Hepcidin levels in Diamond-Blackfan anemia reflect erythropoietic activity and transfusion dependency

Diamond-Blackfan anemia (DBA) is a rare congenital red cell aplasia associated with mutations in ribosomal proteins (RP) in 49-71% of cases.[1] DBA is a clinically heterogeneous disorder with one-third of patients developing transfusion-acquired iron overload.[2] The severity of anemia and transfusion dependency in DBA is comparable to transfusion-dependent beta-thalassemia major. However, moderate to severe suppression of erythropoiesis in DBA[2] is in contrast to accelerated ineffective erythropoiesis in β-thalassemia.[3,4] Knowledge of systemic iron regulation in DBA is limited.

In this study, we assessed selected markers of erythropoietic activity and iron metabolism including the key molecule of this process, hepcidin, in DBA patients from the Czech National DBA Registry (Table 1 and *Online Supplementary Table S1*).[6] The cohort was made up of 12 patients receiving regular transfusions with or without chelation therapy, 4 patients on steroids, 7 patients in remission without treatment and 2 patients treated with corticosteroids and occasional transfusions. Nine patients, mostly from the transfusion-dependent group, were concomitantly treated with leucine (*Online Supplementary Table S1*).[7]

In transfusion-dependent patients, reduced number of erythroblasts in the bone marrow (median 0.8%), together with markedly decreased soluble transferrin receptor (sTfR, often under the lower limit of detection), confirmed severely suppressed erythropoiesis that corresponded to substantially elevated erythropoietin (EPO) levels (median 2452 IU/L) (Table 1). Analysis of iron parameters showed increased serum iron, transferrin saturation (TSAT) and high ferritin levels (median 1290 ng/mL) (Table 1). This suggests that erythropoiesis of these patients is not able to utilize transferrin-bound iron effectively. Liver biopsy in 5 selected transfusion-dependent patients consistently showed markedly elevated liver iron concentration (LIC) (Table 1). Moreover, massive iron stores were detected in both hepatocytes and Kupffer cells (Figure 1A), distinguishing DBA from β-thalassemia major with iron deposits predominantly in macrophages.[4] The macrophage iron loading can be attributed to non-effective erythrocyte-derived iron recycling.[8,9] Iron deposits in hepatocytes likely resulted from increased iron uptake by these cells. The involvement of non-transferrin-bound iron (NTBI) needs to be considered, as NTBI can be found in the plasma of patients with oversaturated transferrin and liver is the primary site for deposition of free iron from plasma.[10,11] Since 2009, all our transfusion-dependent patients have been monitored for potential cardiac, liver and pancreatic iron overload by magnetic resonance imaging (MRI).[12]

Contrary to transfusion-dependent DBA patients, patients who are currently in disease remission or on steroids showed improved erythropoiesis as documented by near normal/higher number of bone marrow erythroblasts and the levels of sTfR within the normal range (Table 1). These patients had slightly increased or even normal levels of ferritin and near normal serum iron and TSAT

Table 1. Parameters of iron status and erythropoietic activity in subgroups of Diamond-Blackfan anemia patients.

	TDP (n=12)	RP (n=7)	SP (n=4)	S,T* (n=2)	Reference range
Age (years)	7.6 (1.0-27.9)	26.9 (13.3-35.8)	32.6 (27.0-42.9)	25.8 25.9	–
Hepcidin (ng/mL)	341.5 (165.1-572.6)	72.1 (29.5-153.3)	24.7 (8.5-100.1)	173.9 144.9	27.6 (13.1-104.8)[5]
Ferritin (ng/mL)	1290 (343-3747)	177 (42-1079)	188 (44-637)	3150 868	22-275
Hepcidin/ferritin ratio	0.240 (0.09-1.35)	0.222 (0.09-1.04)	0.151 (0.10-0.19)	0.06 0.17	0.35 (0.2-2.2)[5]
Fe (μmol/L)	41.5 (35.8-59.0)	20.0 (10.0-30.6)	21 (14.7-33.4)	39.6 43.2	7.2-29
TSAT (%)	91 (61-100)	38 (25-55)	64 (63-65)	93 94	21-48
LIC (mg/g d.w.)	7.3# (4.6-16.3)	*ND*	*ND*	*ND*	0.3-1.4
GDF15 (pg/mL)	676.2 (352.1-1694.8)	927.0 (556.3-2824.8)	1127.6 (408.8-2190.8)	4221.5 3497.1	223 (166-344)[5]
EB (%)	0.8 (0.4-8.2)	21.4 (8.4-34.0)	20.4 (16.8-33.4)	10.6 20.8	15.0-25.0
EPO (IU/L)	2452 (837-6476)	137 (20-1913)	441 (287-500)	615 4000	4.3-29.0
sTfR (mg/L)	*ND*† <0.5-1.2	2.3 (1.3-3.2)	2.4 (1.8-2.8)	1.4 1.4	1.9-4.4[6]

