

Hepcidin and Chronic Hepatitis C Virus: Exploring the Controversy

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Abstract: Background and aim of the work: There are currently about 170 million hepatitis C virus (HCV) infected persons worldwide. Hepcidin has a central role in iron homeostasis, as it decreases iron release from macrophages and iron absorption from intestinal enterocytes, its correlation to chronic hepatitis C (CHC), hepatic iron load, and fibrosis is controversial between different studies. Thus, we aim to assess plasma hepcidin levels in CHC patients and to investigate the association of this molecule with iron parameters, hepatic inflammatory and fibrotic scores. **Patients and methods:** 38 CHC patients mean age 42.0 ± 7.4 years 34 males and 4 females (group 1) and 38 age and sex matched healthy controls (group 2) were included in the study. Both groups were subjected to blood tests for serum ferritin, serum iron, serum hepcidin, complete blood picture (CBC). Group 1 patients were subjected to ultrasound guided liver biopsy for assessment of hepatic activity and fibrosis scores according to METAVIR as well as hepatic iron stores. **Results:** Mean serum hepcidin and ferritin levels were significantly higher among cases with HCV compared to controls (77.4 ± 48.5 ng/ml vs 43.9 ± 22.9 ng/ml $p=0.000$ and 165.9 ± 120.9 ng/ml vs 98.5 ± 77.6 ng/ml $p=0.005$, respectively). There is a significant positive correlation between serum hepcidin level and serum ferritin level ($r=0.563$), Metavir fibrosis score ($r=0.666$), Metavir activity scores ($r=0.36$) and hepatic iron stores ($r=0.378$). Serum Hepcidin was significantly higher among CHC patients with fibrosis stage (F3-F4) than those with fibrosis stage (F0-F2) (117.4 ± 51.4 ng/ml vs 51.3 ± 21.9 ng/ml respectively $p=0.000$), also it was significantly higher among CHC patients with activity scores (A2-A3) than among those with activity score (A0-A1) (200.6 ± 117.2 vs 153.6 ± 121.9 respectively $p=0.03$). Serum Hepcidin could predict stage 3 fibrosis at a cut off level of 73.8 with a sensitivity of 80% and specificity of 83%. **Conclusion:** Serum Hepcidin is significantly higher among CHC patients than normal controls and is positively correlated to stage of hepatic fibrosis, grade of hepatic inflammation and serum ferritin

Key words:

INTRODUCTION

Hepatitis C is a major worldwide health problem affecting 170 million persons all over the world which accounts for about 3% of the global human population (Wasmuth, 2009). Pathological iron deposits have been observed in about 50% of patients with chronic HCV infection (KO *et al.*, 2007) which affects the course of HCV infection through various mechanisms, where iron negatively affects cell-mediated immune pathways weakening Th1-mediated effector mechanisms (Weiss *et al.*, 1999, Recalcati *et al.*, 1998) and induces hepatotoxicity through oxygen free radicals. Moreover, iron deposition within Kupffer cells and oxidative stress enhances the release of profibrogenic and proinflammatory cytokines, that activate the stellate cells to produce collagen and other extracellular matrix components resulting in fibrosis (Pietrangolo, 1998). The underlying mechanisms of the hepatic iron accumulation in HCV-infected liver are still poorly understood. Hepcidin is a polypeptide synthesized mainly in the liver and found in the serum and urine, while measurable amounts of hepcidin mRNA and protein are found in cells and tissues other than liver as heart, kidney, retina, monocytes and macrophages, splenocyte and alveolar cells, stomach, adipocytes, prostate gland, tonsils, salivary gland, trachea and pancreatic β -cells. (Theurl *et al.*, 2008). Hepcidin plays a central role in iron homeostasis, as it decreases iron release from macrophages and iron absorption from intestinal enterocytes through its binding to the iron exporter protein, ferroportin. (Xia, 2008). Several studies revealed decreased hepcidin levels and hepcidin/ferritin ratio among HCV infected patients as compared to controls (Girelli *et al.*, 2009, Fujita *et al.*, 2008; Franchini *et al.*, 2008) and increased levels of transferrin receptor 2 (which is located on the hepatocyte

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membrane and is involved in the uptake of iron by hepatocytes) ((Kageyama *et al.*, 2000) resulting in increased iron delivery to hepatocytes from macrophage iron stores and intestinal mucosa (Franchini *et al.*, 2008).

