Liver expression of hepcidin and other iron genes in two mouse models of β-thalassemia

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Background and Objectives. Homozygous β-thalassemia patients may develop iron overload even if untransfused, due to inappropriately high intestinal iron absorption. Reduction of hepcidin synthesis has been reported both in patients and in animal models. We have measured liver hepcidin and other iron gene transcripts in two different mouse models of β-thalassemia at different ages.

Design and Methods. Mice Hbb$^{th/th}$, characterized by spontaneous homozygous deletion of the major *b1* globin gene were studied at 2 and 8 months. Mice *Hbb*$^{th3/+}$, characterized by the heterozygous deletion of *b1* and *b2* globin genes were studied at 4 and 10 months. Hematologic data were obtained and iron overload estimated by Perls' staining of the liver. Expression of liver hepcidin, Tfr2, Hjv, Fpn and Hfe RNA was assessed by real-time polymerase chain reaction. Levels of serum cytokines (interleukin-6, IL-1β, IL-10, granulocyte-macrophage colony-stimulating factor) levels were assayed by enzyme-linked immunosorbent assay.

Results. Hemoglobin, hematocrit and mean corpuscolar volume were significantly reduced in both β-thalassemia models, more significantly in *Hbb*$^{th3/+}$, which have the greater, age-dependent, iron overload. Hepcidin RNA was not increased despite iron overload in both strains. Fpn RNA was increased and Tfr2 was decreased in older animals. Inflammatory cytokine levels were striking variable and unrelated to hepcidin levels.

Interpretation and Conclusions. Although anemia is reported to inhibit hepcidin expression, *normal* hepcidin synthesis was maintained in both thalassemic models studied. However, hepcidin levels were inappropriate for the body iron, especially in *Hbb*$^{th3/+}$ 10-month-old animals. As we previously reported in wild type mice after parenteral iron overload, Tfr2 is reduced and Fpn RNA increased in thalassemic mice. Inflammatory cytokines did not play a major role in increasing hepcidin levels or in modifying iron homeostasis in this study.

Key words: iron metabolism, thalassemia, hepcidin, anemia.

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β-thalassemia is an inherited recessive disorder due to defective β-globin chain synthesis, which impairs erythrocyte hemoglobinization and leads to ineffective erythropoiesis and severe anemia. Homozygous patients may exhibit the transfusion-dependent picture of thalassemia major or the less severe transfusion-independent features of thalassemia intermedia. Homozygous thalassemia is characterized by increased iron absorption in spite of high total body iron, a feature shared with other congenital anemias with high degrees of ineffective erythropoiesis, globally called *iron-loading anemias*. Recent advances in the understanding of the regulation of systemic iron homeostasis have allowed new approaches to studying the molecular basis of this deregulation. Hepcidin, a liver peptide hormone that inhibits iron release from duodenal cells and macrophages to circulating transferrin is central to systemic iron regulation.[1] In mice inactivation of hepcidin causes iron overload, whereas its overexpression causes severe iron deficiency.[2,3] In humans mutations of hepcidin cause juvenile hemochromatosis,[4] a recessive disease characterized by early onset of iron overload and iron-related clinical complications. Hepcidin levels, measured either as liver mRNA or as levels of the active peptide excreted in the urine, are reduced in all types of genetic hemochromatosis, both in animal models and in human patients.[5] This finding indicates that the hemochromatosis gene products (*HFE, TFR2*, hemojuvelin and hepcidin) are components of the same iron regulatory pathway and that hepcidin is the pathway final effector. Hepcidin is negatively regulated by anemia and hypoxia.[6] Since hepcidin is an acute phase protein, its levels are also significantly increased by inflammatory cytokines, especially interleukin (IL)-6.[7] Recently a link has been established between hepcidin and the cellular iron

