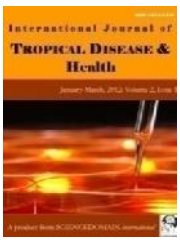

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Hepcidin: A New Noninvasive Biomarker for Histological Cirrhosis in Hepatitis C Virus Patients

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Authors' contributions

This work was carried out in collaboration between all authors. Authors HEB and LA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors HA and AT managed the analyses of the study. Author RAAED managed the literature searches and design the practical protocol. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: To investigate the potential role of serum hepcidin as a biomarker for histological cirrhosis in hepatitis C virus patients.
Methods: Serum hepcidin was measured in 80 patients with hepatitis C virus and 15 age and sex matched healthy subjects as control. Liver biopsy was available for 50 patients only. All subjects were divided as follows:
Group I: 50 patients with chronic HCV waiting for treatment decision. They were subdivided according to liver fibrosis stage into:
Group I (a): 39 patients with fibrosis stage F1, F2 and F3.
Group I (b): 11 patients with histological cirrhosis F4.
Group II: 30 HCV cirrhotic patients.

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Group III: 15 age- and sex-matched healthy subjects as control.
Results: Serum hepcidin concentration was significantly lower in patients with chronic HCV, histological cirrhosis and cirrhotic patients than healthy control and was lowest in those with cirrhosis (P<0.001). Serum hepcidin level was significantly positively correlated with serum ferritin, transferrin saturation, ALT, AST, hemoglobin level, albumin and hepatic iron but negatively correlated with serum bilirubin. Serum hepcidin decrease with progression of liver fibrosis and was lowest in those with histological cirrhosis and clinically proven cirrhosis. In contrast, serum ferritin increased progressively with increasing stages of fibrosis and was highest in those with histological cirrhosis and cirrhotic patients.
Conclusions: Increasing hepatic fibrosis is associated with decreased hepcidin serum level. This indicates that hepcidin may serve as a potential biomarker for fibrosis and cirrhosis. Hepcidin is positively correlated with hepatic iron and liver enzymes but negatively correlated with the stage of fibrosis.

Keywords: Hepcidin; iron overload; HCV; cirrhosis.

1. INTRODUCTION

Persistent infection with hepatitis C virus (HCV) is a major cause of chronic liver disease, with an estimated 170 million infected people worldwide [1,2]. It is well established that about 205 of patients with chronic HCV will progress to cirrhosis within 20 year from infection, further resulting in an annual risk of 3-7% of hepatocellular carcinoma (HCC) [3]. Chronic HCV patients frequently develop mild to moderate iron overload [4]. Many experimental and clinical studies suggest that excessive iron in chronic HCV is a cofactor promoting the progression of liver damage and increasing the risk of fibrosis, cirrhosis and HCC [5,6]. Hepcidin is a peptide hormone and the principal regulator of systemic iron homeostasis [7]. The primary site of hepcidin synthesis is the liver and serum levels correlate with hepatic mRNA expression [8,9]. Being an antimicrobial peptide, its synthesis is increased in response to inflammation and systemic infection [10]. Studies have reported a significant correlation between hepcidin and ferritin in the serum [11]. In the absence of liver disease, it is therefore expected that hepcidin and ferritin concentrations should increase in a concordant fashion and that the hepcidin:ferritin ratio should remain stable. In advanced fibrosis and cirrhosis, however, it is possible that this relationship is altered and that decreased hepcidin synthesis from progressive hepatocyte injury may cause a corresponding fall in the hepcidin:ferritin ratio [12,13].

2. PATIENTS AND METHODS

This study was carried out on 50 Egyptian patients with chronic HCV, 30 clinically proven cirrhosis with HCV and 15 age- and sex-matched healthy subjects as control. Patients were followed up at outpatient clinics of Tropical medicine department, Tanta University Hospital, Tanta, Egypt during the period between January 2012 and December 2012 .Diagnosis of chronic HCV was based on persistence of HCV antibodies and HCV RNA in the serum of the patients for more than 6 months. Chronic hepatitis was confirmed by liver biopsy. Diagnosis of cirrhosis was established by clinical, laboratory, ultrasonographic findings.

