Transfusion suppresses erythropoiesis and increases hepcidin in adult patients with beta-thalassemia major – a longitudinal study

Sant-Rayn Pasricha1,2,3
David M Frazer4
Donald K Bowden1
Gregory J Anderson4

1Medical Therapy Unit (Thalassaemia Service), Southern Health, Clayton Road, Clayton, Australia

2Nossal Institute for Global Health, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, Carlton, Australia

3MRC Human Immunology Unit, MRC Weatherall Institute of Molecular Medicine, University of Oxford, United Kingdom

4Iron Metabolism Laboratory, Queensland Institute of Medical Research, Brisbane, Queensland, Australia

Corresponding Author

Dr Sant-Rayn Pasricha
Nossal Institute for Global Health
Faculty of Medicine, Dentistry and Health Sciences
The University of Melbourne
Carlton Victoria 3010 Australia
Tel: +61 3 8344 9299 Fax: +61 3 9347 6872
sant-rayn.pasricha@unimelb.edu.au
Current Address:
Molecular Immunology Unit
Weatherall Institute of Molecular Medicine
University of Oxford
John Radcliffe Hospital
Oxford OX3 9DS United Kingdom
Tel: +44 1865 222443 Fax: +44 1865 222737

Key Points:

• In β-thalassemia major, hepcidin levels are simultaneously associated with erythropoiesis and iron loading pre- and post-transfusion.
• Transfusion improves anemia, suppressing erythropoiesis and in turn increasing hepcidin in patients with β-thalassemia major.
Abstract

β-thalassemia major causes ineffective erythropoiesis and chronic anemia, and is associated with iron overload due to both transfused iron and increased iron absorption, the latter mediated by suppression of the iron-regulatory hormone hepcidin. We sought to determine whether, in β-thalassemia major, transfusion-mediated inhibition of erythropoiesis dynamically affects hepcidin. We recruited 31 chronically transfused patients with β-thalassemia major and collected samples immediately before and 4-8 days post-transfusion. Pre-transfusion hepcidin was positively correlated with hemoglobin and ferritin, and inversely with erythropoiesis. The hepcidin-ferritin ratio indicated hepcidin was relatively suppressed given the degree of iron loading. Post-transfusion, hemoglobin increased, erythropoietin and growth differentiation factor-15 (GDF-15) fell, and hepcidin rose. By multiple regression, pre- and post-transfusion hepcidin concentrations were both associated positively with hemoglobin, inversely with erythropoiesis, and positively with ferritin. Although males and females had similar pre-transfusion hemoglobin, males had significantly increased erythropoiesis and lower hepcidin, received a lower transfusion volume per liter blood volume, and experienced a smaller post-transfusion reduction in erythropoiesis and hepcidin rise. Age of blood was not associated with post-transfusion hemoglobin or ferritin change. Hepcidin levels in patients with β-thalassemia major dynamically reflect competing influences from erythropoiesis, anemia and iron overload. Measurement of these indices could assist clinical monitoring.

Keywords:
Thalassemia, Hepcidin, Erythropoiesis, Iron, Transfusion
Introduction

The mainstay of therapy for β-thalassemia major is lifelong red-cell transfusion to improve anemia and suppress ineffective, expanded erythropoiesis. Ineffective erythropoiesis causes iron overload due to suppression of the liver-derived hormone hepcidin that regulates iron absorption and recycling via its effects on ferroportin, the cellular iron export protein. Low hepcidin preserves ferroportin and permits increased intestinal iron absorption and enhanced macrophage iron release (and hence iron recycling), whereas elevated hepcidin causes degradation of ferroportin, thus decreasing iron absorption and recycling. Hepcidin is suppressed by hypoxia and iron deficiency, and is elevated by iron loading and inflammation. Erythropoiesis is perhaps the most potent suppressor of hepcidin, although the mechanism remains uncertain. The existence of a secreted factor from the erythroid marrow that suppresses hepcidin synthesis has been hypothesized. One candidate, Growth Differentiation Factor-15 (GDF-15), is elevated in ineffective erythropoiesis (especially in thalassemia) and suppresses hepcidin expression in vitro.

Ineffective erythropoiesis in thalassemia is suppressed by transfusion. Cross-sectional studies suggest that transfused patients with β-thalassemia major have higher hepcidin than non-transfusion dependent patients with β-thalassemia intermedia. Transfusion increases urinary hepcidin in patients with β-thalassemia major. If erythropoiesis measurably fluctuates over the inter-transfusion interval, thalassemia could be a valuable model to study the dynamic regulation of hepcidin in the face of competing influences of coexistent anemia, expanded erythropoiesis and iron loading. Although hemoglobin (Hb) concentrations in non-thalassemic males are
generally higher than those for females, current transfusion dosing guidelines do not account for these potential differences.

In a cohort of chronically transfused patients with β-thalassemia major, we hypothesized 1) that pre- and post-transfusion hepcidin concentrations are simultaneously influenced by anemia and erythropoiesis, iron loading and inflammation, 2) that transfusion-mediated increases in Hb suppress erythropoietic drive which, in turn, de-suppresses (raises) hepcidin, and 3) that there are differences in males and females with regard to pre-transfusion erythropoiesis and response to transfusion. We found that hepcidin levels in patients with β-thalassemia major are associated with anemia, erythropoiesis and iron stores; that suppression of erythropoiesis by transfusion is associated with an increase in hepcidin; and that compared with females pre- and post-transfusion, males had increased erythropoiesis and lower hepcidin.
Methods

Patients

The Southern Health thalassemia service (Melbourne, Australia) is the state center for management of thalassemia. Patients receive red cell transfusions and iron chelators in accordance with international recommendations. Adults with transfusion-dependent β-thalassemia were eligible for this study. Blood was collected immediately prior to transfusion, and patients were asked to return five days following transfusion for post-test sampling, when erythroid suppression was expected to correlate with maximal suppression. Data on the transfused units were collected.

