

Hepcidin Levels and Iron Status in β -Thalassemia Major Patients with Hepatitis C Virus Infection

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β -thalassemia is an inherited anemia in which synthesis of the hemoglobin β -chain is decreased. Clinical features of β -thalassemia include variably severe anemia and iron overload due to increased intestinal iron absorption, which may result in damage to vital organs. The hepatic peptide, hepcidin is a key regulator of iron metabolism in mammals. The present study aimed to determine the relationship between hepcidin expression and iron status in β -thalassemia patients with hepatitis C virus infection. The study included 50 patients diagnosed as β -thalassemia major (21 of them were HCV infected and 29 were HCV negative), in addition, 20 healthy subjects were enrolled in the study. The hepatic iron and hepcidin mRNA concentration in liver biopsy samples were measured, as well as serum ferritin, serum iron, hemoglobin and levels and serum hepcidin. Result showed remarkable decrease of serum and liver hepcidin mRNA expression in thalassemic patients as compared to controls, and showed a positive correlation with hemoglobin concentration, but negatively correlated with serum ferritin level and hepatic iron index (HII). In HCV infected patients, serum and liver hepcidin mRNA were markedly depressed in HCV positive β -thalassemia cases, and positively correlated serum albumin and prothrombin concentrations, but inversely correlated with HII and fibrosis score. In HCV positive β -thalassemia major patients, the hepcidin mRNA level was positively correlated with the synthetic function of the liver (namely serum albumin and prothrombin concentration) and with serum hepcidin level. While, both serum and hepcidin mRNA level was inversely correlated with HII and fibrosis score in these patients. These results suggest a possible role of hepcidin expression in iron overload in β -thalassemia major, consequent disease progression and development of liver fibrosis.

Thalassemias are a heterogeneous group of inherited anemias resulting from reduced or absent synthesis of α - or β -globin chains of hemoglobin. Patients with β -thalassemia major have a reduction or a lack of synthesis of the β -chain of hemoglobin (Gu & Zeng, 2002), the increased a chain form unstable aggregates that affect erythrocyte membrane plasticity. This leads to premature red blood cell (RBC) hemolysis in the bone marrow and spleen, resulting in severe anemia, increased erythrocyte turnover, and ineffective erythropoiesis (Hoffbrand, 2001). To treat the anemia, patients need regular blood transfusions that, in addition to their increased intestinal iron absorption, result in

iron accumulation and severe damage and fibrosis of vital organs, particularly of the heart, liver, and endocrine organs (Rachmilewitz & Schrier, 2001).

Recent studies indicate that it is the liver produced peptide hormone hepcidin that plays this important role in the regulation of systemic iron homeostasis (Ganz & Nemeth, 2006; Swinkels *et al.*, 2006). Hepcidin inhibits duodenal iron absorption, iron release from the reticuloendothelial system macrophages, and iron transport across the placenta (Ganz, 2003), by binding directly to the iron exporter ferroportin, leading to its internalization and degradation (Nemeth *et al.*, 2004a). Hepcidin-deficient mice develop

iron overload, while excess hepcidin results in iron deficiency anemia in mice (Nicolas *et al.*, 2002a) and humans (Weinstein *et al.*, 2002). Hepcidin found in human plasma and urine and synthesized in the liver (Park *et al.*, 2001). Studies in mice, humans, and cell cultures demonstrated that hepcidin mRNA levels were regulated by iron stores (Pigeon *et al.*, 2001), inflammation (Nemeth *et al.*, 2004b), anemia (Nemeth *et al.*, 2003) and hypoxia (Nicolas *et al.*, 2002b).

There are three putative upstream pathways (i.e., iron store related, erythropoietic activity, and inflammation related) as well as a mandatory signaling pathway, which are in a way presumed to be interconnected (Babitt *et al.*, 2006). These pathways are all found to interact with liver cells to control the production of sufficient hepcidin for a proper maintenance of iron homeostasis (Pak *et al.*, 2006).

Adamsky *et al.*, (2004) have shown previously that hepcidin liver mRNA expression is decreased in the liver of β -thalassemia intermedia mouse model and a remarkable decrease in hepcidin liver expression in the β -thalassemia major mouse model, as well as changes in the expression of other iron metabolism-related genes (Weizer-Stern *et al.*, 2006).

In this study, we aimed to determine the levels of serum and hepatic hepcidin to find its relationship with iron status in patients with β -thalassemia major infected by hepatitis C virus.

Patients and Methods

The study included 50 patients diagnosed as β -thalassemia major (34 males and 16 females, with age ranged from 10-29 years), attending the Hematology & Oncology Unit of Internal medicine-Ain Shams Faculty of Medicine, Tropical Medicine-Al Azhar Faculty of Medicine for Girls, and Pediatric Hepatology of National Liver Institute-Menoufyia University and Pediatric Department of Ahmed Maher Teaching Hospital.

Exclusion criteria for both groups: Patients with known genetic hemochromatosis as those are expected to exhibit abnormal hepcidin regulation, anaemia of chronic disorders or any haemolytic anaemias other than thalassemia previous, history of any malignancy, renal impairment, autoimmune diseases or any genetic diseases as well as patients with chronic liver disease other than HCV related.

