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【 ORIGINAL ARTICLE 】

Male-specific Association between Iron and Lipid Metabolism Changes and Erythroferrone after Hepatitis C Virus Eradication

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Abstract:

Objective Hepatitis C virus (HCV) eradication is associated with decreased serum ferritin and increased serum low-density lipoprotein-cholesterol (LDL-C) levels, although the mechanisms underlying these changes remain unclear. This study aimed to identify the mechanisms underlying the changes in iron and lipid metabolism after HCV eradication.

Methods We retrospectively investigated iron and lipid metabolism changes in 22 patients with chronic hepatitis or compensated liver cirrhosis with HCV genotype 1b infection after HCV eradication. We measured the serum erythroferrone (ERFE) levels to assess the association with these metabolic changes. Patients were administered ledipasvir 90 mg and sofosbuvir 400 mg once daily for 12 weeks and were observed for 12 more weeks to evaluate the sustained virological response.

Results Half of the patients were men. At baseline, the serum ferritin and ERFE levels were elevated, while the serum LDL-C levels were within the normal range. All patients achieved a sustained virological response at 24 weeks; furthermore, the serum ferritin and ERFE levels were significantly decreased, and the serum LDL-C levels were significantly increased at 24 weeks from baseline ($p<0.001$, all). In men, a decrease in serum ERFE levels was correlated with changes in the serum ferritin and LDL-C levels ($r=0.78$, $p<0.01$; $r=-0.76$, $p<0.01$, respectively). In addition, a decrease in the serum ferritin levels was correlated with an increase in the serum LDL-C levels ($r=-0.89$, $p<0.001$). These correlations were not observed in women.

Conclusion Our results suggest a possible association between iron and lipid metabolism changes and the involvement of ERFE after HCV eradication in men as well as potential sex-related differences.

Key words: ferritin, LDL-C, ERFE, HCV

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Introduction

Hepatitis C virus (HCV) infection is often related to an increase in serum ferritin and a decrease in serum low-density lipoprotein-cholesterol (LDL-C) (1, 2). These metabolic dysfunctions improve after HCV eradication, although the precise integrated mechanism underlying these changes remains unclear (3).

Iron metabolism is regulated by hepcidin, which is produced in the liver. Hepcidin is induced by iron overload and inflammation and is suppressed by erythropoiesis (4). Erythropoiesis is regulated by the hormone erythroferrone (ERFE). ERFE is produced by erythroblasts and stimulated

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by erythropoietin and suppresses hepcidin (5). The suppression of hepcidin by ERFE promotes iron absorption from the intestine, resulting in increased iron storage (4, 5). We previously reported an improvement in iron dysmetabolism associated with the reduction in the ERFE levels after HCV eradication (6). However, another study showed a correlation between increased LDL-C levels and *interleukin-28B* gene polymorphism after HCV eradication (7). These previous studies revealed the individual mechanisms underlying iron and lipid metabolism changes after HCV eradication.

Recent research has revealed that ERFE is identical to myokine and myonectin and belongs to the C1q tumor necrosis factor-related protein (CTRP) family (8). Importantly, myonectin plays an important role in lipid metabolism (9). Therefore, lipid and iron metabolism can be linked to ERFE, which is equivalent to myonectin.

However, these metabolic profiles are not the same in men and women. In women, the proportion of body fat is higher than in men, although the serum triglyceride levels are lower than in men (10, 11). In addition, serum hemoglobin and ferritin levels are higher in men than in women (12). We therefore consider the evaluation of lipid and iron metabolism based on sex to be important.

Little is presently known concerning the role of ERFE in iron and lipid metabolism changes after HCV eradication. Furthermore, the sex-related differences in these changes remain unclear. The present study aimed to examine the involvement of ERFE in iron and lipid metabolism changes and to clarify the interplay between these changes after HCV eradication. In addition, we assessed differences between the sexes regarding these changes.

Materials and Methods

Study design

We previously reported an improvement in iron dysmetabolism after HCV eradication (6) in a prospective, single-center, non-controlled open trial registered with the University Hospital Medical Information Network (registration number UMIN000021011). The present study concerns an additional retrospective analysis of the above-mentioned study.

In the present study, we set the primary outcome as the association between changes in the serum ERFE, ferritin, and LDL-C levels as well as other clinical parameters after HCV eradication.

