Role of Hepcidin in Anemia of Chronic Hepatitis C Patients

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Abstract: This study was done to clarify the role of hepcidin in the regulation of iron homeostasis and development of anemia in chronic hepatitis C (CHC) patients targeting the differentiation of the type of anemia. Patients and methods: This study was conducted on 70 CHC patients. Iron profile and soluble transferrin receptor (sTfR) were measured. Transferrin saturation and transferrin receptor ferritin (TfR-F) Index were calculated. Serum prohepcidine hormone and IL6 levels were measured (ELISA). Histopathological examination and immunohistochemical detection of hepcidin were done. According to the iron profile patients were reclassified into iron deficiency anemia (IDA) group, anemia of chronic disease group (ACD) and combined anemia group (COMBI). Results: 64.3% of patients were of the COMBI group, 10% had ACD and 25.7% had IDA. Hepcidin was increased in Child C group (P<0.05). Hepatic expression of hepcidin showed reduced expression in Child A, B and C groups. Hepcidin level was found to be increased in ACD and COMBI group in comparison to control and IDA group. Stepwise logistic regression demonstrated that sTfR was the most predictive parameter for IDA while hepcidin was the most predictive parameter for ACD and COMBI in CHC patients. Conclusion: hepcidin plays an important role in the pathogenesis of anemia in CHC patients. The role of hepcidin in discriminating different types of anemia in CLD is comparable to that of sTfR/logFn index. An appropriate combination of both tests provides evidence for iron depletion or reflects excessive production of hepcidin which will help to establish a correct diagnosis of IDA, ACD or combined anemia in patients with CHC. [Journal of American Science. 2010;6(12):145-154]. (ISSN: 1545-1003).

Key words: Hepcidin, CHC, IDA, ACD, Anemia

1. Introduction

The prevalence of hepatitis C virus (HCV) infection varies throughout the world, with the highest number of infections reported in Egypt [1]. Anemia of diverse etiology occurs in about 75% of chronic liver disease (CLD) patients and this frequent association provides a rationale for examining the contribution of the liver in the development of anemia in those patients [2].

Anemia of chronic illness is frequent among patients with inflammatory disease without apparent blood loss e.g. rheumatoid arthritis, renal failure or chronic hepatitis [3].

Increased iron requirements, limited external supply, and increased blood loss may lead to iron deficiency (ID) and iron-deficiency anemia (IDA). In chronic inflammation, there is hypoferremia and iron-restricted erythropoiesis, despite normal iron stores (functional ID). Anemia of chronic disease (ACD) can evolve into combined ACD and true ID (COMBI anemia) [4].

The liver performs three main functions in iron homeostasis. It is the major site of iron storage, it regulates iron traffic into and around the body through its production of the peptide hepcidin, and it is the site of synthesis of major proteins of iron metabolism such as transferrin and ceruloplasmin [5].

At the core of iron homeostasis is hepcidin, a small acute phase anti-microbial peptide that now appears to synchronously orchestrate the response of iron transporter, ferroportin and regulatory genes [6]. Liver hepcidin is regulated by a number of factors such as iron overload, inflammation, hypoxia and anemia [7].

The effects of inflammation on hepcidin levels are best understood and are mediated at least in part by interleukin 6 (IL6) [8]. Thus, in chronic inflammation, the excess of hepcidin decreases iron absorption and prevents iron recycling [9].

Laboratory tests provide evidence of iron depletion in the body, or reflect iron-deficient red cell production. The appropriate combination of these laboratory tests help to establish a correct diagnosis of ID status and anemia [3].

Measurement of serum ferritin is currently the accepted laboratory test for diagnosing iron deficiency, and a ferritin value of 12 μg/l is a highly specific indicator of iron deficiency. Other commonly used laboratory tests such as serum iron, total iron-binding capacity, mean corpuscular volume, and transferrin saturation provide little additional diagnostic value over ferritin [10]. However, serum ferritin levels are increased in several pathological conditions associated with chronic inflammatory conditions.

The transferrin receptor (TfR) mediates cellular uptake of iron by binding the iron carrier-protein

transferrin (Tf). The soluble form of TfR in serum is used as a surrogate marker of bone marrow erythropoiesis and red cell production [11]. Because serum ferritin reflects the storage iron compartment and sTfR reflects the functional iron compartment, these two values can be combined into a ratio, which is reciprocally regulated. The special value of the sTfR/log ferritin index (TfR-F Index) in the differential diagnosis of IDA has already been documented [12].