exporter ferroportin 1 (*FPN*). In the HEK293 cell line transfected with *FPN*, hepcidin binds to FPN, causing its internalization and lysosomal degradation.[8] In this way FPN behaves *in vitro* as the hepcidin receptor, defining a loop of iron regulation based on iron/hepcidin/FPN. Decreased hepcidin might be responsible for increased iron absorption in iron-loading anemias. However, available data in patients are scanty. Low levels of urinary hepcidin have been recorded in a few adult β-thalassemia homozygotes and in two patients with congenital dyserythropoietic anemia type I,[9] in agreement with the hypothesis that the iron needs of an expanded erythron overtake the inhibitory signal of the iron stores on iron absorption. The study of a larger series of thalassemic patients of different ages revealed a more complex pattern. Hepcidin levels were reduced, if compared with ferritin levels (hepcidin/ferritin ratio), but were otherwise *normal* and increased after blood transfusions in thalassemia major.[10] Low hepcidin levels were documented in untransfused thalassemia intermedia.[10] Studies in patients are complex to interpret. Several factors, besides the inhibitory effect of anemia,[6] may affect hepcidin production: iron chelation and its kinetics, splenectomy, and different degrees of tissue hypoxia .[10]

The availability of mouse models for human β-thalassemia enables these problems in evaluating hepcidin expression to be overcome. Red cells of adult wild-type (*wt*) mice contain two β globin chains, each encoded by an adult globin gene. The β major gene (*b1*) provides 80% of the total β hemoglobin chains, whereas the β minor gene (*b2*) contributes the remaining 20%.[11,12] Among the different mouse models of β thalassemia, we focused on two different strains. The first one, results from the spontaneous DNA deletion of *b1* ($Hbb^{th/th}$) and is characterized by the absence of β major globin chain but shows some compensatory β-minor globin chain synthesis.[11,13] The second, artificially created by deleting both *b1* and *b2* loci ($Hbb^{th3/+}$), does not show any production of adult β-globin chains.[14] Previous studies on these two β-thalassemic models have suggested that Hbbth/th is the counterpart of human β-thalassemia intermedia and $Hbb^{th3/+}$ of the more severe β^0-thalassemia. Limited data, mainly from the latter strain,[14] are available on iron overload in these animals. Recently, a single preliminary report showed lower levels of liver hepcidin mRNA in adult $Hbb^{th-3/+}$ mice than in *wt* controls.[15]

Here we report the expression of hepcidin and other liver iron genes in both $Hbb^{th/th}$ and $Hbb^{th3/+}$ mice at different ages, in relation to hematologic parameters and iron overload. We compare the results to those obtained in known non-thalassemic models of iron overload. To exclude the effect of co-existing inflammation in the induction of hepcidin synthesis, we measured the levels of inflammatory cytokines, since these have been shown to induce hepcidin synthesis.[7]

Design and Methods

Animals

Mice homozygous for β thalassemia $Hbb^{th/th}$ were obtained from breeding performed in the animal facility (CIRSAL) of the University of Verona (Italy). Female mice were studied at 2 and 8 months of age. The original β-thalassemia mutation in DBA/2J was back-crossed in C57BL/6 for more than 11 generations. Wt littermates were used as control group.

Mice heterozygous for the deletion of both b1 and b2 globin genes $Hbth^{-3/+}$ on a C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Animals were selected for the study at 4 and at 10 months of age. Wt littermates were used as controls. Female mice weighing 25 to 28 grams were used.

Institutional and national guidelines for the care and use of laboratory animals were followed. The study protocol was reviewed and approved by the University of Verona, Animal Care and Use Committee (CIRSAL) and the Italian Ministry of Health. All mice were given a standard diet containing 250 mg/Kg iron (diet number 48, Piccioni laboratory, Milan, Italy).

Four to six female animals were used in each experiment and four were used as controls. After sacrifice each animal's liver was dissected and snap-frozen immediately for RNA analysis. Only females were used because of the observed significant differences in iron metabolism between sexes in mice.[16]

Hematologic parameters

Blood was collected from isofluorane-anesthetized mice by retro-orbital venipuncture into heparinized microhematocrit tubes. Hemoglobin (Hb) concentration was determined by spectroscopic measurement of the cyanmet derivative. The hematocrit (Hct) was determined by centrifugation in a micro-hematocrit centrifuge. Erythrocyte and reticulocyte cellular indices were calculated on an ADVIA 120 hematology analyzer, using a mouse-specific software program (Bayer Diagnostics, Tarrytown, NY, USA).