Patients and schedule

Twenty-four Japanese patients with chronic hepatitis or compensated liver cirrhosis with HCV genotype 1b infection were enrolled between February 2016 and July 2017 at our institution. Patients were administered a combination tablet of ledipasvir (LDV) 90 mg and sofosbuvir (SOF) 400 mg once daily for 12 weeks. They were observed for an additional 12 weeks after the end of therapy to evaluate the sustained virological response at 24 weeks. Patients were not given any restrictions regarding their lifestyle, including their diet.

Exclusion criteria were hepatitis B virus infection, autoimmune liver disease, chronic inflammatory disease, persistent anemia, viable hepatocellular carcinoma, severe renal dysfunction, uncontrolled cardiac disease or diabetes, and patients who received medications for dyslipidemia.

We measured the serum ERFE, iron, ferritin, and transferrin saturation levels and serum lipid profiles in addition to routine laboratory test findings before and after treatment. The serum LDL-C levels were measured using a direct method. The serum Mac-2 binding protein glycan isomer (M2BPGi) levels were assessed as a liver fibrosis marker (13).

The changes in clinical parameter levels were set as the difference at 24 weeks (12 weeks after the end of LDV/SOF administration) from baseline, as the lipid metabolism can be influenced by LDV/SOF administration (14).

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

Ethics

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 principles of the Declaration of Helsinki. All participants provided their written informed consent.

ERFE and M2BPGi measurements

Serum ERFE levels were measured with an enzyme-linked immunosorbent assay (ELISA) using a human Erythroferrone/Myonectin/CTRP15 ELISA kit (SK00393-19, Aviscera Bioscience, Santa Clara, USA). A previous study showed that mean serum ERFE level in healthy controls was 12±10 ng/mL (15).

Serum M2BPGi levels were measured using a glycan-based immunoassay (Sysmex, Kobe, Japan), which detects fibrosis-related glyco-alterations in hyperglycosylated Mac-2 binding protein. A serum M2BPGi value ≥3.00 indicates liver cirrhosis (13).

Statistical analyses

Wilcoxon signed rank-sum tests were performed to compare the medians of continuous variables, and Spearman rank correlation coefficients were calculated to determine the relationship between the medians of the continuous variables. We did not perform a multiple linear regression analysis because of the small number of patients.

In Table 1, we described the measured values for all clinical parameters to show the patient characteristics. However, in Table 2-5, we determined the logarithm of serum ferritin and ERFE levels for statistical analyses because of several outliers. We did not use logarithms in any other parameters because there were no notable outliers.

Missing values were imputed using the last observation

carried forward method. The threshold for significance was set at $p<0.05$. All statistical analyses were conducted using the JMP software program, version 11 (SAS Institute, Cary, USA).

Table 1. Baseline Patient Characteristics (n=22).

Characteristics	
Men	11/22 (50%)
Age (year)	60.5 (55-69)
BMI (kg/m^2)	21.0 (20.1-26.2)
Hemoglobin (g/dL)	14.3 (13.4-15.8)
Platelet count (×10^9/L)	149 (102-195)
Albumin (g/dL)	4.1 (3.9-4.3)
ALT (IU/L)	66 (46-92)
M2BPGi (COI)	1.72 (1.22-3.75)
TC (mg/dL)	165 (148-182)
LDL-C (mg/dL)	90 (76-106)
HDL-C (mg/dL)	48 (41-52)
TG (mg/dL)	115 (75-158)
Iron (μg/dL)	157 (124-193)
Ferritin (ng/mL)	273 (148-408)
TSAT (%)	45 (36-58)
ERFE (ng/mL)	266 (51-744)

Categorical data are presented as the number of patients (%). Continuous data are presented as median (interquartile range).

BMI: body mass index, ALT: alanine aminotransferase, M2BPGi: Mac-2 binding protein glycan isomer, COI: cutoff index, TC: total cholesterol, LDL-C: low-density lipoprotein-cholesterol, HDL-C: high-density lipoprotein-cholesterol, TG: triglyceride, TSAT: transferrin saturation, ERFE: erythroferrone

Results

Patient characteristics

Twenty-four patients were enrolled in this study. Two patients were excluded because of sinus bradycardia at 1 week in one and hepatocellular carcinoma onset at 12 weeks in the other. Another patient was lost to follow-up at 24 weeks, and the missing data for this patient were imputed using the last observation carried forward method. This resulted in 22 patients being included in the analyses.

