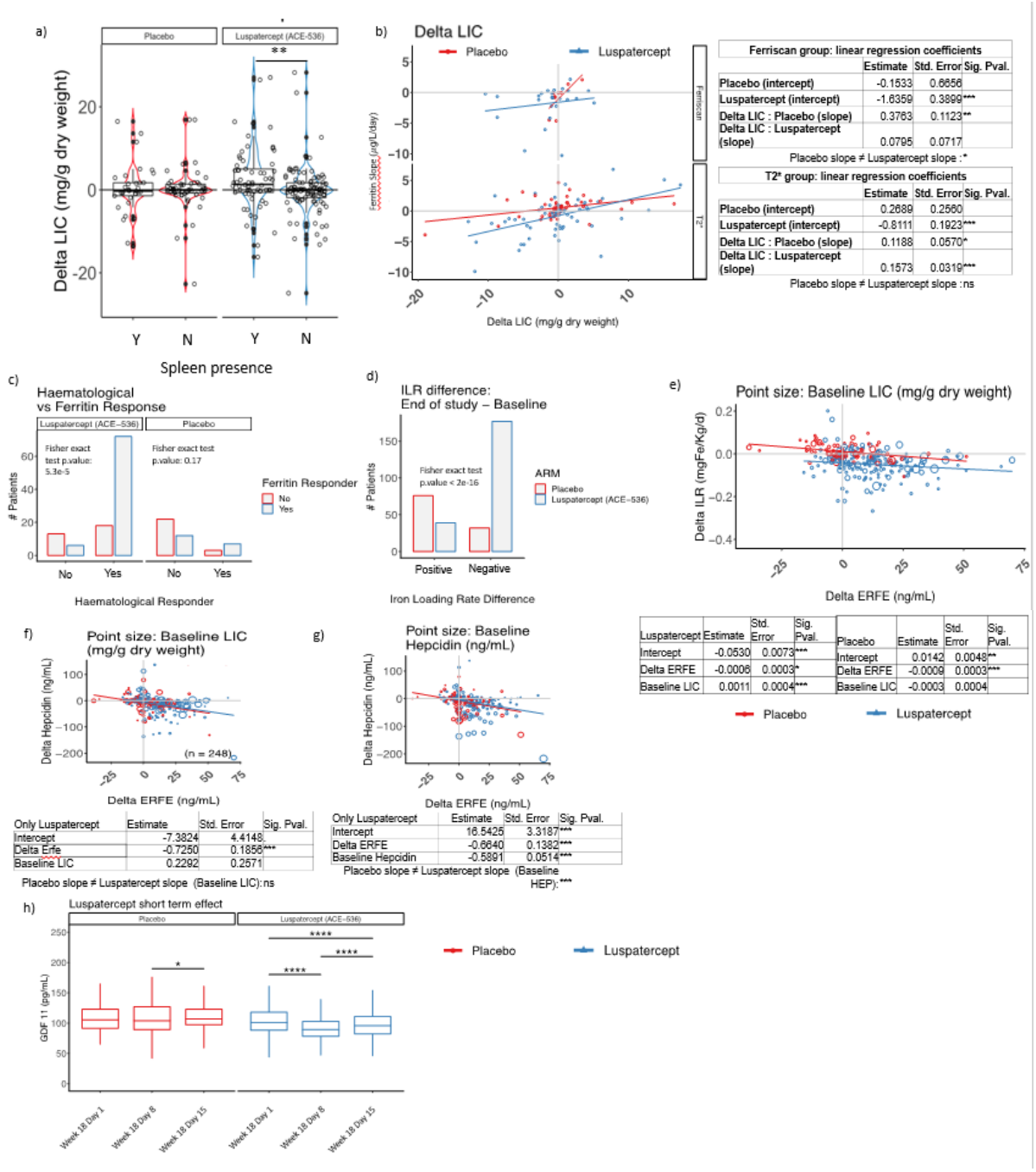
FIGURE S7 Delta ERFE versus delta hepcidin. Coefficients shown in the table were computed using multiple linear regressions. Significant difference between the placebo and luspatercept slopes was obtained using a separate regression model (not shown) with the interaction delta biomarker – arm. Regression lines are shown separately for placebo (red) and luspatercept (blue). * $P \leq .05$; *** $P \leq .001$. ERFE, erythroferrone; NS, not significant; SE, standard error.



