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Changes in the Serum Hepcidin-to-ferritin Ratio with Erythroferrone after Hepatitis C Virus Eradication Using Direct-acting Antiviral Agents

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Short title: Iron metabolism and HCV

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Abstract

Aim: Hepcidin is a master iron regulator hormone produced by the liver, but precise mechanism underlying its involvement in iron overload in hepatitis C virus (HCV) infection remains unclear. We investigated the serum hepcidin levels against iron overload before and after HCV eradication.

Methods: We prospectively investigated the iron metabolism characteristics in 24 patients with HCV genotype 1b infection before and after treatment. We also assessed the serum erythroferrone (ERFE) levels to investigate its association with iron metabolism changes. Patients were treated with Ledipasvir 90 mg and Sofosbuvir 400 mg once daily for 12 weeks and observed for 12 more weeks in order to evaluate their sustained virological response.

Results: Serum hepcidin levels at baseline were in the normal range, although serum ferritin levels were increased. After HCV eradication, both serum ferritin and hepcidin levels were significantly decreased at 24 weeks from baseline ($p<0.001$, $p=0.006$, respectively). However, the serum hepcidin-to-ferritin ratios were significantly increased ($p<0.001$). In addition, the serum ERFE levels were significantly decreased ($p<0.001$). Increases in the serum hepcidin-to-ferritin ratios were correlated with decreases in the serum ERFE levels ($\rho=-0.422$, $p=0.039$).

Conclusions: Serum hepcidin levels were relatively low against ferritin levels in HCV infection. However, after HCV eradication, the serum hepcidin-to-ferritin ratios were increased. These results indicate the improvement of inadequate hepcidin secretion against iron overload after HCV eradication. Downregulation of ERFE may have affected the improvement of iron metabolism.

Key words: Hepcidin, Ferritin, Hepatitis C virus, Direct-acting antiviral agents, Erythroferrone

Introduction

Chronic hepatitis C (CHC) is frequently associated with iron overload (1). Liver production of the iron regulator hormone hepcidin plays a critical role in iron metabolism, and its reduced secretion is involved in hepatitis C virus (HCV) infection (2). Impaired hepcidin secretion accelerates iron absorption and causes excess iron storage (3). However, the exact mechanism underlying the iron overload observed in HCV infection remains unclear.

Hepcidin is regulated by diverse factors, such as iron overload, inflammation, erythropoiesis and hypoxia (4). Erythroferrone (ERFE), a suppressor of hepcidin, is stimulated by erythropoietin (EPO) and secreted from erythroblasts for erythropoiesis (5) (6) (7) (8). Little is currently known about the relationship of ERFE with HCV infection, and the changes in hepcidin and ERFE after HCV eradication are poorly understood.

A previous study showed the improvement of hepcidin secretion with a reduction in iron overload in sustained virological response (SVR) CHC patients after the administration of interferon alpha (IFN α) and Ribavirin (RBV) (9). However, IFN α has been shown to induce hepcidin expression in a human hepatoma cell line (10) as well as in mice (11). In addition, RBV administration often induces hemolytic anemia (12) and affects iron metabolism because of reactive erythropoiesis (13,14).

At present, IFN-free, direct-acting antiviral agents (DAAs) are a standard therapy in HCV eradication. We consider DAA therapy more appropriate than IFN α and RBV therapy for evaluating the relationship between iron metabolism and HCV dynamics because IFN α induces hepcidin upregulation, RBV-related hemolytic anemia induces changes in iron metabolism, and the dietary iron intake is reduced because of long-term adverse reactions. In addition, identifying the mechanism underlying the changes in iron metabolism after HCV eradication is crucial. We therefore measured the serum ERFE levels to investigate their association with changes in the iron metabolism.

The aim of this study was to clarify the role of hepcidin in iron metabolism and the mechanism underlying the change in its levels after HCV eradication using DAAs.

Methods

Study design

This study was a prospective, interventional, single-center, non-randomized, and non-case controlled open trial. It was registered as a prospective trial, titled, “The analysis of iron metabolism during Ledipasvir/Sofosbuvir treatment in patients with genotype 1 hepatitis C virus infection and compensated cirrhosis,” with the University Hospital Medical Information Network registration number UMIN000021011.

Patients and schedule

Twenty-six Japanese patients with genotype 1b HCV in chronic hepatitis or compensated liver cirrhosis were enrolled from February 2016 to July 2017 at Fukuoka University Hospital. In this trial, patients were administered a Ledipasvir (LDV) 90 mg and Sofosbuvir (SOF) 400 mg combination tablet once daily for 12 weeks. They were observed for 12 more weeks after the end of therapy to evaluate the SVR12 with no restriction on lifestyle, including diet.