Histology and Perls' staining

For histological studies and iron staining mouse livers were fixed in 4% (vol/vol) buffered formalin and embedded in paraffin. For assessment of non-heme iron, slides of liver sections were stained using the Perls' Prussian blue method. Hematoxylin-eosin counterstain was performed to mark nuclei and cytoplasm by standard procedures.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total cellular RNA was isolated from *snap frozen* mice livers by using the RNAeasy Kit (Quiagen Sciences,

Maryland, USA). Levels of gene expression were measured by qRT-PCR, as previously described.[17] For the reverse transcription 1 μg of total RNA, random hexamers at a concentration of 25 μM and 100 U of the reverse transcriptase (Applera, Milan, Italy) were added to the reaction mixture.

PCR reactions and fluorescence measurements were made on an iCycler (Bio-Rad Laboratories, Hercules, CA, USA). qRT-PCR assays (Assays-on-Demand, Gene Expression Products), supplied by Applied Biosystems (Foster City, CA, USA), were used to evaluate the amounts of hepcidin, Tfr2, Hjv, Fpn and Hfe transcripts. In all the assays, in order to avoid co-amplification of genomic DNA, primer sets were designed complementary to exonic sequences separated by introns.

For the PCR reaction, 100 ng cDNA were added to 15 μL of the PCR reaction mix containing 10 μL of TaqMan Universal PCR Master Mix (Applera), 1 μL of assay in a final volume of 20 μL. The PCR procedure started with a step of 2 min at 50°C to activate the UNG enzyme, followed by 10 min at 95°C to inactivate the UNG enzyme and to provide a *hot start* activating the AmpliTaq polymerase. Fifty cycles of denaturation (95°C for 15 seconds), followed by annealing and extension (60°C for 60 s) were performed. All analyses were carried out in triplicate. Results showing a discrepancy greater than one cycle threshold in one of the wells were excluded.

Data analysis

The values obtained were normalized using the β-glucuronidase gene (*GUS*) as a control. The results were analyzed using the ΔΔCt method, as the efficiencies of amplification of both the target and reference genes were found to be approximately equal. Briefly, the threshold cycle (C_T) indicates the cycle number at which the amount of the amplicon reaches the fixed threshold. ΔC_T is the difference between threshold cycle for the gene of interest and the reference gene (ΔC_{Target}-$\Delta C_{Reference}$). ΔΔCt is the difference between the ΔC_T of the sample and the ΔC_T of a RNA calibrator. A pool of normal mice liver RNA was used as the calibrator. The final result is expressed as $2^{-(\Delta\Delta Ct)}$.[17]

Hepcidin 1/hepcidin 2 ratio

The commercial assay used for hepcidin dosages contains primers that do not discriminate between hepcidin 1 and hepcidin 2. Therefore, in order to assess their separate contribution, we developed a method that relies on the different sizes of the amplified products. The same primers (forward 5'-ATGGCACTCAGCACTCGGA-3' and reverse - made fluorescent - 5'ATCGTCTTTATTTCAAGG-3' were used for amplification. The resulting fragments differ in size by four nucleotides.[18] PCR was performed on retrotranscribed cDNA under standard conditions. PCR products were electrophoresed in an automatic sequencer (ABI Prism 3100 Genetic Analyzer; Applied Biosystem). Hepcidin 1 and 2 fragments were discriminated by size and the height of the peaks used to quantify the relative proportions of the two fragments expressed as the hepcidin 1/hepcidin 1+ hepcidin 2 ratio.

Serum cytokine measurements

The serum levels of IL-6, IL-1β, IL-10 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were determined using the commercial Bio-Plex Mouse Cytokine 17-Plex assay (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions.