2.1 Inclusion Criteria

Patients with positive HCV antibodies by ELISA and HCV RNA by Real Time polymerase chain reaction (RT- PCR) and histological evidence of chronic hepatitis. HCV cirrhotic patients were diagnosed by by clinical, laboratory, ultrasonographic findings.

2.2 Exclusion Criteria

Presence of chronic liver diseases other than HCV related, pregnancy, malignancy, coinfection with human immunodeficiency virus, respiratory or cardiac diseases. Patients with coexisting conditions that could influence the interpretation of iron parameters, such as acute or chronic inflammatory disease, haematological disorders and haemochromatosis were excluded.

We used a group of 15 healthy individuals with normal transaminase levels, negative serologic results for hepatitis B and hepatitis C and normal iron indices as the control group. All subjects were divided as follows:

Group I : 50 patients with chronic HCV waiting for treatment decision. They were subdivided according to liver fibrosis stage into:
Group I (a) : 39 Patients with fibrosis stage F1,F2 and F3.
Group I (b) : 11 Patients with histological cirrhosis F4.
Group II : 30 HCV cirrhotic patients.
Group III : 15 age- and sex-matched healthy subjects as control.

All patients and control were subjected to:

2.2.1 Blood sampling and laboratory tests

Blood chemistry values including complete blood count, alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum albumin, serum bilirubin, prothrombin time, INR were determined on the liver biopsy day. Serum iron, serum ferritin, transferring saturation levels were measured in stored serum samples of all patients and control.

2.2.2 Quantitative assessment of HCN- RNA by RT- PCR for HCV patients

Quantitative detection of HCV RNA by real-time PCR was performed using the light cycler taqman master mix kit on Roche Light cycler version 2.0 (Roche Diagnostic, GmbH, Mannheim, Germany). The cycle conditions of RT-PCR include 95°C for 20 seconds, followed by a further 40 cycles at 95° C for 10 seconds, 58° C for 15 seconds and 72° C for 10 seconds.

Serum iron was mea¬sured by atomic absorption spectrophotometer (Varian Model) after digesting the serum samples according to the manufacturer's instructions [14]. Ferritin level in the serum was measured by Ferritin ELISA Kit (IBL Immuno-Biological Laboratories, Minneapolis, MN, USA) according to the manufacturer's instructions [15]. Hepcidin Serum level was determined using a commercial ELISA kit (DRG Instruments, GmbH, Marburg, Germany) according to the manufacturer's protocol. The dynamic range of the assay is between 0.9 to 140 ng/mL. The serum level of apparently normal healthy adults was 13.3 to 54.4 ng/mL according to the manufacturer [15].

2.2.3 Transferrin saturation ratio

The percentage of serum transferrin saturation was calculated by dividing the serum iron level by the total iron-binding capacity [15].

2.2.4 Liver biopsy for chronic HCV patients

Liver injury, activity of inflammation and fibrosis were assessed by pathologist using the METAVIR scoring system on haematoxylin/eosin stained sections, where Fibrosis stage (F) was scored as F0 (absent), F1 (portal fibrosis), F2 (portal fibrosis with few septa), F3 (septal fibrosis) and F4 (cirrhosis) [16,17]. Hepatic iron deposits were evaluated by Perl's stain and were graded from 0 to 4 according to standard protocol [18].

All patients and control gave their informedconsent and the study was approved by Ethical, and Research Committee, Tanta Faculty of Medicine, Tanta, Egypt.

2.3 Statistical Analysis

The statistical data are reported as the mean±SD, frequencies (number) and percentages when appropriate. A comparison of the numerical variables between groups was performed using a one-way analysis of variance test to compare normal data, and the Kruskal–Wallis test was used when the data were not normal. Spearman rank correlation was used to quantify the association between continuous or ordered categorical variables. A P-values less than 0.05 were considered statistically significant. All statistical calculations were performed using the computer program SPSS (Statistical Package for the Social Science; SPSS, Chicago, IL, USA) version 15 for Microsoft Windows.

3. RESULTS

There was no statistical difference between patients' groups and control as regard age and gender (P> 0.05).

Statistically significant difference between groups as regard serum billirubin, ALT, AST, albumin and heamoglobin was detected. Serum billirubin, ALT, AST was significantly higher in chronic hepatitis and cirrhotic patients than control ($p < 0.05$) while serum albumin and hemoglobin were significantly lower in cirrhotic patients than control ($p < 0.05$) (Table 1).

