Increased levels of ERFE-encoding *FAM132B* in patients with Congenital Dyserythropoietic Anemia type II

Roberta Russo[1,2], Immacolata Andolfo[1,2], Francesco Manna[2], Gianluca De Rosa[2], Luigia De Falco[1,2], Antonella Gambale[1,2], Mariasole Bruno[2,3], Alessandro Mattè[3], Paolo Ricchi[4], Domenico Girelli[3], Lucia De Franceschi[3], Achille Iolascon[1,2]

[1]Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, Napoli, Italy

[2] CEINGE Biotecnologie Avanzate, Napoli, Italy

[3] Department of Medicine, University of Verona and AOUI-Verona, Verona, Italy

[4] UOSD Malattie rare del globulo rosso, AORN A. Cardarelli, Napoli, Italy

Running heads: Erythroferrone expression in CDAII

Correspondence:

Roberta Russo, PhD

CEINGE – Biotecnologie Avanzate

Via Gaetano Salvatore 486

80145 Naples (Italy)

Email: roberta.russo@unina.it

Recessive mutations in *SEC23B* gene cause congenital dyserythropoietic anemia type II (CDAII),[1] a rare hereditary disorder hallmarked by ineffective erythropoiesis, iron overload, and reduced expression of hepatic hormone hepcidin.[2,3] Some erythroid regulators have been proposed as pathological suppressors of hepcidin expression, such as growth differentiation factor 15 (GDF15) in thalassemia, CDAI and II,[4-6] even if alone it seems not necessary for physiological hepcidin suppression.[7] The most recently described is the erythroblast-derived hormone erythroferrone (ERFE), a member of TNF-α superfamily that specifically inhibits hepcidin production. ERFE-encoding *FAM132B* is an erythropoietin (EPO)-responsive gene in experimental models.[8] However, the function of ERFE in humans remains to be investigated.

To evaluate ERFE expression in CDAII patients we enrolled 37 *SEC23B*-related non-transfusion dependent patients (Table S1). Ten new cases from 9 unrelated pedigrees were described (Table S2). All patients were young adults (19.1±3.0 years at diagnosis), with high transferrin saturation (71.4±5.5%). For case-control study, 29 age and gender matched healthy controls (HC) were also included (Table S1). See Methods in Supplemental data available on the BloodWeb.

We observed a statistically significant overexpression of *FAM132B* gene either in peripheral blood leukocytes (PBL) (Figure 1a) or in reticulocytes (Figure S1a) from CDAII patients compared to HC. Indeed, a statistically significant correlation between PBL and reticulocyte *FAM132B* expression from the same patients was observed (Spearman $\rho=0.78$, $p=0.02$) (Figure S1b). Similarly, a marked up-regulation of *Erfe* in both erythroblasts and PBL from $Hbbt^{h3/+}$mice compared to wild type ones was observed (Figure S1c). Of note, approximately 0.03% of erythroblasts (CD71+/CD45- cells) were found in PBLs from CDAII patients (data not shown).

Consistent with previous studies and with the loss of function of *SEC23B* mutations,[9] we observed a reduced *SEC23B* expression in our patients compared to HC (Figure S2a). Indeed, *FAM132B* and *SEC23B* gene expression exhibited an inverse correlation (Figure S2b). Interestingly, we did not observe in our patients the same trend of expression of *FAM132A* paralog gene, which shares 69% of transcript identity with *FAM132B* (Figure S2c). This suggests a possible involvement of ERFE-encoding *FAM132B* in the pathogenesis of hemosiderosis in CDAII.

During the preparation of this study, two articles reporting measurement of human ERFE protein were published.[10,11] Accordingly to gene expression analysis, we found an increased ERFE expression in CDAII patients compared to HC (Figure 1b).

Of note, when we analyzed *ERFE* expression in β-thalassemia (BT)-intermedia patients, exhibiting iron overload likewise for CDAII patients (Table S1), we observed similar results compared to CDAII. Conversely, only a slight increase of *ERFE* expression was observed in patients with mild well-compensated anemia, such as hereditary spherocytosis (HS) (Figure 1c). These data suggested

that the marked increased ERFE expression observed in both CDAII and BT-intermedia patients is mainly due to the ineffective erythropoiesis.