The aim of this study was to clarify the role of hepcidin (serum and tissue) in the regulation of iron homeostasis and development of anemia in patients with CHC targeting the differentiation of the type of anemia (ID, ACD or combined) in those patients. We also aimed at investigating the iron profile and tissue iron score and its relevance to the diagnosis of anemia in CLD patients with different clinical stages in an attempt to choose the best diagnostic markers for type of anemia.

2. Patients and Methods

This study was conducted on 70 CLD patients admitted to Hepatology Department Theodor Bilharz Research Institute in addition to 20 healthy controls who had normal biochemical tests, CBC, liver function tests and were negative for hepatic viral infection both by ELISA and by polymerase chain reaction (PCR) in addition to normal ultrasonograph configuration of the liver Patients were enrolled in this study if they had CLD as evidenced by 2-3 folds increased liver function tests for more than 6 months and the etiology of CLD was chronic hepatitis C (CHC) virus as diagnosed by HCV antibody detection in serum (ELISA) and PCR viral detection both quantitative and qualitative tests. Patients were excluded if they had hepatitis B or schistosomiasis co-infection or history of hematemesis or melena in the last 21 days. None of the patients received blood or blood components transfusion therapy in the past 21 days. Patients were classified according to modified Child Pugh classification (1973) [13] into child A (25 patients), Child B (17 patients) and Child C (28 patients).

After taking their written consent according to Helsinki guidelines of ethical research, Samples from all patients and controls were withdrawn in suitable vacutainers and subjected to liver function tests (AST, ALT, Albumin, Bilirubin and Prothrombin time), complete blood count (CBC) including Hb concentration, MCV, MCH, MCHC and RDW using hematology analyzer Celtac-MEK 8118 (Nihon Kohden, Japan), HCV antibody by ELISA and PCR detection of HCV both qualitative and quantitative. Liver biopsies of which pathological examination, tissue iron quantification and immunohistochemical

detection of prohepcidine expression using monoclonal antibody was done. The same procedures were done on liver biopsies taken from patients during cholecystectomy operation after taking their written consents. Those acted as control for immunohistochemistry. Biopsies from controls were negative for hepatitis markers both B and C and didn't show any evidence of inflammation.

Iron profile (serum iron, TIBC, serum ferritin and transferrin) was measured by ELISA technique using commercially available kits (Bayer Diagnostics, U.K.) Transferrin saturation was calculated as serum iron (μg/dl) x 70.9/ serum transferrin (mg/dl). sTfR receptor (sTfR) level was measured by ELISA using Quantikin human sTfR kit (R&D systems Inc, USA). Calculation of the ratio TfR/log ferritin (TfR-F Index) was used as a determinant of body iron stores. If the Index was equal or more than 2, the patients were considered iron deficient. Patients were considered iron replete if Index was less than or equal to1 [14].

Serum prohepcidine hormone levels were measured by using DRG hepcidin prohormone ELISA kit (DRG, Germany). IL6 was evaluated by ELISA using Quantikine IL-6 (Quantikine IVD, minipolos, USA) test kits. Blood samples were collected from all patients at the same time during routine sampling to avoid diurnal variation of hepcidin.

According to the results of the iron profile and markers of inflammation, patients were regrouped into 3 groups; group 1: patients with iron deficiency anemia (IDA, n=18). Criteria of inclusion in this group were: low Hb (male<13 g/dl and female <12 g/dl), TSAT (<20%) and ferritin concentrations (<30 ng/ml). Group 2: patients with anemia of chronic disease (ACD, n=7). Criteria of inclusion in this group were: evidence of chronic inflammation (high CRP level>1mg/dl), Hb concentration <13 g/dl for male and<12 g/dl for female and low TSAT <20% but normal or increased serum ferritin concentration (>100 ng/ml) or low serum ferritin concentration (30-100 ng/dl) and sTfR/ log ferritin ratio< 1. Group 3 was compromised of patients who had combined ID and ACD (COMBI, n=45). Criteria of inclusion were: chronic inflammation (high CRP level >1mg/dl), hemoglobin concentration < 13 g/dL for male and < 12 g/dL for female, low TSAT < 20%, serum ferritin concentration > 30 and < 100 ng/mL, and a sTfR/log ferritin ratio > 2 [4].
Histopathological examination and tissue Iron evaluation:

Histological sections were processed and stained with hematoxylin and eosin (H&E) and Masson trichrome was used to examine the histopathological changes. Perls Prussian blue staining for iron was performed. Liver specimens were scored for grade of inflammatory activity according to the classification of Desmet et al. [15] The histological quantification of

iron was done according to Deugnier et al. [16] by scoring iron separately within hepatocytes (hepatic iron score (HIS) 0 to 36), sinusoidal cells (sinusoidal iron score (SIS) 0 to 12), and portal tracts or fibrotic tissue (portal iron score (PIS) 0 to 12). The total iron score (TIS) 0 to 60, was the sum of these scores.
Immunohistochemical procedure:

The standard avidin-biotin immunoperoxidase technique was used [17]. Hepcidin was detected using a primary antibody against hepcidin (Santa Cruz Biotechnology, Santa Cruz, CA., USA). The number of positively stained cells with positive intracytoplasmic brown stain was recorded within ten successive fields (X400), the final value represented 10 readings per case. Zero percentage was given to unstained sections. Hepcidin expression sites were examined in hepatocytes, intra-lobularly and in peri-portal areas.

Statistical methods:

All data are reported as mean +/- SD. The data were analyzed by computer using the statistical package SPSS for windows version 11 (software). Comparing means was performed by one-way ANOVA Post HOC LSD test. Correlations between different parameters were determined by bivariate Pearson correlation test. The linear relationship between variables was assessed by linear regression analysis. To determine the best predictors of ID, ACD and COMBI anemia multivariate analysis was done using stepwise logistic regression and odd ratios were estimate for each of the independent variables in the model. For all tests P values <0.05 were considered statistically significant.

3. Results:

The results of the biochemical tests of the control and patient groups are shown in table 1
The hematological results of the control and patients groups are shown in table 2.

Results of the iron profile (serum iron, TIBC, transferrin saturation and serum ferritin), sTfR, sTfR/Fn index, prohepcidin hormone, IL6, tissue expression of hepcidin, tissue iron scores and liver histology in the control, Child A, Child B and Child C groups are shown in Table 3.
Histopathological grading of inflammation and hepcidin detection:

In Child A, all cases were with mild inflammatory activity. All Child B cases showed moderate inflammatory activity and all 28 cases in Child C expressed severe activity.
Tissue expression of hepcidin was mainly in hepatocytes, expressed as cytoplasmic brownish stain (figures 1, 2 and 3). Figure 4 shows iron stain in a Child C patient.

Correlation studies:

In Child A group, there was a negative correlation between hepcidin and sTfR (r=-0.52) and a positive correlation between hepcidin and Fn (r= 0.059). Negative correlations were found between Fn index and hepcidin (r=-0.58) and between sTfR and Fn (r=-0.57) in Child A. In Child B group, there were two positive correlations between hepcidin and each of IL6 (r=0.54) and ferritin (r=0.49) while IL6 revealed a negative correlation with sTfR (r=-0.66) and a positive correlation with ferritin (r=0.93) in the same group. Correlation studies also showed an inverse correlation between sTfR and ferritin (r=-0.71) and between ferritin index and IL6 (r=-0.76) in Child B group. In Child C group there was a positive correlation between ferritin index and IL6 (r=0.39). The results of IDA group, ACD group and COMBI group are shown in table 4.

In Child A group, 10/25 (40%) of patients were categorized under IDA group, 5/25 (20%) under ACD group while 10/25 (40 %) were of the COMBI group. In Child B group 8/17 (47%) were found to have IDA, 2/17 (11.7%) were found to be in the ACD group while 7/17 (41.1%) were found to be of the COMBI group. As regard Child C group, all 28 patients (100%) were found to have combined IDA and ACD. In all diseased groups 18/70 (25.7%) had IDA, 7/70 (10%) had ACD and 45/70 (64.3%) had combined anemia.

Correlation studies revealed a negative correlation between hepcidin and sFe (r=-0.83) in ACD group. In COMBI group, positive correlations were expressed between IL6 and ferritin (r=0.37), Fn index and hepcidin (r=0.36) while negative correlations were noted between hepcidin and TfR (r=-0.46), IL6 and serum Fe (r=-0.32), Fn index and serum Fe (r=-0.42).

Univariate regression analysis comparing variables other than those used for classification of IDA, ACD and COMBI anemia revealed that sTfR (P<0.001) and ferritin (P<0.001) were the most predictive parameters for IDA, while hepcidin was the most predictive parameter for both ACD (P<0.05) and COMBI group (P<0.001). Stepwise multivariate logistic regression analysis demonstrated that sTfR was the most predictive parameter for IDA anemia while hepcidin was the most predictive parameters for ACD and COMBI in CLD patients. Odd ratios (OR) estimated for sTfR and hepcidin were considered significant. The 95% confidence interval (CI) for OR was 1.6 [lower bound (L) 1.2 and upper bound (U) 1.84] and 0.003 (Lower 0.0001and Upper 0.68) respectively with P<0.0014 and 0.043 respectively. This was confirmed by stepwise discriminate analysis (p<0.0001 for both parameters).

