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Serum prohepcidin reflects the degree of liver function impairment in liver cirrhosis

JERZY JAROSZEWICZ, MAGDALENA ROGALSKA, & ROBERT FLISIAK

Department of Infectious Diseases, Medical University of Bialystok, Poland

Abstract

In the past few years the role of hepcidin metabolism disturbances, a recently described key regulator of iron metabolism, has been raised in patients with chronic liver diseases. The aim of this study was to assess the serum concentrations of prohepcidin in liver cirrhosis of various aetiologies and their possible relationship with the disease activity. Prohepcidin was measured in the sera of 70 patients with liver cirrhosis of various aetiologies by an immunoassay technique. The serum concentrations of prohepcidin were compared with the degree of liver insufficiency and biochemical markers of iron metabolism. A significant decrease in serum prohepcidin was observed in patients with liver cirrhosis compared with healthy individuals (52.6 ± 1.9 vs 79.5 ± 9.7 ng ml^{-1}, $p<0.01$); this was most prominent in patients with hepatitis C virus and alcohol-related liver cirrhosis. The association between serum prohepcidin and the degree of liver dysfunction was observed in alcoholic liver cirrhosis, as illustrated through the inverse correlation with the Child–Pugh score ($r = -0.41$, $p<0.01$). In conclusion, serum prohepcidin concentration is lowered in liver cirrhosis, which at least to some extent, may be a result of impaired liver function.

Keywords: *hepcidin, liver cirrhosis, liver insufficiency, iron metabolism*

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Introduction

Iron accumulation is inevitably associated with chronic liver damage. Humans cannot eliminate iron and any excess of this microelement results in deposition in parenchymal organs, mainly the liver. Iron-induced oxidant stress is involved in cell damage and liver fibrogenesis. Iron-related hepatic cellular injury is induced by reactive oxygen species generation (ROS) and peroxidation of membranes lipids (Shaw et al. 1988). Moreover iron acts as an activator of hepatic stellate cells and thus stimulates the production of collagens and other extracellular matrix proteins (Friedman 1995, Guo et al. 2006). Continuous stimulation and self-perpetuation of these processes may lead to accumulation of connective tissue and liver cirrhosis development.

A recently discovered iron regulatory hormone, hepcidin (Park et al. 2001), has changed the current understanding of iron metabolism regulation. The name hepcidin

Correspondence: Jerzy Jaroszewicz, Department of Infectious Diseases, Medical University of Białystok, 15-540 Bialystok, Zurawia str., 14, Poland. Tel/Fax: +48 85 7416 921. E-mail: jerzy.jr@gmail.com

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was used because of the predominant liver production and antimicrobial properties of this protein. Hepcidin, an acute-phase protein, is induced by infection and inflammatory responses and results in rapid plasma iron decrease (Nicolas et al. 2002, Nemeth et al. 2003). This limits iron availability to microorganisms, and therefore contributes to host defence. The mechanisms of iron metabolism regulation by hepcidin have recently been clarified. It was established that it supresses intestinal absorption of iron through binding to ferroportin, which is strongly expressed by enterocytes and liver macrophages (Nemeth et al. 2004). The mechanism of ferroportin inhibition includes its internalization and degradation (De Domenico et al. 2007). Hepcidin production is increased by iron overload and decreased by anaemia, hypoxia-inducible factors and ROS (Choi et al. 2007, Peyssonnaux et al. 2007). Lack of hepcidin gene expression in animal studies resulted in severe tissue iron overload (Nicolas et al. 2001). In addition, inadequate production of this hormone was observed in patients with hereditary haemochromatosis (Piperno et al. 2007).