*Values are shown as medians and the full range of variation. TDP: transfusion-dependent patients; RP: patients in disease remission; SP: patients treated with steroids; S,T: patient on steroids and occasional transfusion; Fe: serum iron; TSAT: transferrin saturation; LIC: liver iron concentration, d.w.: dry weight; GDF15: growth differentiation factor 15; EB: erythroblasts in the bone marrow; EPO: serum erythropoietin; sTfR: soluble transferrin receptor; ND: not determined. *individual values are shown; #values available for 5 patients; †median could not be calculated as 9 of 12 patients had sTfR below the limit of detection (less than 0.5 mg/L). Detailed description of individual methods can be found in the Online Supplementary Appendix. Pre-transfusion samples were collected for TDP.*

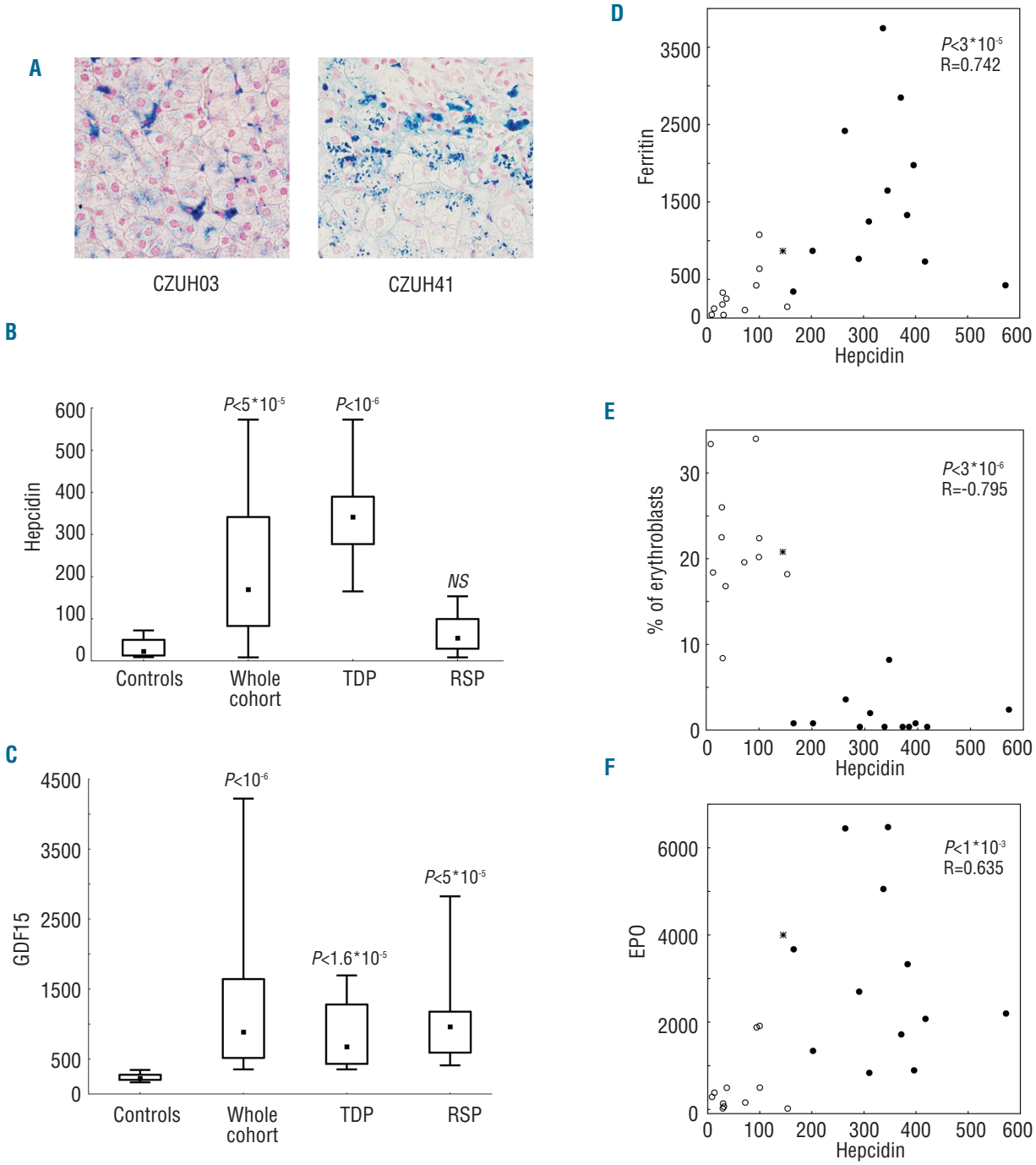

Figure 1. Hepatic iron distribution, hepcidin and GDF15 levels and correlation analyses. (A) Perl's staining showing iron deposits in the liver of 2 transfusion-dependent DBA patients; CZUH03 at the age of 10 (LIC, 4.6 mg/g d.w.) and CZUH41 at the age of 5 (10.3 mg/g d.w.). Massive iron stores (blue stain) can be found in both hepatocytes and Kupffer cells. Similar pattern of iron staining was obtained for the remaining 3 transfusion-dependent DBA patients analyzed (*data not shown*). (B) Significantly increased hepcidin levels in comparison with healthy controls were detected for the whole DBA cohort (n=25) and transfusion-dependent DBA patients (TDP, n=12); DBA patients in remission or treated with