In addition, 15 healthy subjects were selected from potential liver donors with histological normal liver (15 males, with age ranged from 21-32 years). As well as 5 apparently healthy females with age ranged from 12-17 years, were selected and added to control group to be matched with patient's age and sex. All 20 control individuals had normal values of serum alanine aminotransferase (ALT) and were seronegative for antibodies to HCV, and all HCV- positive and HCV-negative cases included in this study were seronegative for hepatitis B surface markers (HBs Ag, HBe Ag and HBc Ab).

The patients and controls were subjected to the followings:

- 1- Full history includes: family history, history of blood transfusion, iron chelation history and history of complications (hepatic, endocrinal, renal and cardiac).
- 2- Clinical examination includes: general examination, examination of long bone and skull, cardiac and abdominal examination stressing on splenomegaly, hepatomegaly or splenectomy).
- 3- Routine laboratory tests including: Serum levels of AST and ALT, albumin, total and direct bilirubin, serum iron, TIBC were measured using Integra-400 (Roche-Germany). Serum ferritin was measured by an automated chemiluminescences using ACS-180SE (Chiron, Diagnostic-Germany). Transferrin saturation was calculated and expressed as a percentage (serum iron/TIBC \times 100%). Complete blood cell counts were measured by Sysmix K-21 automatic cell counter and reticulocyte counts was done manually. Hemoglobin electrophoreses was done using Helena system. HCV antibodies was assayed by EIA (COBAS-Amplicore, Germany). HCV-RNA levels were analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) using a commercial kit (Roche Diagnostic, Branchburg, NJ) according to the manufacturer's instructions.
- 4- Serum hepcidin was measured by the DRG hepcidin ELISA kit supplied by DRG diagnostic- GmbH, Germany. It is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The microtiter wells are coated with a monoclonal antibody directed towards the antigenic site

of the bioactive hepcidin 25 molecule. Endogenous hepcidin of a patient sample competes with the added hepcidin-biotin conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. Incubation with a streptavidin-peroxidase enzyme complex and a second wash step follows. The addition of substrate solution results in a colour development which is stopped after a short incubation. The reaction was terminated by the addition of a sulphuric acid solution and the colour change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of hepcidin in the samples was determined by comparing the optical density of the samples to the standard curve and put by ng/ml. The intensity of colour developed is reverse proportional to the concentration of hepcidin in the patient sample (Park *et al.*, 2001).

5- Liver biopsy and histological examination of the liver: Liver biopsies under ultrasonic guidance were performed for 37 patients and 15 control cases. Approximately 2 mm of the sample was snap-frozen in liquid nitrogen immediately and stored at -80°C until RNA extraction for measurement of hepcidin messenger ribonucleic acid (mRNA). While, most of the specimens were fixed in 10% saline-buffered formalin and processed for routine diagnostic histopathological examination. Paraffin embedded liver biopsies were stained with Hematoxylin-Eosin, Mason-Trichrome, Orcein and Perls' stains. Histological grading and staging were performed using a modified Knodell scoring system by a pathologist blinded to the clinical data (Ishak *et al.*, 1995). Biopsies were scored for hepatic fibrosis stage from 0-6 according to modified Knodell score, as follows: Fibrosis scale from 0-6, 0=no fibrosis, 1-2=portal fibrosis, 3-4=bridging fibrosis and 5-6= cirrhosis (Knodell *et al.*, 1981).

6- The liver iron concentration (LIC) was evaluated, briefly, the liver tissue fragment (approximately 15 mm of biopsy) was weighed and dried at 85°C for 2 hours in decontaminated quartz vessel and then digested in concentrated nitric oxide (2 ml). The resulting solution was then diluted to 5 ml with deionized water. Quantitative analysis was performed by inductively coupled plasma-atomic emission spectrometry to determine hepatic iron content (Barry & Sherlock, 1971). Hepatic iron index (HII) was calculated by dividing LIC in $\mu\text{mol/gm}$ dry weight by the patient's age. Any value higher than 1.9 for hepatic iron index was considered positive for hemochromatosis (Summers *et al.*, 1990).

7- Real-time PCR for quantification of hepcidin mRNA: For real-time PCR, liver biopsy was stored in liquid nitrogen immediately and kept at -80°C until RNA extraction. Total RNA from each sample was isolated using a Total RNA Isolation Kit (Roche -Mannheim,

Germany). Reverse transcription reactions were performed using a Rever Tra Ace alpha-First Strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). Briefly, 1 μg of total RNA, 1 μL of oligo dT-primer, and 1 μL of dNTPs were incubated at 65°C for 5 min, then 10 μL of a cDNA synthesis mixture was added and the mixture was incubated at 50°C for 50 min. The reaction was terminated by adding 1 μL of RNaseH and incubating the mixture at 37°C for 20 min.

Real-time quantification of hepcidin mRNA transcripts was performed using Roche LightCycler-2.0 TM (Mannheim, Germany). The PCR reaction was carried out in a final volume of 2 μL cDNA, 12.5 μL 2 \times SYBR Green (Applied Biosystems), 0.5 μL of 25 nM sense and antisense primers, and H₂O up to 25 μL . The PCR conditions consisted of 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds.