Laboratory analysis

Samples were tested for Hb and reticulocyte count (Beckman Coulter LH750), ferritin, soluble transferrin receptor (sTfR) and erythropoietin (EPO) by immunoassay (Beckman Coulter DXi 800), C-reactive protein (CRP) (Beckman Coulter DXc 800), GDF-15 (ELISA, R&D Systems) and hepcidin (EIA, Bachem).

Normal values and definitions

The reference range for the Beckman Coulter sTfR assay established by the manufacturer in 189 healthy adults is 0.90-2.01 mg/L, mean 1.35mg/L. For EPO, the manufacturer’s upper limit of normal is 18.5mIU/mL. Tanno et al suggested GDF-15 levels in normal individuals fall between 200-1150 pg/mL. In a study investigating effects of altitude on erythropoiesis and hepcidin in healthy participants of similar mean age (40 years) to patients in our study, Piperno et al reported baseline mean EPO of 11.5mU/mL and using the same assay, GDF-15 of 338.7 pg/mL. Thus,
erythroid activity was evaluated by patient sTfR divided by 1.35 mg/L, \(^{18}\) patient EPO divided by 11.5 mLU/mL, and patient GDF-15 divided by 338.7 pg/mL. Absolute hepcidin values vary between assays.\(^{19}\) Using the same assay (Bachem) used in our study, Talbot et al reported mean hepcidin concentrations of 14 ng/mL (standard deviation 11 ng/mL) in healthy adults,\(^4\) and Choi et al identified a range of 3.2–66.9 ng/mL in non-iron deficient Korean children.\(^{20}\) The hepcidin-ferritin ratio was calculated to normalize hepcidin relative to concomitant iron loading, and should be approximately one in normal controls.\(^{12,21}\) \(\beta\)-gene mutations were classified as \(\beta^0\) or \(\beta^+\) according to an international database.\(^{22}\) Blood volume was estimated using Nadler’s formula based on sex, weight and height.\(^{23}\)

**Statistical considerations**

Variables with a right-skewed distribution were log-transformed following addition of 1 to approximate a normal distribution, a standard procedure to enable parametric analysis of skewed data.\(^{17}\) Geometric means were calculated by exponentiation of the arithmetic mean of the log-variable, followed by subtraction of 1. Student’s t-test was used to compare means between groups or pre- and post-transfusion. Pearson’s correlation was used to assess associations between indices. Change from pre to post-transfusion was evaluated by calculation of difference (post-transfusion minus pre-transfusion) where variables were normally distributed, and percentage change where variables were skewed. Univariate and then multiple linear regression was used to evaluate the independent relationship between included variables and hepcidin pre- and post-transfusion. Standardized (beta) coefficients were calculated (where variables are standardized to have a variance of 1) to enable comparison between variables. The study had a power of 0.80 to detect a difference of 25% in normally
distributed variables (assuming a standard deviation of 30% of the value of the mean of the variable) by t-test and a power of 0.80 to be able to detect correlations between variables with r exceeding 0.5.

*Ethics approval*

The Southern Health Human Research Ethics Committee approved the study. All patients provided written consent in accordance with the Declaration of Helsinki.
Results

Pre-transfusion

Thirty-one (male n=16; female n=15) patients with β-thalassemia major (β⁰/β⁰=3, β⁰/β⁺=14, β⁺/β⁺=11, Eβ-thalassemia=1, unknown=2) provided pre-transfusion samples (Table 1). The mean duration since previous transfusion was 22 days [range 13-36]. Twenty-seven patients were receiving chelation with deferasirox (daily or twice-daily) and four with desferrioxamine (four to six nights per week).

Figure 1 summarizes pre-transfusion Hb together with indices of erythropoiesis and iron and shows change post-transfusion. Pre-transfusion, mean Hb was 101.9g/L and Hb for 29 of 31 patients exceeded 90g/L, including 18 for whom it exceeded 100g/L. Mean reticulocyte count was 83.5×10⁹/L (within the normal range). Based on the sTfR, mean relative erythroid activity was 2.1. EPO (4.8×mean) and GDF-15 (22.3×mean) levels were also increased. Pre-transfusion hepcidin concentrations were within the range reported in normal adults and children, however, the hepcidin-ferritin ratio was markedly below 1 in all patients (mean 0.018), indicating suppression of hepcidin out of proportion with the degree of iron loading.¹²,²⁴

There were no associations between duration since previous transfusion and any pre-transfusion index. The 4 patients (3 female, 1 male) receiving desferrioxamine had lower pre-transfusion Hb (mean 88.9g/L vs. 103.9g/L, p<0.005, t-test) compared with those taking deferasirox, but there were no differences in age or pre-transfusion ferritin, EPO, sTfR, GDF-15 or hepcidin. Age was inversely associated with ferritin (r=-0.47, p=0.0085) but not with hepcidin or any other index. By ANOVA, severity of the β-globin genotype was not associated with Hb, erythropoiesis or hepcidin.
**Associations between pre-transfusion indices and hepcidin**

Figure 2 presents associations between pre-transfusion Hb, indices of erythropoiesis and hepcidin. Pre-transfusion Hb was inversely associated with indices of erythropoiesis (EPO and GDF-15 but not sTfR). Table 2 presents results of univariate and multiple linear regression for associations with pre-transfusion hepcidin. Hepcidin was positively associated with Hb and ferritin, and inversely with EPO, sTfR and GDF-15. Women had higher hepcidin levels.