Table 1. Baseline characteristics of the studied groups

Parameters	Control n = 15	CHC n = 50	Cirrhosis n = 30	P- value
Age	37.3±1.1	42.7±1.6	51±0.3	>0.05
Gender (M/F)	10/5	34/16	27/13	>0.05
Hb (gm/dl)	14.2±1.8	13.1±1.2	10.9±1.5	<0.05*
AST (IU/L)	21.1±2.7	81.5±7.3	69±4.8	<0.05*
ALT (IU/L)	21.6±1.9	94.9±6.2	78±3.1	<0.05*
Bilirubin (mg/dl)	0.6±0.2	2.1±0.8	3.4±2.3	<0.05*
Albumin (g/dl)	4.4±0.5	3.2±0.5	2.6±0.8	<0.05*

*Hb,hemoglobin; ALT, alanine aminotransferase; AST, aspartate aminotransferase, * ignificant.*

There was a significant decrease in serum hepcidin concentration in patients with chronic HCV, histological cirrhosis and cirrhotic patients than healthy control and was lowest in those with cirrhosis (P<0.001). Also, there was a significant decrease of hepcidin concentration in cirrhotic (P<0.001)and histological cirrhosis patients (P<0.049) than chronic HCV patients. Significant high serum iron, ferritin concentration and transferin saturation levels were detected in patients with chronic HCV, histological cirrhosis and cirrhotic patients compared to control (<0.05). Serum iron, ferritin and transferritin saturation levels were higher in cirrhotic patients compared to chronic HCV (<0.05) Table 2.Hepcidin initially increased in early stages of fibrosis (23.2±5.4 in F1 Vs 25.8±4.3 in F2 ng/ml), but declined thereafter in the late stages of fibrosis 21.3±6.2 ng/ml in F3 and was lowest in those with F4 fibrosis 19.8±5.6 ng/ml and clinically proven cirrhosis 17.4±8.8 ng/ml. In contrast, serum ferritin increased progressively with increasing stages of fibrosis (146.5±4.4 in F1, 153.3±3.2 in F2 and 159.6±6.6 ng/ml in F3) and was highest in those with, F4 191.4±41.4 and cirrhotic patients 234.5±52.6 ng/ml.

Table 2. Comparison of serum iron, ferritin, hepcidin andtransferin saturation% in the studied groups

Parameters	Control n=15	CHC n=50		Cirrhosis n = 30	P- value
		CHC (F1-F2-F3) n=39	Histological cirrhosis (F4) n=11		
S. iron (ug/dl)	87.2±9.1	115.8±12.4	148.4±3.6	196.5±7.1	0.002*
S. ferritin (ng/ml)	93.3±13.9	146.8±30.8	191.4±41.4	234.5±52.6	<0.001*
Tf. Sat (%)	26.2±1.4	31.5±0.9	39.9±1.7	48.1±3.6	<0.001*
S. hepcidin (ng/ml)	39.9±11.8	26.6±10.4	19.8±5.6	17.4±8.8	<0.001*

*Tf, transferin saturation; S, serum * significant.*

Serum hepcidin concentration was significantly positively correlated with serum ferritin (r=0.53, P=<0.001), serum iron (r=0.42,P=<0.001), transferrin saturation (r=0.24, P=0.003), ALT(r=0.19, P = 0.002), AST (r=0.15, P =<0.001), hemoglobin level (r=0.37, P=<0.001) and albumin (r=0.31, P=<0.001), hepatic iron stores (r=0.44, P=<0.001) but negatively correlated with serum bilirubin (r =- 0.26, P =<0.001) and stage of fibrosis (r=- 0.49, P =<0.001) Table 3.

Table 3. Correlation between serum hepcidin in HCV patients and different laboratory and histological parameters

Parameters	R	p- value
S. ferritin	0.53	<0.001*
S. iron	0.42	0.001*
Tf saturation	0.24	0.003*
Haemoglobin	0.37	0.001*
S. albumin	0.31	<0.001*
S. bilirubin	-0.26	<0.001*
ALT	0.19	0.002*
AST	0.15	<0.001*
Stage of fibrosis	-0.49	<0.001*
Necroinflammatory activity	0.32	0.014
Steatosis	0.34	0.021
Hepatic iron	0.44	<0.001*

*R, correlation, Tf, transferin saturation; S, serum * significant*

Hepatic iron stores in CHC patients showed positive iron stain in 17 patients out of 50. The distribution of patients according to semi-quantitative assessment of hepatic iron was as follows: Grade 0 (n = 33), grade 1 (n = 11), grade 2 (n = 5) and grade 3 or 4 (n = 1) patients. Table 4.