steroids (RSP, n=11) had hepcidin levels comparable with the controls. (C) GDF15 levels were significantly elevated in the whole DBA cohort (n=25), TDP (n=12) and RSP (n=11) when compared to healthy controls. (D-F) Hepcidin levels showed the following correlations: positive with ferritin (D), negative with the number of erythroblasts in the bone marrow (E) and positive with serum EPO (F). Closed and open circles depict TDP and RSP, respectively. Asterisk shows one patient on steroids and occasional transfusions; the other similarly treated patient (CZUH37) was excluded from statistical analyses due to concomitant occurrence of DBA and HFE C282Y homozygous mutation. The image in (A) was visualized with an Olympus IX 71 light microscope (Olympus, Hamburg, Germany), original magnification 400× and acquired with an Olympus DP 50 camera driven by DP controller software (provided by Olympus). Data in (B and C) are shown in a box plot depicting median (closed square), 25-75% range (box), and the highest and lowest value (the highest and the lowest whiskers, respectively). Image was assembled and labeled using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA). *NS*: not significant.

(Table 1). This indicates that, in patients who achieved a remission state or who are hematologically stable on steroids, the improved erythropoietic activity increases iron utilization and consequently leads to amelioration of hyperferritinemia. The effect of leucine treatment on iron metabolism seems to be indirect (via improved erythropoiesis).[7]

