

HEPCIDIN SERUM LEVELS IN HCV CHRONICALLY MONO-INFECTED NAÏVE PATIENTS, COMPARED TO HEALTHY INDIVIDUALS

Ari Ben-Hur Stefani Leão^{1,2}, Maria da Graça Ferronato²,
Carlos Kupski¹, Fernando Comunello Schacher¹,
Denise Cantarelli Machado³, Mário Reis Álvares-da-Silva^{2,4}

ABSTRACT

Introduction: Metabolism of iron is altered in patients infected with chronically Hepatitis C. The aim of this study is to compare the hepcidin levels in between individuals chronically infected with HCV and uninfected individuals. The aim of this study is to compare the hepcidin serum levels between individuals chronically infected with HCV and uninfected individuals.

Methods: A cross-sectional study evaluating hepcidin serum levels of mono-infected HCV (n=29), naive, non-diabetic, non-cirrhotic and non-obese patients by means of ELISA, compared to uninfected patients (n=9) with the same characteristics. The degree of liver fibrosis, according to the METAVIR scale on liver biopsies, the lipid profile, the resistance insulin level, as calculated on HOMA-IR (homeostatic model assessment for insulin resistance), the interleukin-6 (IL-6) and the ferritin serum levels were also measured.

Results: The levels of hepcidin were significantly lower in HCV patients compared to controls (8.4 pg/mL (± 4.94) vs. 19.51 pg/mL (± 5.51)) with $p < 0.001$. The levels of ferritin and hepcidin did not show any relation. There was no difference between hepcidin levels in relation to viral genotype, viral load, IL-6 and degrees of fibrosis within HCV infected individuals.

Conclusion: It is possible that hepatic iron overload in this population is explained by suppressed levels of hepcidin in patients with HCV.

Keywords: *Hepcidin; HCV; interleukin-6 (IL-6); mono-infected*

Iron is an essential ion for many cellular metabolic processes in the human body, participating in processes of hematopoiesis, DNA synthesis and mitochondrial respiration. On the other hand, its excess can stimulate cellular damage by promoting oxidative stress^{1,2}. This element of homeostasis depends primarily on hepcidin, a polypeptide hormone with 25 amino acids synthesized in the liver, which controls its absorption, storage and recirculation^{2,3}. Chronic hepatitis C virus (HCV) infection is a major global public health problem. It is estimated that the RNA virus of the *Flaviviridae* family and *Hepacivirus*⁴⁻⁷ genus chronically infects about 170 million people, and about 20% of these will progress to cirrhosis and its consequences^{8,9}. In individuals with HCV, 30-40% have high iron, ferritin and transferrin saturation serum levels¹⁰⁻¹². The increased cellular and systemic iron levels have been associated with a worse prognosis in these patients¹¹⁻¹³. At present, the HCV is a known activator of several signaling pathways that cause the production of reactive oxygen species (ROS) leading to oxidative stress in both hepatocytes and liver macrophages¹⁴. The exact mechanisms by which HCV influences the iron homeostasis modulation are still unknown and its influence on the production of hepcidin remains controversial². At the molecular level, there is a link between hepcidin and ferroportin, a transmembrane protein expressed in enterocytes and macrophages, which is responsible for iron efflux. Their association culminates with the internalization and degradation of ferroportin, reducing iron

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1 Departamento de Gastroenterologia e Endoscopia, Hospital São Lucas, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS). Porto Alegre, RS, Brasil.

2 Programa de Pós-graduação em Gastroenterologia e Hepatologia, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul (UFRGS). Porto Alegre, RS, Brasil.

3 Laboratório de Biologia Celular e Molecular, Instituto de Pesquisa Biomédica, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS). Porto Alegre, RS, Brasil.

4 Departamento de Hepatologia, Hospital de Clínicas de Porto Alegre (HCPA). Porto Alegre, RS, Brasil.

Corresponding author:

Ari Ben-Hur Stefani Leão
absleao@hotmail.com
Hospital São Lucas, Pontifícia
Universidade Católica do Rio Grande do
Sul (PUCRS)
Av. Ipiranga, 6690.
90610-000, Porto Alegre, RS, Brasil.

entry in the plasma compartment^{15,16}. Factors such as iron reserve, hypoxia, anemia and inflammatory processes influence this metabolic process¹⁵⁻¹⁷, therefore we included only non-diabetic and non-obese patients with low degrees of hepatic fibrosis by the METAVIR system (an international validated scale for assessing the degree of fibrosis in patients with Hepatitis C) to reduce the confounding factors. In this study, we compared the hepcidin serum levels between individuals chronically infected with HCV and uninfected individuals.