Table 4. Histological findings in CHC patients

Parameters	CHC N=50	
	N	%
Positive iron stain	17	34
Iron score		
Grade 0	33	66
Grade 1	11	22
Grade 2	5	10
Grade 3	1	2
Grade 4	0	0
Fibrosis stages		
F0	0	0
F1	16	32
F2	13	26
F3	10	20
F4	11	22
Necroinflammatory activity		
A0	5	10
A1	15	30
A2	17	34
A3	13	26
Steatosis		
0	12	24
1	15	30
2	14	28
3	9	18

CHC, chronic hepatitis C

As regard correlation between liver iron and fibrosis stage. out of 16 patients with fibrotic stage F1 2 of them have grade 1 liver iron and 14 grade 0. Fibrosis stage F2 in 13 patients have one patient with hepatic iron grade 1 and one with grade 2 liver irons. 10 patients have fibrotic stage F3, 4 patients have grade 1 liver iron and one patient grade 2 liver irons while the remaining 5 patients have grade 0 liver iron. 11 patients with F4 fibrosis stage, 4 patients have grade 1 liver iron, 3 patients grade 2, one patient grade 3 and 3 patients grade 0 liver ironTable 5.

As regard Fig. 1 and Fig. 2 they show iron deposition grade 2 in patients with fibrosis score F2 and F3 respectively while Fig. 3 shows iron deposition grade 3 in chronic hepatitis C virus with fibrosis score F4.

Table 5. Correlation between liver iron and fibrosis stage in CHC patients

Liver iron grade	CHC n=50					P value
	F0	F1	F2	F3	F4 Histological cirrhosis	
	0/0	2/16	2/13	5/10	8/11	
Grade 0	0	14	11	5	3	0.03*
Grade 1	0	2	1	4	4	
Grade 2	0	0	1	1	3	
Grade 3	0	0	0	0	1	
Grade 4	0	0	0	0	0	

*CHC, chronic hepatitis C; *significant.*

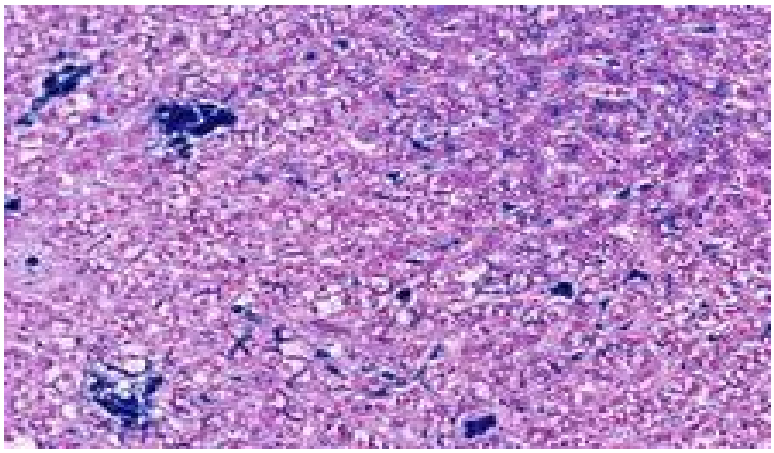

Fig. 1. Iron deposition grade 2 in F2 stage chronic hepatitis case (x100)