Four multiple linear regression models adjusted for sex were developed to evaluate simultaneous co-regulation of hepcidin by iron loading (ferritin) and anemia/erythropoiesis (measured by Hb, EPO, GDF-15 and sTfR respectively) (Table 2). One index of erythropoiesis or anemia was included per model. As CRP was not associated with hepcidin, it was not considered further. The models show that controlling for sex, iron stores and erythropoiesis simultaneously influence hepcidin, with these factors explaining about half the variance in hepcidin (as indicated by the $R^2$).

**Post-transfusion**

Twenty-six patients returned after a mean of six days for post-transfusion sampling. There were no differences in age, sex, or pre-transfusion iron loading, Hb, indices of erythropoiesis and hepcidin between patients who did and did not return. Change in Hb and indices of erythropoiesis, ferritin, CRP and hepcidin are presented in Figure 1. In every patient, Hb and hepcidin increased (other than one in whom hepcidin remained undetectable) and EPO and GDF-15 fell. Change in sTfR was
heterogeneous, with a reduction in 15 of 26 patients, while mean sTfR did not change
(2.1×mean). EPO was 2.2×mean, whereas GDF-15, although reduced, remained
approximately 14.7×mean. Mean hepcidin rose to 45.6ng/mL, more than two standard
deviations above the mean reported in normal individuals by Talbot⁴ although within
the range reported in healthy children.²⁰ The hepcidin-ferritin ratio rose following
transfusion but remained below 1 (mean=0.054).

**Associations between post-transfusion indices and hepcidin**

Reflecting the pre-transfusion situation, post-transfusion Hb was inversely associated
with indices of erythropoiesis (EPO and GDF-15, but not sTfR) (Figure 2). Post-
transfusion hepcidin was positively associated with Hb and ferritin, and inversely
with EPO, sTfR and GDF-15 (Table 2). Again, women had higher hepcidin
concentrations. Multiple linear regression showed that, controlling for sex,
erythropoiesis continued to influence hepcidin post-transfusion, while ferritin was
only associated when GDF-15 was used to indicate erythropoiesis. The R² for each
model was similar, indicating a similar effect for each measure of
anemia/erythropoiesis on hepcidin. The effect of ferritin on hepcidin appeared weaker
post-transfusion.

**Associations between change in Hb, erythropoiesis and hepcidin**

Associations between changes pre- to post-transfusion were examined for variables
that changed significantly post-transfusion (i.e. Hb, EPO, GDF-15, ferritin and
hepcidin) (Figure 3). The volume (mL) of red cells transfused per liter blood volume
(and the volume (mL) per kg body weight [r=0.53, p=0.006], but not the absolute
number of units transfused [p=0.5598]) was associated with the post-transfusion
increase in Hb. In turn, the change in Hb was associated with the reduction in EPO and increase in hepcidin. EPO and hepcidin changes were inversely correlated. Associations between changes in GDF-15 and hepcidin were not evident, nor was change in ferritin and hepcidin associated.

Comparisons between males and females

Male and female patients were of similar age, duration since previous transfusion, time to return post-transfusion, and pre-transfusion Hb and reticulocyte count. Male patients were heavier, taller and had higher blood volumes than females (Table 1). Although male patients were transfused a similar number of units (absolute and mL/kg) compared with females, male patients received a lower transfusion volume per liter blood volume compared with their female counterparts.

Comparisons of Hb, indices of erythropoiesis, ferritin and hepcidin between the sexes are presented in Table 3. Pre-transfusion, male patients had higher sTfR and GDF-15 (indicating higher relative erythropoiesis), lower ferritin and lower hepcidin compared with females. Post-transfusion, males had significantly lower Hb, higher reticulocyte count, lower ferritin, higher sTfR and GDF-15 (with EPO approaching significance, p=0.053), and lower hepcidin compared with females.

Age of blood

Finally, post-hoc analysis was performed to study associations between age of the red cell units (defined as duration between collection date and transfusion date) and changes in Hb, erythropoiesis and ferritin. The mean age of units transfused was 16.1 days. In one patient, all transfused units were 7 days or younger; in a further 6
patients all units were 14 days or younger. There were no associations between mean unit age and change in Hb, ferritin, CRP, EPO, sTfR, GDF-15 or hepcidin, nor did patients who exclusively received units ≤14 days experience different changes in these parameters.
Discussion

Our study is the first to examine the effect of transfusion on erythropoiesis and in turn, the effect of modulation of erythropoiesis on serum hepcidin in patients with β-thalassemia major. We find that hepcidin concentrations reflect competing influences from erythropoiesis, anemia and iron overload, and are dynamic over the inter-transfusion interval, with changes reflecting suppression of erythropoiesis by transfusion-related increases in Hb. Furthermore, despite similar pre-transfusion Hb levels, males have increased erythropoiesis compared with females, compounded by smaller post-transfusion Hb increments due to smaller transfusate volumes per liter blood volume.