METHODS

Patients

This study was approved by the Research Ethics Committee of the Institution, and the individuals signed the free informed consent form. A cross-sectional study was conducted at the Hospital de Clínicas de Porto Alegre (HCPA), a tertiary hospital in southern Brazil, between March 2011 and December 2013, comparing the assessment of liver fibrosis on liver biopsies through METAVIR score¹⁸, serum hepcidin levels, lipid profile, ferritin values of homeostasis model for insulin resistance (HOMA-IR), body mass index (BMI), serum levels of IL-6, and demographic variables among 29 HCV mono-infected patients, naïve, with low degrees of hepatic fibrosis score, and 9 uninfected patients, aged 18-60. Virus-related variables such as viral load and genotype and their relation with the expression of hepcidin were also analyzed. Patients with clinical, laboratory and or histopathological evidence of liver disease not associated with HCV, severe chronic diseases, obesity (Body Mass Index, BMI ≥ 30 Kg/m²), excessive alcohol consumption in the last six months (up to 40g/day), diabetes, and users of corticosteroids and/or other immunosuppressant or lipid-lowering drugs were excluded. Patients with current malignant disease, active opportunistic infections, transplanted, and pregnant women were also excluded. In the uninfected group were included HCV-uninfected individuals aged 18-60, regardless of sex and using the same factors of exclusion of infected ones.

Hepcidin and Interleukin-6 quantitation

The hepcidin serum level was performed by collecting blood from a peripheral vein (cubital), with pre-established fasting for 12 hours.

The blood was centrifuged immediately after collection and the serum samples stored at -20 °C, then transferred to a -80 °C freezer up to hepcidin measurement. The hepcidin levels were determined using the ELISA kit for human Hep C® (Uscn Life Science Inc., Wuhan, China), and all samples analyzed in duplicate. The IL-6 serum level was determined using the ELISA Kit Human IL-6

(Invitrogen™, Camarillo, USA) also in duplicate. The HOMA-IR dosages of fasting glucose in mg/dL were calculated and serum insulin was considered in mU/mL. The final value of the HOMA-IR was obtained by multiplication between serum insulin by fasting glucose divided by the constant 405. Insulin resistance was defined as HOMA-IR > 2.7, according to the Brazilian threshold¹⁹. The viral detection and determination of viral load was performed by real-time polymerase chain reaction in peripheral blood with a detection limit of 43 IU/mL. Genotyping was determined by RT-PCR with reverse hybridization (Roche™). The METAVIR^{19,20} score was analyzed in samples with 8 or more portal tracts by an expert pathologist. The biochemical tests were conducted in laboratory routine in the tertiary center and the anthropometric measurements using calibrated and measured equipment by the Brazil National Institute of Metrology, Quality and Technology (INMETRO).

Statistical Analysis

Data were entered into Excel and then exported to SPSS version 18.0 for statistical analysis. Categorical variables were described as frequencies and percentages. Quantitative variables with normal distributions were described by mean \pm SD and those with asymmetric distribution were described with the median and interquartile range. Categorical variables were compared using Fisher's exact test. Quantitative variables with normal distributions were compared between groups by Student's *t* test for independent samples. Variables with asymmetric distribution were compared between groups by Mann-Whitney to evaluate the correlation between variables; the Pearson correlation coefficient was used (we used the normal distribution according to the usual range worldwide. Therefore, we use the Pearson correlation). Due to the lack of previous evidence according to the relationship between ferritin and hepcidin, it was obtained a convenience sample in a 3 cases: 1 control ratio. A significance level of 5% was considered for the established comparisons.

RESULTS

Participants included a total of 38 individuals, 29 patients chronically infected with HCV and 9 uninfected. Demographic and clinical characteristics of the individuals analyzed did not differ significantly in sex, race, BMI, lipid profile (expressed by quantifying the levels of total cholesterol and triglycerides), values of HOMA-IR and serum ferritin levels. There was a significant difference in the age groups having the infected group presented a mean age of 46.9 (± 7.07) years versus 33.8 (± 5.2) years (Table 1). The cases had a IL-6 median of 33.8 pg/mL (interquartile range: 0.01 to 66.5 pg/mL)

Table 1: Demographic data of patients chronically infected with Hepatitis C.