The median serum ERFE level at baseline in this study was 266 ng/mL, which was greater than that in previously reported healthy controls (15). Seventeen patients had increased serum ferritin levels (men >465 ng/mL, women > 138 ng/mL) or elevated transferrin saturation levels (both men and women >40%). The serum lipid profiles were within the normal range for most patients. Serum alanine aminotransferase (ALT) levels were moderately elevated, but the liver function was maintained in most patients. However, six patients had serum M2BPGi levels of ≥3.00, which was indicative of liver cirrhosis. The patient characteristics are summarized in Table 1.

Half of the patients were men. The serum hemoglobin, platelet count, and triglyceride levels were significantly higher in men than in women. The proportion of patients above the reference interval of serum ferritin level was significantly higher in women than men (8 vs. 3 out of 11, chi-squared test, p=0.033). There was no significant difference in the serum $\log_{10}$ ERFE levels or other clinical parameters between men and women. The differences in patient charac-

Table 2. Differences in Baseline Patient Characteristics by Sex.

Characteristics	Men (n=11)	Women (n=11)	p value
Age (year)	60 (50-62)	64 (56-73)	0.15
BMI (kg/m^2)	21.2 (20.2-25.4)	20.8 (19.4-29.1)	0.81
Hemoglobin (g/dL)	15.8 (14.6-16.7)	13.5 (12.8-14.2)	0.001
Platelet count (×10^9/L)	162 (137-212)	11.5 (8.5-16.5)	0.042
Albumin (g/dL)	4.1 (3.9-4.2)	4.0 (3.7-4.3)	0.84
ALT (IU/L)	64 (39-93)	66 (47-91)	0.79
M2BPGi (COI)	1.69 (1.03-1.87)	1.74 (1.53-5.3)	0.24
TC (mg/dL)	177 (157-183)	156 (144-169)	0.13
LDL-C (mg/dL)	100 (82-107)	78 (69-98)	0.14
HDL-C (mg/dL)	45 (36-49)	51 (44-54)	0.24
TG (mg/dL)	156 (110-165)	96 (73-115)	0.022
Iron (μg/dL)	169 (124-203)	145 (123-183)	0.38
$\log_{10}$ ferritin (ng/mL)	2.56 (2.37-2.67)	2.26 (2.05-2.61)	0.066
TSAT (%)	48 (37-62)	41 (30-57)	0.32
$\log_{10}$ ERFE (ng/mL)	2.17 (1.67-2.80)	2.48 (1.85-3.31)	0.25

Wilcoxon signed-rank sum test. Continuous data are presented as median (interquartile range).

BMI: body mass index, ALT: alanine aminotransferase, M2BPGi: Mac-2 binding protein glycan isomer, COI: cutoff index, TC: total cholesterol, LDL-C: low-density lipoprotein-cholesterol, HDL-C: high-density lipoprotein-cholesterol, TG: triglyceride, TSAT: transferrin saturation, ERFE: erythroferrone

Table 3. Changes in Clinical Parameters at 24 Weeks from Baseline in All Patients (n=22).

Parameters	Baseline	24 weeks	p value
Hemoglobin (g/dL)	14.3 (13.4-15.8)	14.2 (13.2-15.7)	0.24
Platelet count ($\times10^9$/L)	149 (102-195)	161 (114-213)	<0.001
Albumin (g/dL)	4.1 (3.9-4.3)	4.3 (4.1-4.6)	<0.001
ALT (IU/L)	66 (46-92)	18 (13-32)	<0.001
M2BPGi (COI)	1.72 (1.22-3.75)	0.84 (0.59-1.54)	<0.001
TC (mg/dL)	165 (148-182)	185 (171-203)	<0.001
LDL-C (mg/dL)	90 (76-106)	102 (89-120)	<0.001
HDL-C (mg/dL)	48 (41-52)	52 (43-60)	<0.001
TG (mg/dL)	115 (75-158)	114 (81-176)	0.084
Iron (μg/dL)	157 (124-193)	129 (89-167)	0.004
Log_{10} ferritin (ng/mL)	2.44 (2.17-2.61)	2.07 (1.93-2.34)	<0.001
TSAT (%)	45 (36-58)	37 (26-46)	0.002
Log_{10} ERFE (ng/mL)	2.42 (1.71-2.87)	2.09 (0.89-2.78)	<0.001

Wilcoxon signed-rank sum test. Continuous data are presented as median (interquartile range).