	Estimate	Std. Error	Sig. Pval.
Placebo (intercept)	-7.2599	3.4424	*
Luspatercept (intercept)	-4.3719	3.0881	
Delta Erfe : Placebo (slope)	-0.8930	0.2514	***
Delta Erfe : Luspatercept (slope)	-0.8463	0.1515	***

Placebo slope ≠ Luspatercept slope : ns

FIGURE S8 Association between biomarkers in the iron metabolism cycle. Coefficients shown in tables were computed using multiple linear regressions. Significant difference between placebo and luspatercept slopes (A, C-E) was obtained using a separate regression model (not shown) with the interaction delta biomarker arm. In every plot, regression lines are shown separately for placebo (red) and luspatercept (blue). (A) LIC change on placebo (red) and luspatercept (blue) in patients with and without spleen, box-and-whisker plots; n = 253, unpaired Wilcoxon test *P* value shown. (B) s-ferritin slope (all, significant, and not significant slopes) explained by delta LIC. The relationship was evaluated separately for FerriScan and T2* LIC quantification methods; n = 195. (C) Treatment-wise association between hematological response and s-ferritin response (red = No, blue = Yes) using Fisher's exact test; n = 332. (D) Association between hematological response (as ILR difference) and treatment using Fisher's exact test; n = 332; red = placebo, blue = luspatercept. (E) Delta ILR explained by delta ERFE and baseline LIC; alternative representation of supplemental Figure 7. Point sizes represent baseline LIC; n = 279. (F) Delta hepcidin explained by Delta ERFE; point sizes represent baseline LIC; n = 248. (G) Delta hepcidin explained by Delta ERFE; point sizes represent baseline hepcidin; n = 248. (H) GDF11 levels within luspatercept and placebo dosing cycles, pre-dose (immediate), 1 week, and 2 weeks post dose shown; paired and unpaired Wilcoxon tests shown. * $P \leq .05$; **** $P \leq .0001$. EPO, erythropoietin; ERFE, erythroferrone; GDF, growth differentiation factor; ILR, iron loading rate; LIC, liver iron content; s-ferritin, serum ferritin.



Ferriscan group: linear regression coefficients	Estimate	Std. Error	Sig. Pval.
Placebo (intercept)	-0.1533	0.6656	
Luspatercept (intercept)	-1.6359	0.3899	***
Delta LIC : Placebo (slope)	0.3763	0.1123	**
Delta LIC : Luspatercept (slope)	0.0795	0.0717	

Placebo slope ≠ Luspatercept slope : *

T2* group: linear regression coefficients	Estimate	Std. Error	Sig. Pval.
Placebo (intercept)	0.2689	0.2560	
Luspatercept (intercept)	-0.8111	0.1923	***
Delta LIC : Placebo (slope)	0.1188	0.0570	^
Delta LIC : Luspatercept (slope)	0.1573	0.0319	***

Placebo slope ≠ Luspatercept slope : ns

Luspatercept	Estimate	Std. Error	Sig. Pval.
Intercept	-0.0530	0.0073	***
Delta ERFE	-0.0006	0.0003	*
Baseline LIC	0.0011	0.0004	***

Placebo	Estimate	Std. Error	Sig. Pval.
Intercept	0.0142	0.0048	**
Delta ERFE	-0.0009	0.0003	***
Baseline LIC	-0.0003	0.0004	

Only Luspatercept	Estimate	Std. Error	Sig. Pval.
Intercept	-7.3824	4.4148	.
Delta Erfe	-0.7250	0.1856	***
Baseline LIC	0.2292	0.2571	