Statistical analysis

Values are expressed as means plus or minus the standard deviation. The statistical significance of the differences of mRNA expression between wt and thalassemic mice was evaluated using Student's t test (unpaired, two tailed). Correlations between variables were analyzed by Spearman's rank coefficient. A p value of less than 0.05 was considered statistically significant.

Results

Hematologic parameters

Hematologic data and red cell indices of all the strains studied are shown in Tables 1 and 2. As previously shown,[19] both β-thalassemic strains showed highly significant reductions of Hct, Hb, mean corpuscular volume (MCV) and increases of red blood cell distribution width (RDW), hemoglobin concentration distribution width (HDW) and total reticulocyte count, as compared with their *wt* littermates (p<0.01 Tables 1 and 2). Marked reductions of reticulocyte MCV (MCVr), Hb content (CHr) and HDW (HDWr) were also evident.

A comparison of the hematologic data between younger and older mice of the same strain showed no significant differences in Hb concentrations or red cell indices in $Hbb^{th/th}$ mice of different ages (Table 1). In contrast, 10-month old $Hbb^{th\text{-}3/+}$ mice had significantly lower Hb and MCV levels and higher reticulocyte counts than had 4-month old animals (p<0.05; Table 2), such that the age-related severity of the thalassemic phenotype increased in this strain.

Finally we compared the hematologic data of the oldest β-thalassemic animals of the two models. As shown in Table 2, Hct and Hb levels were significantly lower and the fraction of dense damaged red cells (indicated by RDW) significantly higher in 10-month-old $Hbb^{th\text{-}3/+}$ than in the oldest $Hbb^{th/th}$ mice studied.

Histology

Young $Hbb^{th/th}$ mice did not show iron overload, whereas at 8 months some iron deposition was

Table 1. Hematologic data, hepcidin 1/hepcidin 2 ratios and serum cytokine levels in *Hbb*$^{th/th}$ mice.

	Wild-type		*β-thalassemia*	
	2 months	*8 months*	*2 months*	*8 months*
Hct (%)	44.7±1.2	46±1.4	31.3±1.2°	30.6±1.2°
Hb (g/dL)	15.1±0.2	14.9±0.1	9.5±0.6°	9.3±0.4°
MCV (fL)	51.8±1.3	51.5±0.7	38.9±1.6°	39.2±1.9°
MCHC (g/dL)	13.9±0.5	13.8±0.2	9.3±0.4°	9.4±0.4°
RDW (%)	12.2±0.3	12.2±0.2	32.9±1.8°	32.5±1.3°
HDW (g/dL)	1.7±0.2	2.0± 0.1	4.3±0.3°	4.3±0.2°
Retics (%)	2.2±0.9	2.5±0.8	30.1±0.8°	22.5±2.4°
MCVr (fL)	53.6±0.7	54±1.4	44.4±1.7°	45.0±1.6°
CHr (pg)	14.1±0.2	14.1±0.2	10.3±0.2°	10.2±0.4°
HDWr (%)	1.6±0.1	1.7±0.1	3.9±0.19°	4.0± 0.1°
Hepc1/Hepc1 +Hepc2 %	63.4±3.28	N.D.	52.71±9.0	N.D.
IL-6 pg/mL	220±243	120±66	109±125	29.4±26.8*
IL-1β pg/mL	238±279	65.0±42	104±129	50.1±23.1
IL-10 pg/mL	18.6±17.5	26.7±10	10.8±9.0	8.8±4.5**
GM-CSF pg/mL	53.9±39.2	42.3±39	17.3±12.7	26.9±39.9

p<0.05 and °p<0.01 vs wild-type controls of comparable age. N.D.: not determined.

Table 2. Hematologic data, hepcidin 1/hepcidin 2 ratios and serum cytokine levels in *Hbb*$^{th-3/+}$ mice.