The sequences of the primers were as follows (Iso *et al.*, 2005):

GAPDH: sense-primer 5'-CCACCCAGAAGACTGTGGAT-3'
anti-sense 5'-TTCAGCTCAGGGATGACCTT-3'

Hepcidin: sense-primer 5'-CACAAACAGACGGGACAACCTT-3'
anti-sense 5'-GCAGCAGAAAATGCAGATG-3'

The level of hepcidin expression was calculated using Sequence Detector software. A standard curve was established with cDNA derived from an arbitrarily chosen liver RNA sample and used as a calibrator in each assay. Hepcidin mRNA was recorded by copies relative to the standard measurements.

Statistical Analysis

Statistical package for SPSS (statistical package for social science) program version 13 for windows and Epi info computer program was used for data analysis. Quantitative variables were summarized using Mean \pm SD. Student's t-test was done to compare two normally distributed variables and Mann Whitney test was done to compare two of non normally distributed variables. Correlation coefficients (r) were calculated using the Pearson's correlation analysis. *p* value was significant at <0.05 level.

Results

Results of the study are demonstrated in tables from 1 to 5, and figures from 1-3. Table (1) showed a demographic and clinical features of patients and control groups.

Table 1. Demographic and Clinical Features of β -Thalassemia and Control Groups.

Studied variables	β -Thalassemia major group (N=50)	Control group (N=20)
	Range	Range
Age (year)	10-29	12-32
<i>Hb electrophoresis (%)</i> :		
Hb A	12.8-52.3	95.7-98.2
Hb F	42.5-85.7	0.3-0.6
Hb A2	1.5-5.2	1.2-4.0
<i>Hb (g/dl)</i>	4.8-9.10	11.7-15.1
<i>Retics (%)</i>	2.3-6.5	0.6-2.5
<i>Ht (%)</i>	14.9-29	36-45
<i>Trans. Sat. (%)</i>	32-46	15-22
<i>Iron (μg/dl)</i>	203-312	59-128
<i>Ferritin (μg/dl)</i>	280-2640	98-215
<i>HII (μmol/gm /age)</i>	0.42-1.64	0.27-0.61
<i>Hepcidin mRNA (copies/ml)</i>	1780-4580	10800-19620
<i>Serum Hepcidin (ng/ml)</i>	15-154	176-298
	Number (%)	Number (%)
<i>Gender :</i>		
<i>Male</i>	34 (68%)	15 (75%)
<i>Female</i>	16 (32%)	5 (25%)
<i>Splenomegaly</i>	33 (66%)	-----
<i>Hepatomegaly</i>	37 (74%)	-----
<i>Splenoectomy</i>	9 (18%)	-----

When comparing between β -thalassemia major patients with the control group, there was a significant decrease in hemoglobin concentration, hematocrite value, while, markers of iron indices (serum iron, serum ferritin, transferrin saturation and HII were

significantly increased in β -thalassemia major patients compared to control group. Also, a significant decrease in both hepcidin mRNA expression (Figure 1) and serum hepcidin levels was found in β -thalassemia major patients compared to control group (Table 2).

Table 2. Comparison between of β -Thalassemia major patients and controls

Studied variables	β -Thalassemia major group (N=50) Mean \pm SD	Healthy Control group (N=20) Mean \pm SD	P-Value
Age (years)	13.9 \pm 6.64	16.9 \pm 8.56	NS
Hb (g/dl)	6.81 \pm 1.18	13.5 \pm 0.86	< 0.01
Retics (%)	3.89 \pm 1.09	1.44 \pm 0.46	< 0.05
Ht (%)	21.47 \pm 3.53	42 \pm 2.51	< 0.01
Trans. Sat. (%)	36.62 \pm 14.31	18.2 \pm 3.87	< 0.01
Iron (μ g/dl)	249.9 \pm 33.4	90.5 \pm 20.2	< 0.01
Ferritin (μ g/dl)	1252 \pm 623	145 \pm 36	< 0.01
HII (μ mol/gm /age)	0.88 \pm 0.38	0.43 \pm 0.1	< 0.01
Hepcidin mRNA (copies/ml)	2485 \pm 632	15046 \pm 2123	< 0.01
Serum Hepcidin (ng/ml)	76.46 \pm 44.57	226.05 \pm 30.44	< 0.01

P>0.05 is non significant, NS= not significant.