Hepcidin has been previously measured in patients with β-thalassemia. In the only previous longitudinal study, Kearney et al reported that transfusion increased urinary hepcidin in patients with β-thalassemia major, reflecting our findings.\textsuperscript{12} Cross-sectional studies\textsuperscript{10,11} have shown that hepcidin in non-transfusion dependent β-thalassemia is lower, and erythropoiesis higher, than in transfused β-thalassemia major, with inverse associations between indices of erythropoiesis and hepcidin. Studies in patients with β-thalassemia major have reported that post-transfusion hepcidin is relatively normal,\textsuperscript{25,26} but that hepatic hepcidin expression and urinary hepcidin were suppressed for the degree of iron loading, and chiefly influenced by erythropoiesis,\textsuperscript{27} reflecting our findings.

Observed differences in patterns of pre-transfusion levels and post-transfusion changes of EPO, sTfR and GDF-15 may reflect the different physiology and clinical significance of these indices. EPO closely correlated (inversely) with Hb pre- and
post-transfusion, and EPO percentage change inversely correlated with Hb change.

EPO rapidly rises in anemia as a result of anemia-induced hypoxia and falls as Hb is corrected. EPO levels are highest when anemia is due to marrow hypoplasia and are lower when anemia is due to dyserythropoiesis (i.e. in thalassemia), perhaps due to erythroblast uptake of EPO. sTfR and GDF-15 both reflect erythropoiesis, but we observed different behavior between them. Transferrin receptors are chiefly expressed by erythroblasts, increasing from early to intermediate stages and declining with maturation. Thus, transferrin receptor mass reflects numbers of immature erythroid cells. We did not observe a significant pre- to post-transfusion change in sTfR in the group as a whole, and we did not identify associations between Hb and sTfR either pre- or post-transfusion. In our cohort, pre-transfusion Hb exceeded 100g/L in 18 patients, potentially considerably suppressing erythropoiesis (indicated by sTfR) both pre-transfusion and over the inter-transfusion interval. Relative stability of erythroid activity over the transfusion cycle in chronically transfused adult patients has been observed previously. Conversely, although GDF-15 fell following transfusion, it was markedly elevated pre- and remained so post-transfusion. Expression of GDF-15 peaks in late erythroblasts and is also associated with erythroblast apoptosis, a feature of ineffective erythropoiesis in β-thalassemia. Ineffective erythropoiesis with tissue hypoxia and erythroblast apoptosis may induce the high levels of GDF-15 seen in thalassemia. Reflecting our data, GDF-15 has been found to be markedly elevated in β-thalassemia syndromes (e.g. 11,512-127,254pg/mL), and moderately elevated in other dyserythropoietic conditions, including congenital dyserythropoietic anemia and pyruvate kinase deficiency. Conversely, as discussed below, GDF-15 does not appear to increase as dramatically where erythropoiesis is physiologically increased (for example, altitude or following administration of EPO). Thus, the divergent
patterns of EPO, sTfR and GDF-15 may reflect differing physiologies of these parameters and provide insights into transfusion effects on erythropoiesis in thalassemia. EPO closely reflects anemia but may also reflect erythroid suppression. sTfR reflects overall erythropoietic activity, but not necessarily ineffective erythropoiesis/ dyserythropoiesis with apoptosis, which appears best reflected by GDF-15. Differential expression of these parameters may indicate that even adequately transfused patients with β-thalassemia, despite stable overall erythroid suppression, have ongoing ineffective erythropoiesis with apoptosis that fluctuates with transfusion.

In all patients, the hepcidin-ferritin ratio was markedly below one both pre- and post transfusion, indicating suppression of hepcidin out of proportion to the degree of iron loading, and implying a suppressive effect from erythropoiesis. Although ferritin rose post-transfusion, it is unlikely that increasing iron stores caused the rise in hepcidin as percentage changes in hepcidin and ferritin were not correlated. Furthermore, the rise in the hepcidin-ferritin ratio following transfusion despite the increase in ferritin indicates that hepcidin increased out of proportion to the increase in ferritin.

The mechanism for suppression of hepcidin in patients with thalassemia and other conditions with increased erythropoiesis remains uncertain but several lines of evidence suggest an erythropoiesis-derived signal that inhibits hepcidin production.

Sera from thalassemic patients suppresses Hamp expression in hepatoma cell lines, indicating the presence of the signal in serum and activity in vitro. The suppressive signal appears to override iron loading induced-BMP6 mediated signaling.
Suppression of hepcidin by anemia and hypoxia is contingent on erythroid activity. For example, mice with induced anemia require an active erythroid compartment to suppress hepatic hepcidin expression. Hypoxia does not induce suppression of hepcidin if erythropoietin mediated erythropoiesis is inhibited. Our study has emulated these animal data by using transfusion to physiologically correct anemia, suppress erythropoiesis and increase hepcidin.

Two molecules, GDF-15 and Twisted gastrulation protein homolog 1 (TWSG1), have been identified through transcriptome analysis as putative erythroblast-derived factors that modulate hepcidin. Tanno et al showed that recombinant GDF-15 inhibited expression of hepcidin in hepatic cell lines, while depletion of GDF-15 in thalassemic serum reverses the suppressive effect on hepcidin expression. However, the relationship between GDF-15 and hepcidin regulation remains equivocal. For example, Gdf-15−/− mice subjected to phlebotomy appropriately suppress hepcidin, and Hbb th3+/+ mice (a thalassemia model) do not express increased bone marrow Gdf-15. Hepcidin suppression in pregnancy is not associated with increased GDF-15, even though expanding maternal erythroid mass is one of the reasons for the increased iron requirement. Following stem cell transplantation, recovery of erythropoiesis is associated with suppression of hepcidin which is not associated with rising GDF-15. Finally, when serum from thalassemic patients immunodepleted of GDF-15 was applied to hepatoma cells, the suppressive effect of serum on hepcidin expression was only partially reversed. GDF-15 may play a particular role in hepcidin suppression in ineffective erythropoiesis, but appears to be only one of the factors involved. In vitro, TWSG1 interferes with BMP-mediated hepcidin expression in human hepatocytes through inhibition of BMP dependent SMAD-phosphorylation, and expression is
increased in thalassemic mice. Human correlative studies are not yet available. Our
findings do not provide evidence that hepcidin is regulated directly by GDF-15 or any
other specific factor. Rather, our data confirm that erythroid activity is closely and
dynamically associated with hepcidin, consistent with a potential secreted ‘erythroid’
factor that suppresses hepcidin.