Using reverse-phase liquid chromatography,[5] we observed significantly higher hepcidin levels for the whole DBA cohort in comparison with healthy controls (median 165.1 *vs.* 27.6 pg/mL; $P<0.00005$) (Figure 1B). When hepcidin was assessed in individual subgroups of DBA patients, patients on regular transfusions showed significantly elevated hepcidin (median 341.5 pg/mL), as previously shown for transfusion-dependent β-thalassemia major patients.[4] Increased hepcidin above the median value of controls was detected even in the youngest patients aged 1.0 and 2.7 years (6-times and 20-times, respectively). This suggests that the absent or diminished erythropoiesis in the bone marrow, together with transfusions, is the driving factor for iron overload from early childhood. In contrast, DBA patients in remission or on steroids had hepcidin levels comparable to controls (medians 72.1 and 24.7 pg/mL, respectively) (Figure 1B). As erythropoiesis is known to produce a signal for hepcidin suppression,[8,9] DBA patients with restored erythropoietic activity are likely to be able to attenuate hepcidin expression and thus increase the iron pool available for improved erythropoiesis. In agreement with this concept, we detected a trend towards lower hepcidin-ferritin ratio in patients on steroids (median 0.151) or patients in remission (median 0.222) when compared to transfusion-dependent patients (median 0.240) (Table 1), although these individual values were not significantly different from the hepcidin-ferritin ratio of healthy controls (median 0.35).[5] Indeed, the hepcidin-ferritin ratio, which indicates suppression of hepcidin proportional to iron loading, is much higher in transfused DBA patients (range 0.09-1.35) than the hepcidin-ferritin ratio reported for transfusion-dependent β-thalasemia major patients (range 0.02-0.3),[4] suggesting that the erythroid drive suppressing hepcidin is much stronger in β-thalassemia and not completely attenuated by transfusions. On the other hand, the bone marrow of DBA patients receiving transfusions is probably not releasing the putative erythroid suppressor of hepcidin production.

In the 2 patients evaluated independently, hepcidin-ferritin ratio was more comparable with patients on steroids, reflecting their improved erythropoiesis (Table 1). Nevertheless, patient CZUH37 inherited a homozygous C282Y HFE mutation (*Online Supplementary Table S2*), which may contribute to inappropriately low levels of hepcidin (173.9 ng/mL) for the observed hyperferritinemia (3150 ng/mL).[13]

We next examined the levels of growth differentiation factor 15 (GDF15), a candidate negative regulator of hepcidin in β-thalassemia and a marker of ineffective erythropoiesis.[3] Significantly increased levels of GDF15 were detected for the whole DBA cohort as well as for the groups receiving different treatments when compared to normal controls (Figure 1C). We suppose that elevated levels of GDF15 in DBA patients may reflect the increased apoptosis of bone marrow erythroblasts that we observed in DBA patients selected for the TUNEL assay (*Online Supplementary Figure S1*).[3]

Lastly, we assessed which of the aforementioned signals/markers contribute to the regulation of hepcidin synthesis in DBA. Hepcidin positively correlated with ferritin ($P=0.00003$) (Figure 1D), reflecting hepcidin stimulation by the patients' iron overload. An inverse correlation between the percentage of bone marrow erythroblasts and hepcidin levels ($P=0.000003$) (Figure 1E) is consistent with negative regulation of hepcidin synthesis by erythropoietic activity. Although a negative correlation between EPO and the number of erythroblasts in the bone marrow (*data not shown*) confirms that EPO is stimulated in response to suppressed erythropoiesis and hypoxia, a positive correlation between hepcidin and EPO ($P=0.001$) (Figure 1F) demonstrates that hepcidin suppression by EPO requires active erythropoiesis in the bone marrow.[14,15] Similarly, no correlation between hepcidin or hepcidin-ferritin ratio and GDF15 indicates that GDF15 is not playing a hepcidin-regulatory role in DBA.

We conclude that DBA patients with different severities of anemia and different treatment strategies have diverse levels of hepcidin and iron overload. Hepcidin production in DBA reflects variable erythropoietic activity in the bone marrow and further contributes to the heterogeneity of this disease. It will be important to address whether some of these dissimilarities can be attributed to different types of disease-causing RP mutations.

Dagmar Pospisilova,[1] Dusan Holub,[2] Zuzana Zidova,[3] Lucie Sulovska,[1] Jiri Houda,[1] Vladimir Mihal,[1] Ivana Hadacova,[4] Lenka Radova,[5] Petr Dzubak,[2] Marian Hajduch,[2] Vladimir Divoky,[3] and Monika Horvathova[3]

[1]Department of Pediatrics, Faculty of Medicine and Dentistry, Palacky University and University Hospital Olomouc; [2]Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University, Olomouc; [3]Department of Biology, Faculty of Medicine and Dentistry, Palacky University; [4]Department of Pediatric Hematology and Oncology, Charles University, 2nd Faculty of Medicine, Prague; and [5]Central European Institute of Technology, Masaryk University, Brno, Czech Republic

Correspondence: monika.horvathova@upol.cz dagmar.pospisilova@fnol.cz

doi:10.3324/haematol.2014.104034

Key words: Diamond Blackfan anemia, hepcidin levels, erythropoietic activity, transfusion dependency.