	Wild-type		*β-thalassemia*	
	4 months	*10 months*	*4 months*	*10 months*
Hct (%)	48.8±0.9	48.7±0.8	28.7±0.7**	27.9±0.9**^^
Hb (g/dL)	15.5±0.4	15.3±0.4	8.3±0.5**	7.5±0.4**°^^
MCV (fL)	51.9±1.4	50.6±0.9	39.9±2.1**	36.9±0.1**°
MCHC (g/dL)	13.9±0.2	13.6±0.2	9.8±0.3**	9.1 ± 0.1**
RDW (%)	12.2±0.2	12.5±0.7	33.9±0.7**	35.1±0.8**^^
HDW (g/dL)	1.7±0.2	1.7±0.1	4.3±0.1**	4.7±0.1**^
Retics (%)	2.8±0.7	2.8±0.2	18.6±5.2**	23.4±5.1**
MCVr (fL)	54.7±1.2	53.5±0.8	44.2±1.9**	44±1.4**
CHr (pg)	14.5±0.2	14.2±0.2	10.3±0.2**	9.9±0.2**
HDWr (%)	1.7±0.1	1.7±0.05	3.9±0.07**	3.8±0.3**
Hepc1/Hepc1 +Hepc2 %	82.2±11.2	72.75± 7.8	91.0±8.44	96.75±4.72
IL-6 pg/mL	121±76.8	126±53.4	193±186	293±404
IL-1β pg/mL	42.7±39.1	41.1±33.1	111±146	75.0±81.4
IL-10 pg/mL	23.0±7.0	23.4±8.5	27.0±14.5	57.0±63.4
GM-CSF pg/mL	19.7±3.9	22.2±1.2	27.7±18.5	48.5±72.7

**p<0.05 and **p< 0.01 vs controls of comparable age; °p<0.05 vs 4-month-old thalassemic mice; ^p<0.05 and ^^ p<0.01 vs 8-month old Hbb$^{th/th}$ mice.*

observed, prevalently in macrophages (*not shown*). Perls' staining of 4- and 10-month old *Hbb*$^{th-3/+}$ mice is shown in Figure 1, in comparison with age-matched wt animals. Significant iron accumulation is evident in thalassemic animals especially at 10 months of age. Liver structure was normal and fibrosis was not observed.

Liver expression of iron genes

In *Hbb*$^{th/th}$ mice mean hepatic hepcidin RNA levels, expressed in arbitrary units (U), were similar to those of *wt* mice (Figure 2A) at all ages, but with striking individual variations both in thalassemic and in *wt* animals. Mean hepcidin RNA levels of 4-month old *Hbb*$^{th-3/+}$ mice were greater than those of *wt* animals of the corresponding age ($p<0.05$), whereas levels of 10-month old *wt* and *Hbb*$^{th-3/+}$ mice were similar (Figure 2B). Considering liver iron accumulation (Figure 1), hepcidin levels appeared inappropriate to regulate iron homeostasis in the oldest thalassemic animals. When we measured the products of hepcidin 1 and 2 genes we found that some amounts of hepcidin 2, which is unrelated to iron metabolism,[29] were present in *Hbb*$^{th/th}$ mice (Table 1). *Hbb*$^{th-3/+}$ mice had a higher proportion of hepcidin 1 (Table 2). Differences between thalassemic and *wt* ani-