Aim of the Work:

To assess plasma hepcidin levels in chronic hepatitis C patients and to investigate the association of this molecule with iron parameters, histological activity index, and liver fibrosis scores.

Patients and Methods:

Study Design:

This is a case control cross sectional prospective study.

Sample Size Calculation:

Based on mean serum hepcidin 83.7 ± 21.5 in CHC patients and 90.9 ± 76.1 in controls in the study done by Girelli *et al.* (Girelli *et al.*, 2009) the minimum required sample size is 60 individuals divided into 30 patients and 30 controls.

Alpha error 1% and power of the study 90%.

After having the ethical committee approval, the study was carried on throughout the period from January 2011 to January 2012, in collaboration between Tropical Medicine Department Ain Shams University Hospital and National research center (Internal Medicine Department/ Clinical and Chemical Pathology Department/Pathology Department) according to the Good Clinical Practice(GCP) guidelines. Known chronic HCV patients followed up at the Tropical medicine department of Ain Shams University & the Hepatology and gastroenterology out patient clinic of Doaah hospital were screened where 40 patients (group 1) were included according to the following inclusion/exclusion criteria after signing an informed consent

Inclusion:

- 1.Age: 20-60 years old.
- 2.Gender: Both sexes.
- 3.Chronic hepatitis C virus infection as proven by PCR.

Exclusion:

- 1.Decompensated cirrhotic patients.
- 2.History of alcohol or drug abuse.
- 3.Patient with known other chronic liver diseases: hemochromatosis, 4.Wilson's disease, autoimmune hepatitis.
- 5.Malignancy of whatever nature.
- 6.Systemic failure (Renal, Cardiac, Respiratory..etc).
- 7.History of standard of care for HCV infection.
- 8.Concomitant HBV infection.
9. History of organ transplantation.
10. Anemia (hemoglobin < 13 in men & < 11.5 in women).
11. Vitamins or iron supplementations.

In addition to 40 age and sex matched apparently healthy subjects as a control group (group 2). 2 patients and 2 controls were excluded due to hypoferritinemia despite normal CBC and serum iron, as an early stage of iron deficiency anaemia could not be ruled out. Thus, we were left finally with 38 patients in group 1 of mean age 42.0 ± 7.4 years 34 males and 4 females and 38 age and sex matched controls in group 2.

Group 1 was subjected to complete history taking and thorough clinical examination. Both groups were subjected to the following laboratory analysis performed at clinical & chemical pathology department of the National research center:

- 1.Complete blood picture measured with cell DYN 900 electronic counter.
- 2.Serum iron & ferritin.
- 3.Serum hepcidin.
- 4.Serum HCV-Antibodies.

5.Ultrasound guided liver biopsy was done for group 1 in Tropical Department Ain Shams University & Radiology Department of Doaah hospital then examined histopathologically in the Pathology Department of the National research center for determination of the following:

Activity index and fibrosis scores according to Metavir ((The French METAVIR Cooperative Study Group 1994).

Hepatic iron deposits according to Deugnier *et al* score (Deugnier *et al.*, 1993).

Methods:

1-Laboratory Methods:

10 ml blood samples were collected after an overnight fasting from controls & patients (on the liver biopsy day) and were divided into EDTA tube for complete blood picture & plain vacutainer for serum separation. Sera were stored frozen in duplicate Eppendorf aliquots at -80 °C until analysis to avoid freezing and thawing. The following tests were performed:

A-Serum HCV-Antibody:

The presence of serum HCV-Antibodies was measured for all the normal controls using commercially available kits (PPC Pharm-Tec-GmbH, HCV Enzyme immunoassay Kit catalog #: EL-2048) according to the manufacturer's instructions

B-Serum iron:

It was done by commercially available kits (Stanbio laboratory, Stanbio Iron and Total Iron Binding Capacity, procedure NO.0370). The procedure was done according to the manufacturer's instructions with normal values ranging for male (65-170) g/dl & females (50-170) g/dl.

C-Serum Ferritin:

By ELISA technique using commercially available kits (Calbiotech Catalog No. FR065T 96 Test) according to the following assay procedure:

Prior to assay, reagents were allowed to stand at room temperature and gently mixed before use.