VARIABLE	N (%)	HEPCIDIN (pg/ml)	p
SEX	29 (100)		0.803
MALE	16 (55)	8.18 (± 5.17)	
FEMALE	13 (45)	8.66 (± 4.65)	
BMI (kg/m²)	29 (100)		0.258
<25	17 (59)	9.2 (± 5.7)	
≥25 and <30	12 (42)	7.1 (± 3.47)	
HOMA-IR	27 (100)		0.727
<2.71	23 (85)	7.66 (± 4.01)	
≥2.71	4 (15)	8.41 (± 3.27)	
TOTAL CHOLESTEROL (mg/dl)	27 (100)		0.740
<200	24 (89)	8.73 (± 5.29)	
≥200	3 (11)	7.71 (± 3.31)	
TRYGLICERIDES (mg/dl)	29 (100)		0.684
<150	26 (89)	8.53 (± 5.29)	
≥150	3 (11)	8.27 (± 3.31)	
VIRAL GENOTYPE	29 (100)		0.550
GENOTYPE 1	19 (65)	8.8 (± 5.17)	
GENOTYPE 2 AND 3	10 (35)	7.62 (± 4.05)	
GENOTYPE 2	2 (7)		
GENOTYPE 3	8 (28)		
VIRAL LOAD (UI/ml)	23 (100)		0.610
<600.000	16 (70)	5.93 (± 2.08)	
≥600.000	7 (30)	8.42 (± 3.89)	
METAVIR	29 (100)		0.742
F0	8 (27.5)	7.21 (± 4.22)	
F1	20 (69)	8.84 (± 5.35)	
F2	1 (3.5)	-	

and the controls 41.6 pg/mL (interquartile range 23.5 to 75.0 pg/mL), with no statistically significant difference between the groups (p=0.460).

The serum levels of hepcidin in patients with Hepatitis C virus was of 8.4 pg/mL (± 4.94) vs. 19.5 (±5.51) in uninfected individuals with p<0.001. There was no statistically significant difference in the analysis of hepcidin-infected population levels in relation to sex, BMI (stratified as <25 and ≥25 and <30 kg/m²), viral load (stratified <600,000 IU/mL and ≥600,000 IU/mL), viral genotype (stratified on genotype 1 and non-1), levels of HOMA-IR (stratified as <2.71 and ≥2.71) and degrees of fibrosis METAVIR classification (stratified in fibrosis grade 0, 1, and 2) (Table 2). In both groups, the correlation between hepcidin and ferritin levels was not statistically significant: infected r=0.337, p=0.074 and uninfected r=0.275, p=0.475 (Figure 1). The relation between IL-6 and hepcidin level of the

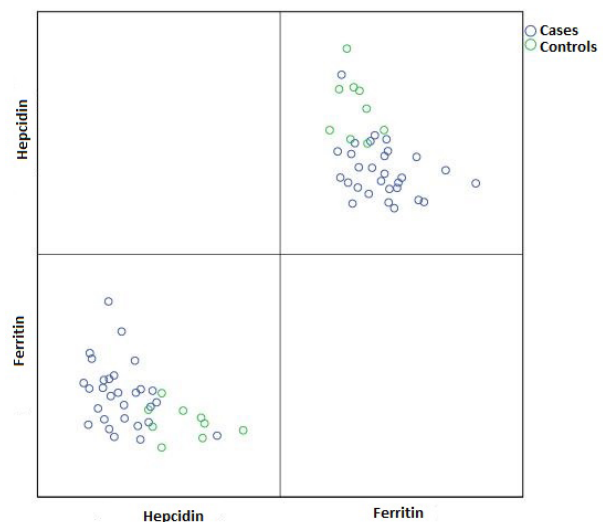


Figure 1: Correlation between hepcidin and ferritin levels.

Table 2: Group demographic data.