Table 1: Results of the biochemical tests of the control and patient groups.

	Control (n=20)	Child A (n=25)	Child B (n=17)	Child C (n=28)
Albumin gm/dl	4.08±0.32	4.48±. 35	3.43±0.48	2.53±. 76
Prothrombin concentration%	90.04±11.37	84.4±15.7	73.18±9.6	51.14±9.63*
Total bilirubin mg/dl	1.17±. 67	1.46±1.02	2.86±1.54	4.78±2.49
Direct bilirubin mg/dl	1.06±. 3	1.05±1.02	1.981+0.7	2.75±1.78
Alkaline phosphatase u/L	227.6±120.22	227.08±82.93	223.12±109.23	287.57±42.45*°
ALT u/L	97.8±61.34	101.72±60.79	112.94±65.75	134.96±31.02*°
AST u/L	105.6±99.9	118.96±42.35	129.88±80.45	142.78±39.3*°
Alpha feto protein	0.78±0.26	0.93±0.58	2.66±0.46	11.6±1.5
PCR IU	_	150 000±5000	300 000±100 000	600 000±150 000
Gamma GT	120±74.75	162±72.4	117.29±57.23	139.7±83.34
CRP mg/dl	0.2±0.7	2.3±0.6	4.7±1.3	12.4±1.5

* Statistical significant difference compared to control group (P<0.05).

° Statistical significant difference compared to Child A group (P<0.05).

 Statistical significant difference compared to Child B group (P<0.05).

Table 2: Hematological results of the control and patients groups.

	Control (n=20)	Child A (n=25)	Child B (n=17)	Child C (n=28)
WBC (×103/L)	8.6±0.75	6.25±0.59*	6.48±1.02*	5.41±0.54*°
RBC (× 1012/L)	5.2±0.24	4.9±0.4*	4.5±0.54*	3.93±0.38*°
Hb g/dl	13.8±0.27	11.69±0.37*	10.61±0.42*°	8.74±0.41*°
RDW %	15± 0.34	18±0.98*	19±0.67*	19±1.12*
MCV (fl)	86.45±3.49	76.4±3.7*	72.42±2.78*	67.76±3.95*°
MCH pg/L	28.53±1.23	25.67±1.24*	23.26±1.25*°	21.54±2.12*°
MCHC g/dl	34.14±1.1	33.02±1.2	30.23±1.53*	29.98±0.85*°
Platelet (×103/L)	200.2±8.28	186.16±21.11*	150±2.9±25.39*°	100.5±33.18*°

* Statistical significant difference compared to control group (P<0.05).

° Statistical significant difference compared to Child A group (P<0.05).

 Statistical significant difference compared to Child B group (P<0.05).

Table 3: Results of the iron profile, sTfR, sTfR/Fn index, prohepcidin hormone, IL6, tissue expression of hepcidin, tissue iron scores and liver histology in the control and diseased groups.

	Control(n=20)	Child A (n=25)	Child B (n=17)	Child C (n= 28)
Serum iron ug/dl	94.78±15.59	82.65±13.74*	78.84±13.3*	70.62±13.98*°
TIBC	345±69.96	355.44±56.56*	409.18±63.26*°	430.18±64.53*°
TSAT%	37.28±1.45	19.12±2.32*	15.24±3.13*	11.58±1.71*
s ferritin ng/ml(Fn)	133.07±21.9	128.82±105.17	104.78±94.98	82.64±19.52*
sTfR nmol/L	3.2±1.03	4.04±1.25*	3.95±1.43*	4.6±1.18*
sTfR/log Fn index	1.53	2.14*	0.95*	2.36*
Serum prohepcidin	76.88±11.45	74.91±19.95	81.15±23.17	91.88±9.05*°
IL6 (pg/mL)	9.28±1.27	9.91±1.38	11.75±4.97	13.49±2.2*°
Tissue expression of hepcidin	0.0	70.9±9.9*	57.06±8.9*°	26.32±8.2*°
Tissue iron score	0.0	2.4±03*	5.2±2.2*°	8.4±3.2*°
Inflammatory activity	10/0/0/0	0/25/0/0	0/0/17/0	0/0/0/28

*Statistically significant difference compared to Control group (P<0.05).