Guidelines for the management of β-thalassemia major advise transfusion to maintain
a pre-transfusion Hb at 90-105g/L, based on studies indicating that, compared with
hyper-transfusion regimes, patients maintained in this range balance satisfactory
suppression of erythropoiesis with manageable transfusional iron loading and reduced
transfusion requirements. Cazzola et al reported that erythroid proliferation
(measured by sTfR) was 1-2 times normal in patients with pre-transfusion Hb 100-
110g/L, 1-4 times normal for Hb 90-100g/L, and 2-6 times normal in patients with Hb
85-90g/L. The authors concluded that maintaining Hb concentrations above 90g/L
should sufficiently balance the need to suppress erythropoiesis with transfused iron.

Adoption of these pre-transfusion Hb targets reduced transfusion requirements and
eased iron loading. In our study, patients were receiving an individualized, stable,
regular transfusion regimen based on their tolerable transfusion volume and a
clinically and logistically acceptable inter-transfusion interval. Pre-transfusion
erythroid activity (defined by sTfR) was 1-4 times normal in 24 of 28 patients with
Hb>90g/L, thus our data support current recommendations.

Previous studies reporting erythropoiesis and hepcidin in thalassemia did not present
comparisons between males and females. Multiple regression indicated that pre- and
post-transfusion sex differences in hepcidin levels are partially mediated by
differences in erythropoiesis. We observed evidence of increased erythropoiesis in males compared with females. We hypothesize that two mechanisms contribute to this difference. Firstly, in non-thalassemic populations, despite similar EPO concentrations,\textsuperscript{45} Hb levels in males are generally higher than females, presumably due to androgen effects on erythropoiesis.\textsuperscript{28} Males may thus require higher Hb concentrations to suppress erythropoietic drive. Secondly, post-transfusion, smaller increments in Hb in males appear to perpetuate differences in erythropoiesis and hepcidin. The smaller rise in Hb in men may be explained by male patients receiving a lower transfusion dose per unit blood volume. Current guidelines do not account for sex-wise differences in blood volume, which may result in males receiving a relatively smaller transfusion volume. We also noted that male patients had lower ferritin concentrations compared with females. Although relatively lower transfusion volumes could be one explanation, differences in dosing and adherence to iron chelators also need to be considered. Although weight and height were inversely associated with hepcidin and positively with sTfR and GDF-15 (data not shown), these associations were no longer seen when controlling for sex. As the number of subjects in our study was relatively small, studies in other thalassemia patient groups are needed to establish whether sex-wise differences observed here are present more generally.

Earlier studies in transfused children with β-thalassemia major showed that when children were anemic and transfusions delayed, iron absorption from ferrous sulfate and food is increased, whereas absorption is normal when measured shortly following transfusion when Hb is raised, predicting an interaction between erythropoiesis and hepcidin many years before hepcidin was discovered.\textsuperscript{46,47} Hepcidin regulates
intestinal iron absorption and its levels can predict erythrocyte incorporation of dietary iron. However, correlations between hepcidin and iron utilization in patients with β-thalassemia have not been evaluated and thus specific predictions of iron absorption based on hepcidin cannot be made. In chronically transfused patients with thalassemia, hepcidin levels resemble those in non-thalassemic individuals, potentially implying relatively normal dietary iron absorption, especially post-transfusion. Interestingly, the coefficient between hepcidin and Hb remained constant pre- and post-transfusion, indicating stability in the relationship between these indices.

Compared with transfusion of fresh blood, aged (40-42 day old) blood has been found to produce greater increases in serum ferritin, transferrin saturation and non-transferrin bound iron in healthy volunteers. We did not find evidence of an effect of blood storage age on changes in ferritin. However, only few patients received exclusively fresh units and further studies are needed to evaluate the effects of blood storage in β-thalassemia.

Unlike mouse models of thalassemia, age was not associated with hepcidin levels. This association may be mediated by iron loading and may be modified by chelator adherence. One patient appeared an outlier in our dataset: a 40-year-old male with pre-transfusion Hb=66g/L; EPO=244.7IU/mL, sTfR=4.1mg/mL, GDF-15=33,464pg/mL, hepcidin undetectable; post-transfusion Hb=84g/L, EPO=160.5IU/mL, GDF-15=21,931pg/mL, hepcidin remained undetectable. The patient was retained in the analysis as he fulfilled inclusion criteria and recorded data were accurate. If this patient was excluded from the analysis, pre-transfusion multiple
regression models 2 and 4, and post-transfusion model 4, remained significant; associations between transfusion volume and change in Hb, and between change in Hb and change in EPO and hepcidin remained significant; and differences between males and females in pre-transfusion sTfR, GDF-15 and hepcidin, and post-transfusion reticulocyte count, GDF-15 (with sTfR approaching significance p=0.055) and hepcidin, persisted; indicating our findings are robust.