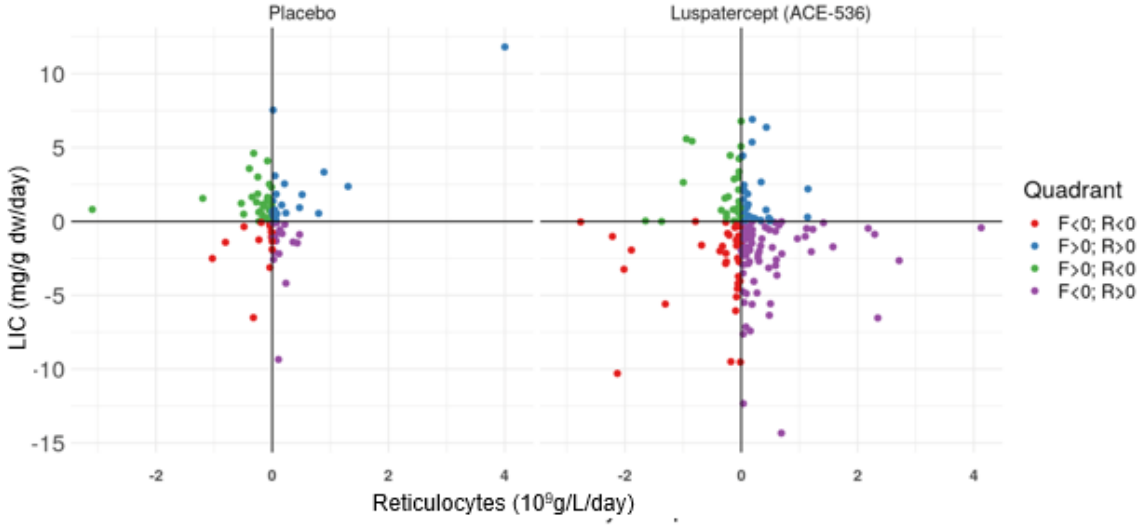
Placebo slope ≠ Luspatercept slope (Baseline LIC): ns

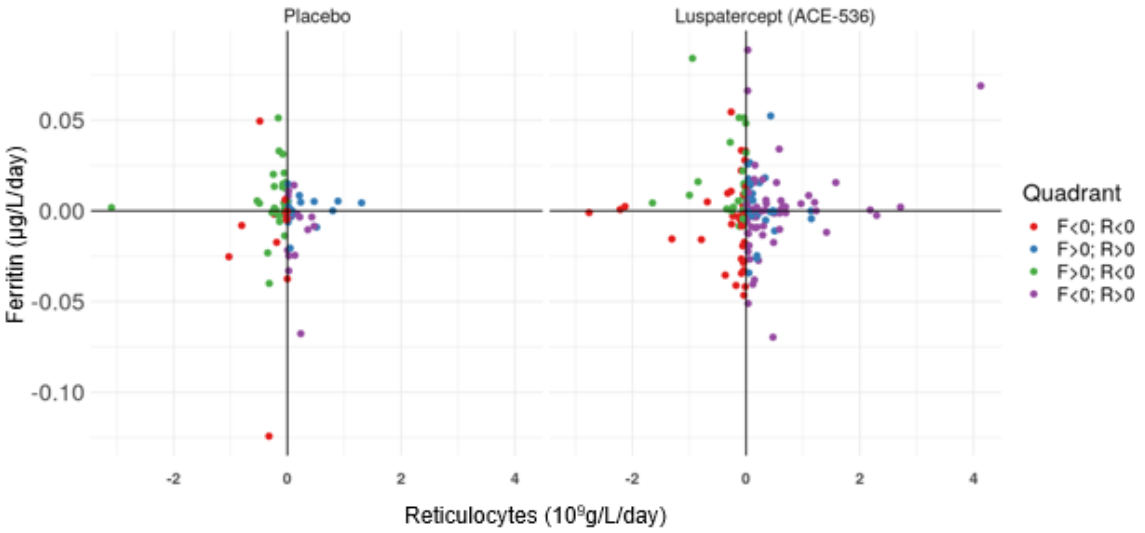
Only Luspatercept	Estimate	Std. Error	Sig. Pval.
Intercept	16.5425	3.3187	***
Delta ERFE	-0.6640	0.1382	***
Baseline Hepcidin	-0.5891	0.0514	***

Placebo slope ≠ Luspatercept slope (Baseline HEP): ***

FIGURE S9 Ferritin reduction in reticulocyte responders does not predict their LIC reduction. Top panel: serum ferritin change (as patient-wise slope)) plotted against reticulocyte change (as patient-wise slope) for placebo (left) and luspatercept arm (right). Each quadrant is color-defined and represented in the bottom panels.

