Involvement of essential trace elements in the pathogenesis of hepatitis C virus-related chronic liver disease and nonalcoholic steatohepatitis

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Abstract. Essential trace elements are involved in the pathogenesis of chronic liver disease (CLD), which causes hepatic inflammation, steatosis and fibrosis. The present study investigated the roles of essential trace elements in the pathogenesis of hepatitis C virus-related CLD (CLD-C) and nonalcoholic steatohepatitis (NASH), and compared the levels of these trace elements between the two groups. Serum zinc (Zn), selenium (Se), copper (Cu) and ferritin levels were measured in patients with CLD-C (n=66) and NASH (n=26). Subsequently, the correlations between the levels of these essential trace elements in patient sera and the biochemical or pathological parameters of patients with CLD-C and NASH were determined. The results demonstrated that the serum ferritin levels were significantly correlated with serum alanine aminotransferase levels in both the CLD-C and NASH groups. In both groups, the serum Zn and Se levels were significantly associated with serum albumin levels, and inversely associated with the stages of hepatic fibrosis. Furthermore, serum ferritin levels were positively associated, and serum Zn levels were inversely correlated with the grades of hepatic steatosis in patients with CLD-C, whereas serum Se levels were closely associated with the grades of hepatic steatosis only in patients with NASH. In both groups, serum ferritin levels were positively correlated, and serum Zn levels were inversely correlated with homeostasis model for the assessment of insulin resistance (HOMA-IR) values, and serum Se was negatively correlated with the HOMA-IR values in patients with CLD-C only. In conclusion, these results indicated that the involvement of essential trace elements in insulin resistance and hepatic steatosis may differ slightly between patients with CLD-C and those with NASH.

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Introduction

Several essential trace elements are required for the maintenance of numerous physiological functions, since the elements act as important components of metalloproteins and metalloenzymes (1). It is well established that deficient or excessive levels of essential trace elements can result in the development of chronic liver diseases (CLDs), such as viral chronic hepatitis/cirrhosis, alcoholic liver disease, nonalcoholic fatty liver disease (NAFLD) and autoimmune liver diseases (2,3). Metabolic abnormalities, including insulin resistance and dyslipidemia, are closely associated with the development of hepatic steatosis and hepatic fibrosis (4). Such metabolic abnormalities are also related to disorders of the metabolism of some trace elements, including zinc (Zn), selenium (Se) and iron (Fe) (5).

Strong correlations between impaired trace element metabolism and insulin resistance, hepatic steatosis or hepatic fibrosis have been observed in patients with hepatitis C virus (HCV)-related CLD (CLD-C) (6-9). Recent studies have also revealed that serum Zn and Se levels are inversely associated with the severity of hepatic fibrosis in patients with NAFLD or nonalcoholic steatohepatitis (NASH) (10,11), whereas serum ferritin levels have been shown to be increased in parallel with the degree of hepatic fibrosis in such patients (12). Furthermore, it has been demonstrated that serum copper (Cu) levels are decreased as disease activity becomes more severe in patients with NASH (13,14).

Patients with NASH have some similar common clinical features as patients with CLD-C, including insulin resistance, hepatic steatosis and iron overload (15,16). However, to the best of our knowledge, it is not yet known as to whether the involvement of essential trace elements in the pathogenesis of NASH is identical to that in CLD-C.

The present study aimed to verify the involvement of four essential trace elements (Fe, Zn, Se and Cu) in the pathogeneses of CLD-C and NASH, and to compare the involvement of these trace elements between groups of patients with CLD-C and NASH.

Materials and methods

Study population. The present study was conducted retrospectively. A total of 66 patients with CLD-C and 26 patients with NASH were randomly selected from patients admitted to the Hospital of Kagawa University Faculty of Medicine (Miki, Japan) between January 2015 and December 2019. The pathological diagnosis of NASH was determined on the basis of Matteoni's classification (17).

All of the selected patients with CLD-C had detectable serum HCV-RNA as determined by PCR, and exhibited histological findings compatible with chronic hepatitis or liver cirrhosis.

The study protocol complied with all of the provisions of The Declaration of Helsinki. The design of the study was approved by the Ethical Committees of both the Kagawa Prefectural University of Health Sciences (approval no. 305; Takamatsu, Japan) and Kagawa University Faculty of Medicine (approval no. 2020-045). Written informed consent was obtained from each individual.

Laboratory assessments. HCV-RNA was quantitatively detected with the COBAS TaqMan HCV assay (Roche Molecular Diagnostics) as previously described (18). Serum albumin (Alb) and alanine aminotransferase (ALT), ferritin, glucose and insulin levels were measured using standard laboratory techniques. Insulin resistance was estimated based on the homeostasis model for the assessment of insulin resistance (HOMA-IR) value using the following equation: HOMA-IR value=fasting insulin (μU/m) x fasting glucose (mg/dl)/405. The normal ranges of serum Alb level, ALT level and HOMA-IR value were 3.5-5.5 g/dl, <35 IU/l and <1.6, respectively. Serum Zn, Se and Cu levels were determined by atomic absorption spectrometry, as previously described (19). The serum Zn levels were measured in the morning after the patients underwent an overnight fast due to its circadian rhythm.