Our data may have clinical implications. Erythropoiesis varies over the transfusion cycle, thus timing of measurement of indices for clinical and research purposes must be standardized (e.g. immediately pre-transfusion) or at least documented. Secondly, monitoring of EPO, sTfR and GDF-15 could have a potentially valuable clinical role in the future in quantitatively and qualitatively evaluating erythropoiesis and thus optimizing transfusion dosing. Further studies that identify trends in these indices over time in patients, and correlate these indices with clinical implications of expanded erythropoiesis (for example, extra-medullary hematopoiesis, osteoporosis, growth), may clarify the clinical role of these parameters. As hepcidin appears to integrate erythropoietic and iron-loading signals, clinical measurement of hepcidin (together with the hepcidin-ferritin ratio) may become a useful indicator of erythropoiesis and iron kinetics in complex patients. Our data also suggest that, reflecting the situation in non-thalassemic individuals, optimal pre-transfusion hemoglobin levels in males with β-thalassemia may be higher than for female patients, although corroboration in other cohorts is necessary. In all patients, the hemoglobin increment is related to the transfusion volume relative to patient blood volume, and thus incorporation of the recipient blood volume into transfusion dosage calculations may be useful.
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Authorship contributions
SP designed the research, collected, analyzed and interpreted the data, performed statistical analysis, and wrote the manuscript. DF performed the hepcidin and GDF-15 assays, and wrote the manuscript. DB designed the research and wrote the manuscript. GA designed the research, performed the hepcidin and GDF-15 assays, and wrote the manuscript. All authors approved the final manuscript.

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### Table 1: Summary of patients included in the study

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<th>Overall</th>
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<th>Females</th>
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<tr>
<td><strong>Weight (kg)</strong></td>
<td>62.3</td>
<td>67.3</td>
<td>56.9</td>
<td>0.0462</td>
</tr>
<tr>
<td>[56.9, 67.6]</td>
<td>[61.6, 72.9]</td>
<td>[47.8, 66.1]</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Height (cm)</strong></td>
<td>159.6</td>
<td>166.3</td>
<td>153.0</td>
<td>0.0006</td>
</tr>
<tr>
<td>[155.4, 163.9]</td>
<td>[161.1, 171.4]</td>
<td>[147.7, 158.4]</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Days since previous transfusion</strong></td>
<td>22 [20, 24]</td>
<td>22 [18, 25]</td>
<td>23 [20, 25]</td>
<td>0.5701</td>
</tr>
<tr>
<td><strong>Number of red cell units transfused</strong></td>
<td>2.8 [2.6, 3.1]</td>
<td>2.9 [2.5, 3.2]</td>
<td>2.8 [2.4, 3.2]</td>
<td>0.7835</td>
</tr>
<tr>
<td><strong>Transfusion volume (mL/kg)</strong></td>
<td>11.9</td>
<td>11.0</td>
<td>12.9</td>
<td>0.1273</td>
</tr>
<tr>
<td><em>Assuming each bag contains 260mL red cells (Australian Red Cross Blood Service red cell unit mean volume = 259mL ± 23mL)</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[10.7, 13.2]</td>
<td>[9.3, 12.7]</td>
<td>[11.0, 14.8]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[4.14, 4.78]</td>
<td>[3.97, 3.74]</td>
<td>[3.36, 3.74]</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Estimated mL transfused per L blood volume†</strong></td>
<td>196.5 [175.2, 217.9]</td>
<td>171.3 [143.4, 197.2]</td>
<td>221.0 [190.9, 251.2]</td>
<td>0.0122</td>
</tr>
<tr>
<td>[175.2, 217.9]</td>
<td>[143.4, 197.2]</td>
<td>[190.9, 251.2]</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean age of transfused unit (days)</strong></td>
<td>16.1 [14.2, 18.0]</td>
<td>15.7 [13.0, 18.4]</td>
<td>16.5 [13.4, 19.6]</td>
<td>0.6865</td>
</tr>
</tbody>
</table>

Arithmetic mean [95% Confidence interval]; p calculated from two sample t-test

* Assuming each bag contains 260mL red cells (Australian Red Cross Blood Service red cell unit mean volume = 259mL ± 23mL)*

† Nadler's formula for total blood volume (TBV):  

\[
\text{Males: } TBV \ [mL] = 604 + (367 \times \text{height [m]}^3) + (32.2 \times \text{weight [kg]})
\]

\[
\text{Females: } TBV \ [mL] = 183 + (356 \times \text{height [m]}^3) + (33.1 \times \text{weight [kg]})
\]
Table 2: Associations with hepcidin pre- and post-transfusion