Bottom panel: LIC change (as patient-wise LIC slope) plotted against reticulocyte change with quadrant color code from the top paned, Compare how purple quadrant (reduction of ferritin and increase in reticulocytes) distribution differs between the placebo and luspatercept arm with respect to LIC change. On placebo these ferritin responders typically reduce LIC, while on luspatercept more than half of these ferritin responders increase LIC (purple points distribute nearly equally above and below the x-axis). dw, dry weight; LIC, liver iron content;





FERRITIN vs. RETICULOCYTES				LIC vs. RETICULOCYTES		
	Placebo	Luspatercept (ACE-536)			Placebo	Luspatercept (ACE-536)
F<0; R<0	14	42		F<0; R<0	14	40
F>0; R>0	22	28		F>0; R>0	20	28
F>0; R<0	33	28		F>0; R<0	31	26
F<0; R>0	22	79		F<0; R>0	20	71

FIGURE S10 Reticulocyte and HbF time courses

Top panel: Absolute reticulocyte counts (×10^9/L) at each visit (expressed as weeks after first dose) for luspatercept arm (blue) and placebo arm (red) are shown as box-and-whiskers with median. Comparison at each time point between luspatercept and placebo with Wilcoxon-Mann-Whitney test's *P* value indicated.

Bottom panel: Percentage HbF values at each visit (expressed as weeks after first dose) for luspatercept arm (blue) and placebo arm (red) are shown as box-and-whiskers with median. Comparison at each time point between luspatercept and placebo with Wilcoxon-Mann-Whitney test's *P* value indicated. HbF, fetal hemoglobin.