As recently reported the ablation of Erfe in thalassemic mice induces a slight amelioration of ineffective erythropoiesis but does not improve the anemia.[12] In order to explore the possible interplay between the levels of erythroferrone and the degree of anemia in CDAII patients, we divided our cohort into two sub-groups: low- and high-FAM132B. Unlike thalassemic Erfe-haploinsufficient mice, we observed a statistically significant reduction in hemoglobin (Hb) level in the high-FAM132B subset compared to the low-FAM132B one. Of note, we focused on non-transfusion dependent patients, thus the expression level of FAM132B was not biased by transfusion regimen; however, ERFE expression did not correlate even with the occasional need of transfusions (Table 1). The higher expression of ERFE seems to reflect the increase of iron demand for Hb production as well as the expanding abnormal erythropoiesis, characterizing CDAII, as attested by the increased levels of sTfR and EPO in high-FAM132B patients. This in turn leads to reduced hepcidin and hepcidin/ferritin ratio in high-FAM132B group compared to low-FAM132B one, resulting in augmented iron delivery to the erythron. Indeed, an inverse correlation between *FAM132B* expression and hepcidin levels was observed (Spearman r -0.49, p = 0.004). However, the iron balance data do not differ significantly between the two CDAII sub-groups, even if a trend to increased transferrin saturation was observed in high-FAM132B patients (Table 1). Thus, ERFE up-regulation may contribute to the inappropriate suppression of hepcidin, but not to account alone for the subsequent hemosiderosis observed in CDAII patients.

A high expression of *FAM132B* in *ex vivo* EPO-differentiating human erythroblasts has been documented.[8] Accordingly, we confirmed a progressive increase of *FAM132B* expression in healthy CD34+ progenitors cells EPO-induced to erythroid differentiation. Particularly, we observed a significant increase of *FAM132B* in the transition from basophilic (9d, 3.93±1.32) to polychromatic erythroblasts (11d, 14.30±2.46; p=0.02) (Figure 1d). Of note, the onset of multinuclearity in *in vitro* model of CDAII involves polychromatic and orthochromatic precursors.[13] Thus, the accumulation of highly expressing-*FAM132B* polychromatic erythroblasts in CDAII marrow could account for the pathological over-expression observed in CDAII patients. Our observation is consistent with the demonstration that polychromatic erythroblasts are the main source of Erfe in BT mice. Indeed, similarly to CDAII erythropoiesis, BT is characterized by an accumulation of polychromatic erythroblasts that do not mature to the orthochromatic stage. Thus, the increased Erfe expression in

erythroblasts from $Hbb^{Th3/+}$ mice compared to WT mice is mainly due to the relative abundance of polychromatic erythroblasts.[12]

CDAII is still lacking of a reliable animal model, because SEC23B deficiency results in different phenotypes in humans and mice.[14,15] So far the only reliable *in vitro* model for CDAII is the SEC23B-silenced K562 cells.[1] We established K562 stable clones silenced for SEC23B gene by lentiviral shRNAmir targeting human SEC23B, which ensures a highly efficiency of silencing. When we induced to erythroid differentiation K562 stable clones silenced for SEC23B, we observed a higher expression of *FAM132B* at 5 days of erythroid differentiation in K562 SEC23B-silenced cells compared to not-silenced ones (Figure 1e). Conversely, *SEC23B* expression was lower in both K562 SEC23B-stably silenced cell lines compared to K562 sh-CTR cells at 2 and 5 days of differentiation (Figure S2d). Thus, we confirmed the *ex vivo* data about inverse correlation between *FAM132B* and *SEC23B* expression observed in our patients.

Literature data lacks of information about human ERFE protein. In order to validate our data at protein level, we firstly evaluated the good performance of the commercial anti-FAM132B antibody we used (Figure S3a-c). We also provided the first demonstration that ERFE protein is N-glycosylated; indeed, the higher band obtained by WB determination is the glycosylated isoform of the same protein, as demonstrated by the digestion pattern after PGNase treatment (Figure S3d). Accordingly to gene expression data, we observed an increased level of secreted ERFE in the extracellular medium of K562 SEC23B-silenced cells compared to not-silenced ones at 5 days of erythroid differentiation, whereas ERFE intracellular level was lower (Figure 1f).