We excluded patients with hepatitis B virus infection, autoimmune liver disease, chronic inflammatory disease, persistent anemia, viable hepatocellular carcinoma (HCC), severe renal dysfunction, uncontrolled cardiac disease or diabetes. We did not confirm the absence of the C282Y mutation on the hemochromatosis gene because it is rare in the Japanese population (15).

The primary outcome was the change in the serum hepcidin level and iron metabolism at SVR12 from baseline. The serum iron, ferritin, total iron binding capacity, transferrin, transferrin saturation (TSAT) and hepcidin levels were measured at baseline and 1, 4, 12 and 24 weeks (SVR12) after the administration of LDV/SOF.

We assessed the serum Mac-2 binding protein glycan isomer (M2BPGi) levels as a liver fibrosis marker at baseline and at 4, 8, 12 and 24 weeks. The HCV-RNA levels and genotype were assessed in addition to routine laboratory tests before treatment. We also assessed the serum levels of ERFE, which suppresses hepcidin (5) (6) (7) (16). The serum ERFE levels were measured at baseline and at 12 and 24 weeks. In addition, we assessed the urinary 8-hydroxydeoxyguanosine (8-OHdG) levels as a reactive oxygen species marker, which can affect hepcidin, induced by iron overload (17) (18) (19). These levels were measured at baseline and at 12 and 24 weeks.

Blood and spot urine samples were collected in the morning after overnight fasting. Sera were immediately separated by centrifuging and stored at -80 °C until use. Untreated urine samples were stored at -30 °C until use.

The study protocol was approved by the institutional review board of Fukuoka University Hospital (reference number 15-12-02). The study was conducted in compliance with the Declaration of Helsinki. All participants provided their written informed consent.

Hepcidin analyses

The serum hepcidin-25 level was measured using high-performance liquid chromatography-tandem mass spectrometry (20), performed by Medical Care Proteomics Biotechnology (Kanazawa, Japan).

ERFE analyses

The serum ERFE level was measured by an enzyme-linked immunosorbent assay (ELISA) using a human Erythroferrone/Myonectin/CTRP15 ELISA kit (SK00393-19, Aviscera Bioscience, Santa Clara, CA, USA).

Urinary 8-OHdG analyses

The urinary creatinine-corrected 8-OHdG level was measured by an ELISA (21) using spot urine, performed by NIKKEN SEIL (Tokyo, Japan).

Sample size calculation

The average of normal serum hepcidin-25 level was 22.2 ng/mL, standard deviation (SD) 12.3 (20,22), and the intra-subject coefficient variation was 19% (23). Therefore, the intra-subject SD was estimated to be 4.2. The primary endpoint was the change in hepcidin (Δ hepcidin) at 24 weeks from baseline, and we estimated variation of SD for more than $(\sqrt{2}) \times 4.2$. We conservatively set the SD as 8 with an average variation of 5 ng/mL. Thus, a sample size of 23 patients had 80% power using a 2-sided *t*-test with a significance level of $p < 0.05$. Finally, we set the sample size at 25 patients after taking dropouts into account.

Statistical analyses

We used *t*-tests to compare the parameters between two groups if the normality assumption held. Otherwise, we used Wilcoxon's signed-rank sum test. Missing values were imputed by the last observation carried forward (LOCF) method. The primary endpoint of Δ hepcidin at 24 weeks from baseline was also analyzed in the completers set as a sensitivity analysis. Family-wise errors were adjusted using the Bonferroni method.

Pearson's correlation coefficients between continuous variables were calculated if the normality assumption held. If the normality assumption did not hold, we used Spearman's rank correlation coefficients.

We performed a linear regression analysis to extract the factors associated with the Δ hepcidin-to-ferritin ratio. Variables with $p < 0.20$ in a univariate analysis were included in the multiple linear regression analysis. The threshold significance level was $p < 0.05$. All statistical analyses were conducted using the JMP software program, version 11 (SAS Institute, Cary, NC, USA).

Results

Patient characteristics

Twenty-six patients were enrolled. Two were excluded because of sinus bradycardia at 1 week and HCC onset at 12 weeks. Another was lost to follow-up at 24 weeks, and the missing data were imputed using the LOCF method. This resulted in 24 patients included in the analyses, and the SVR12 rate was 100%.

Most patients had moderately elevated serum alanine aminotransferase (ALT) levels. However, the liver function was maintained, and the serum M2BPGi levels were not severely elevated.

Twelve patients (50%) had increased serum ferritin levels, and 14 (58%) had increased TSAT levels. Six patients (25%) had normal ferritin and TSAT levels. Only one patient underwent a liver biopsy. Patients' characteristics are summarized in Table 1.