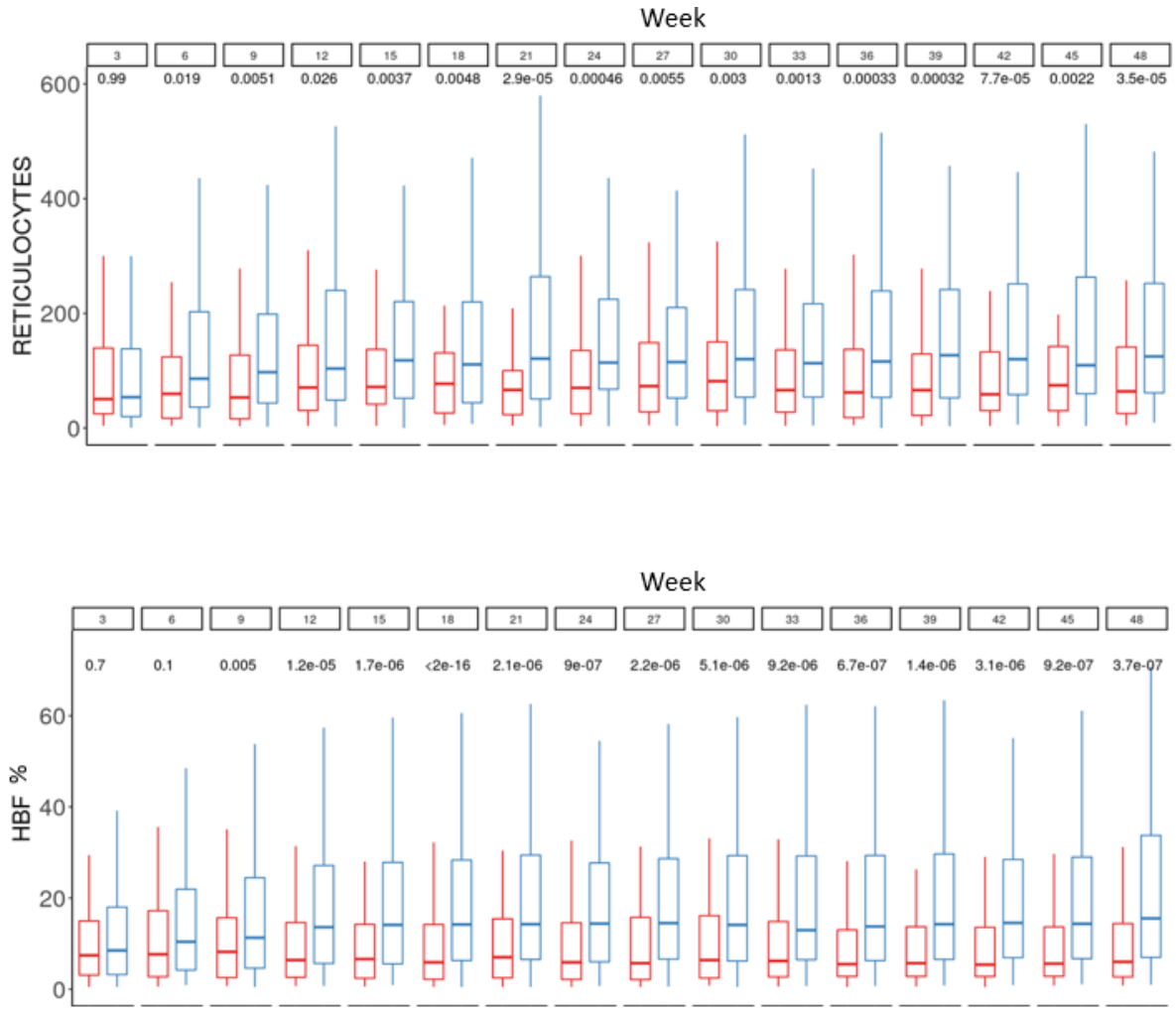
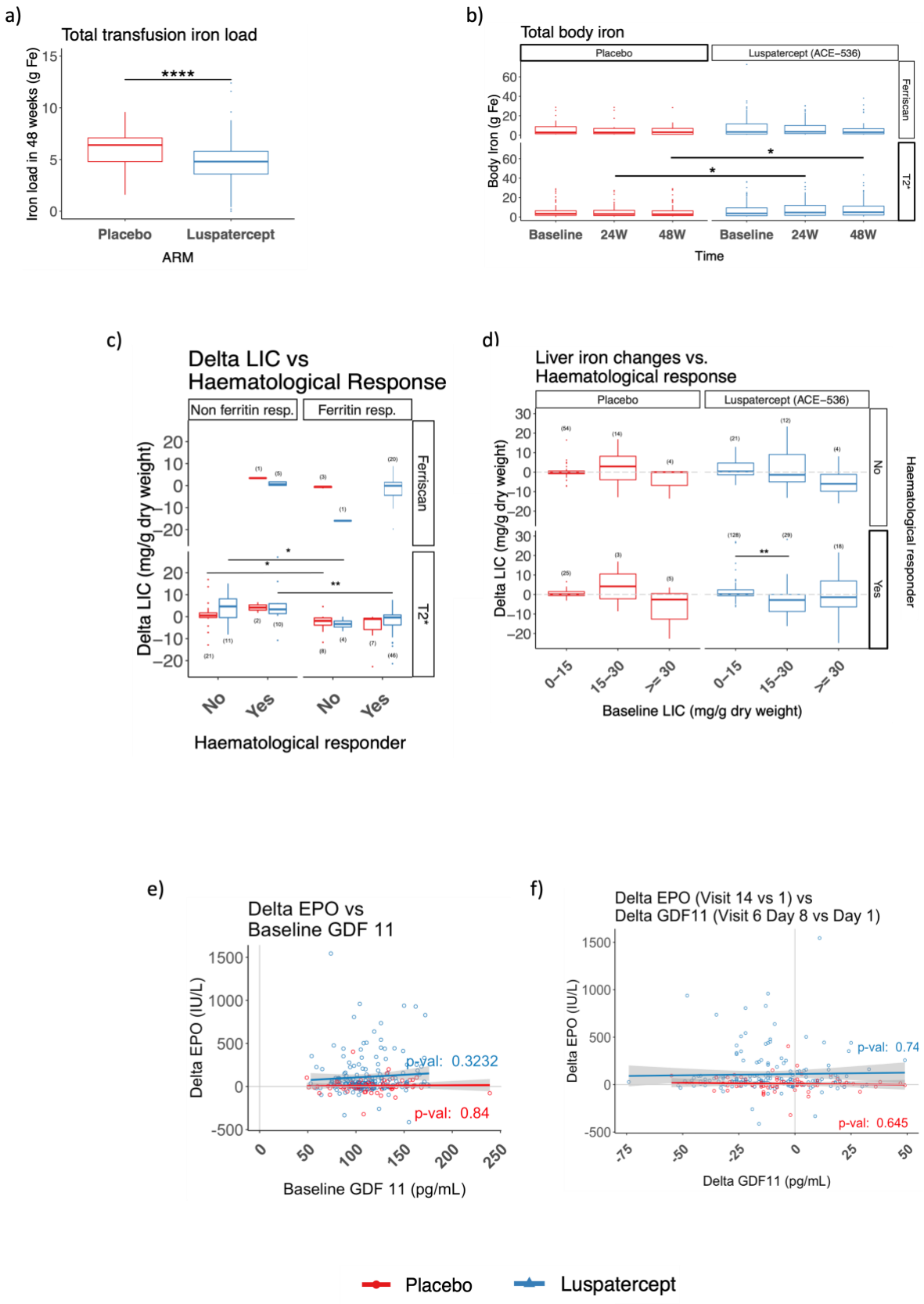