Although the mechanisms of hemin-induced differentiation are quite different from EPO-induced ones, we can hypothesize that ERFE over-expression is related to the maturative arrest and the subsequent increased number of erythroid precursors.

This study provides the first analysis on ERFE expression in human model of dyserythropoietic anemia with ineffective erythropoiesis, such as CDAII. Our *ex vivo* and *in vitro* data indicate that ERFE over-expression in CDAII patients might be most likely related to both physiological and pathological mechanisms leading to hepcidin suppression in condition of dyserythropoiesis. Indeed, we clearly demonstrated that in two different genetic conditions sharing common clinical findings and similar pathogenesis, such as CDAII and BT-intermedia, *FAM132B* over-expression is related to the abnormal erythropoiesis. Nevertheless, the absence of a clear correlation between erythroferrone levels and CDAII iron balance suggest that ERFE cannot be the only erythroid regulator of hepcidin suppression, at least in CDAII patients.

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Author contributions

RR and AI designed and conducted the study, and prepared the manuscript; IA established K562 SEC23B silenced clones, performed FAM132B dosages, and prepared the manuscript; LDFa prepared CD34+ cultures; FM performed qRT-PCR and sequencing analysis; MB performed GDF15 and EPO dosages; AG cared for CDAII patients; GDR performed WB analysis; AM provided $Hbb^{th3/+}$ and WT mice; PR cared for BT-intermedia patients; DG performed hepcidin dosage; LDFr designed mouse model experiment and contributed to critical review of the manuscript.

Conflict of interest statement

The authors have declared that no conflict of interest exists.

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FIGURE LEGEND

Figure 1. ERFE expression in CDAII patients and during erythroid differentiation in normal and SEC23B-deficient cells

a) *FAM132B* mRNA levels (normalized to *β-actin*) in PBL from 37 CDAII patients and 29 HC. Over expression of *FAM132B* gene in CDAII patients (9.11 ± 0.10) compared to HC (8.32 ± 0.12, $p < 0.0001$) was observed. Data are presented as mean ± SEM. P value by Student t test. **b)** Human protein ERFE evaluation in plasma samples from 29 CDAII patients and 12 HC. Data are presented as mean ± SEM. P value by Student t test. **c)** *FAM132B* mRNA levels (normalized to *β-actin*) in PBL from 37 CDAII, 21 BT-intermedia, 13 HS patients and 29 HC. Data are presented as mean ± SEM. **$p<0.0001$; *$p<0.05$ vs HC group (Student t test). **d)** *FAM132B* mRNA levels (normalized to *GADPH*) in normal CD34+ cells induced to erythroid differentiation by EPO at 7, 9, 11, and 13 days. Data are presented as mean ± SEM of 3 experiments. P value by ANOVA test. **e)** *FAM132B* mRNA levels (normalized to *GADPH*) in stable clones of K562 silenced for SEC23B at 2 and 5 days of erythroid differentiation by hemin. Data are presented as mean ± SEM of 3 experiments. *$p<0.05$ (Student t test). **f)** Immunoblot of ERFE protein in total cell lysate and medium samples from SEC23B-silenced K562 cells induced to erythroid differentiation at 2 and 5 days; GAPDH was loading control for total cell lysate, while medium samples was normalized by Ponceau red staining of the blots. Densitometric analysis of the blot showed on the left (5 days): ERFE protein levels of both SEC23B-silenced K562 clones were normalized on sh-CTR K562 clone. O.D., optical density.