Zn deficiency was defined as a serum Zn level <60 μg/dl (20) and Se deficiency was defined as a serum Se level <10 μg/dl (21). The serum ferritin level in each patient was also measured as a serological hallmark of iron storage in the liver. Iron overload was defined as a serum ferritin level exceeding 1.5 times the upper normal range (>300 ng/ml in women and >450 ng/ml in men) (22). The normal range of serum Cu levels was 70-132 μg/dl.

Histological assessments. Each liver biopsy was conducted under the guidance of ultrasound prior to treatment, using 16-gauge needles to obtain the liver specimen. The tissue samples were fixed in 10% formalin and embedded in paraffin. The tissue sections were stained with hematoxylin and eosin for pathological evaluation, as previously described (23). The stage of hepatic fibrosis was based on the New Inuyama Classification system (24), which provides the standard criteria for the histological assessment of chronic hepatitis in Japan. Briefly, the staging of hepatic fibrosis was scored as follows from F_0 to F_4.: F_0, no fibrosis in the liver specimen; F_1, portal expansion; F_2, bridging fibrosis; F_3, bridging fibrosis with lobular distortion; and F_4, liver cirrhosis. The severity of hepatic steatosis was classified into grade 0 to 3, based on the classification proposed by Brunt *et al* (25). Hepatic steatosis in 0, <33, 33-66 and >66% of hepatocytes was defined as grade 0, 1, 2 and 3, respectively.

Statistical analysis. Statistical analyses were conducted using JMP 14 (SAS Institute, Inc.). Data are presented as the mean ± standard deviation. The Mann-Whitney U-test and the Kruskal-Wallis test were used to compare variables between two groups and more than three groups, respectively. When significant differences were identified by the Kruskal-Wallis test, the Dunn-Bonferroni test was used for pairwise comparisons. The correlation between quantitative variables was analyzed by Pearson's test. Fisher's exact probability test was used to compare differences in frequencies. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical characteristics. Table I summarizes the clinical characteristics of the patients. No significant differences in age or sex were identified between the CLD-C and NASH groups; however, patients with NASH had significantly higher body mass index (BMI) values compared with those of patients with CLD-C. The serum ALT levels and HOMA-IR values were also significantly higher in the NASH group compared with those in the CLD-C group. The serum Alb levels were similar between the two groups, indicating that hepatic reserve in patients with NASH was similar to that in patients with CLD-C. Histologically, patients with NASH had significantly more severe hepatic steatosis than patients with CLD-C, whereas the severity of hepatic fibrosis was nearly identical between the two groups.

Distribution of circulating trace elements. The present study investigated the distribution of serum Zn, Se, Cu and ferritin levels in the CLD-C and NASH groups, and compared them (Fig. 1). Eight of the 66 (12.1%) patients with CLD-C and three of the 26 (11.5%) patients with NASH were diagnosed as having Zn deficiency. The mean serum Zn levels in the CLD-C group were similar to those in the NASH group (70.8±12.3 vs. 73.9±12.3 μg/dl; P=0.3058). Similarly, seven of the 66 (10.6%) patients with CLD-C and two of the 26 (7.7%) patients with NASH were diagnosed with Se deficiency, and mean serum Se levels were almost identical between the groups (13.1±2.5 vs. 13.3±2.8 μg/dl; P=0.9593). These Zn- and Se-deficient patients were free from symptoms. The mean serum Cu levels in the NASH group tended to be higher than those in the CLD-C group (115.9±20.6 vs. 106.3±19.0 μg/dl; P=0.0865). The frequencies of hyperferritinemia (>300 ng/ml in women and >450 ng/ml in men) in the CLD-C and NASH groups were 10.6 and 11.5%, respectively. The mean serum ferritin levels in the CLD-C group was almost identical to those in the NASH group (219.0±241.5 vs. 255.9±187.0 ng/ml; P=0.4970).

Table I. Characteristics of patients with CLD-C and NASH.

Characteristic	CLD-C (n=66)	NASH (n=26)	P-value
Age, years[a]	59.3±8.5 (35-81)	60.8±11.6 (36-81)	0.4483
Sex (male/female), n	38/28	10/16	0.0776
BMI, kg/m^{2}[a]	23.9±3.4 (16.6-34.1)	27.9±3.8 (20.7-35.3)	0.0001
ALT, IU/l[a]	70.8±55.3 (15-287)	102.8±65.0 (23-301)	0.0057
Alb, g/dl[a]	4.0±0.5 (2.7-5.1)	4.2±0.4 (3.6-5.1)	0.1792
HOMA-IR[a]	2.06±1.22 (0.52-5.74)	4.49±2.90 (0.89-13.0)	<0.0001
Hepatic steatosis (Gr 0/1/2/3), n	32/24/10/0	0/10/10/6	<0.0001
Hepatic fibrosis (F1/2/3/4), n	19/22/15/10	13/2/11/0	0.1637

[a]Data is shown as mean ± standard deviation (range). CLD-C, hepatitis C virus-related chronic liver disease; NASH, nonalcoholic steatohepatitis; BMI, body mass index; ALT, alanine aminotransferase; Alb, albumin; HOMA-IR, homeostasis model for the assessment of insulin resistance.