In recent years various authors have reported hepcidin regulation disturbances in animal models of liver injury and in patients with chronic liver diseases. These included liver cirrhosis, alcoholic liver disease and chronic hepatitis C (Nagashima et al. 2006, Fujita et al. 2007, Valenti et al. 2007). However the precise influence and clinical significance of alterations in hepcidin production in those disorders remains to be clarified. In the current study we measured prohepcidin levels, a hepcidin precursor protein, which is predominantly synthesized in the liver. In the hepatocytes prohepcidin undergoes two cleavages and is rapidly secreted from the cells as a mature, 25 amino acid peptide. It was shown, that some amount of prohepcidin is also secreted from heptocytes (Valore & Ganz 2008). The significance of this process as well as the possible association with circulating hepcidin concentrations remains unclear. The aim of this study was to assess the serum concentrations of prohepcidin in individuals with the liver cirrhosis of various aetiologies and their possible association with disease severity, as estimated by use of the Child–Pugh classification.

Material and methods

Study population

Prohepcidin was measured in the sera of 70 patients with liver cirrhosis (28 females and 42 males; median age 51.5, min–max 31–83 years). Alcohol-related liver cirrhosis (ALC) was diagnosed in 40, primary biliary cirrhosis (PBC) in nine, and hepatitis C virus-related liver cirrhosis (HCV-LC) in eight, whereas the role of hepatitis B virus (HBV-LC) as an aetiological factor was established in 13 subjects. Degree of liver insufficiency was established according to the Child–Pugh classification (Pugh et al. 1973). Ascites, encephalopathy, prothrombin index, and concentrations of bilirubin and albumin were evaluated for this purpose. Patients were scored as follows: 5–6 points as class (group) A, 7–9 points as class (group) B, and 10–15 points as class (group) C. In addition, serum iron, total iron-binding capacity (TIBC), transferrin saturation and ferritin were measured in liver cirrhosis patients. The clinical characteristics of the study population are presented in Table I. Serum prohepcidin concentration from cirrhotic groups was also compared with that measured in the sera of 14 healthy volunteers (8 females and 6 males, with a median age of 41 years, min–max 26–58 years). Informed consent was obtained from each patient.

Table I. Clinical characteristics of study subjects.

Age (years) (median, min–max)	51.5 (31–83)
Men/women	42/28
Liver cirrhosis aetiology, *n*	
ALC	40
HBV-LC	13
HCV-LC	8
PBC	9
Child–Pugh class, *n*	
A	15
B	28
C	27
Child–Pugh score (median, min–max)	9 (5–13)
ALT ($U\,l^{-1}$) (mean ± SE)	66.9 ± 7.4
Bilirubin ($mg\,dl^{-1}$) (mean ± SE)	5.8 ± 1.0
International normalized ratio (mean ± SE)	75.2 ± 1.9
Albumin ($g\,dl^{-1}$) (mean ± SE)	3.1 ± 0.1
Haemoglobin ($g\,dl^{-1}$) (mean ± SE)	12.3 ± 0.3
Iron ($\mu g\,dl^{-1}$) (mean ± SE)	103.6 ± 6.9
TIBC ($\mu g\,dl^{-1}$) (mean ± SE)	273.9 ± 10.5
Ferritin ($\mu g\,dl^{-1}$) (mean ± SE)	268.1 ± 34.8

ALC, alcohol-related liver cirrhosis; PBC, primary biliary cirrhosis; HCV-LC, hepatitis C virus-related liver cirrhosis; HBV-LC, hepatitis B virus-related liver cirrhosis.

Prohepcidin measurement

Venous blood was collected on ice using vacutainer tubes and centrifuged at 2500*g* at 4°C within 30 min of collection. Serum samples were assayed using a commercially available quantitative sandwich enzyme kit (DRG Instrument GmbH, Marburg, Germany) according to the manufacturer's instructions. The employed antibody detects both the proregion and prohepcidin (aa 25–84). The sensitivity of the assay was 3.95 ng ml^{-1}, intra-assay variation CV 4.69% and inter-assay variation CV 4.82%.

The procedures were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983. The study was approved by the Bioethical Committee of the Medical University of Bialystok. Informed consent was obtained from each patient.

Statistical analyses

Values were expressed as mean ± standard error of mean (SE). The significance of differences was calculated by the non-parametric Mann–Whitney *U* and Kruskall–Wallis ANOVA tests. For correlation analysis, the Spearman non-parametric correlation was used. Statistical analyses were performed with Statistica 5.0 for Windows software (Statsoft Inc., Tulsa, USA). Values of $p<0.05$ were considered to be statistically significant.