° Statistically significant difference compared to Child A group (P<0.05)

 Statistically significant difference compared to Child B group (P<0.05).

Intensity of necro-inflammatory lesions: 0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity.

Table 4: The results of control, IDA, ACD and COMBI groups

	Control (n=20)	IDA (n=18)	ACD (n=7)	COMBI (n=45)
CRP mg/dl	0.20±1.4	0.3±0.12	0.5±0.4	2.5±1.2*°
Hb g/dl	13.8±0.27	11.0±2.3*	8.5±2.1*°	10.8±1.5*
sFe µg/dl	101.58±42.06	79.1±11.47*	72.71±9.23*	81.16±17.67*
Fn ng/ml	118.8±83.19	63.89±11.38*	296.31±78.01*°	79.77±12.24*
Ferritin index	0.9±5.5	2.51±0.6*	0.95±0.3°	2.43±0.59*
sTfR mg/L	3.5±1.03	4.4±0.95*	2.3±0.74*°	4.63±1.08*
prohepcidin(ng/mL)	77.08±14.37	55.23±11.65*	97.44±7.88*°	91.1±8.5°
IL-6 (pg/mL)	9.16±3.28	9.27±1.6	13.74±*	10.25±1.6°

*Statistically significant difference compared to Control group (P<0.05).

° Statistically significant difference compared to Child A group (P<0.05)

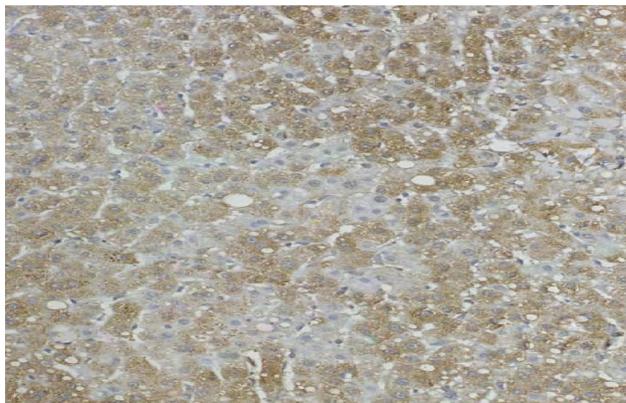 Statistically significant difference compared to Child B group (P<0.05).

Figure 1: A case of HCV, Child A, showing expression of Hepcidin in about 60% of hepatocytes as brownish cytoplasmic colour (IHC, DABx200) (20mcm).
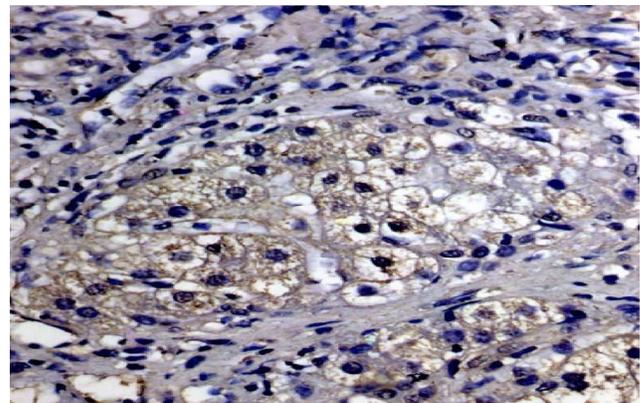

Figure 3: A case of HCV, Child C, showing cirrhotic nodule, expressing Hepcidin in about 40% of hepatocytes as brownish cytoplasmic color (IHC, DABx400) (50mcm).
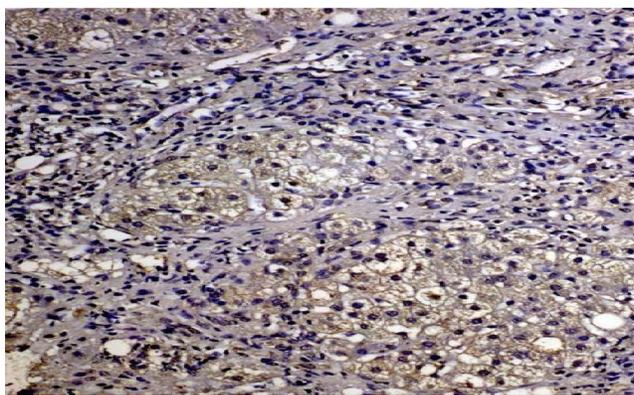