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre-Transfusion</th>
<th>Post-Transfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient [95% CI]</td>
<td>Beta coefficient</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Univariate regression</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>-0.02 [-0.07, 0.03]</td>
<td>-0.14</td>
</tr>
<tr>
<td>Sex</td>
<td>-0.99 [-1.69, -0.30]</td>
<td>-0.48</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>0.05 [0.01, 0.08]</td>
<td>0.47</td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.70 [0.30, 1.11]</td>
<td>0.56</td>
</tr>
<tr>
<td>CRP</td>
<td>0.01 [-0.53, 0.56]</td>
<td>0.01</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>-0.67 [-1.17, -0.17]</td>
<td>-0.48</td>
</tr>
<tr>
<td>GDF-15</td>
<td>-0.97 [-1.51, -0.42]</td>
<td>-0.56</td>
</tr>
<tr>
<td>sTfR</td>
<td>-1.18 [-2.16, -0.20]</td>
<td>-0.43</td>
</tr>
<tr>
<td><strong>Multiple regression</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>-0.66 [-1.26, -0.07]</td>
<td>-0.32</td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.49 [0.13, 0.86]</td>
<td>0.40</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>0.04 [0.02, 0.07]</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>Ferritin</td>
</tr>
<tr>
<td>------</td>
<td>--------------</td>
<td>----------</td>
</tr>
<tr>
<td><strong>Model 2</strong></td>
<td></td>
<td>-0.46 [-1.09, 0.17]</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ferritin</td>
<td>0.58 [0.17, 0.98]</td>
</tr>
<tr>
<td></td>
<td>Erythropoietin</td>
<td>-0.59 [-1.00, -0.18]</td>
</tr>
<tr>
<td><strong>Model 3</strong></td>
<td></td>
<td>-0.12 [-0.82, 0.58]</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ferritin</td>
<td>0.58 [0.21, 0.96]</td>
</tr>
<tr>
<td></td>
<td>GDF-15</td>
<td>-0.76 [-1.30, -0.21]</td>
</tr>
<tr>
<td><strong>Model 4</strong></td>
<td></td>
<td>-0.28 [-1.01, 0.45]</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ferritin</td>
<td>0.60 [0.19, 1.00]</td>
</tr>
<tr>
<td></td>
<td>sTfR</td>
<td>-0.89 [-1.80, 0.03]</td>
</tr>
</tbody>
</table>

Hepcidin, sTfR, Ferritin, Erythropoietin, CRP all log transformed following addition of 1

Sex coded: Female=0, Male=1

Beta-coefficient: coefficient using variables standardized to have variance of 1
Table 3: Differences between males and females in pre- and post-transfusion indices of hematology, erythropoiesis and iron physiology

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre-transfusion</th>
<th>Post-transfusion</th>
<th>p difference between sexes</th>
<th>Males</th>
<th>Females</th>
<th>p difference between sexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>16</td>
<td>15</td>
<td></td>
<td>12</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/L)†</td>
<td>102.0 [95.6, 108.4]</td>
<td>101.8 [97.0, 106.6]</td>
<td>0.9583</td>
<td>116.9 [108.6, 125.3]</td>
<td>126.8 [120.4, 133.2]</td>
<td>0.0486</td>
</tr>
<tr>
<td>Reticulocytes: absolute (10^9/L)†</td>
<td>97.4 [64.9, 129.8]</td>
<td>69.7 [34.6, 104.9]</td>
<td>0.2255</td>
<td>110.7 [65.7, 155.7]</td>
<td>55.3 [23.2, 87.4]</td>
<td>0.0349</td>
</tr>
<tr>
<td>Ferritin (ng/mL)†</td>
<td>716.1 [481.8, 1064.0]</td>
<td>1362.2 [866.1, 2145.5]</td>
<td>0.0298</td>
<td>796.3 [549.6, 1154.7]</td>
<td>1420.4 [912.0, 2215.2]</td>
<td>0.0435</td>
</tr>
<tr>
<td>C-Reactive Protein (mg/L)†</td>
<td>1.53 [0.90, 2.36]</td>
<td>2.06 [0.83, 4.13]</td>
<td>0.4896</td>
<td>1.53 [0.66, 2.87]</td>
<td>2.22 [0.86, 4.56]</td>
<td>0.4725</td>
</tr>
<tr>
<td>Soluble transferrin receptor (mg/L)†</td>
<td>3.66 [2.80, 4.72]</td>
<td>2.35 [1.82, 2.97]</td>
<td>0.0127</td>
<td>3.40 [2.55, 4.45]</td>
<td>2.33 [1.81, 2.95]</td>
<td>0.0351</td>
</tr>
<tr>
<td>Erythropoietin (mIU/mL)†</td>
<td>60.7 [38.8, 97.1]</td>
<td>49.6 [34.2, 71.8]</td>
<td>0.4737</td>
<td>33.2 [22.3, 49.0]</td>
<td>20.9 [15.2, 28.7]</td>
<td>0.0548</td>
</tr>
<tr>
<td>Hepcidin (ng/mL)†</td>
<td>11.3 [5.6, 22.0]</td>
<td>32.2 [22.0, 47.0]</td>
<td>0.0067</td>
<td>19.9 [10.1, 43.3]</td>
<td>76.1 [55.0, 105.3]</td>
<td>0.0009</td>
</tr>
<tr>
<td>GDF-15 (pg/mL)†</td>
<td>10267.0 [7007.9, 14915.1]</td>
<td>5561.0 [4691.5, 6530.5]</td>
<td>0.0039</td>
<td>7406.0 [5039.0, 9783.0]</td>
<td>3543.4 [2933.1, 4153.5]</td>
<td>0.0006</td>
</tr>
<tr>
<td></td>
<td>15041.9]</td>
<td>6591.6]</td>
<td>10885.6]</td>
<td>4280.6]</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hepcidin –</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ferritin ratio</strong></td>
<td>0.025 [0.014, 0.037]</td>
<td>0.030 [0.015, 0.044]</td>
<td>0.6116 [0.020, 0.057]</td>
<td>0.038 [0.020, 0.057]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.038 [0.020, 0.057]</td>
<td>0.068 [0.039, 0.098]</td>
<td>0.0838 [0.057, 0.118]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Arithmetic mean [95% Confidence interval]; p calculated from t-test*

†Geometric mean [95% Confidence interval]; p calculated from t-test of log-transformed data
Figure Legends

Figure 1: Pre and post-transfusion indices of hematology, erythropoiesis and iron physiology. There were 31 patients pre-transfusion and 26 post-transfusion.