Results

A significant decrease in serum prohepcidin was observed in patients with liver cirrhosis compared with healthy individuals (52.6 ± 1.9 vs 79.5 ± 9.7 ng ml^{-1},

$p<0.01$). Serum concentrations of prohepcidin were not associated with age ($r=0.21$, $p=0.19$), sex ($p=0.45$), markers of liver injury – aminotransferases (ALT: $r=0.06$, $p=0.6$) or markers of iron metabolism disturbances (serum iron: $r=-0.03$, $p=0.83$; ferritin: $r=-0.06$, $p=0.58$; TIBC: $r=0.2$, $p=0.18$). In contrast, a gradual, but not statistically significant, decrease of prohepcidin levels with the degree of liver insufficiency was observed. The lowest values were observed in patients with end-stage disease: Child–Pugh class C (A: 57.7 ± 6.1 ng dl^{-1}, B: 53.8 ± 2.4 ng dl^{-1}, C: 47.9 ± 2.7 ng dl^{-1}). Furthermore, the prohepcidin/ferritin ratio, which is an established indicator of iron metabolism alterations, was significantly decreased in Child–Pugh class C compared with classes A and B (Table II) and correlated with the Child–Pugh score ($r=0.38$, $p=0.01$). In addition, serum prohepcidin correlated positively with albumin concentration ($r=0.28$, $p<0.05$) and negatively with international normalized ratio (INR) ($r=-0.24$, $p<0.05$) in individuals with liver cirrhosis.

Evaluation of serum prohepcidin concentrations in particular groups indicated an association with the aetiology of liver cirrhosis. The lowest levels were observed in patients with HCV-LC (39.3 ± 5.0 ng dl^{-1}, $p<0.01$) and ALC (53.9 ± 2.1 ng dl^{-1}, $p<0.01$), while the groups with HBV-LC (54.9 ± 5.7 ng dl^{-1}, $p=0.08$) and PBC (54.8 ± 5.9 ng dl^{-1}, $p=0.05$) were not significantly different compared with healthy individuals (Figure 1). The most prominent association between serum prohepcidin and the degree of liver dysfunction was observed in ALC. This was illustrated through the inverse correlation with the Child–Pugh score ($r=-0.41$, $p<0.01$; Figure 2), positive correlation with serum albumins ($r=0.39$, $p<0.01$) and negative correlation with INR ($r=-0.35$, $p<0.05$). In the group of patients with hepatic encephalopathy, serum prohepcidin significantly increased (56.1 ± 2.5 vs 46.9 ± 1.9 ng dl^{-1}, $p<0.01$). These associations resulted in a significant, gradual decrease of serum prohepcidin in the ALC group with respect to the Child–Pugh class (A: 71.3 ± 9.5 ng dl^{-1}, B: 55.8 ± 2.2 ng dl^{-1} and C: 48.3 ± 2.8 ng dl^{-1}, $p<0.05$) (Figure 3).

Discussion

The recently discovered hormone, hepcidin, plays a crucial role in iron homeostasis. Following infectious or inflammatory stimuli and iron overload its production increases, resulting in a decrease of plasma iron by negative regulation of iron uptake by duodenal enterocytes and sequestration by macrophages. In contrast, down-regulation of hepcidin is an important factor facilitating iron deposition in

Table II. Serum concentrations of prohepcidin in liver cirrhosis patients according to liver function as assessed by Child–Pugh classification (A–C).