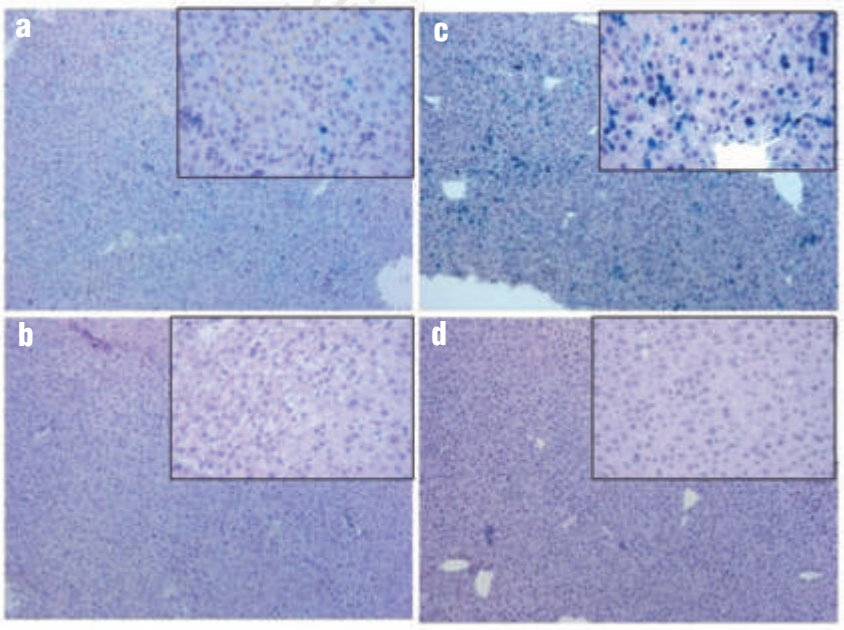

Figure 1. Perls' staining of liver in *Hbb*$^{th-3/+}$ and *wt* mice of different ages at 10× magnification. The insets are higher power views (40×). A, at a 4-month old *Hbb*$^{th-3/+}$ mouse. B, a comparable age *wt* mouse. C, a 10-month old *Hbb*$^{th-3/+}$ mouse. D, a *wt* mouse of the same age.