Figure 2: A case of HCV, Child C showing cirrhotic nodule expressing Hepcidin in about 40% of hepatocytes as brownish cytoplasmic color (IHC, DABx200) (20mcm).
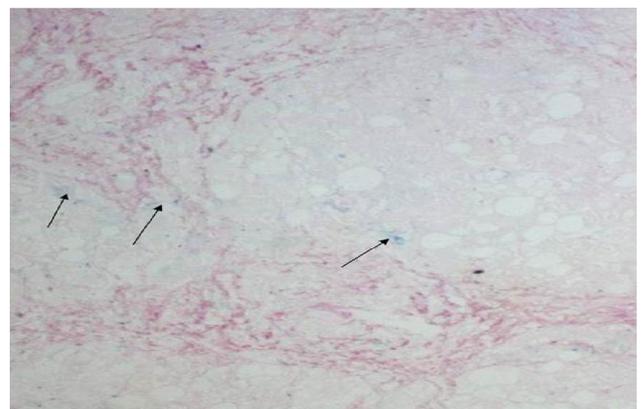

Figure 4: A case of HCV, Child C, showing some hepatocytes revealed positive expression of iron as bluish intracytoplasmic granules (Perl's iron stain, x100) (10mcm).

4. Discussion:

Hepatitis C virus (HCV) infection is a major cause of liver diseases [18] in which anemia is a prevalent feature. Hepcidin is the central regulator of systemic iron homeostasis. Hepcidin deficiency is the cause of iron overload in hepatitis C while hepcidin excess is associated with anemia of inflammation and iron-refractory iron deficiency anemia [19].

According to the Hb and RBCs indices, patients had microcytic hypochromic anemia with anisocytosis in all diseased groups. Since active bleeding was excluded in these patients, anemia could be attributed to iron deficiency either functional (FID) or true (TID). True iron deficiency in CLD could be due to increased requirements or diminished supplies. This may result from hidden blood loss or low-grade gastrointestinal bleeding which also may be compounded by decreased oral iron absorption because of dietary restrictions. Blood loss could be caused by defects of blood coagulation as a consequence of endothelial dysfunction, thrombocytopenia or deficiencies of coagulation factors. Anemia could also be due to secondary hemolysis caused by splenomegaly, which is usually caused by portal hypertension in patients with CLD. In some patients, bone marrow failure and aplastic anemia develop after an episode of hepatitis. Finally, anemia could be due to ribavirin-induced hemolysis as a complication of treatment of CHC with a combination of interferon and ribavirin [2].

The iron profile denotes that patients suffered from decreased iron in all groups. A significant reduction in ferritin was noted only in Child C. Low ferritin levels due to iron deficiency could be counteracted by increased ferritin synthesis which is induced by inflammatory cytokines [20]. The inflammatory state detected in those patients induces ferritin synthesis, probably through hepcidin secretion, masking the reduced ferritin levels due to iron deficiency [21]. Elevated serum ferritin and hepatic iron concentrations are frequently observed in CHC which may be related to hepcidin [22].

The level of tissue iron showed a statistical significant increase in all diseased groups. Increased serum hepcidin levels explain the inappropriate low hepatic hepcidin expression in an iron-over loaded cirrhotic liver. While the cirrhotic liver tissue is unable to secrete hepcidin, extra hepatic sources of hepcidin increases. Irrespective of the source of hepcidin, the downstream consequences are likely to be internalization and degradation of the cellular iron export protein ferroportin in the background of an elevation in the cellular iron import proteins such as transferrin receptor 1. This culminates in cellular iron accumulation; a phenotype which have previously been reported in HCV patients. This raises the possibility that hepcidin may have a dual effect contributing to the systemic anemia whilst acting locally at liver cells leading to iron accumulation. [23].

The soluble form of TfR (sTfR) is proportional to the total amount of surface TfR [24] and the erythroblasts rather than reticulocytes are the main source of serum sTfR [25]. The interpretation of the iron state according to sTfR has been controversial by many authors. Some believe that increased sTfR concentrations indicate ID even during ACD, and sTfR levels, was found to be considerably elevated in iron deficiency anemia but remain normal in the anemia of inflammation [24]. Others claim that elevated sTfR levels are also the characteristic feature of functional iron deficiency (FID) [25]. Increased sTfR may also indicate increased erythropoietic activity without ID, whereas lower sTfR concentrations may reflect decreased numbers of erythroid progenitors [24].

sTfR was found to be significantly increased in all patients compared to the controls (P<0.05). According to these results, all Child groups suffered from iron deficiency either solely or on top of ACD. It might also indicate the increased erythroid synthesis compensating for hemolysis or hidden gastrointestinal bleeding.