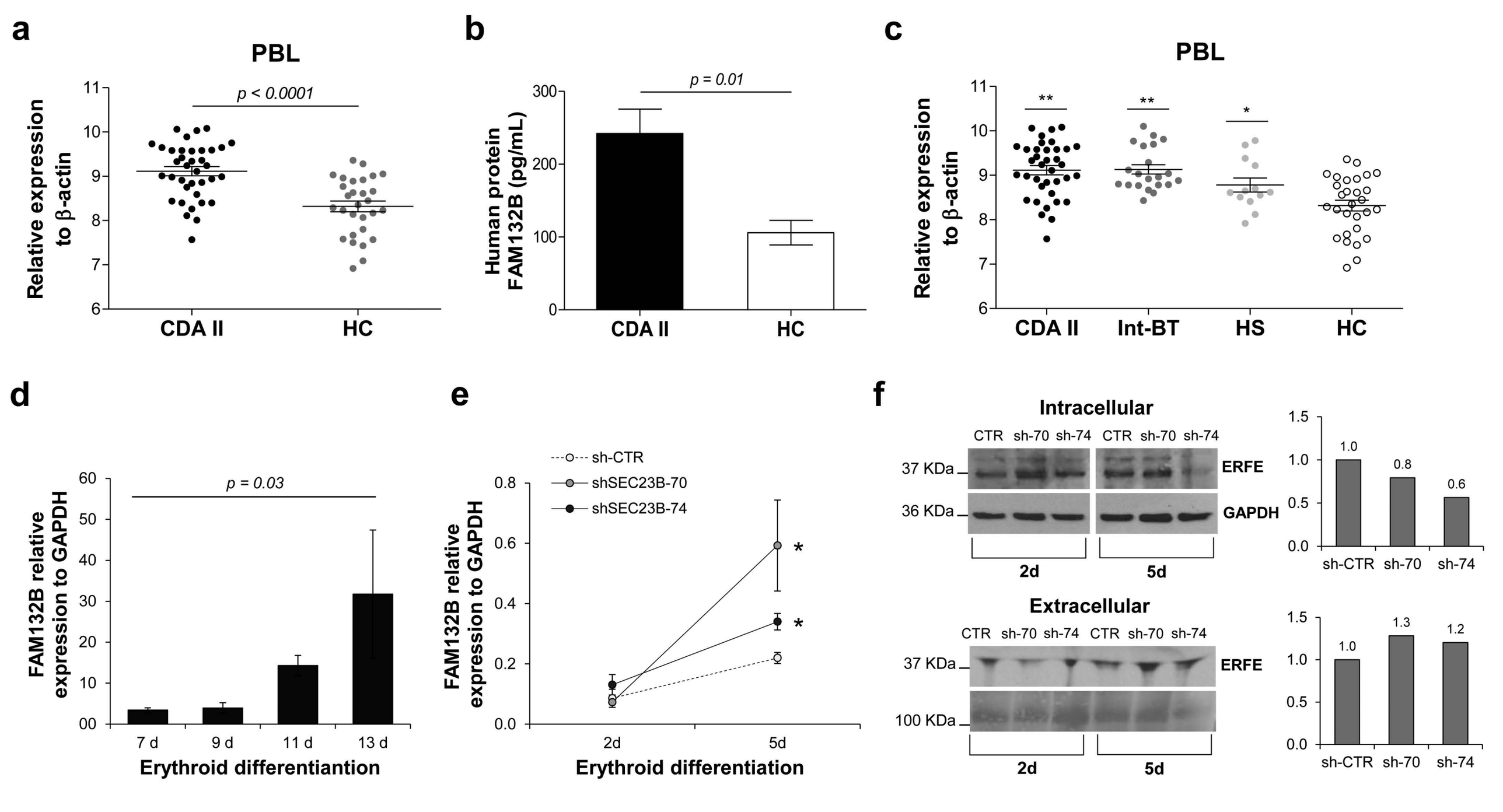
Table 1. *FAM132B* expression and clinical correlations in CDAII patients