Then:

1. 10 l was pipetted of Ferritin standards, control and patient's sera.
2. 100 l of enzyme conjugate was added to all wells.
3. The plate was covered and incubated for 60 minutes at room temperature (18-26°C).
4. Liquid was removed from all wells, and then wells were washed three times with 300 l of 1X wash buffer and blotted on absorbent paper towels.
5. 100 l of TMB substrate was added to all wells.
6. Wells were Incubated for 10 minutes at room temperature.
7. 50 l of stop solution was added to all wells.
8. We Read absorbance on ELISA Reader at 450 nm within 15 minutes after adding the stopping solution.

D-Serum Hcpidin:

Serum hepcidin was measured by ELISA technique using commercially available kits (Uscn Life Science Inc. Catalog No. E91979Hu 96 Tests) according to the following assay procedure:

1. 100µL of blank, samples and standard dilutions were added into the appropriate wells then Incubated for 2 hours at 37°C.
2. The liquid of each well was removed without wash.
3. 100µL of Detection Reagent A was added then incubated for 1 hour at 37°C.
4. Liquid was aspirated and washed 3 times.
5. 100µL of prepared Detection Reagent B was added then incubated for 30 minutes at 37°C.
6. Liquid was aspirated and washed 5 times.
7. 90µL Substrate Solution was added then incubated 15-25 minutes at 37°C.
8. 50µL of Stop Solution was added to each well. Then, the microplate reader was run and conducted measurement at 450nm immediately.

N.B:

Sample dilutions were taken in consideration when calculating the results by multiplying the dilution factor (which was 100 fold dilutions in the present study) by the concentrations read from the standard curve.

2-Histological Methods:

All liver biopsies had an adequate specimen of 1 cm in length and were blindly evaluated by a single liver histopathologist.

Liver biopsies were fixed in 10 % formalin solution for 24 hours then processed in ascending grades of ethyl alcohol (70%-90%-100%), then in xylene and wax for preparing paraffin blocks.

The paraffin wax sections were cut at 5 microns. Then the following stains were applied:

A-Hematoxylin And Eosin Stain:

For assessing the severity and the activity of histological lesions by a pathologist blinded to the clinical data according to the classification system Metavir. ((The French METAVIR Cooperative Study Group 1994)

B-Perl's stain:

The Hepatic iron score was assessed by a pathologist blinded to both clinical data and to the results of severity and activity of the histological lesions using the system proposed by Deugnier and co-workers in 1993 that was well validated in both hemochromatotic and nonhemochromatotic iron overload disorders. Iron deposits were assessed according to both their amount and their cellular and lobular location in Rappaport's acinus, (Rappaport 1980) using the grading of (Deugnier *et al.*, 1993) modified in order to take into account the heterogeneity of iron distribution. We classified hepatic iron scores according to Turlin and Deugnier (Turlin and Deugnier 1997) into mild, moderate and severe (47.4, 26.3 and 26.3 respectively)

Statistical Methods:

The data was collected, coded and entered to a personal computer (P.C.) IBM compatible 2.6 GHZ. The data was analyzed with the program (SPSS) statistical package for social science under windows version 11.0.1.

Student T test was used to compare means, Pearson Correlation coefficient (r) test: to indicate the extent that two variables change with one another in a linear fashion. One way Analysis of variance (ANOVA) was used for comparison between multiple groups with Quantitative continuous variables. Least significant difference test: (LSD) was used after we determine that differences exist among the means by ANOVA test, post hoc range tests and pair wise multiple comparisons can determine which means differ. Chi square test to determine the extent that a single observed series of proportions differs from a theoretical or expected distribution of proportions or the extent that two or more series, proportions or frequencies differ from one another based on the chi-square distribution. Linear regression was used to estimate the coefficients of the linear equation, involving one or more independent variables.

Ethical Considerations:

This study was conducted according to the GCP guidelines.

Results:

On comparing the general characteristics of the 2 groups table (1) group 1 showed a statistically significant higher mean MCV, MCH and Haemoglobin than group 2 while the later showed statistically significant higher mean RBCs and platelets counts. Group 1 showed significantly higher mean Hepcidin level than group 2 (77.4±48.5 ng/ml vs 43.9 ±22.9 ng/ml respectively p=0.000). There is no statistically significant difference between the two studied groups as regards the mean serum iron, but there was a significantly higher mean serum ferritin among group 1 than group 2 (165.9±120.9 ng/ml vs 98.5 ±77.6 ng/ml respectively p=0.005).