FIGURE S11 Iron redistribution in patients treated with lupatercept. (A) Total transfusion iron load on study (i.e., over 48 weeks). Distributions for placebo (red) and luspatercept (blue) were compared by unpaired Wilcoxon test. Box-and-whisker plot was used for representation; n = 332. (B) Total body iron at baseline, 6 months (24 weeks), and end of study (48 weeks) for placebo (red) and luspatercept (blue). Box-and-whisker plot and Wilcoxon test used; n = 332. (C) Delta LIC association with hematological and s-ferritin responses by LIC method. Box-and-whisker plot and Wilcoxon test used; number of patients given in parentheses. (D) Delta LIC as a function of baseline LIC for placebo (red) and luspatercept (blue) subdivided by hematological response. Box-and-whisker plot and Wilcoxon test were used; number of patients given in parentheses. (E) Delta EPO versus baseline GDF11. Linear regression lines shown separately for placebo (red) and luspatercept (blue); *P* values correspond to slope coefficient; n = 266. (F) Delta EPO versus (short-term) delta GDF11. Linear regression lines shown separately for placebo (red) and luspatercept (blue); *P* values correspond to slope coefficient; n = 263. * $P \leq .05$; ** $P \leq .01$. EPO, erythropoietin; GDF, growth differentiation factor; LIC, liver iron content; s-ferritin, serum ferritin.


a)
Total transfusion iron load
Iron load in 48 weeks (g Fe)
****
Placebo
Luspatercept
ARM
b)
Total body iron
Placebo
Luspatercept (ACE−536)
Ferriscan
T2*
Body Iron (g Fe)
Baseline
24W
48W
Time
c)
Delta LIC vs
Haematological Response
Non ferritin resp.
Ferritin resp.
Delta LIC (mg/g dry weight)
No
Yes
Haematological responder
d)
Liver iron changes vs.
Haematological response
Placebo
Luspatercept (ACE−536)
Haematological responder
Baseline LIC (mg/g dry weight)
0−15
15−30
>= 30
e)
Delta EPO vs
Baseline GDF 11
Delta EPO (IU/L)
p-val: 0.3232
p-val: 0.84
Baseline GDF 11 (pg/mL)
f)
Delta EPO (Visit 14 vs 1) vs
Delta GDF11 (Visit 6 Day 8 vs Day 1)
Delta EPO (IU/L)
p-val: 0.74
p-val: 0.645
Delta GDF11 (pg/mL)
Placebo
Luspatercept