Urinary 8-OHdG levels before and after treatment

The mean urinary creatinine-corrected 8-OHdG level at baseline was 10.9 ± 3.4 ng/mg, which was within the normal range (0.0–16.4 ng/mg) (24). Urinary 8-OHdG levels were positively correlated with serum ferritin and negatively correlated with transferrin levels (Pearson's correlation test, $r=0.581$, $p=0.003$ and $r=-0.570$, $p=0.004$, respectively). However, there was no correlation between the urinary 8-OHdG levels and other clinical parameters. In addition, there was no significant change in the urinary 8-OHdG levels after treatment (data not shown).

Serum ERFE levels at baseline

The median serum ERFE level at baseline was 193.3 ng/mL, and the interquartile range was 45.8–714.3, which was greater than that noted in previously reported healthy controls (mean 12 ± 10 ng/mL) (16). There was no correlation between serum ERFE levels and other clinical parameters.

Correlations between serum hepcidin levels and clinical parameters at baseline

The mean serum hepcidin level at baseline was 23.8 ± 12.1 ng/mL, which was within the normal range. Only 4 patients (17%) had decreased serum hepcidin levels. Two of these patients had hepatitis, and two had cirrhosis; none showed any marked clinical differences in parameters such as the serum ERFE levels from other patients.

The serum hepcidin levels were positively correlated with the serum ferritin levels and negatively correlated with the serum transferrin levels but not correlated with the serum ALT or M2BPGi levels. The serum hepcidin levels were significantly higher in men than in women. The correlations between serum hepcidin levels and clinical parameters at baseline are summarized in Table 2.

Correlations between serum hepcidin-to-ferritin ratios and clinical parameters at baseline

The serum hepcidin-to-ferritin ratios were positively correlated with age and negatively correlated with the serum ALT and hemoglobin levels. The serum hepcidin-to-ferritin ratios were significantly higher in women than in men. Correlations between the serum hepcidin-to-ferritin ratios and clinical parameters at baseline are summarized in Table 3.

Changes in the serum hepcidin levels and clinical parameters after treatment

After LDV/SOF administration, the serum iron levels at 1, 4, 12 and 24 weeks from baseline were significantly decreased (paired *t*-test, $p=0.009$, 0.006 , 0.009 and 0.003 , respectively), as were the TSAT levels (paired *t*-test, $p=0.002$, 0.002 , 0.005 and 0.003 , respectively). The serum ferritin levels were also significantly decreased after treatment (Fig. 1A), and although the serum hepcidin levels were significantly decreased (Fig. 1B), the serum hepcidin-to-ferritin ratios were significantly increased (Fig. 1C).

Changes in the serum ERFE levels and clinical parameters after treatment

The serum ERFE levels significantly decreased after treatment (Fig. 2). Importantly, the Δ serum ERFE levels were negatively correlated with the Δ serum hepcidin-to-ferritin ratios (Fig. 3).

There was no correlation between the Δ serum ERFE levels and other clinical parameters after treatment. The serum hemoglobin levels showed no significant change after treatment, while the serum ALT and M2BPGi levels were significantly decreased (baseline vs. 24 weeks, paired *t*-test, $p<0.001$ for both).

The Δ hepcidin-to-ferritin ratio and clinical parameters at baseline

The serum hepcidin-to-ferritin ratios were significantly increased after treatment (Fig. 1C). Several clinical parameters at baseline were predictors of the Δ hepcidin-to-ferritin ratio using the univariate linear regression model (Table 4A). The serum hepcidin and hemoglobin levels at baseline were independent predictors of the Δ hepcidin-to-ferritin ratio using the multiple linear regression model (Table 4B).

Discussion

In this study, we prospectively investigated the changes in hepcidin levels against iron overload with serum ERFE levels before and after HCV eradication using DAAs. The results showed that iron parameters decreased, but serum hepcidin levels were downregulated after HCV eradication. However, the serum hepcidin-to-ferritin ratios were increased. In addition, the serum ERFE levels were decreased after HCV eradication. Importantly, increases in the serum hepcidin-to-ferritin ratios were correlated with decreases in the serum ERFE levels.

A previous study demonstrated recovery of hepcidin secretion in SVR-achieving CHC patients treated by IFN α and RBV (9). In that study, iron parameters were decreased in both SVR and non-SVR patients. The results of this previous study suggest that IFN α and RBV administration reduced iron storage without HCV eradication. In addition, IFN α administration was shown to be capable of inducing hepcidin secretion (10) (11). These findings suggest that the recovery of hepcidin secretion in the previous study may have been caused by IFN α administration. However, the influence of LDV/SOF administration on iron metabolism would be smaller than that of IFN-related therapy for the above-mentioned reasons.