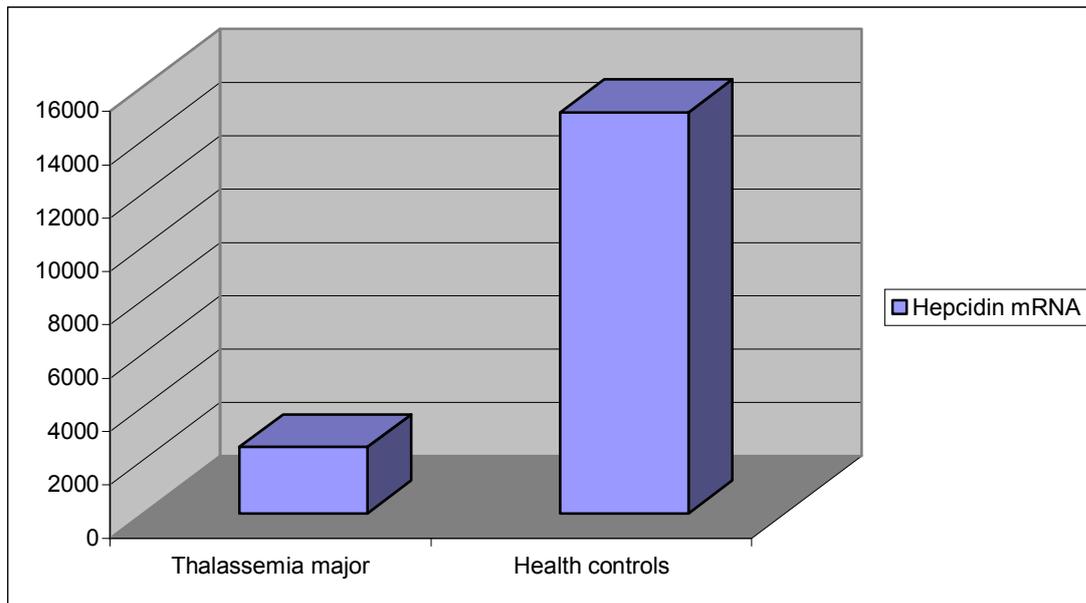


Figure 1. Shows the mean value of hepcidin mRNA levels in thalassemia major patients versus healthy controls

The comparison between HCV positive β -thalassemia major and HCV negative β -thalassemia major patients showed a significant increase in the AST, ALT, reticulocyte counts, serum ferritin and HII in HCV positive thalassemia major compared to HCV negative cases. While, hemoglobin concentration, hematocrite value and prothrombin concentration were significantly decreased, and no significant difference was found as regard transferrin saturation and serum iron. Furthermore, the levels of both hepcidin mRNA (Figure 2) and serum hepcidin were significantly lower in HCV positive thalassemia major compared to HCV negative cases (Table 3).

Table (4) showed a positive correlation between hepcidin mRNA level and both of

hemoglobin concentration, hematocrite value and serum, hepcidin level in β -thalassemia major patients. While, an inverse correlation was found between hepcidin mRNA level and each of reticulocyte counts, serum ferritin level and HII. In addition, hepcidin mRNA was not correlated with transferrin saturation or serum iron. Also, serum, hepcidin level was positively correlated with hemoglobin concentration and hematocrite value in the same patient group and an inverse correlation was found between serum hepcidin level and reticulocyte counts, serum ferritin level and HII. However, serum hepcidin mRNA was not correlated with neither transferrin saturation or serum iron.

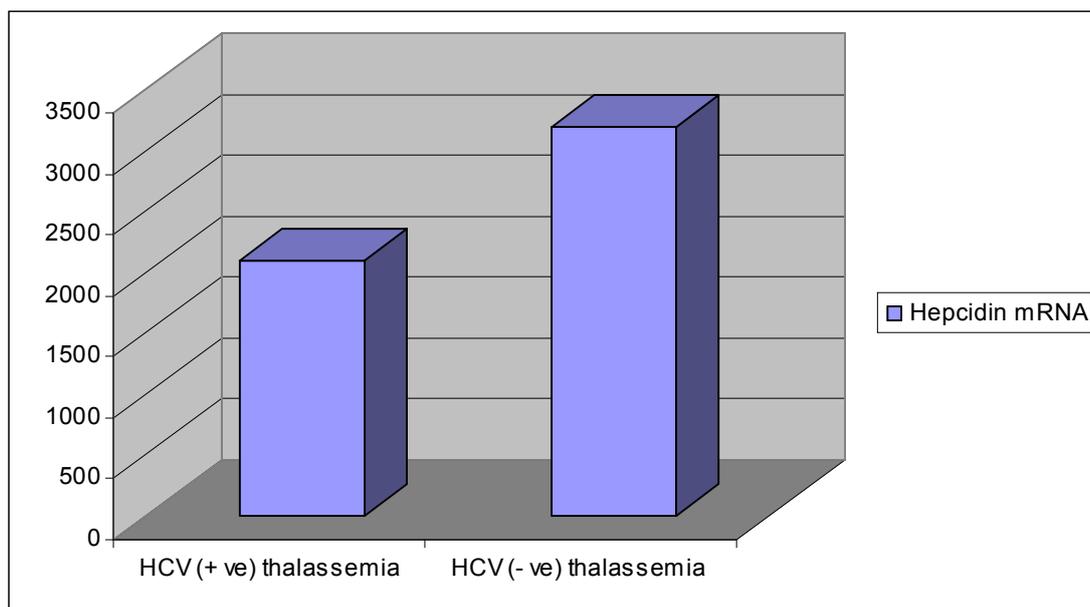


Figure 2. Shows the mean value of hepcidin mRNA levels in HCV positive β -thalassemia versus HCV negative β -thalassemia major patients

Table 3. Comparison between of HCV positive and HCV negative Thalassemia major patients