Following transfusion, mean (A) Hb increased (mean pre-transfusion 101.9 g/L [98.1, 105.7], post-transfusion 122.2 g/L [117.0, 127.4], p<0.0001; change 20.5 g/L [16.1, 24.8]), (B) erythropoietin decreased (pre-transfusion 54.7 mIU/mL [41.3, 72.4], post-transfusion 25.6 mIU/mL [20.0, 32.8], p<0.0001, percentage change -49.4% [-58.4, -40.5]), (C) GDF-15 decreased (pre-transfusion 7556.2 pg/mL [6016.5, 9490.8], post-transfusion 4979.6 pg/mL [3910.8, 6340.5], p<0.0001, percentage change -35.6% [-41.3, -29.9]), (D) sTfR did not significantly vary (pre-transfusion 2.95 mg/L [2.43, 3.54], post-transfusion 2.79 mg/L [2.30, 3.35], p=0.0937, percentage change -2.0% [-5.3, 1.3]), (E) ferritin increased (pre-transfusion 987.7 ng/mL [726.2, 1343.2], post-transfusion 1086.3 ng/mL [806.2, 1463.6], p=0.0021, percentage change 18.7% [8.3, 29.1]); (F) CRP did not vary significantly (pre-transfusion 1.78 mg/L [1.11, 2.68], post-transfusion 1.88 mg/L [1.06, 3.02], p=0.7423, percentage change 22.9% [-8.8, 54.6]); (G) hepcidin increased (pre-transfusion 19.2 ng/mL [12.7, 29.8], post-transfusion 41.3 ng/mL [26.3, 64.5], p<0.0001, percentage change 154.6% [108.9, 200.2]); and (H) hepcidin-ferritin ratio increased (pre-transfusion 0.027 [0.019, 0.036], post-transfusion 0.054 [0.036, 0.072], p=0.0001, percentage change 129.7% [75.0, 184.5]). Reticulocyte count (not shown) did not change (pre-transfusion 83.6×10^9/L [60.6, 106.6], post-transfusion 80.8×10^9/L [53.4, 108.3], p=0.8252).
**Figure 2: Associations between Hb and indices of erythropoiesis and hepcidin pre-transfusion and post-transfusion.**

Associations between Hb, erythropoiesis, iron stores and hepcidin were evaluated in both the pre-transfusion and post-transfusion state. (A) Pre-transfusion Hb concentration was associated with indices of erythropoiesis GDF-15 \( (r=-0.54, p=0.0019) \) and EPO \( (r=-0.61, p=0.0004) \), but not sTfR (not shown, \( r=-0.14, p=0.4682 \)), and also with hepcidin \( (r=0.47, p=0.0094) \). (B) Pre-transfusion hepcidin was inversely associated with erythropoiesis: GDF-15 \( (r=-0.57, p=0.0011) \); EPO \( (r=-0.48, p=0.0101) \); and sTfR \( (r=-0.43, p=0.0199) \). (C) Pre-transfusion hepcidin was positively associated with iron loading (ferritin, \( r=0.56, p=0.0015 \)) but not with inflammation (CRP, \( r=0.01, p=0.9627 \)). (D) Post-transfusion Hb was associated with erythropoiesis \( (GDF-15 (r=-0.58, p=0.0018), EPO (r=-0.58, p=0.0026)) \) and hepcidin \( (r=0.60, p=0.0011) \), but not sTfR (not shown, \( r=-0.29, p=0.1563 \)). (E) Post-transfusion hepcidin was inversely associated with erythropoiesis: GDF-15 \( (r=-0.65, p=0.0004) \); EPO \( (r=-0.71, p=0.0001) \); and sTfR \( (r=-0.50, p=0.0101) \). (F) Post-transfusion hepcidin was positively associated with iron loading (ferritin, \( r=0.44, p=0.0258 \)); but not with inflammation (CRP, \( r=0.17, p=0.4045 \)).
Figure 3 Associations between change from pre- to post-transfusion. Associations between indices that changed significantly from pre to post-transfusion were evaluated (i.e. Hb, ferritin, EPO, GDF-15, hepcidin). (A) Transfused volume of red cells normalized for patient blood volume was positively associated with post-transfusion change in Hb ($r=0.56$, $p=0.0033$). (B) Post-transfusion change in Hb was inversely associated with change in EPO ($r=-0.56$, $p=0.0034$) and positively with change in hepcidin ($r=0.44$, $p=0.0300$). (C) Percentage change in EPO was inversely associated with change in hepcidin ($r=-0.41$, $p=0.0489$). Changes in GDF-15 and sTfR were not associated with change in hepcidin, nor was change in ferritin associated with change in hepcidin (not shown).
Figure 1

A. Hemoglobin (g/L) before and after transfusion, with a p-value of <0.0001.

B. Erythropoietin (mIU/L) before and after transfusion, with a p-value of <0.0001.

C. GDF-15 (pg/mL) before and after transfusion, with a p-value of <0.0001.

D. sTFR (mg/L) before and after transfusion, with a p-value of 0.0937.

E. Ferritin (ng/mL) before and after transfusion, with a p-value of 0.0021.

F. CRP (mg/L) before and after transfusion, with a p-value of 0.7423.

G. Hepcidin (ng/mL) before and after transfusion, with a p-value of <0.0001.

H. Hepcidin:Ferritin ratio before and after transfusion, with a p-value of 0.0001.
Transfusion suppresses erythropoiesis and increases hepcidin in adult patients with beta-thalassemia major: a longitudinal study

Sant-Rayn Pasricha, David M. Frazer, Donald K. Bowden and Gregory J. Anderson