	Low *FAM132B* *(n = 20)*	High *FAM132B* *(n = 17)*	P‡
Age (years)	25.3 ± 4.9 (16.0; 19)	12.1 ± 2.5 (10.0; 17)	0.03
Onset symptoms (years)	7.5 ± 2.5 (5.0; 16)	3.2 ± 1.3 (1.3; 16)	0.14
Gender (Female/Male)	9 (45.0)/11 (55.0)	9 (52.9)/8 (47.1)	0.63
Complete blood count			
RBC (10^6/µL)	3.6 ± 0.2 (3.5; 20)	3.2 ± 0.1 (3.3; 17)	0.05
Hb (g/dL)	10.7 ± 0.5 (10.4; 20)	9.2 ± 0.4 (9.5; 17)	0.02
Ht (%)	31.7 ± 1.4 (30.6; 20)	27.5 ± 1.2 (28.0; 17)	0.03
MCV (fL)	89.7 ± 1.8 (90.2; 20)	86.0 ± 2.2 (84.7; 17)	0.20
MCH (pg)	30.6 ± 0.7 (31.0; 18)	28.9 ± 0.9 (27.9; 17)	0.12
MCHC (g/dL)	33.8 ± 0.4 (33.5; 19)	33.3 ± 0.3 (33.1; 16)	0.32
RDW (%)	19.9 ± 2.5 (18.9; 12)	21.8 ± 1.2 (22.0; 15)	0.48
PLT (10^3/µL)	373.0 ± 41.1 (290.0; 17)	459.2 ± 69.2 (390.0; 17)	0.30
Retics abs count (10^3/µL)	67.4 ± 9.2 (59.2; 20)	87.3 ± 17.5 (79.7; 16)	0.30
Retics (%)	2.0 ± 0.3 (1.5; 20)	2.7 ± 0.6 (2.2; 16)	0.25
Reticulocyte Index	1.3 ± 0.2 (1.2; 20)	1.7 ± 0.3 (1.5; 16)	0.38
Iron balance			
Hepcidin/ferritin	0.04 ± 0.01 (0.02; 16)	0.01 ± 0.003 (0.006; 16)	0.01
Hepcidin (nM)	5.8 ± 1.9 (2.7; 17)	1.0 ± 0.3 (0.6; 16)	0.02
Ferritin (ng/mL)	372.1 ± 107.7 (200.0; 19)	168.5 ± 36.0 (99.8; 17)	0.10
Ferritin level/dosage age§	32.9 ± 17.2 (14.9; 18)	26.1 ± 8.6 (12.7; 17)	0.73
Transferrin saturation (%)	67.7 ± 6.8 (62.5; 19)	81.8 ± 7.8 (86.0; 8)	0.23
Serum iron (µg/dL)	157.8 ± 13.6 (159.5; 18)	162.7 ± 20.4 (172.0; 13)	0.84
sTfR (mg/L)	3.7 ± 0.4 (3.7; 12)	5.1 ± 0.5 (5.7; 8)	0.04
Laboratory data and transfusion regimen			
EPO (mIU/mL)	82.5 ± 19.1 (61.9; 14)	154.3 ± 14.5 (170.1; 13)	0.01
GDF15 (pg/mL)	814.9 ± 251.1 (503.5; 13)	781.9 ± 140.6 (804.0; 9)	0.92
Total bilirubin (mg/dL)	3.7 ± 0.8 (2.5; 19)	2.3 ± 0.3 (2.1; 16)	0.15
Unconjugated bilirubin (mg/dL)	3.1 ± 0.8 (2.2; 17)	1.9 ± 0.3 (1.5; 12)	0.22
Transfusion need (Yes/No)	7 (46.7)/8 (53.3)	10 (58.8)/7 (41.2)	0.49

Data are not available for all patients. For quantitative variables data are presented as average ± SEM (median; n). For qualitative variables data are presented as n (%)/n (%)

‡ Student t test for quantitative unpaired data; chi square test for categorical data

§ Normalization of ferritin by means of "Ferritin level/dosage age ratio"[16]


a
PBL
p < 0.0001
Relative expression to β-actin
11
10
9
8
7
6
CDA II
HC
b
p = 0.01
Human protein FAM132B (pg/mL)
300
200
100
0
CDA II
HC
c
PBL
**
**
*
Relative expression to β-actin
11
10
9
8
7
6
CDA II
Int-BT
HS
HC
d
p = 0.03
FAM132B relative expression to GAPDH
60
50
40
30
20
10
00
7 d
9 d
11 d
13 d
Erythroid differentiantion
e
sh-CTR
shSEC23B-70
shSEC23B-74
FAM132B relative expression to GAPDH
0.8
0.6
0.4
0.2
0.0
*
*
2d
5d
Erythroid differentiation
f
Intracellular
CTR sh-70 sh-74 CTR sh-70 sh-74
37 KDa
ERFE
36 KDa
GAPDH
2d
5d
1.5
1.0
0.5
0.0
1.0
0.8
0.6
sh-CTR sh-70 sh-74
Extracellular
CTR sh-70 sh-74 CTR sh-70 sh-74
37 KDa
ERFE
100 KDa
2d
5d
1.5
1.0
0.5
0.0
1.0
1.3
1.2
sh-CTR sh-70 sh-74

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