ALT: alanine aminotransferase, M2BPGi: Mac-2 binding protein glycan isomer, COI: cutoff index, TC: total cholesterol, LDL-C: low-density lipoprotein-cholesterol, HDL-C: high-density lipoprotein-cholesterol, TG: triglyceride, TSAT: transferrin saturation, ERFE: erythroferrone

Table 4. Changes in Clinical Parameters at 24 Weeks from Baseline in Men (n=11) and Women (n=11).

Parameters	Sex	Baseline	24 weeks	p value
ALT (IU/L)	Men	66 (47-91)	23 (14-38)	0.002
	Women	64 (39-93)	16 (11-24)	0.001
M2BPGi (COI)	Men	1.69 (1.03-1.87)	0.66 (0.53-0.95)	<0.001
	Women	1.74 (1.53-5.3)	0.92 (0.68-2.23)	<0.001
LDL-C (mg/dL)	Men	100 (82-107)	109 (90-119)	0.006
	Women	78 (69-98)	97 (85-122)	0.012
Log_{10} ferritin (ng/mL)	Men	2.56 (2.37-2.67)	2.19 (2.06-2.69)	0.007
	Women	2.26 (2.05-2.61)	2.01 (1.61-2.16)	<0.001
Log_{10} ERFE (ng/mL)	Men	2.17 (1.67-2.80)	1.84 (0.89-2.72)	0.001
	Women	2.48 (1.85-3.31)	2.20 (1.80-2.93)	0.032

Wilcoxon signed-rank sum test. Continuous data are presented as median (interquartile range).

ALT: alanine aminotransferase, M2BPGi: Mac-2 binding protein glycan isomer, COI: cutoff index, LDL-C: low-density lipoprotein-cholesterol, ERFE: erythroferrone

teristics according to sex are summarized in Table 2. In addition, there was no correlation between the clinical parameters in men, women, and the entire patient group (data not shown).

Changes in clinical parameters after HCV eradication (24 weeks from baseline)

In the total patients, the sustained virological response rate was 100% at 24 weeks. After HCV eradication, the median serum log_{10} ERFE level at 24 weeks from baseline was significantly decreased. Likewise, the iron metabolism parameters, liver inflammation, and fibrosis parameters were also significantly decreased, and the lipid profiles were significantly increased. These changes are summarized in Table 3.

In both men and women, the serum ALT, M2BPGi, log_{10} ferritin, and log_{10} ERFE levels were significantly decreased, and the serum LDL-C levels were significantly increased. These changes are summarized in Table 4.

Correlations between changes in clinical parameters (24 weeks from baseline)

In the total patients, the changes in the serum log_{10} ferritin levels were correlated with changes in the serum LDL-C, ALT, and M2BPGi levels. The changes in the serum LDL-C levels were correlated with changes in the serum M2BPGi levels (Table 5).

For men, the changes in the serum log_{10} ERFE levels were correlated with the changes in the serum log_{10} ferritin and LDL-C levels. The changes in the serum log_{10} ferritin

Table 5. Correlations between Changes in Clinical Parameters in All Patients (n=22).

Variables	Log_{10} ERFE	Log_{10} Ferritin	LDL-C
Log_{10} ERFE	-		
Log_{10} Ferritin	0.18	-	
LDL-C	-0.025	-0.62**	-
ALT	0.35	0.65***	-0.26
M2BPGi	-0.096	0.47*	-0.53*

Spearman rank correlation coefficient test. *p<0.05, **p<0.01, ***p<0.001

ERFE: erythroferrone, LDL-C: low-density lipoprotein-cholesterol, ALT: alanine aminotransferase, M2BPGi: Mac-2 binding protein glycan isomer

Table 6. Correlations between Changes in Clinical Parameters in Men (n=11).