DP, DH and ZZ contributed equally to this manuscript.

The online version of this article has a Supplementary Appendix.

Funding: this work was supported by grant No. NT/11059 (Ministry of Health, Czech Republic) and partially by grants No. LF_2013_010 and LF_2013_015 (Internal Grant Agency of Palacky University). DH, PD and M Hajduch were supported by CZ.1.05/2.1.00/01.0030 (Ministry of Education, Youth and Sports, Czech Republic), M Horvathova, LS and VD were supported by NT/13587 (Ministry of Health, Czech Republic) and M Horvathova and VD partially by P305/11/1745 (Czech Science Foundation). LR was supported by 306242 NGS-PTL (the FP7 grant).

Acknowledgments: we thank Zuzana Prouzova, M.D. (Department of Clinical and Molecular Pathology, University Hospital Olomouc) for photomicrographs of the liver.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Boria I, Garelli E, Gazda HT, Aspesi A, Quarello P, Pavesi E, et al. The ribosomal basis of Diamond-Blackfan Anemia: mutation and database update. Hum Mutat. 2010;31(12):1269-79.
2. Vlachos A, Ball S, Dahl N, Alter BP, Sheth S, Ramenghi U, et al.

Diagnosing and treating Diamond Blackfan anaemia: results of an international clinical consensus conference. Br J Haematol. 2008;142(6):859-76.
3. Tanno T, Noel P, Miller JL. Growth differentiation factor 15 in erythroid health and disease. Curr Opin Hematol. 2010;17(3):184-90.
4. Origa R, Galanello R, Ganz T, Giagu N, Maccioni L, Faa G, et al. Liver iron concentrations and urinary hepcidin in beta-thalassemia. Haematologica. 2007;92(5):583-8.
5. Mojzikova R, Koralkova P, Holub D, Zidova Z, Pospisilova D, Cermak J, et al. Iron status in patients with pyruvate kinase deficiency: neonatal hyperferritinaemia associated with a novel frameshift deletion in the PKLR gene (p.Arg518fs), and low hepcidin to ferritin ratios. Br J Haematol. 2014;165(4):556-63.
6. Pospisilova D, Cmejlova J, Ludikova B, Stary J, Cerna Z, Hak J, et al. The Czech National Diamond-Blackfan Anemia Registry: clinical data and ribosomal protein mutations update. Blood Cells Mol Dis. 2012;48(4):209-18.
7. Pospisilova D, Cmejlova J, Hak J, Adam T, Cmejla R. Successful treatment of a Diamond-Blackfan anemia patient with amino acid leucine. Haematologica. 2007;92(5):e66-7.
8. Ganz T, Nemeth E. Hepcidin and iron homeostasis. Biochim Biophys Acta. 2012;1823(9):1434-43.
9. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of mammalian iron metabolism. Cell. 2010;142 (1):24-38.
10. Brissot P, Ropert M, Le Lan C, Loréal O. Non-transferrin bound iron: a key role in iron overload and iron toxicity. Biochim Biophys Acta. 2012;1820(3):403-10.
11. Hoffbrand AV, Taher A, Cappellini MD. How I treat transfusional iron overload. Blood. 2012;120(18):3657-69.
12. Berdoukas V, Nord A, Carson S, Puliyel M, Hofstra T, Wood J, et al. Tissue iron evaluation in chronically transfused children shows significant levels of iron loading at a very young age. Am J Hematol. 2013;88(11):E283-5.
13. van Dijk BA, Laarakkers CM, Klaver SM, Jacobs EM, van Tits LJ, Janssen MC, et al. Serum hepcidin levels are innately low in HFE-related haemochromatosis but differ between C282Y-homozygotes with elevated and normal ferritin levels. Br J Haematol. 2008;142(6):979-85.
14. Vokurka M, Krijt J, Sulc K, Necas E. Hepcidin mRNA levels in mouse liver respond to inhibition of erythropoiesis. Physiol Res. 2006;55(6):667-74.
15. Pak M, Lopez MA, Gabayan V, Ganz T, Rivera S. Suppression of hepcidin during anemia requires erythropoietic activity. Blood. 2006;108(12):3730-5.