VARIABLE	INFECTED (N=29)	UNINFECTED (N=9)	P
SEX			0.273
MALE	19 (55.2%)	7 (77.8%)	
FEMALE	13 (45.8%)	2 (22.2%)	
AGE (YEARS)	46.9 (\pm 7.07)	33.8 (\pm 5.2)	< 0.001
COLOR (WHITE)	27 (93.1%)	9 (100%)	0.989
TOTAL CHOLESTEROL (mg/dl)	174.2 (\pm 36.9)	223.2 (\pm 48.2)	0.303
TRYGLICERIDES (mg/dl)	92 (66.5-112.5)	125 (88-239)	0.074
HOMA-IR	1.74 (1.28-2.27)	1.48 (1.18-2.08)	0.72
FERRITIN	372.3 (\pm 132.4)	259.3 (\pm 67.3)	0.125
BMI (Kg/m²)	24.2 (\pm 2.42)	23.9 (\pm 2.48)	0.707
IL-6	33.8 (0.01-66.5)	41.6 (23.5-75)	0.460

infected group did not show a statistically significant relation ($r=-0.17$ $p=0.440$).

Former figure 1 was excluded due to irrelevant necessity.

DISCUSSION

This study attempted to reduce the confounding factors related to the expression of hepcidin, selecting infected and non-infected patients based on the exclusion of hepcidin expression modulating factors. This strategy aimed to assess the relationship of chronic infection by HCV and its influence on serum expression of hepcidin in the most reliable way. The selection of patients with low degrees of fibrosis (METAVIR) comes from the fact that patients with cirrhosis present a proinflammatory state with higher circulating levels of IL-6 leading to a higher hepcidin expression, as well as the obese, diabetic, alcoholic beverages users and those with chronic, neoplastic and/or infectious diseases.

We chose to study naïve patients to avoid any interference with the natural course of the infection. There was no difference in race, sex and BMI between the two groups, supporting the assertion that this is a homogeneous group, reducing the effect of any confounding factor. Regarding the lipid profile analyzed, the serum levels of total cholesterol and triglyceride groups, were similar, as were the values of HOMA-IR. In relation to the ferritin levels there was no statistically significant difference between the two groups, evidencing only a tendency to higher levels of ferritin in individuals with chronic hepatitis C virus infection. This finding may be related to the type II error (β) in the sample size and/or is a consequence of infected patient samples present only in early stages of liver fibrosis, thus suggesting shorter time of disease evolution and, consequently, lower levels of hepatic accumulation of iron. Regarding hepcidin, it became evident that the group chronically infected with HCV showed suppressed hormone levels compared

to the uninfected group. This result confirms the theory that the hepatitis C virus per se modulates the expression of hepcidin directly influencing the iron recirculation mechanism and assisting in hepatic iron accumulation, increasing oxidative stress and thereby contributing to liver fibrogenesis as suggested in previous studies, both in animals and humans²⁰⁻²². Girelli et al. examined the expression of serum hepcidin levels by specific ELISA, analyzing 57 uninfected and 81 HCV patients. They demonstrated that hepcidin levels were significantly reduced in individuals carrying the virus²².

Nevertheless, this study included all degrees of fibrosis, and may have underestimated the true expression of hepcidin, since there is a lower mass of viable hepatocytes for the production of hepcidin and a higher accumulation of hepatic iron as well as a greater inflammatory process and IL-6, which may decrease the expression of hepcidin in cirrhotic livers with advanced fibrosis. Another study evaluated the levels of hepcidin in 27 patients before and after treatment for 48 weeks with PEG-interferon with ribavirin and demonstrated that prior to drug therapy, levels of hepcidin were reduced and after successful virus eradication, the levels of hepcidin increased²¹, thus reinforcing the theory that the virus is an independent factor for iron load. In relation to viral factors, such as viral genotype (segmented in genotype 1 and not 1 (2 and 3)), and viral load values using the cut-off point ≤ 600.000 IU/mL as low, and values > 600.000 IU/mL as high, no statistically significant differences were detected, suggesting that these factors are not related to the hepcidin expression, corroborating the data from previous studies^{21,22}. In relation to IL-6 serum levels, there were no significant differences between serum levels of hepcidin, in line with findings already described in the literature^{20,23}.

Despite the small number of patients enrolled in this study, it was enough to show statistical power in correlating hepcidin and ferritin. Yet, our study has

limitations: we intended to minimize the confounding factors that increase the ferritin levels. However, since the sample was composed of few patients, there may have been other factors that could have influenced the ferritin or hepcidin levels that were not analyzed.

Therefore, our study reinforces the view that the hepatitis C virus is an important independent factor in the modulation of hepcidin expression. A better understanding of iron's mechanism in patients with HCV may contribute to the development and potentiation of the effects of the antiviral agent and strategies to

reduce the progression of hepatic fibrogenesis and hepatocellular carcinoma.

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Conflicts of Interest

The authors declare no conflicts of interest.

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