	Prohepcidin (ng ml^{-1})			Prohepcidin/ferritin ratio		
Aetiology group	A	B	C	A	B	C
ALC	71.3 ± 9.5	55.8 ± 2.2	$48.3\pm2.8^{\star\dagger}$	0.81 ± 0.3	1.32 ± 0.3	$0.57\pm0.2^{\dagger}$
HBV-LC	54.5 ± 12.9	52.7 ± 6.7	53.2 ± 10.4	0.71 ± 0.4	0.46 ± 0.2	$0.13\pm0.5^{\star}$
HCV-LC	–	43.6 ± 6.7	32.2 ± 6.7	–	0.22 ± 0.3	0.11 ± 0.4
PBC	50.1 ± 7.8	63.1 ± 22.6	56.9 ± 0.7	1.69 ± 0.9	1.87 ± 1.6	0.27 ± 0.3
Liver cirrhosis – all	57.7 ± 6.1	53.8 ± 2.4	47.9 ± 2.7	1.04 ± 0.3	1.1 ± 0.2	$0.45\pm0.2^{\star\dagger}$

$^{\star}p<0.05$ in comparison to Child–Pugh class A; $^{\dagger}p<0.05$ in comparison to Child–Pugh class B.

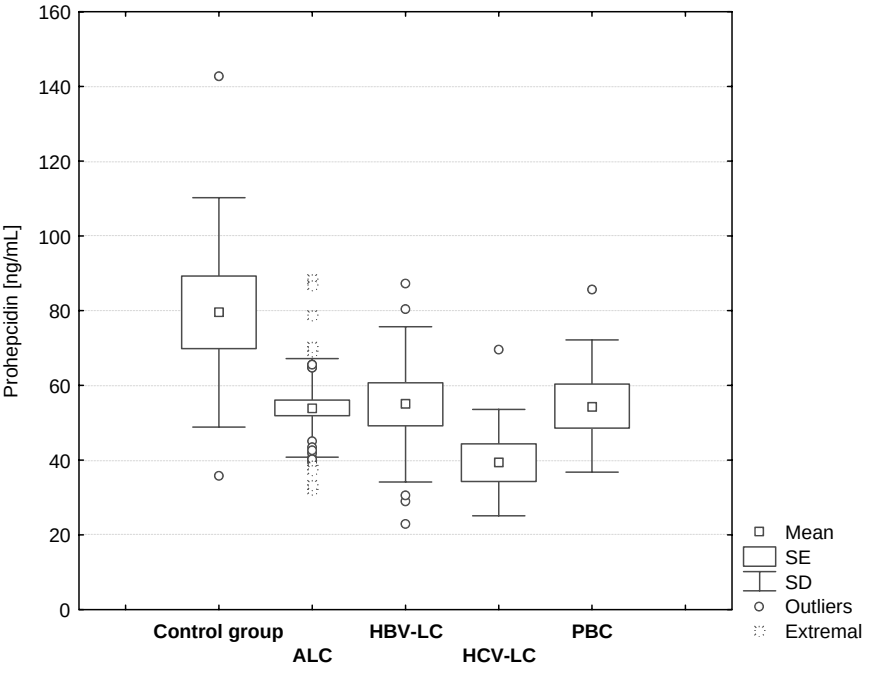

Figure 1. Serum concentration of prohepcidin in the control group and in patients with liver cirrhosis of various aetiologies. ALC, alcohol-related liver cirrhosis; PBC, primary biliary cirrhosis; HCV-LC, hepatitis C virus-related liver cirrhosis; HBV-LC, hepatitis B virus-related liver cirrhosis.