On breaking both groups according to ferritin levels into 4 quartiles each, we found a significantly higher mean serum hepcidin level among second and fourth quartile groups among group 1 as compared to the same quartiles in group 2 (79.7 ±37.5 ng/ml vs 44.3±17.8 ng/ml p=0.01 and 114.5±58.3 ng/ml vs 57.2 ±13.0 ng/ml p=0.01 respectively) as shown in table 2.

We studied the correlation between serum Hepcidin and different studied parameters within group 1 (table 3) where we found a highly significant positive correlation between serum hepcidin level and ;serum ferritin levels, Metavir fibrosis and activity scores and hepatic iron score.

Table 1: General characteristics of the 2 studied groups

	Group 1 N=38	Group 2 N=38	Test T, x ²	P value
Age	42(±7.4)	39.4(±7.7)	T 1.5	0.1
Gender				
Male	34(89.5%)	33(86.8%)	x ² 0.1	0.7
Female	4(10.5%)	5(13.2%)		
Blod indices				
RBCs(10 ⁶ /ml)	5.0(±0.4)	7.1(±2.1)	T 5.9	0.000**
WBCs(10 ³ /ml)	6.3(±1.8)	4.8(±0.4)	T 4.7	0.000**
MCV(fl)	86.0(±5.2)	82.1(±3.6)	T 3.8	0.000**
MCH(pg/cell)	29.41(±.7)	28.6(±1.2)	T 2.3	0.02*
MCHC(g/dl)	34(±1.4)	34.6(±1.6)	T 1.5	0.1
Hemoglobin(g/dl)	14.7(±1.4)	13.9(±1.0)	T 2.7	0.000**
Platelets(10 ³ /ml)	210.25(±7.5)	278.7(±71.0)	T 4.6	0.000**
Hepcidin(ng/dl)	77.4(±48.5)	43.9(±22.9)	T 3.8	0.000**
Serum Iron(ug/dl)	136.1(±86.2)	118.1(±99.9)	T 0.8	0.4
Ferritin(ng/dl)	165.9(±120.9)	98.5(±77.6)	T 7.5	0.005**
Hepcidin/Ferritin	0.58(±0.3)	0.60(±0.4)	T 0.1	0.8

RBCs= Red Blood cells, MCV=mean corpuscular volume, MCH=mean corpuscular hemoglobin, MCHC=mean corpuscular hemoglobin concentration in, WBCs=white blood cells .

Table 2: Comparison of groups 1 and 2 Ferritin quartiles as regards Hcpidin levels

	Group1	Group 2	t	p
Ferritin Q1 (ng/ml)	(0-79.1)	(0-42.07)	0.9	0.3
Hcpidin (ng/ml)	33.7(±11.4)	25.7(±21.7)		
Ferritin Q2 (ng/ml)	(79.11-120.5)	(42.08-83.8)	2.6	0.01*
Hcpidin (ng/ml)	79.7(±37.5)	44.3(±17.8)		
Ferritin Q 3 (ng/ml)	(120.5-214.1)	(83.81-118.6)	2.1	0.05
Hcpidin (ng/ml)	81.2 (±42.8)	47.9 (±27.2)		
Ferritin Q 4 (ng/ml)	(214.11-459.6)	(118.61-383.4)	2.8	0.01*
Hcpidin (ng/ml)	114.5(±58.3)	57.2(±13.0)		

Table 3: Correlation coefficient between serum hepcidin level and other studied parameters among group 1:

Serum iron	r= 0.216 p=0.06
Serum ferritin	r=-0.563 P=0.000**
Hepatic Activity (Metavir A)	R=0.360 P=0.02*
Hepatic Fibrosis (Metavir F)	R=0.666 P=0.000**
Hepatic iron score	R=0.378 P=0.01*
Haemoglobin	R=0.121 P=0.4

Table 4: Comparison between grade of liver activity according to Metavir & mean hepcidin level, ferritin & hepcidin ferritin ratio (HF ratio):

	Activity(0-1) N=28 Mean(±SD)	Activity (2-3) N=10 Mean(±SD)	T	P
Hcpidin ng/ml	153.6(±121.9)	200.6(±117.2)	2.1	0.03*
Ferritin ng/ml	67.6(±41.1)	104.9(±59.0)	1.0	0.2
HF ratio	0.58(±0.2)	0.59 (±0.3)	0.1	0.8