Studied variables	β -Thalassemia major group (n=50)		P-Value
	HCV Positive (N=21) Mean \pm SD	HCV Negative (N=29) Mean \pm SD	
AST(U/L)	74.7 \pm 26.6	41.1 \pm 14.8	< 0.01
ALT(U/L)	86.3 \pm 29.8	62.4 \pm 47.1	< 0.01
ALB (g/dl)	3.51 \pm 0.28	4.04 \pm 0.29	< 0.05
P.C (%)	70.5 \pm 6.31	88.9 \pm 1.92	< 0.01
Hb (g/dl)	6.03 \pm 0.68	7.91 \pm 0.77	< 0.05
Retics (%)	4.34 \pm 1.22	3.3 \pm 0.43	< 0.05
Ht (%)	19.23 \pm 2.28	24.6 \pm 2.34	< 0.01
Trans. Sat. (%)	48.31 \pm 9.23	37.51 \pm 7.32	NS
Iron (μ g/dl)	246.8 \pm 33.1	254.2 \pm 35.2	NS
Ferritin (μ g/dl)	1414 \pm 855	543 \pm 425	< 0.01
HII (μ mol/gm /age)	1.11 \pm 0.32	0.55 \pm 0.12	< 0.01
Hepcidin Mrna (copies/ml)	2100 \pm 175	3185 \pm 703	< 0.01
Serum Hepcidin (ng/ml)	57.6 \pm 29.3	134.7 \pm 26.2	< 0.01

P>0.05 is non significant, NS= not significant.

Table 4. Pearson's Correlation between Hepcidin mRNA, serum hepcidin and all studied variables in β -thalassemia major patients

Studied Variables	Hepcidin mRNA		Serum Hepcidin	
	r	P-value	r	P-value
Hb	0.86	< 0.01	0.97	< 0.01
Retics	- 0.45	< 0.05	- 0.53	< 0.05
Ht	0.76	< 0.01	0.94	< 0.01
Transferrin Sat.	- 0.04	NS	- 0.16	NS
Iron	0.08	NS	0.14	NS
Ferritin	- 0.61	< 0.01	- 0.72	< 0.01
HII	- 0.74	< 0.01	- 0.76	< 0.01
Serum Hepcidin	0.89	< 0.01	-----	-----

P>0.05 is non significant, NS= not significant.

Furthermore, in HCV positive β -thalassemia major patients, the hepcidin mRNA level was positively correlated with the synthetic function of the liver (namely serum albumin and prothrombin concentration) and with serum hepcidin level (Figure 3). While, hepcidin mRNA level was inversely

correlated with HII and fibrosis score in these patients. No correlation was found as regard transaminases levels (AST or ALT). Also, serum hepcidin level was inversely correlated with both HII and liver fibrosis score (Table 5).

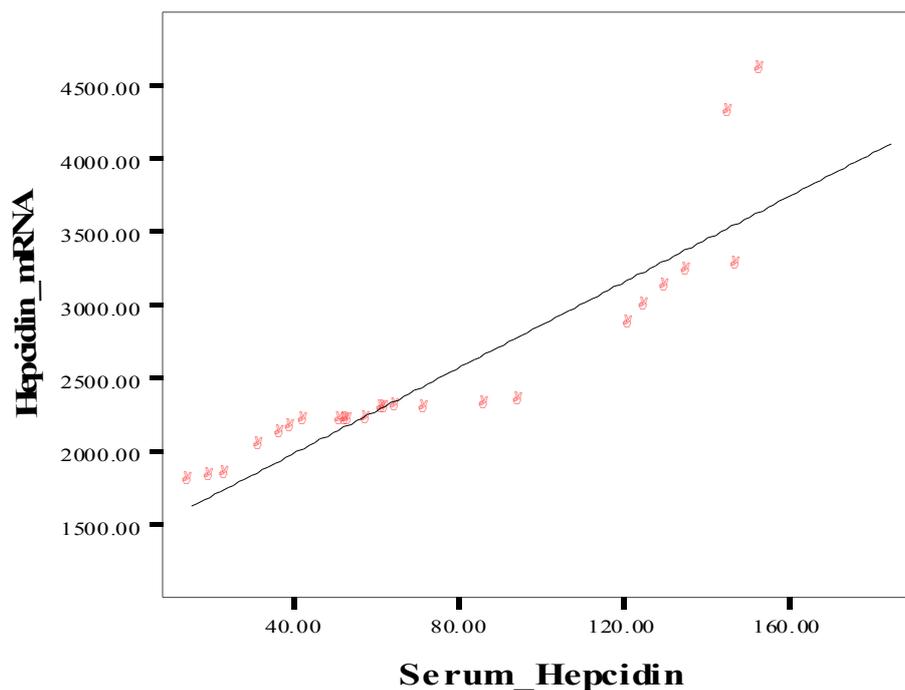


Figure 3. Shows a positive correlation between hepcidin mRNA levels and serum hepcidin in β -thalassemia major patients.