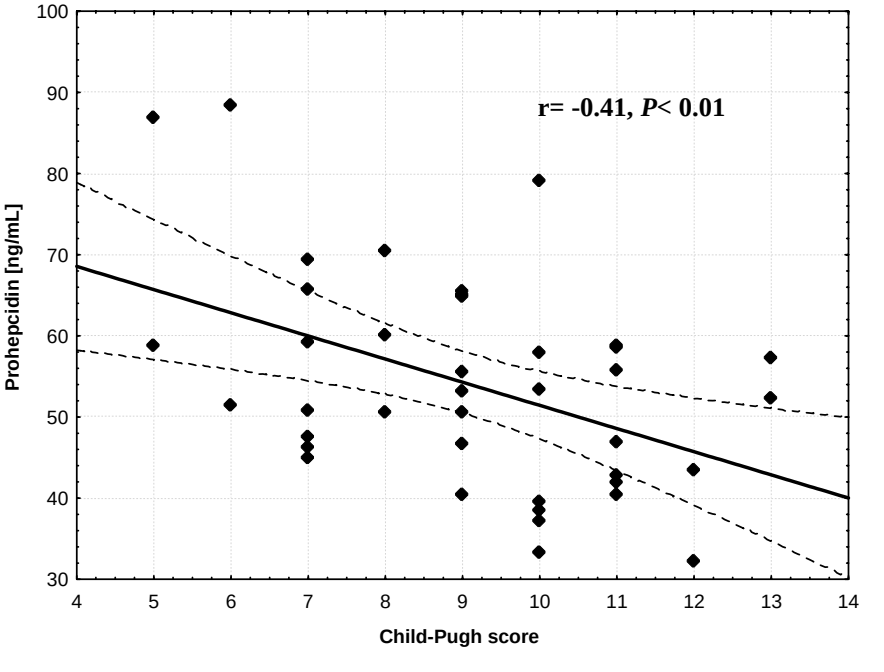

Figure 2. The correlation between serum prohepcidin and Child–Pugh score in the alcoholic liver cirrhosis group.

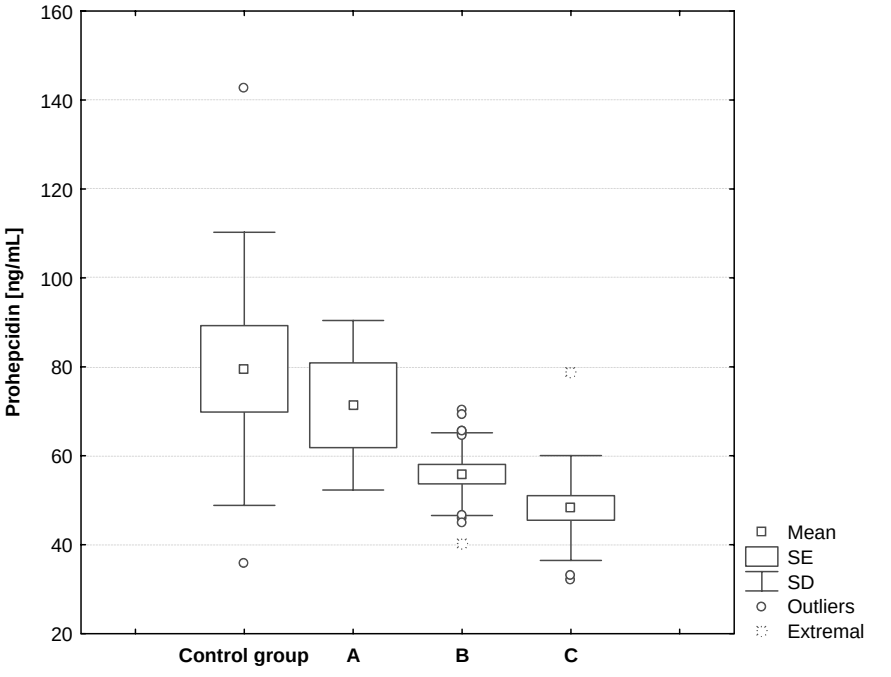

Figure 3. Serum concentration of prohepcidin in the alcoholic liver cirrhosis group in respect to Child–Pugh class.

parenchymal organs, especially liver. Recent reports documented deregulation of hepcidin expression in chronic liver diseases, mainly alcoholic liver disease (ALD) and chronic hepatitis C. Ohtake et al. (2007) showed a greater than twofold decrease in serum prohepcidin – proregion sequence of an inactive form of hepcidin, in patients with ALD compared with healthy individuals. Moreover, the authors found a lower expression of hepcidin in the livers of ethanol-fed mice. Nagashima et al. (2006) observed decreased levels of serum prohepcidin in chronic hepatitis C patients with a further decline in HCV-positive liver cirrhosis. Interestingly, prohepcidin serum concentration was significantly lower in HCV- than in HBV-infected subjects. Those observations were confirmed by Fujita et al. (2007), who found lower hepcidin mRNA expression in livers of HCV-infected patients compared with those of patients with chronic hepatitis B and healthy individuals.