HF ratio: Hcpidin to ferritin ratio

Table 5: Comparison between liver fibrosis and mean hepcidin level, ferritin and HF ratio according to Metavir:

	Fibrosis (0 to 2) N=23 Mean(±SD)	Fibrosis (3 to 4) N=15 Mean(±SD)	T	P
Hcpidin ng/ml	51.3(±21.9)	117.4(±51.4)	5.4	0.000**
Ferritin ng/ml	129.6 (±108.3)	221.6(±121.5)	2.4	0.02*
HF ratio	0.56 (±0.2)	0.62 (±0.3)	0.5	0.5

HF ratio: Hcpidin to ferritin ratio

Table 6: Sensitivity and specificity of serum hepcidin level in detection of fibrosis scores of Metavir F3-F4 among group 1 :

S.hepcidin ng/ml	Sensitivity	Specificity
61.2	100	74
62.9	93	74
68.2	86	79
73.8	80	83
	Area under the curve	95% Confidence interval
Serum hepcidin	0.916	0.831-1.00

We found a significantly higher mean serum hepcidin level among HCV cases with moderate to severe Metavir hepatic activity(A2-A3) compared to cases with no to mild activity(A0-A1) (153.6 ± 121.9 ng/ml vs 200.6 ± 117.2 ng/ml respectively p=0.03) (table 4) while serum ferritin did not differ significantly between the two hepatic activity groups. Also, our results showed a highly significant mean serum hepcidin and ferritin levels among cases with higher Metavir hepatic fibrosis score(F3-F4) compared to cases without or early fibrosis(F0-F2) (117.4± 51.4 ng/ml vs 51.3±21.9 ng/ml p=0.000 and 221.6(±121.5) ng/ml vs 129.6 (±108.3) ng/ml p=0.02 respectively)(table 5) .Finally, we tested Hcpidin as a marker for fibrosis (table 6) that showed a cut off value of 61.2 ng/ml of hepcidin could be a fibrosis marker with the highest sensitivity (100%) while a hepcidin value of 73.8 ng/ml carries the highest specificity(83%) for fibrosis.

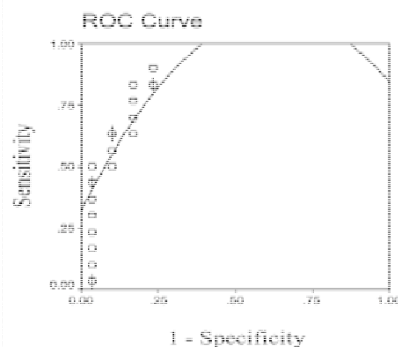


Fig. 1: ROC curve for sensitivity and specificity of serum hepcidin level in detection of fibrosis scores of Metavir F3 or above among group 1:

Discussion:

The main objectives of this study was to assess plasma hepcidin levels in CHC patients and to investigate its association with iron parameters, histological activity index, and liver fibrosis scores.

Serum hepcidin was significantly higher in the CHC group than the control group which could be attributed to two factors; inflammation & iron stores. Inflammation & infection increases hepcidin synthesis through inflammatory cytokines mainly IL-6 (Ganz, 2006) where a positive correlation was found between inflammatory markers and serum hepcidin (Tsochatzis *et al.*, 2010) and between prohepcidin and IL-6 (Lee *et al.*, 2010), besides, IL-6 was found to be the necessary and sufficient cytokine for the induction of hepcidin during inflammation (Nemeth *et al.*, 2004). Serum IL-6 levels are significantly elevated in CHC patients compared to healthy controls (Migita *et al.*, 2006) owing to the fact that HCV induces IL-6 production via inducing Toll-like receptor 4 expression *in vitro* (Machida *et al.*, 2006) or Toll-like receptor 2 expression *in vivo* (Feldman *et al.*, 2006). Although IL-6 was not evaluated in our study, yet the positive correlation between hepcidin and Metavir histological activity we found is worth noting as the Metavir activity score reflects the inflammatory liver condition.