Variables	Log_{10} ERFE	Log_{10} Ferritin	LDL-C
Log_{10} ERFE	-		
Log_{10} Ferritin	0.78**	-	
LDL-C	-0.76**	-0.89***	-
ALT	0.60	0.84**	-0.63*
M2BPGi	0.47	0.63*	-0.67*

Spearman rank correlation coefficient test. *p<0.05, **p<0.01, ***p<0.001

ERFE: erythroferrone, LDL-C: low-density lipoprotein-cholesterol, ALT: alanine aminotransferase, M2BPGi: Mac-2 binding protein glycan isomer

Table 7. Correlations between Changes in Clinical Parameters in Women (n=11).

Variables	Log_{10} ERFE	Log_{10} Ferritin	LDL-C
Log_{10} ERFE	-		
Log_{10} Ferritin	-0.35	-	
LDL-C	0.29	-0.28	-
ALT	0.19	0.41	0.17
M2BPGi	-0.28	0.20	-0.17

Spearman rank correlation coefficient test.

ERFE: erythroferrone, LDL-C: low-density lipoprotein-cholesterol, ALT: alanine aminotransferase, M2BPGi: Mac-2 binding protein glycan isomer

levels were correlated with the changes in the serum LDL-C, ALT and M2BPGi levels. The changes in the serum LDL-C levels were correlated with the changes in the serum ALT and M2BPGi levels (Table 6).

For women, the correlations above were not noted (Table 7).

Discussion

To our knowledge, this is the first report to clarify the involvement of ERFE in both iron and lipid metabolism changes after HCV eradication. In addition, we demonstrated the differences in these associations by sex.

Previous studies have shown a close relationship between HCV infection and iron metabolism, in addition to lipid metabolism. Iron is associated with HCV replication, although results have been mixed (16-19). However, HCV infection is associated with iron overload, and HCV eradication reduces iron overload (1, 3). In contrast, lipids are essential for HCV replication (20, 21). HCV infection is associated with a decrease in the serum LDL-C level, and HCV eradication induces an increase in the serum LDL-C levels (22, 23).

These metabolic changes are considered to occur individually after HCV eradication. However, in terms of the similarity of ERFE and myonectin, we considered these changes potentially linked to ERFE, as ERFE is involved in iron metabolism, while myonectin is involved in lipid metabolism (9, 24). Myonectin reduces circulating free fatty acids by promoting their uptake into adipose tissue, which is produced by skeletal muscle, and its production is stimulated by muscle contraction (9).

In the present study, iron parameters were elevated, and serum ERFE levels were markedly elevated at baseline. It is plausible that elevated serum ERFE levels downregulated hepcidin secretion, resulting in iron overload. At baseline, the median serum ferritin level tended to be higher in men than in women. However, the reference interval of the serum ferritin level is greater in men than in women (25). The proportion of patients with serum ferritin levels exceeding the reference interval was significantly higher in women than men. In addition, the median serum hemoglobin level was in the normal range in both men and women, as we excluded patients with anemia. These results suggest that iron dysmetabolism was relatively advanced in women.

In contrast, lipid profiles were within the normal range for most patients at baseline. Previous studies have shown a negative correlation between the myonectin and LDL-C levels, in addition to the effect of excess iron on the reduction in the LDL-C level (26, 27). These previous reports suggest that elevated serum ERFE and ferritin levels may have affected the serum LDL-C levels at baseline in this study.

After HCV eradication, the serum LDL-C levels were significantly increased at 24 weeks from baseline. In addition, the serum ferritin, ALT, and M2BPGi levels were significantly decreased. Of note, the serum ERFE levels were significantly decreased. These changes were observed in both men and women.

For men, there was a correlation between changes in the serum ERFE levels and ferritin levels. Decreased serum ERFE levels may have upregulated hepcidin secretion, resulting in decreased serum ferritin levels. In addition, there were correlations between changes in the serum LDL-C levels and the ALT, M2BPGi levels, and ferritin levels. These results suggest that an increase in serum LDL-C levels was associated with reduced liver inflammation and fibrosis along with a reduction in iron overload. Importantly, the changes in the serum LDL-C levels were also correlated with changes in the serum ERFE levels. These findings indicate the existence of a close relationship between iron and lipid metabolism changes and the involvement of ERFE af-