In the present study, the mean serum hepcidin levels at baseline were within the normal range, although 75% of patients had increased serum ferritin or TSAT levels. The serum hepcidin levels being in the normal range may have been caused by the liver function being maintained and the exclusion criteria of anemia. Importantly, the serum hepcidin levels were correlated with the serum ferritin levels (Table 2), suggesting that hepcidin responded to iron overload but in an inadequate way in HCV infection. Of note, the serum hepcidin levels being in the normal range cannot be resulted in iron overload. The results of the present study therefore suggest that other mechanisms underlying the development of iron overload exist.

At the baseline, urinary 8-OHdG levels were correlated with serum ferritin levels. This finding indicates that iron overload affected the reactive oxygen species status. However, the urinary 8-OHdG levels at baseline were in the normal range and did not change after HCV eradication. In addition, there was no correlation between the urinary 8-OHdG levels and serum hepcidin or ERFE levels or the hepcidin-to-ferritin ratio. These results indicate the restrictive involvement of urinary 8-OHdG in iron metabolism.

Of note, the serum hepcidin-to-ferritin ratio at baseline was correlated with reduced serum ALT levels (Table 3), suggesting that hepcidin secretion against iron overload was reduced in severe liver inflammation in HCV infection.

A previous study showed that the indirect inhibition of hepcidin expression was caused by EPO (25) and increased EPO in CHC patients (26). A recent study showed that ERFE, a suppressor of hepcidin, was stimulated by EPO (6). In the present study, the serum ERFE levels at baseline were markedly increased compared with previously reported healthy controls, which may have affected the inadequate hepcidin secretion. It is plausible that the

elevated serum ERFE levels were caused by increased EPO.

After the administration of LDV/SOF, all patients achieved SVR12, and the iron parameters were significantly decreased. However, the serum hepcidin levels were also significantly decreased, which was unexpected (Fig. 1B). These findings suggest that decreased iron storage was not directly caused by hepcidin. Furthermore, the decreased serum hepcidin levels may have been caused by preceding reductions in iron storage. Of note, the serum hepcidin-to-ferritin ratios were significantly increased (Fig. 1C). These results suggest that inadequate hepcidin secretion against iron overload was improved after HCV eradication.

The serum ERFE levels were significantly decreased after treatment (Fig. 2). Importantly, increases in the serum hepcidin-to-ferritin ratios were correlated with decreases in the serum ERFE levels (Fig. 3). In other words, greater decreases in serum ERFE levels resulted in greater increases in the serum hepcidin-to-ferritin ratios. These results suggest that ERFE as a suppressor of hepcidin was downregulated after HCV eradication, which affected the improvement of the inadequate hepcidin secretion against iron overload.

In addition, a multiple linear regression analysis indicated that low serum hepcidin and high hemoglobin levels at baseline were associated with an increased serum hepcidin-to-ferritin ratio (Table 4). Conversely, high serum hepcidin and low hemoglobin levels at baseline resulted in smaller increases in the serum hepcidin-to-ferritin ratio after HCV eradication. A high serum hepcidin level, low hemoglobin level and iron overload indicate anemia of chronic disease. Under these conditions, inadequate hepcidin secretion against iron overload may not improve even after HCV eradication.

Several limitations of this study should be acknowledged. First, it was a single-center, non-randomized and uncontrolled trial with a short observation interval. Second, we did not investigate the serum EPO levels. Third, we did not investigate the duodenal ferroportin expression.

In conclusion, our analysis demonstrated the improvement of inadequate hepcidin secretion against iron overload after HCV eradication. Furthermore, the iron metabolism improvement was affected by ERFE downregulation. Iron metabolism improvement through HCV eradication may help prevent cirrhosis progression and HCC development.

Author Contributions

SI and AA designed the study. SI collected the data and performed the analyses. All authors recruited patients to the study. SI, AA, DM, S. Shakado and S. Sakisaka revised the manuscript, and all authors approved the final version.

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Figure Legends

Fig. 1. Changes in serum iron parameters after treatment. Each variable compared with baseline. Family-wise errors were adjusted using the Bonferroni method. (A) Serum ferritin levels were significantly decreased at 1, 4, 12 and 24 weeks (paired *t*-test, * $p < 0.001$). (B) Serum hepcidin levels were significantly decreased at 4 and 24 weeks (paired *t*-test, * $p = 0.012$, ** $p = 0.005$). (C) Serum hepcidin-to-ferritin ratios were significantly increased at 24 weeks (Wilcoxon's signed-rank sum test, * $p < 0.001$).