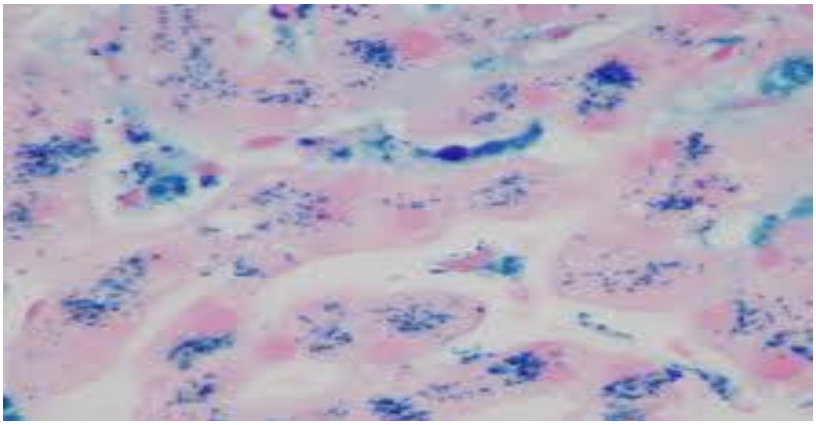

Fig. 2. Iron deposition grade 2 in F3 stage chronic hepatitis case (x200)

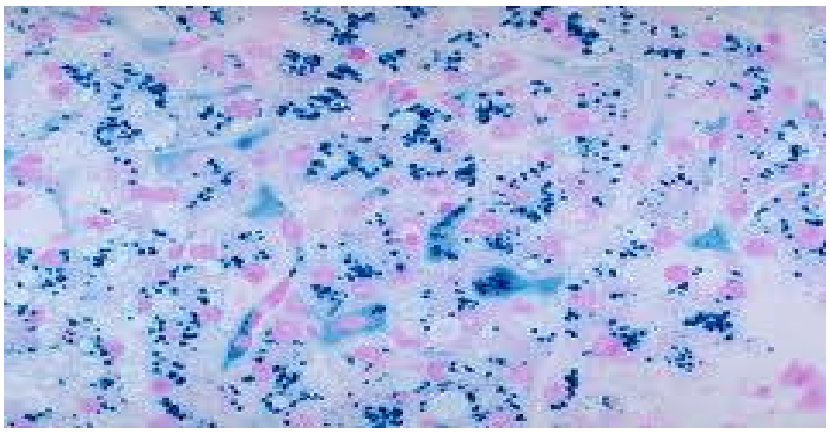

Fig. 3. Iron deposition grade 3 in F4 stage chronic hepatitis case (x200)

4. DISCUSSION

Hepcidin is a recently discovered polypeptide that is present in human serum and urine [8]. It has a central role in iron homoeostasis, as it decrease iron release from macrophages and iron absorption from intestinal enterocytes through its binding to the iron exporter protein, ferroportin [19]. Elevated iron stores, inflammation, hypoxia and iron homoeostasis are the major regulation of hepcidin production [20]. Hepatic expression of hepcidin is decreased in iron deficiency to facilitate iron absorption, and increased in iron repletion to prevent pathological iron overload [21].

This study demonstrated that serum hepcidin concentration was significantly lower in patients with chronic HCV, histological cirrhosis and clinically proven cirrhotic patients than healthy control and was lowest in those with cirrhosis ($P<0.001$). These findings were in keeping with the findings reported by Penkova et al. [22], E Tsochatzis et al. [23] and Girelli et al. [24]. In contrast Terrence et al. [25], reported no significant difference in serum hepcidin levels between CLD patients and healthy control as they analyzed patients with compensated and decompensated disease as a single entity and the heterogeneity of the study population with different etiologies as HBV and alcoholic liver disease.

In this study there was a significant decrease of hepcidin concentration in cirrhotic ($P<0.001$)and histological cirrhosis patients ($P<0.049$) than chronic HCV patients .This could agree with the results of Terrence et al. [25], who detected a significant lower levels of serum hepcidin in cirrhotic compared with chronic hepatitis patients.

Significant high ferritin concentration and transferin saturation levels were detected in our study in patients with chronic HCV, histological cirrhosis and cirrhotic patients compared to control (<0.05). Serum ferritin and transferritin saturation levels were higher in cirrhotic patients compared to chronic HCV(<0.05). These findings were in agreement with Terrence et al. [25] and Girelli et al. [24], they demonstrated high serum ferritin levels in HCV patients with cirrhosis.

Hepcidin down regulation is likely to contribute to liver iron accumulation in this condition. A mild iron overload has been reported to be fairly common in chronic HCV and to be associated with more severe disease [23,25]. Therefore, HCV may represent a complex

condition for hepcidin regulation, as iron overload and HCV protein–induced reactive oxygen species (ROS) with consequent up regulation of an inhibitor to hepcidin [26]. To make the situation more complex, experimental evidence suggests that HCV–induced oxidative stress suppresses hepcidin expression, providing a possible explanation for HCV- induced iron overlod [10,27]. In advanced stages such as cirrhosis, hepcidin may be further decreased by impaired protein synthesis due to markedly reduced functional hepatic mass, contributing to additional parenchymal iron overload [28,29].