Table 5. Pearson's Correlation between both hepcidin mRNA, serum hepcidin and all studied variables in HCV positive β -thalassemia major patients

Studied variables	Hepcidin mRNA		Serum Hepcidin	
	r	P-value	r	P-value
AST	- 0.3	NS	- 0.03	NS
ALT	0.002	NS	0.28	NS
Albumin	0.67	< 0.01	0.30	NS
P.C	0.53	< 0.05	0.24	NS
HII	- 0.69	< 0.01	- 0.89	< 0.01
Serum Hepcidin	0.50	< 0.05	-----	-----
Liver fibrosis	-0.84	< 0.01	- 0.73	< 0.01

$P > 0.05$ is non significant, NS= not significant.

Discussion

Patients with β -thalassemia, like those with genetic hemochromatosis, develop iron overload due to increased iron absorption, and their iron burden is further exacerbated by transfusion therapy (Gu & Zeng, 2002). Hepcidin, a hepatic hormone, regulates systemic iron homeostasis by inhibiting the absorption of iron from the diet and the recycling of iron by macrophages (Ganz, 2003). In turn, hepcidin release is increased by iron loading and inhibited by erythropoietic activity. Hepcidin deficiency is the cause of iron overload in most forms of hereditary hemochromatosis (Origa *et al.*, 2007).

We aimed to determine hepcidin's role in the pathogenesis of iron overload in β -thalassemia patients with and without hepatitis C virus infection. Our results showed a remarkable decrease in both serum and hepcidin liver expression in the β -thalassemia major patients, as well as changes in the expression of other iron indices. These results suggest that hepcidin down-regulation in thalassemia might be influenced by the severity of the disease.

These results are in agreed with Brissot *et al.*, (2008), who reported that hepcidin deficiency in thalassemia major due to ineffective erythropoiesis leads to growth differentiation factor 15 (GDF 15) overexpression by erythroblast, which inhibit hepcidin expression. This could explain why iron overload in hepatocyte can develop in thalassemia in absence of transfusion, and why hepcidin expression is low despite transfusional iron excess. Tano *et al.*, (2007), reported that serum from thalassemia patients suppressed hepcidin mRNA expression in human hepatocyte, and GDF 15 overexpression arising from an expanded erythroid cell contributes to iron overload in thalassemia by inhibiting hepcidin expression.

In the same manner, Kemna *et al.*, (2008), reported that erythropoietin that stimulates erythropoietic activity has been shown to down-regulate liver hepcidin expression. Toledano *et al.*, (2009) mentioned that several disease such as hereditary hemochromatosis and β -thalassemia major are characterized by low hepcidin expression in the liver. The low hepatic hepcidin in these patients is probably responsible for the intestinal absorption of iron.

Thalassemia represents an unusual situation where anemia, which is expected to decrease hepcidin expression, co-exists with iron overload, which usually increases hepcidin expression. In the β -thalassemia mouse models, mice homozygous for this deletion die late in gestation while heterozygotes mice are viable and exhibit mild anemia, abnormal red cell morphology, and splenomegaly and gradually develop spontaneous hepatic iron deposition similar to that found in humans with β -thalassemia intermedia (Rivella *et al.*, 2003).

Adamsky *et al.* (2004) have previously demonstrated decreased hepcidin mRNA expression in the livers of β -thalassemia mice. Weizer-Stern *et al.* (2006) showed that hepcidin mRNA expression in β -thalassemia major mice is down-regulated to a greater extent than in the model of thalassemia intermedia. These findings might suggest that the degree of hepcidin down-regulation points toward the severity of the thalassemia syndrome. Because decreased hepcidin expression results in enhanced intestinal iron absorption and enhanced iron release from the reticulo-endothelial system, we suggest that the decreased liver expression of hepcidin in thalassemia might be induced by a putative "erythropoietic regulator," produced in response to the ineffective erythropoiesis. This may explain the increased absorption of iron in β -thalassemia major.

Transferrin receptor-2 (TfR2) mutations in mouse models result in hemochromatosis phenotype, with decreased hepcidin expression (Kawabata *et al.*, 2005) and failure of hepcidin to up-regulate in response to iron overload (Wallace *et al.*, 2005). Weizer-Stern *et al.* (2006) showed a decreased liver expression of TfR2 in the β -thalassemia mouse models, that might connect TfR2 to the pathway by which anemia affects the liver hepcidin expression and the regulation of iron homeostasis.

Hepcidin mRNA and serum hepcidin levels appeared to be positively correlated, with levels of both parameters increasing in parallel. This result demonstrates that the serum hepcidin assay can be considered a valid reflection of hepatic hepcidin production.

The analysis of the relationship between hepcidin mRNA, serum hepcidin, and other data led us to identify significant correlations, involving iron status, hematologic parameters, and hepatic functional status. Thus, HII appeared to be significantly correlated not only with hepcidin mRNA, as previously reported in untreated hemochromatotic patients (Bridle *et al.*, 2003; De'tivaud *et al.*, 2005) but also with serum hepcidin levels.

As regard a correlation between both serum and hepcidin mRNA expression and other studied parameters, this study demonstrates a relationship between erythroid parameters (Hb and reticulocyte counts) and both serum and hepcidin mRNA expression, supporting the hypothesis of an impact of anemia or hypoxia (or both) on hepcidin mRNA expression. In contrast, Nicolas *et al.* (2002) did not find a significant correlation between erythroid parameters and serum hepcidin concentration, suggesting again additional regulatory mechanisms.