Fig. 2. Changes in serum ERFE levels after treatment. Each variable compared with baseline. Family-wise errors were adjusted using the Bonferroni method. The serum ERFE levels were significantly decreased at 12 and 24 weeks (Wilcoxon's signed-rank sum test, * $p < 0.001$). ERFE, erythroferrone.

Fig. 3. Correlation between the Δ serum hepcidin-to-ferritin ratios and Δ serum ERFE levels. Δ serum hepcidin-to-ferritin ratios were negatively correlated with Δ serum ERFE levels (Spearman's correlation test, $\rho = -0.422$, $p = 0.039$, regression line is shown). ERFE, erythroferrone.

Fig. 1A

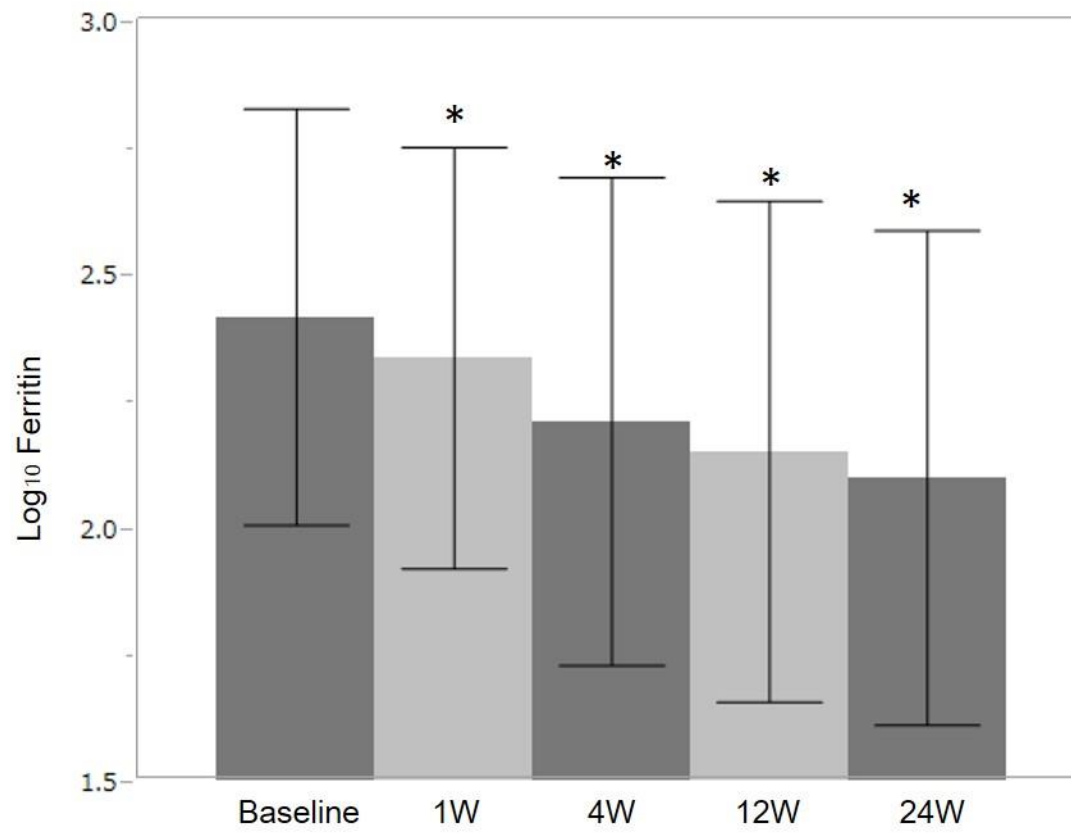


Fig. 1B

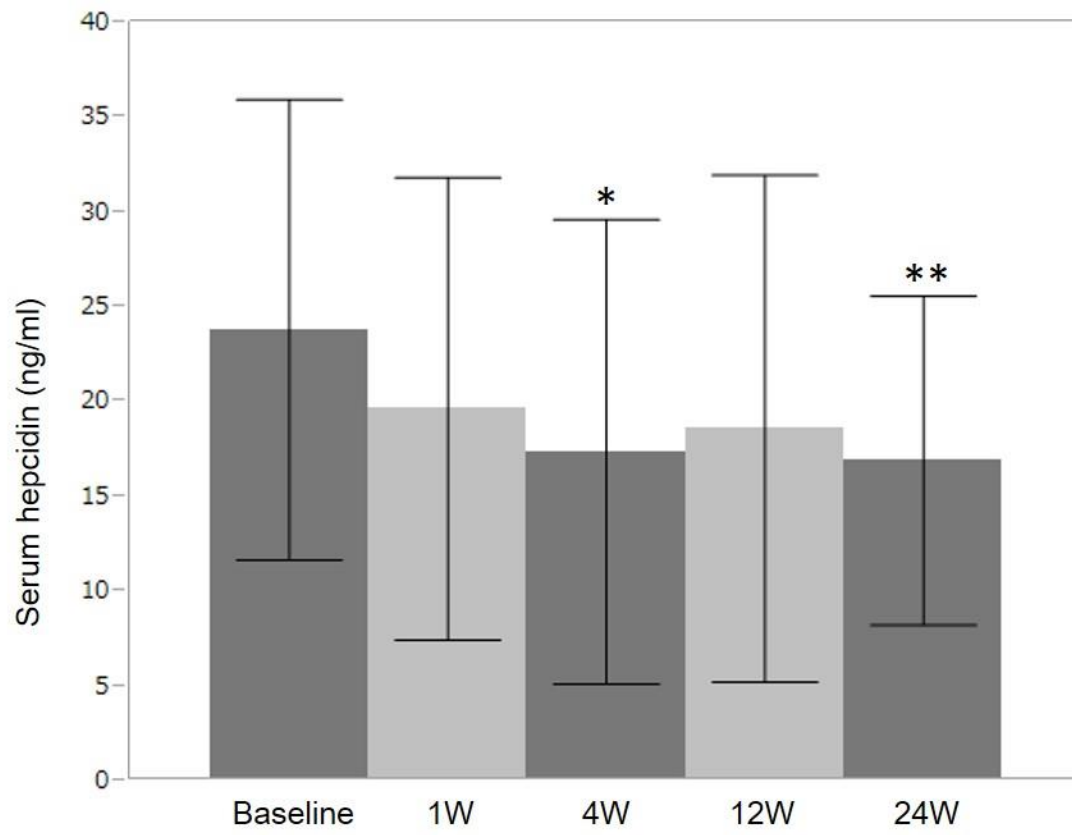


Fig. 1C

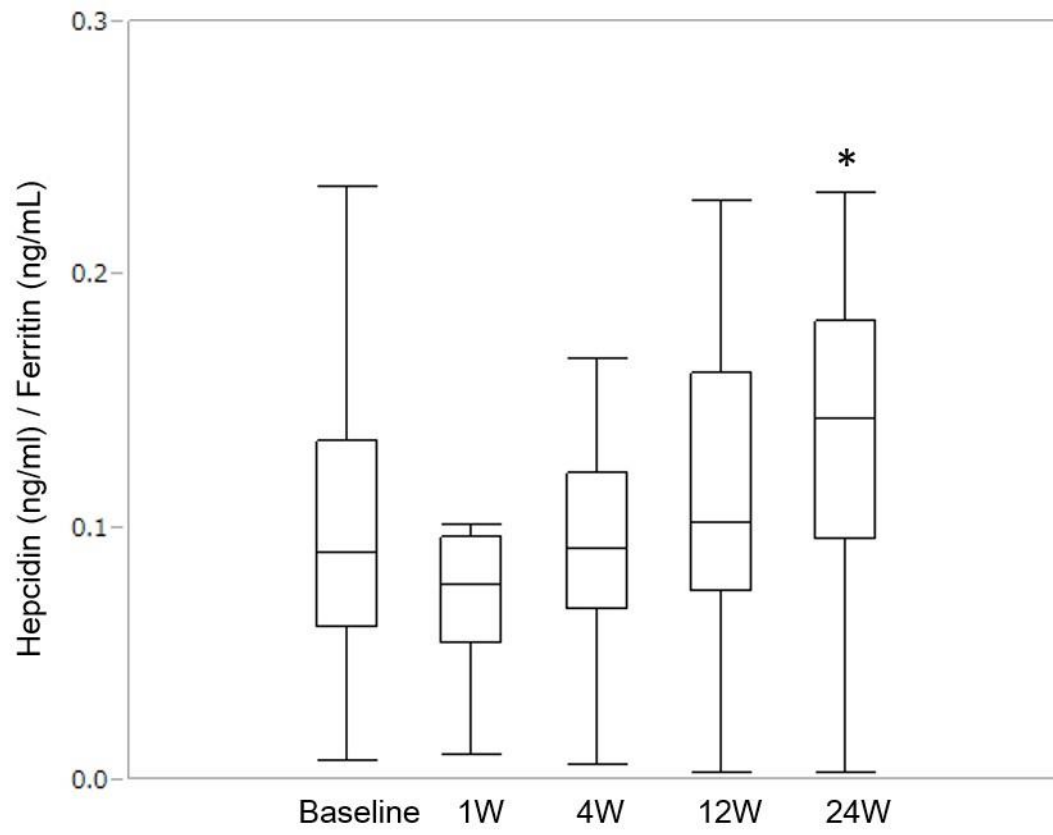


Fig. 2

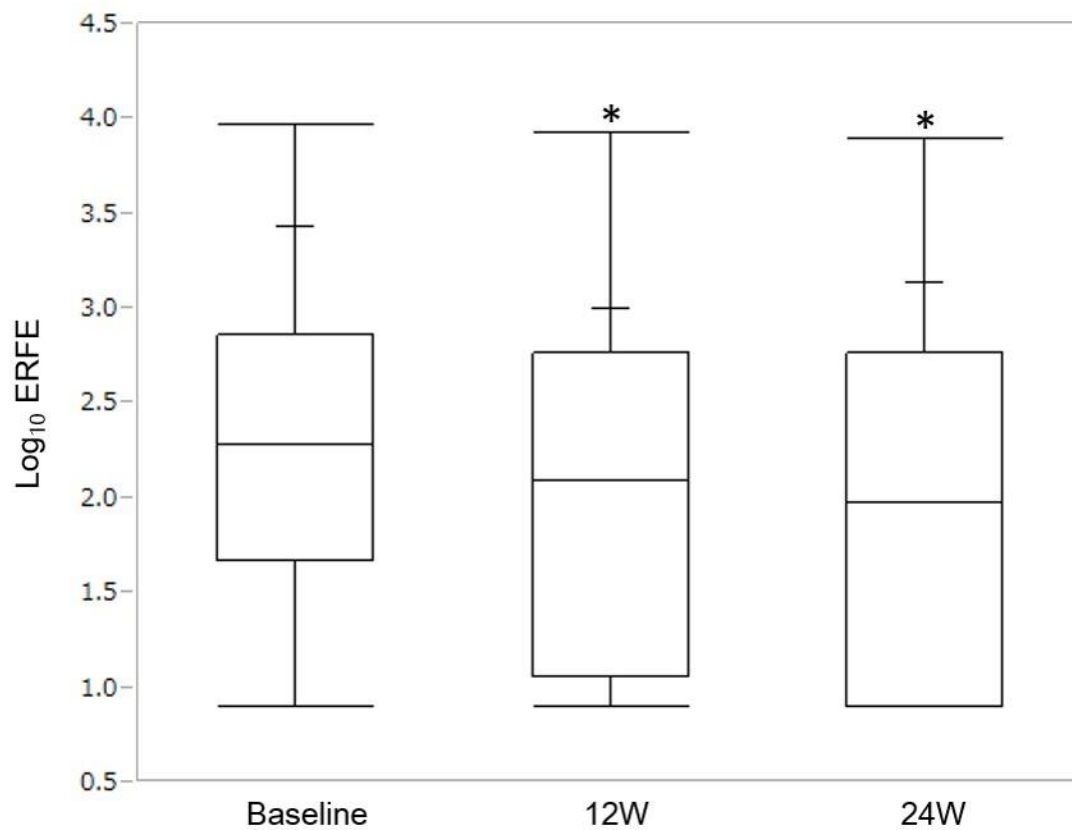


Fig. 3

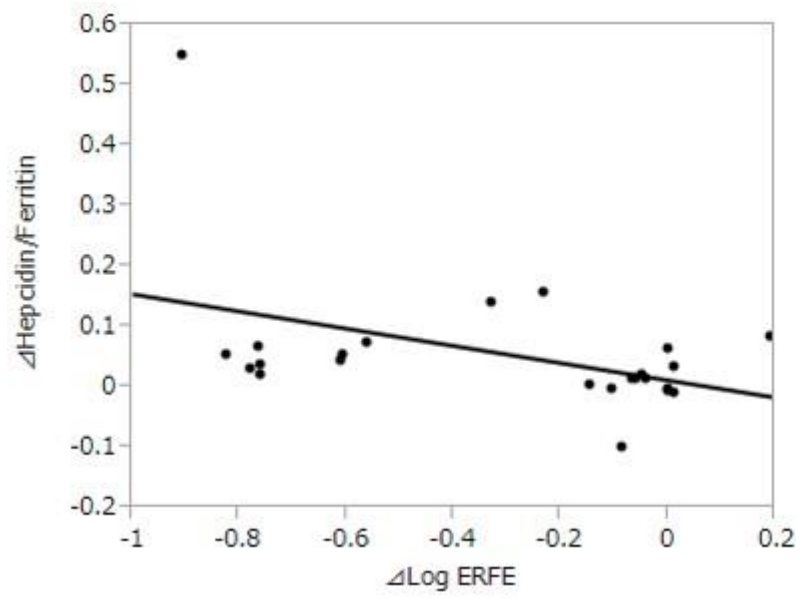


Table 1. Baseline patient characteristics (n=24)