Statistical studies showed that in Child A and C most of the patients (80% and 100% respectively) suffered from iron deficiency either solely or combined with ACD which explains the high ferritin index values. In Child B group although still the percentage of patients suffering from iron deficiency was higher than those with ACD (88.3 versus 11.7), yet the marked high ferritin level of patients with ACD explains the decreased index value in this group. On regrouping patients into IDA, ACD and COMBI we found that after excluding patients with active bleeding the main type of anemia present in CHC patients is a combined form of ACD and IDA (COMBI anemia). All patients in Child C had COMBI anemia denoting that the more the progress of the disease the more patients are liable to acquire both types of anemia probably as a result of increased amount of chronic blood loss together with increased degree of inflammation caused by increased viral influence.

Statistical significant increase was detected in serum hepcidin in Child C group compared to control and Child A groups (P<0.05). On the contrary, hepatic expression of hepcidin showed reduced expression in diseased groups compared to the control group.

High level of hepcidin was detected in the presence of chronic hepatitis C (CHC) in many studies [26, 27]. The discrepancy between serum levels and hepatic expression levels could be due to the fact that hepcidin

expression was also localized in other tissues other than liver as kidney tubular system [28] and macrophages [29]. There is evidence that hepcidin is also expressed in the heart, kidney, adipose tissue, pancreas and haematopoietic cells [30].

Although there was an inflammatory state detected in all patients as proved by inflammatory index and CRP, significant increase in IL6 was detected only in Child C and in COMBI group. The lack of correlation between IL6 and hepcidin except in Child B group denotes that there are other factors contributing to increased hepcidin levels. The inflammatory stimulus to hepcidin secretion could be mediated through other stimulatory cytokines as IL1.

Four putative upstream regulatory pathways are generally thought to control liver hepcidin production: iron store, erythropoietic activity, inflammation, and a mandatory signaling pathway. All are found to interact with liver cells to initiate the production of sufficient hepcidin for correct maintenance of iron homeostasis [31].

Hepcidin transcription is stimulated by iron overload as well as by inflammation. Iron can accumulate in the liver in a variety of conditions, including anemia of chronic disease, hepatitis C and liver-specific iron accumulation of uncertain pathogenesis in cirrhosis [32].

A reduced hepcidin synthesis indeed might be one of the mechanisms leading to iron overload in advanced liver disease of any origin [33]. In a further step, progressive iron retention in the liver induces hepcidin formation to counterbalance hepatic iron accumulation by reducing duodenal iron absorption [34].

The liver plays a central role in maintaining body iron homeostasis not only as a storage tissue and a site of hepcidin production, but also by a relatively specific expression of several other iron-related genes including the hereditary hemochromatosis protein called HFE, transferrin receptor 2, hemojuvelin, bone morphogenetic protein 6, matriptase-2 and Transferrin [29]. Apart from these genes with a relatively specific expression, the liver expresses other genes and molecules involved in cellular iron transport, which are also present in other tissues. They include transferrin receptor 1, divalent metal transporter 1 and ferroportin [35]. The deregulated expression of these proteins in the diseased liver could result in aberrant hepcidin production [36].

Hepatic hepcidin repression might be the result of high circulating erythropoietin level due to ongoing hemolysis and oxidative stress due to accumulation of iron [23]. Hypoxia, anemia, increased erythropoiesis and reduced iron stores all negatively regulate hepcidin expression. The fact that elevated erythropoiesis increases iron absorption regardless of body iron loading could be explained by the presence of a direct connection between EPO and the suppression of hepcidin expression in hepatocytes, which express EPO receptors [29]. Anemia could mediate hepcidin suppression through multiple mechanisms including increased EPO, increased iron demand or liver hypoxia. The nature of the erythropoietic regulator of hepcidin is still uncharacterized, but may include one or more proteins released during active erythropoiesis. Possible candidate proteins include GDF15, secreted by erythroblasts, or soluble hemojuvelin, secreted by skeletal muscle [37]. Hepcidin is suppressed by hypoxia but the mechanisms are still uncertain and conflicting. Hypoxia-inducible factor (HIF)-1 and reactive oxygen species (ROS) have been both implicated [35].

The ongoing hemolysis, hidden blood loss, depressed bone marrow and decreased dietary intake or poor absorption from the intestine might lead to deteriorated iron supply in CHC patients. Iron deficiency state might cause anemia and suppress hepcidin synthesis in those patients [35]. In uncomplicated iron deficiency anaemia, both the anaemia per se and the absent iron stores provide a message to stop production of hepcidin [10].