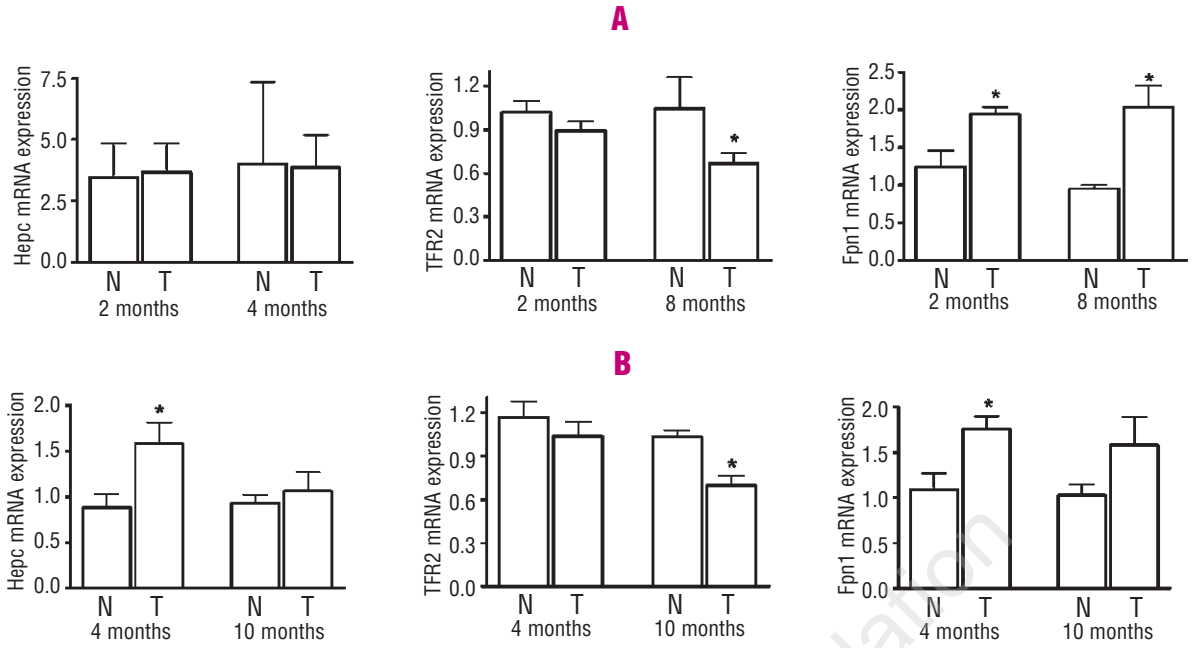

Figure 2. A. Liver RNA expression of hepcidin (Hepc), transferrin receptor 2 (Tfr2) and ferroportin (Fpn) genes in *wt* (N) and *Hbb*$^{th/th}$ (T) animals as measured by real-time PCR. The age of the animals is indicated. Mean and standard deviation values are shown in comparison with β-glucuronidase as the control gene. At least four animals were examined in each group. The asterisk indicates a statistically significant difference (*=p<0.05). **B.** Liver RNA expression of hepidin (Hepc), transferrin receptor 2 (Tfr2) and ferroportin (Fpn) genes in *wt* (N) and *Hbb*$^{th-3/+}$ (T) animals as measured by real-time PCR. The age in months is indicated. Bars indicate mean and standard deviations in comparison with β-glucuronidase as the control gene. At least four animals were examined in each group. The asterisk indicates a statistically significant difference (*=p<0.05).

mals of the same strain were not significant. Tfr2 mRNA levels were significantly lower in the oldest thalassemic animals, than in the *wt* animals in both strains (p<0.05, Figure 2), in parallel with iron loading. This, in theory, suggests a relationship with either the iron loading, the relative hepcidin insufficiency or the degree of anemia. The iron exporter Fpn levels were significantly increased in *Hbb*$^{th/th}$ at 2 and 8 months of age and in 4-month old *Hbb*$^{th-3/+}$ mice as compared with *wt* mice (p<0.05, Figure 2). A trend to an increased level of Fpn was observed also in 10-month old *Hbb*$^{th-3/+}$ mice, although the difference from the level in *wt* mice did not reach statistical significance (p=0.07). Differences of Hfe and Hjv mRNA levels in the different genotypes/strains were not statistically significant (*not shown*). No significant correlation was found between hepcidin levels and Hb, Fpn or IL-6 (*see below*) levels.

Serum cytokine levels

IL-6 and IL-1β levels tended to be higher in *Hbb*$^{th-3/+}$ mice than in *wt* mice, although the differences were not statistically significant, likely because of large individual variability (Table 2). No significant changes were evident in IL-10 or GM-CSF levels in *Hbb*$^{th-3/+}$ mice, as compared to control group. In *Hbb*$^{th/th}$ mice, IL-6 and IL-10 levels were lower than those in the *wt* controls, with this difference being statistically significant in the 8-month old group (Table 1).

Discussion

Intestinal iron absorption in iron-loading anemias is driven by erythropoiesis and is especially high in β-thalassemia in order to meet the huge iron requirements of the abnormally expanded erythron. The most important erythroid expansion is documented in thalassemia intermedia patients, who lack the inhibitory effect of blood transfusions on the expansion of erythropoiesis.

The mice models we studied simulate different thalassemic conditions: heterozygotes for deletion of both *b1* and *b2* loci (*Hbb*$^{th-3/+}$) have severe anemia, strikingly reduced MCV and MCH[14] (Table 2) and splenic enlargment. They develop spontaneous and progressive iron overload In our experience liver iron deposition increased from 4 to 10 months of age (Figure 1). While *Hbb*$^{th-3/+}$ mice are a model of severe β^0-thalassemia intermedia,[14] *Hbbth/th* mice have less severe anemia[21] (Table 1) and are considered a model of mild β-thalassemia intermedia, since their unbalanced globin synthesis is partially corrected by the presence of β-minor globin synthesis.[11] There were striking individual variations of hepcidin levels in both *Hbb*$^{th/th}$ mice and in controls. It seems that the possibility of increasing hepcidin synthesis persists at 4 months of age in *Hbb*$^{th-3/+}$ mice, faced with a modest iron overload, but is lost in older animals, which show inappropriate hepcidin production faced

with excess iron. The latter have hepcidin levels which appear normal, but are inappropriate to their hepatocyte iron stores (Figure 1B). Anemia and microcytosis, which are more severe in the oldest *Hbbth-3/+* mice, could play a role in reducing the ability to increase hepcidin synthesis over basal levels. The inappropriate hepcidin levels in β-thalassemia resemble the low hepcidin levels found in genetic hemochromatosis,[5] underlining that the same mechanisms leading to increased iron absorption operate in both primary and secondary iron overload.