Characteristics	Frequency
Female	13/24 (54%)
Age (y)	62.6 (9.6)
Body mass index (kg/m²)	22.8 (4.5)
Diabetes	5/24 (20%)
HCV-RNA (log₁₀ IU/mL)	6.1 (0.9)
History of interferon treatment	3/24 (12%)
History of hepatocellular carcinoma	2/24 (8.3%)
Hemoglobin (g/L)	144 (15)
Platelet count (x10⁹/L)	155 (54)
Albumin (g/L)	40 (3)
ALT (IU/L)	66 (30)
M2BPGi (COI)	1.72 (1.22-3.19)
Iron (μmol/L)	28.0 (9.5)
Ferritin (ng/mL)	250 (124-404)
Transferrin (g/L)	2.60 (0.37)
Transferrin saturation (%)	46 (17)
eGFR (mL/min/1.73 m²)	77.4 (15.5)

Categorical data are presented as number of patients (%). Continuous data are presented as mean (SD) or median (IQR). HCV, hepatitis C virus; ALT, alanine aminotransferase; M2BPGi, Mac-2 binding protein glycan isomer; eGFR, estimated glomerular filtration rate.

Table 2. Correlations between serum hepcidin levels and clinical parameters at baseline

Characteristics	<i>r</i>	<i>p</i> value
Age (y)	0.085	0.69
Male*		0.049
Body mass index (kg/m²)	-0.041	0.85
HCV-RNA (log₁₀ IU/mL)	0.063	0.77
ALT (IU/L)	-0.139	0.51
Log₁₀ M2BPGi (COI)	-0.219	0.30
Iron (μmol/L)	0.301	0.15
Log₁₀ ferritin (ng/mL)	0.579	0.003
Transferrin (g/L)	-0.433	0.003
Transferrin saturation (%)	0.370	0.075
Hemoglobin (g/L)	0.239	0.26
eGFR (mL/min/1.73 m²)	0.179	0.40
Log₁₀ ERFE** (ng/mL)	-0.036	0.87
Urinary 8-OHdG/creatinine (ng/mg)	0.284	0.18

Pearson's correlation test, *Paired *t*-test, **Spearman's correlation test. HCV, hepatitis C virus; ALT, alanine aminotransferase; M2BPGi, Mac-2 binding protein glycan isomer; eGFR, estimated glomerular filtration rate; ERFE, erythroferrone; 8-OHdG, 8-hydroxydeoxyguanosine.

Table 3. Correlations between serum hepcidin to ferritin ratio and clinical parameters at baseline

Characteristics	ρ	p value
Age (y)	0.534	0.007
Female*		0.048
Body mass index (kg/m²)	-0.089	0.68
HCV-RNA (log₁₀ IU/mL)	-0.238	0.26
ALT (IU/L)	-0.421	0.040
Log₁₀ M2BPGi (COI)	-0.397	0.055
Iron (μmol/L)	-0.258	0.22
Transferrin (g/L)	-0.048	0.82
Transferrin saturation (%)	-0.184	0.39
Hemoglobin (g/L)	-0.427	0.037
eGFR (mL/min/1.73 m²)	0.041	0.85
Log₁₀ ERFE (ng/mL)	0.047	0.83
Urinary 8-OHdG/creatinine (ng/mg)	-0.052	0.81

Spearman's correlation test, *Paired *t*-test. HCV, hepatitis C virus; ALT, alanine aminotransferase; M2BPGi, Mac-2 binding protein glycan isomer; eGFR, estimated glomerular filtration rate; ERFE, erythroferrone; 8-OHdG, 8-hydroxydeoxyguanosine.

Table 4. Baseline factors associated with delta hepcidin to ferritin ratio(A) Univariate linear regression model, $p < 0.20$

	<i>p</i> value
Hepcidin (ng/mL)	0.07
Transferrin (g/L)	0.09
Hemoglobin (g/L)	0.04
Urinary 8-OHdG/creatinine (ng/mg)	0.14

(B) Multiple linear regression model, $R^2 = 0.47$

	β	95% CI	<i>p</i> value
Hepcidin (ng/mL)	-0.45	-0.008 to -0.0005	0.03
Transferrin (g/L)	-0.038	-0.002 to 0.001	0.87
Hemoglobin (g/L)	0.56	0.01 to 0.07	0.006
Urinary 8-OHdG/creatinine (ng/mg)	-0.27	-0.02 to 0.006	0.21

8-OHdG, 8-hydroxydeoxyguanosine.