On the other hand, HCV inhibition to hepcidin was found in several studies (Nagashima *et al.*, 2006, Fujita *et al.*, 2008, Girelli *et al.*, 2009, Nishana *et al.*, 2008, Miura *et al.*, 2008), yet, these were criticized for not taking into account the effect of inflammation (Trinder *et al.*, 2008), which in CHC patients may counteract ROS-induced hepcidin suppression through the known hepcidin upregulation by proinflammatory cytokines, particularly IL-6 (Wrighting *et al.*, 2006 and Verga Falzacappa *et al.*, 2007). Tsochatzis and his co-workers in 2010 had a midway view seeing that HCV infection down-regulates serum hepcidin, while increasing inflammation and/or fibrosis tend to restore its levels and that further studies, with possible determination of oxidative stress markers, are needed to better understand these correlations (Tsochatzis *et al.*, 2010). Serum hepcidin correlated positively with ferritin in this study, which accords with other studies that found ferritin correlated positively with mRNA hepcidin (Aoki *et al.*, 2005, Fujita *et al.*, 2007 and Fujita *et al.*, 2008) and with serum hepcidin (Sugimoto *et al.*, 2009), however some studies found no correlation, between ferritin and prohepcidin (Lin *et al.*, 2009 and Olmez *et al.*, 2010) nor between ferritin and serum hepcidin (Tsochatzis *et al.*, 2010). Moreover, in 2006 Nagashima and his group (Nagashima *et al.*, 2006) found that prohepcidin had negative correlation with serum ferritin. The current study showed a significantly higher serum ferritin level among the CHC group as compared to controls, but there was no significant difference between both groups as regards serum iron, or hepcidin/ferritin ratio. In an attempt to correlate the Hepcidin difference between both groups to ferritin levels we stratified ferritin into four quartiles (Qs) within each group in an ascending ferritin level fashion from Q1 to Q4 where Hepcidin was found to be significantly higher in the CHC subgroups Q2 and Q4. In the CHC group serum hepcidin levels didn't correlate with their hemoglobin values which agrees with several other studies (Aoki *et al.*, 2005, Lin *et al.*, 2009 and Nagashima *et al.*, 2006). In contrast to the results found by Sugimoto and co-workers (Sugimoto *et al.*, 2009) and Girelli and co-workers (Girelli *et al.*, 2009) that there was correlation of serum prohepcidin with serum iron, the CHC group in this study showed no correlation between serum hepcidin and serum iron which was confirmed also by many other studies (Fujita *et al.*, 2008; Jaroszewicz *et al.*, 2010, Nagashima *et al.*, 2006, Olmez *et al.*, 2010 and Tsochatzis *et al.*, 2010). As much as the correlation between Hepcidin and fibrosis has been a subject to many studies it has been as well a matter of controversy. While Tsochatzis *et al.* found a positive correlation between serum hepcidin and fibrosis up to finding hepcidin as an independent predictor of fibrosis (Tsochatzis *et al.*, 2010) other studies found fibrosis negatively correlated with prohepcidin (Nagashima *et al.*, 2006 and Olmez *et al.*, 2010) and mRNA hepcidin (Abdelmonem *et al.*, 2009). To add to the complexity, other studies didn't find any correlation between fibrosis and mRNA hepcidin or with serum hepcidin (Aoki *et al.*, 2005, Fujita *et al.*, 2007). The current study shows a significant association between Hepcidin and the highest hepatic inflammatory and fibrosis scores (A2-A3 and

F3-F4) so, this finding in addition to Tsochatzis' (Tsochatzis *et al.*, 2010) consideration of Hepcidin as a fibrosis predictor tempted us to search for a Hepcidin cut-off value for predicting fibrosis which we found it to be a serum level of 73.8ng/ml that can predict Metavir Fibrosis stage 3 with sensitivity 80% and specificity 83%. In this study we excluded decompensated CHC patients which differs from other studies (Jaroszewicz *et al.*, 2008 and Nagashima *et al.*, 2006) that included cirrhotics and decompensated CHC patients, this hindered us from concluding the relation between serum hepcidin and cirrhosis whether compensated or not. Based upon our findings we strongly recommend further studies on serum and mRNA hepatic Hepcidin in different cohorts representing phases of the natural history of CHC. Also, we recommend putting into consideration the extrahepatic sources of Hepcidin that has not been estimated in almost all studies, and finally we find it quite reasonable to invest in research evaluating Hepcidin as a fibrosis marker.

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