Our results support the observations mentioned in the above studies. We found significantly lower serum concentrations of hepcidin prohormone in cirrhotics compared with healthy individuals. The lowest levels were observed in the HCV-LC group. Serum prohepcidin was also decreased in alcoholic liver cirrhosis. In HBV-LC and PBC groups prohepcidin levels were lower but did not differ significantly compared with the control group. The differences in prohepcidin concentrations in liver cirrhosis of various aetiologies depend on multiregulatory and not fully understood mechanism of hepcidin regulation. In ALD oxidative stress seems to play an important role in hepcidin expression. Choi et al. (2007) suggested that ROS represses the hepcidin gene by preventing C/EBPα and STAT-3 binding to the hepcidin promoter during hypoxia. Hepcidin expression seems to be associated mainly with circulating serum ferritin and free iron (Fujita et al. 2007). However, in a

recent study in mice, Harrison-Findik et al. (2007) found that alcohol intake was capable of suppressing upregulated hepcidin mRNA, despite iron overload. The authors concluded that alcohol may counteract the protective role of hepcidin. This observation may to some extent explain the lack of association between serum concentrations of hepcidin and free iron as well as ferritin in our study.

In chronic hepatitis C the regulation of hepcidin expression seems to be even more complex. As mentioned above, the inflammation is regarded as an important regulator of hepcidin synthesis. However, in the study of Fujita et al. (2007) the differences in alanine aminotransferase activity and hepatic inflammatory score were not sufficient to explain the lower expression of hepcidin in chronic hepatitis C compared with chronic HBV infection. The authors suggested that the upregulation of hepatic hepcidin expression by increased body-stored iron may be relatively diminished by HCV itself. Similar observations of HCV influence on regulation of prohepcidin were presented in the study of Nagashima et al (2006).

The new finding in our study is the association between serum hepcidin and the degree of liver dysfunction. We showed that the decrease in prohepcidin in more advanced liver disease was expressed first of all through positive correlation with serum albumins and negative correlation with INR. Moreover the prohepcidin/ferritin ratio was significantly lowered in end-stage liver cirrhosis. That relationship was most marked in alcoholic liver cirrhosis. Serum prohepcidin correlated inversely with the Child–Pugh score and gradually decreased in accordance with the Child–Pugh class. It is important to state that the liver is the major source of the hepcidin expression and production (Park et al. 2001, Pigeon et al. 2001). Only a limited amount of hepcidin is expressed in the kidneys (Kulaksiz et al. 2004). We hypothesize, that at least to some extent, serum prohepcidin levels may depend on the degree of impairment of liver synthesis function. Diminished production of hepcidin in cirrhotic liver might enhance iron accumulation in this organ and further impact on its function. We are aware however that this hypothesis needs confirmation in further studies.

The limitation of the current study is measurement of the hepcidin prohormone, not a mature peptide, as well as the relatively small sample. It is recognized that serum assessment of prohepcidin does not entirely reflect the levels of the biologically active mature hepcidin peptide. Hepcidin is almost exclusively synthesized in the liver as an 84 amino acid prepropeptide, which is further processed in hepatocytes to a mature form (Pigeon et al. 2001). Recent evidence showed that the proteolytic cleavage of prohepcidin to hepcidin is regulated by the hepatic prohormone convertase furin (Valore & Ganz 2008). The mechanism involved in the transport of hepcidin to the extracellular zone is not fully understood. Valore and Ganz (2008) showed that the larger hepcidin precursor protein undergoes two cleavages (the signal sequence then the proregion) and is rapidly secreted from the cell. Moreover, in primary human hepatocytes, prohepcidin was transiently detected in culture supernatant immediately after labelling but it appeared to be present at low levels compared with mature hepcidin. Interestingly, the inhibition of furin activity prevented the conversion of prohepcidin to hepcidin but also appeared to stabilize the prohepcidin peptide both in the cell and the media. In situations of liver function impairment the prohepcidin synthesis as well as activity or expression of converting enzymes might be altered and affect circulating prohepcidin concentrations.

In conclusion, serum prohepcidin concentration is lowered in liver cirrhosis, which at least to some extent, may be a result of impaired liver function.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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