Despite the fact that hemolysis down regulates hepcidin, Vokurka et al observed an increase in hepcidin expression despite severe hemolytic anemia after suppression of erythropoiesis [35]. This could be the case in patients with CHC in whom erythropoisis could be suppressed due to the presence of immune antibodies. Interferon based therapy can also suppress bone marrow production of erythrocytes [1] thus contributing to increased hepcidin production.

In addition, Merle et al showed that, in contrast to the liver, in which hypoxia results in down-regulation of hepcidin expression, cardiac hepcidin expression is significantly up regulated in response to hypoxia [38]. Extra hepatic sources of hepcidin could be up regulated due to hypoxia resulting in the observed increased levels.

This illustrates potentially differential effects of stimulation of hepcidin perhaps via inflammation and hepatic iron accumulation opposed by repression mediated by increased erythropoisis, anemia, hypoxia, reduced iron stores and oxidative stress. The effect of heamolysis and hypoxia on hepcidin repression could be reversed in our patients due to inhibition of erythropoisis due to interferon therapy and shifting the source of hepcidin to the extra hepatic sites where synthesis of hepcidin is induced by hypoxia. Increased hepatic iron is a double edged weapon which increases hepcidin synthesis as a compensatory mechanism to suppress iron absorption but in the same time it causes

decreased hepatic hepcidin synthesis due to accumulation of ROS.

The level of hepcidin in the three types of anemia is complementary to the pathophysiology of each type. In IDA the decreased iron levels lead to depressed hepcidin secretion while in ACD and COMBI anemia groups increased hepcidin secretion could be a cause or a result of increased hepatic iron accumulation.

It should not be forgotten that chronic disease anemia is a complex phenomenon and hepcidin is not the single molecule playing part in this condition. Numerous cytokines, particularly tumor necrosis factor alpha, which does not induce hepcidin m-RNA, can play an important role as well and the coexistance of iron deficiency may further complicate the process [39].

Univariate analysis study revealed that TfR is the most predicting parameter for the presence of IDA while hepcidin is the most predictive parameter for ACD. COMBI anemia was best predicted by hepcidin. The fact that hepcidin level was increased in ACD and COMBI groups without any statistical difference between these two groups while ferritin index was statistically increased in COMBI compared to ACD group could be used to discriminate between the two types of anemia. This denotes that a combination of the two tests is critical for the accurate determination of the type of anemia.

A positive correlation was found between hepcidin and ferritin levels in Child A and B groups. Hepcidin shifts the iron from the functional compartment to the stored one, part of which is ferritin. In addition ferritin could be the stimulus for hepcidin synthesis. Hepcidin could be the missing link between IL6 and ferritin explaining the positive correlation between the later two detected in Child B and COMBI groups. The negative correlation between hepcidin and ferritin index in Child A group is explained by the increased ferritin levels resulting from increased hepcidin level. The negative correlation between ferritin and sTfR in Child A and B and that between hepcidin and TfR in Child A and COMBI is due to the decreased erythroid synthesis manifested by decreased receptor levels in the presence of high ferritin level due to hepcidin production in ACD patients. The negative correlation between IL6 and TfR in Child B group is linked by increased hepcidin levels as a result of increased IL6 levels. The negative correlation between IL6 and ferritin index in Child B group and between IL6 and serum Fe in COMBI group is due to the stimulatory effect of IL6 on hepcidin production. Hepcidin in return lowers the serum iron level by shifting it to the storage compartment. The negative correlation between hepcidin and serum iron in ACD ($r=-0.83$)

emphasizes the role of hepcidin in controlling the serum iron level in anemia of chronic disease.

5. Conclusion:

It could be concluded that hepcidin plays an important role in the pathogenesis of anemia in CHC patients. Accumulation of iron in hepatocytes together with inflammation induced by IL6 or other factors may cause increased hepcidin secretion whether from normal hepatic tissue or extra hepatic origins. The effect of anemia, hypoxia, ROS and erythropoisis counterbalances increased hepcidin levels. In CHC, combined ACD and ID is the main type of anemia after excluding active bleeding. Hepcidin is a good tool in discrimination of the type of anemia and is comparable to that of sTfR/logFn index. An appropriate combination of both tests provides evidence of iron depletion or reflects excessive production of hepcidin which will help to establish a correct diagnosis and appropriate therapy.

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