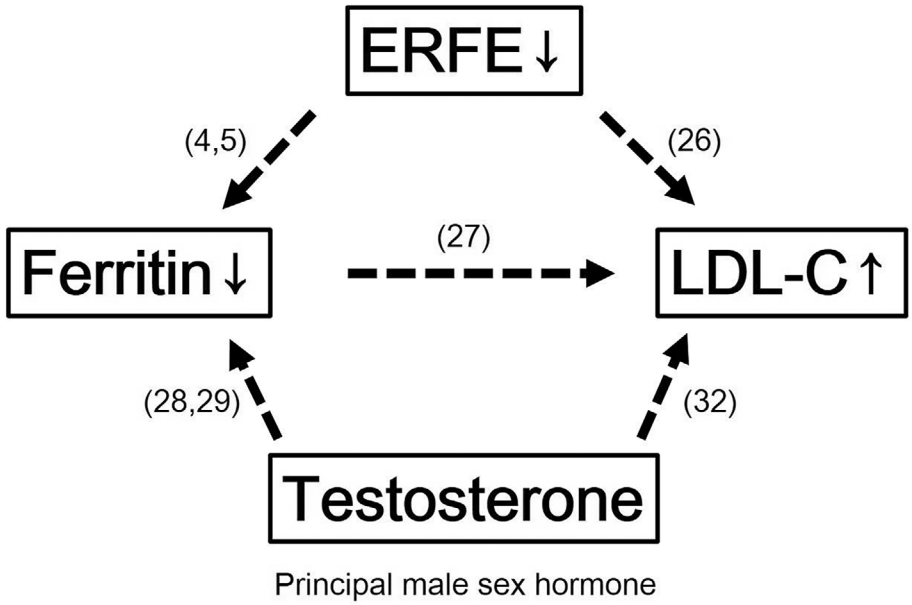

Figure. Possible pathophysiology of the clinical parameter changes after hepatitis C virus eradication in men, based on previous reports (HYPOTHESIS). Numbers in parentheses indicate reference numbers in the main text. ERFE: erythroferrone, LDL-C: low-density lipoprotein cholesterol

ter HCV eradication in men.

In women, no such correlations were noted, although these parameter changes were observed in both sexes. We hypothesized that these sex-related differences might have involved the principal male sex hormone, testosterone, for the following reasons: Testosterone suppresses hepcidin, increases iron availability (28), increases iron turnover, and maintains erythropoiesis (29), thus indicating that the effect of testosterone in iron metabolism is similar to that of ERFE. Furthermore, 5α-dihydrotestosterone treatment was associated with an increased ERFE level in mice (30), suggesting the involvement of testosterone in iron metabolism. Therefore, it is plausible that the association between changes in the serum ERFE and ferritin levels differed by sex because testosterone is a male-dominant hormone.

However, testosterone induces skeletal muscle hypertrophy and a reduction in adipose tissue in men (31). In addition, high endogenous testosterone levels increase serum LDL-C levels (32), indicating the involvement of testosterone in lipid metabolism. Furthermore, another study reported lipid alterations in male myonectin-knockout mice (33), demonstrating the male-specific function of myonectin (equating to ERFE) in lipid metabolism. Therefore, it is also plausible that the association between changes in the serum ERFE and LDL-C levels differed by sex. The possible pathophysiological mechanisms underlying the clinical parameter changes after HCV eradication in men are illustrated in Figure (hypothetical).

Several major limitations of this study should be acknowledged. A small number of patients were included with a short observation period, and the analyses were retrospective. As such, the statistical power may not have been sufficient to detect important differences in this pilot study. In addition, we did not measure the serum testosterone levels.

In conclusion, our analyses suggest a possible association between ERFE, iron, and lipid metabolism changes as well as the interplay between these metabolisms after HCV eradication in men. The decrease in the serum ERFE levels was correlated not only with a decrease in the serum ferritin levels but also with an increase in the serum LDL-C levels. In addition, a decrease in the serum ferritin levels was correlated with an increase in the serum LDL-C levels. These correlations were not observed in women. Therefore, in men, ERFE may be a therapeutic target for iron and lipid dysmetabolism. Further investigations with a larger number of patients are needed to clarify the cause of these sex-related differences.

The authors state that they have no Conflict of Interest (COI).

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