To explore whether the striking individual variations of hepcidin mRNA levels were related to abnormal activation of inflammatory cytokines, we measured serum levels of IL-6, IL-1β, IL-10, and GM-CSF in the different mouse strains (Tables 1-2). The great individual variability of cytokine levels observed in both *wt* and affected animals makes it difficult to interpret the role of inflammatory cytokines in the modulation of hepcidin gene expression *in vivo*. Cytokine levels were not increased, indeed they were even decreased in the $Hbb^{th/th}$ strain, excluding their role. In contrast, IL-6 and IL-1β levels tended to be higher in $Hbb^{th\text{-}3/+}$ mice than in *wt* animals, which may suggest an inflammatory state in this severe β-thalassemic strain. However, no significant correlation was found between IL-6, IL-10 or IL-1β and hepcidin levels. There are several explanations for this. The hepcidin kinetic in response to inflammatory stress may be time-delayed, as previously observed in human models.[22] Alternatively, the inflammatory stimulus might play a role as an acute trigger of hepcidin response, but be less relevant in conditions of chronic inflammation, such as severe β-thalassemia.[23] Indeed in a study of patients with anemia of chronic diseases no correlation was found between hepcidin levels and serum cytokine levels .[24]

Tfr2 RNA levels were low in the oldest animals of both the $Hbb^{th/th}$ and $Hbb^{th\text{-}3/+}$ strains. Significant reductions of Tfr2 RNA were previously observed after parenteral iron dextran administration in both C57BL/6 and DBA/2J non-anemic mice,[17] concomitant with strikingly increased hepcidin levels. Thus we conclude that the reduction of Tfr2 mRNA is not secondary to either anemia or hepcidin levels, but is likely related to the increased macrophage iron turnover present in both conditions.[17]

Fpn was significantly higher in $Hbb^{th/th}$ and in younger $Hbb^{th\text{-}3/+}$ mice than in the *wt* controls, as a result of the increased iron turnover related to the ineffective erythropoiesis/hemolytic condition. Some increase in Fpn levels was observed in the 10 month-old $Hbbth^{\text{-}3/+}$ animals, although the difference from levels in *wt* animals was not statistically significant. Increased Fpn transcription, as well as iron-induced post-transcriptional regulation, which affects Fpn translation, indicate a complex ferroportin regulation, since the protein is also regulated by degradation in the presence of high hepcidin levels.

Hfe RNA levels were unchanged in $Hbb^{th\text{-}3/+}$ mice and modestly reduced in the $Hbb^{th/th}$ model. Hjv mRNA levels were similar at all ages. According to data in the literature, Hjv RNA levels do not change in different experimental conditions,[17,25] whereas iron status influences variations of Hjv protein.[26]

Our results indicate that the kinetics of iron gene expression differ according to the age of the animal and the entity of its iron stores, such that the profile is dynamic over time. Although anemia-increased hepcidin mRNA levels might be a compensatory response to iron loading in younger animals, this becomes ineffective with time. Among the studied models, severe iron overload and the most defective iron regulation were observed in the oldest $Hbb^{th\text{-}3/+}$ animals. This recapitulates the pathogenesis of iron overload, which in thalassemia intermedia is time-dependent and observed only in adult patients.

All Authors meet the criteria for being contributing Authors. LDF and CC outlined the study and wrote the paper. FA was responsible of animal manipulation and haematological data. MEM performed cytokine dosage. AR and FD were responsible for liver histology, molecular studies (RNA extraction and retrotranscription) and statistical analysis. SC performed real-time PCR. All Authors were involved in discussing and interpreting the data; all revised the manuscript and approved the final version.

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