Elevated iron stores, marked by increased iron saturation of transferrin, are predicted to induce hepcidin production in order to decrease iron absorption and demonstrated as

such in healthy volunteers treated with oral iron (Nemeth *et al.*, 2004a). In the present study, the elevated LIC and subsequent HII values in thalassemia major patients were associated with decreased hepcidin levels, this result was in accordance with Kemna *et al.* (2008) findings. The anemia-driven erythropoietic regulation clearly overruled the iron store regulation by decreasing hepcidin production (Pak *et al.*, 2006; Kattamis *et al.*, 2006; Vokurka *et al.*, 2006).

In addition, both hepcidin mRNA and serum hepcidin were negatively correlated with ferritinemia as previously documented (Nemeth *et al.*, 2003; Aoki *et al.*, 2005) these observations document a relationship between hepatic iron and hepcidin levels. However, in our population the two hepcidin parameters did not significantly correlate with either serum iron concentration or with TIBC, De'tivaud *et al.* (2005) reported the same results. Liver iron is frequently elevated in chronic hepatitis C and may contribute to liver injury. The pathophysiology behind this phenomenon may involve hepcidin gene (Aoki *et al.*, 2005). However, the role of hepcidin in the iron loading of patients with hepatitis C is unknown.

Finally, we found that parameters reflecting hepatic function in hepatitis C subgroup were correlated with hepcidin levels. Thus, serum albumin was positively correlated with hepcidin mRNA levels, whereas fibrosis status was negatively correlated with hepcidin mRNA and serum hepcidin levels, this was in agreement with De'tivaud *et al.* (2005) study. Taken together, these results suggest that hepatic function and the effects of disease processes on hepatocytes could modulate iron metabolism. In addition, a relationship between hepcidin mRNA and ferritinemia appears in this group, as previously described (Gehrke *et al.*, 2003; Aoki *et al.*, 2005; De'tivaud *et al.*, 2005). Additionally, our study revealed that, the hepcidin mRNA expression in the liver did

not correlate with aspartate aminotransferase, alanine aminotransferase levels, these findings are in accordance with Aoki *et al.* (2005) results, but on the contrary with us, they found no differences in hepcidin mRNA were found based on the presence of fibrosis.

The decreased hepcidin in β -thalassemia major is significant to our understanding of the mechanisms of iron overload in patients with β -thalassemia. In these patients, increasing iron levels in serum and liver tissue is unable to counteract anemia/ hypoxia-induced down-regulation of the hepcidin gene expression. The decrease in hepcidin expression, despite iron overload, can be considered an inappropriate response that aggravates iron overload and its accompanying pathological conditions.

We concluded that, in β -thalassemia major, the serum and hepatic hepcidin expression were decreased, despite increased iron stores (namely serum ferritin and hepatic iron index), that may aggravate iron overload and its accompanying pathological conditions in these patients. Moreover, the relationship between hepcidin levels and each of synthetic hepatic function and grade of liver fibrosis in thalassemia patients infected with HCV, may suggest a vital role in disease progression. In the future, hepcidin agonists, could be used as a preventive treatment for iron overload in thalassemia patients especially with infected HCV cases to ameliorate disease progression.

References

- Adamsky K, Weizer O, Rachmilewitz E, Breda L, Rivella S, Amariglio N. (2004). Decreased hepcidin mRNA expression in thalassaemic mice. *Br J Haematol*; 124:123–124.
- Aoki CA, Rossaro L, Ramsamooj RR, Brandhagen D, Burritt MF, Bowlus CL (2005). Liver hepcidin mRNA correlates with iron stores, but not inflammation, in patients with chronic hepatitis C. *J Clin Gastroenterol* ; 39:71-74.
- Babitt JL, Huang FW, Wrighting DM, (2006). Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression, *Nat. Genet.* 38:531–9.
- Barry M, Sherlock S. (1971). Measurement of liver-iron concentration in needle biopsy specimens. *Lancet.* 2:100-103.
- Bridle KR, Frazer DM, Wilkins SJ. (2003). Disrupted hepcidin regulation in HFE-associated haemochromatosis and the liver as a regulator of body iron homeostasis. *Lancet.*; 361:669-73.
- Brissot P, Troadec MB, Leroyer P, Ropert, M, Jacquelinet S, Bardou-Jacquet E. (2008). Current approach to hemochromatosis. *Blood Reviews*, 22 (4):195-210.
- De'tivaud L, Nemeth E, Boudjema KB, Turlin B, Leroyer P, Courselaud B, Ganz T, Brissot P, Lore'al O. (2005). Hepcidin levels in humans are correlated with hepatic iron stores, haemoglobin levels, and hepatic function. *Blood.*; 106:746-748.
- Ganz T, Nemeth E. (2006). Regulation of iron acquisition and iron distribution in mammals, *Biochim. Biophys. Acta* 176:690–9.
- Ganz T. (2003). Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood*; 102:783–8.
- Gehrke SG, Kulaksiz H, Herrmann. (2003). Expression of hepcidin in hereditary hemochromatosis: evidence for a regulation in response to serum transferrin saturation and non-transferrin bound iron. *Blood.* 102:371-6.
- Gu X, Zeng Y. (2002). A review of the molecular diagnosis of thalassemia. *Hematology*; 7:203–9.
- Hoffbrand AV. (2001). Diagnosing myocardial iron overload. *Eur Heart J*; 22:2140–41.
- Ishak K, Baptista A, Bianchi L. (1995). Histological grading and staging of chronic hepatitis. *J Hepatol.*; 22:696-9.
- Iso Y, Sawada T, Okada T, Kubota K. (2005). Loss of E-cadherin mRNA and gain of osteopontin mRNA are useful markers for detecting early recurrence of HCV-related hepatocellular carcinoma. *J Surg Oncol*, 92:304-11.
- Kattamis A, Papassotiropou I, Palaiologou D. (2006). The effect of erythropoietic activity and iron burden on hepcidin expression in patients with thalassemia major, *Haematologica* 91:809–12.
- Kawabata H, Fleming RE, Gui D (2005). Expression of hepcidin is down-regulated in TfR2 mutant mice manifesting a phenotype of hereditary hemochromatosis. *Blood*; 105:376–81.