Furthermore, we found that serum hepcidin concentration was significantly positively correlated with serum ferritin, transferrin saturation, ALT, AST, hemoglobin level and albumin but negatively correlated with serum bilirubin which are widely used biochemical markers of liver function. Our results were in agreement with Girelli et al. [24] and Terrence et al. [25]. In contrast Penkova et al. [22] detected inverse relationship between serum hepcidin and the values of serum iron,ferritin, ALT, AST as well as severity of steatosis, inflammation and fibrosis inpatients with alcoholic and non alcoholic liver diseases and chronic HCV patients. They enrolled different other types of CLD patients including HBV, NAFLD, NASH primary biliary cirrhosis and alcoholic liver disease. Tsochatzis et al. [23] reported that, in patients with chronic HCV serum hepcidin levels correlated neither to liver hepcidin nor to serum iron or ferritin saturation levels. This can be explained by that, they excluded decompensated patients from their study.

In consistent with our study Fujita et al. [9,30] reported that in HCV patients serum hepcidin concentration was significantly correlated with hepatic iron stores suggesting that hepcidin may be affected by hepatic dysfunction. Experimental study of Nishina et al. [31] supported our findings, where they found that mRNA of hepcidin, a negative regulator in iron homoeostasis was decreased in cirrhotic mice and was not increased despite iron loading.

In agreement with our study Terrence et al. [25] reported the increase in the liver iron grade was associated with the increase in the fibrosis stage in patients with chronic hepatitis C.

In accordance to our results, Aoki et al. [32] stated that Among patients with hepatitis C, there was a significant correlation of hepcidin mRNA expression in the liver with hepatic iron concentration and serum ferritin. They added that, Hepcidin mRNA expression in the liver did not correlate with aspartate aminotransferase, alanine aminotransferase, HAI, or viral load. No differences in hepcidin mRNA were found based on viral genotype or the presence of fibrosis.

Another study performed by AbdElmonem et al. [33] reported that Hepcidin mRNA had a significant positive correlation with synthetic function of the liver (serum albumin and prothrombin concentration) and haemoglobin. In contrast, hepcidin mRNA was negatively correlated with parameters of iron stores as (serum ferritin and HII) and grade of liver fibrosis in both patient groups. They concluded that, The expression of hepcidin mRNA is decreased in liver tissues of CHC patients and more suppressed in the liver tissues of patients with HCC, suggesting that hepcidin expression appears to be appropriately responsive to iron status and disease progression in cirrhosis and hepatocarcinogenesis.

This study demonstrated that serum hepcidin decrease with progression of liver fibrosis and was lowest in those with histological cirrhosis and clinically proven cirrhosis. In contrast, serum ferritin increased progressively with increasing stages of fibrosis and was highest in those with histological cirrhosis and cirrhotic patients. These findings suggested an association between the presence and the extent of hepatic fibrosis and hepcidin

concentration. Tsochatzis et al. and Girelli et al. findings were consistent with our study, they revealed that in HCV patients, inverse relationship was found between serum hepcidin and the severity of histological lesion [23,24]. This study detected a significant positive correlation between serum hepcidin concentration and hepatic iron which was supported by other studies [9,30]. Hepcidin is known to inhibit iron efflux from macrophages by inducing internalization of ferroportin, an iron exporter [34]. Therefore, decrease hepcidin levels are expected to promote iron efflux from Kupffer cells which may result in predominant accumulation of iron in hepatocytes.

This study had a limitation that the number of patients with histological cirrhosis was small, and future studies for larger numbers of patients, would lend support to our findings.

5. CONCLUSIONS

Increasing hepatic fibrosis is associated with decreased hepcidin serum level. This indicates that hepcidin may serve as a potential biomarker for fibrosis and cirrhosis. Hepcidin is positively correlated with hepatic iron and liver enzymes but negatively correlated with the stage of fibrosis.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this study and accompanying images.

ETHICAL APPROVAL

An ethical approval from Tanta Ethical committee, Faculty of Medicine, was taken before starting our study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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