17. Kemna EHJM, Kartikasari ERA, van Tits LJH, Pickkers P, Tjalsma H, Swinkels WD. (2008). Regulation of hepcidin: Insights from biochemical analyses on human serum samples. *Blood Cells, Molecules and Diseases*, 40:339–346.
18. Knodell RG, Ishak KG, Black WC, Chen TS, Wallman J. (1981). Formulation and application of numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology*; 1981:421-431.
19. Nemeth E, Tuttle MS, Rivera S, Gabayan V, Powelson J. (2004a). Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science*, 306:2090-2093.
20. Nemeth E, Rivera S, Gabayan V, Tuttle MS. (2004b). IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest.*;113:1271-1276.
21. Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A Ganz T. (2003). Hepcidin, a putative mediator of anemia of inflammation, is a type II acute phase protein. *Blood*. 2003;101:2461-2463.
22. Nicolas G, Bennoun M, Chauvet C, Porteu A. (2002a). Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. *Proc Natl Acad Sci USA*; 99:4596–4601.
23. Nicolas G, Chauvet C, Bennoun M, Viatte L. (2002b). The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J Clin Invest*. 110:1037-1044.
24. Origa, R. Galanello T., Ganz, T (2007). Liver iron concentrations and urinary hepcidin in β -thalassemia. *Haematologica* 92:583–588.
25. Pak M, Lopez M.A., Gabayan V. (2006). Suppression of hepcidin during anemia requires erythropoietic activity. *Blood*; 108: 3730–3735.
26. Park CH, Valore EV, Waring AJ, Ganz T. (2001). Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem.*; 276:7806-10.
27. Pigeon C, Ilyin G, Courselaud B. (2001). A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem.*;276:7811-19.
28. Rachmilewitz E, Schrier S. (2001). Pathophysiology of beta-thalassemia. In: Steinberg MH, Forget BG, Higgs DR, Nagel RL, editors. *Disorders of hemoglobin: genetics, pathophysiology, and clinical management*. Cambridge, England: Cambridge University Press; 233–51.
29. Rivella S, May C, Chadburn A, Riviere I and Sadelain M. (2003): A novel murine model of Cooley anemia and its rescue by lentiviral-mediated human beta-globin gene transfer. *Blood*; 101:2932–39.
30. Summers KM, Halliday JW, Powell LW. (1990). Identification of homozygous hemochromatosis subjects by measurement of hepatic iron index. *Hepatology*, 21: 20-25.
31. Swinkels DW, Janssen M.C.H., Bergmans J, Marx J.J.M. (2006). Hereditary hemochromatosis: genetic complexity and new diagnostic approaches, *Clin. Chem*. 52:950–68.
32. Tanno T, Bhanu N.V., Oneal P.A. (2007). High levels of GDF 15 in thalassemia suppress expression of iron regulatory protein hepcidin. *Nature Medicine*, 13:1096-1101.
33. Toledano M, kozer E, Goldstein L.H. (2009). Hepcidin in acute iron toxicity. *American Journal of Emergency Medicine*, 27(7):761-764
34. Vokurka M, Krijt J, Šulc K, Necas E (2006). Hepcidin mRNA levels in mouse liver respond to inhibition of erythropoiesis. *Physiol. Res*. 55:667–74.
35. Wallace DF, Summerville L, Lusby PE, Subramaniam VN. (2005). First phenotypic description of transferrin receptor 2 knockout mouse, and the role of hepcidin. *Gut*; 54:980–6.
36. Weinstein DA, Roy CN, Fleming MD, Andrews NC. (2002). Inappropriate expression of hepcidin is associated with iron refractory anemia: implications for the anemia of chronic disease. *Blood*; 100:3776–81.
37. Weizer-Stern O, Adamsky K, Amariglio N, Rachmilewitz E, Breda L, Rivella S, Rechavi G. (2006). mRNA Expression of iron regulatory genes in β -thalassemia intermedia and β -thalassemia major mouse models. *Am J. Hematol.*, 81:479–83.