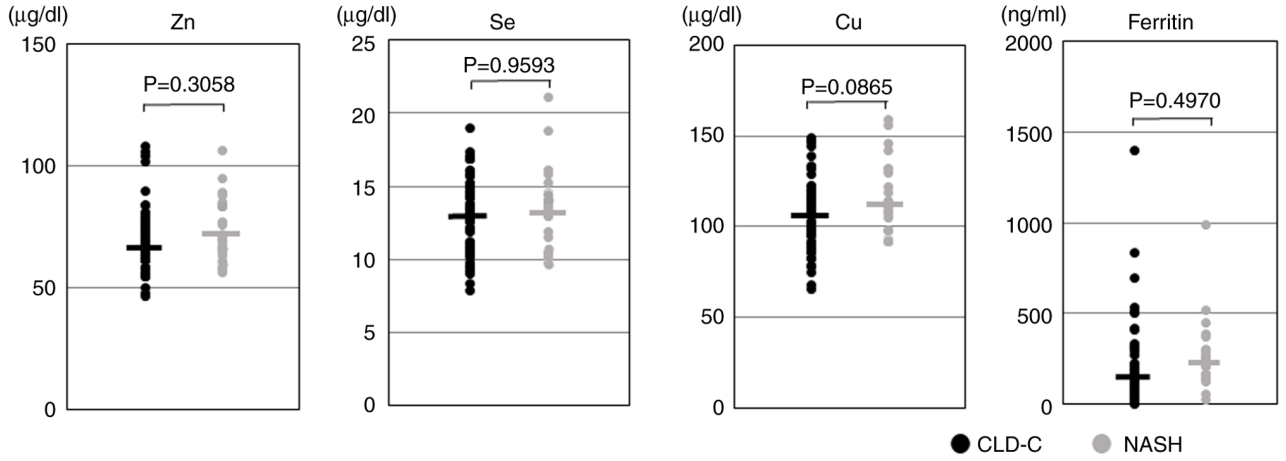

Figure 1. Distributions of serum trace element levels in patients with CLD-C and NASH. The horizontal bars represent the mean value of each serum trace element level. CLD-C, hepatitis C virus-related chronic liver disease; Cu, copper; NASH, nonalcoholic steatohepatitis; Se, selenium; Zn, zinc.

The present study also examined the differences in the trace elements between male and female patients. There were no significant differences in serum Zn or Se levels between male and female patients in both the CLD-C and NASH groups. However, the serum Cu levels were significantly higher in the female patients with CLD-C compared with those in the male patients with CLD-C (113.0±16.5 vs. 101.4±19.4 mg/dl; P=0.0100), whereas the serum ferritin levels were significantly lower in the female patients with NASH compared with those in the male patients (186.4±92.6 vs. 367.1±246.0 ng/ml; P=0.0132) (data not shown).

Biochemical and nutritional factors associated with serum trace elements. The present study analyzed the laboratory factors associated with circulating trace element levels in the patients. As shown in Table II, the serum ALT levels were significantly correlated with the serum ferritin levels in both the CLD-C (r=0.667, P<0.0001) and NASH (r=0.523, P=0.0061) groups. The serum Alb levels were significantly correlated with the serum Zn levels (CLD-C: r=0.475, P<0.0001; NASH: r=0.669, P=0.0002) and serum Se levels (CLD-C: r=0.516, P<0.0001; NASH: r=0.592, P=0.0015) in both groups. A positive correlation was also observed between serum Alb and Cu levels in patients with NASH (r=0.446, P=0.0330).

The HOMA-IR values were positively correlated with serum ferritin levels in both the CLD-C (r=0.419, P=0.0022) and NASH (r=0.411, P=0.0423) groups. There was an inverse association between the HOMA-IR values and serum Zn levels in the CLD-C group (r=-0.290, P=0.0192) and am inverse correlation in the NASH group (r=-0.451, P=0.0309), whereas an inverse association was observed between the HOMA-IR values and serum Se levels in patients with CLD-C only (r=-0.275, P=0.0332). BMI values were not correlated with the levels of any of the four essential trace elements in either patients with CLD-C or NASH.

Histological factors associated with serum trace element levels. Subsequently, the present study investigated which trace element affected hepatic steatosis in CLD-C and NASH patients. As shown in Fig. 2A, the serum Zn levels were significantly decreased as the grade of hepatic steatosis became more severe in patients with CLD-C. The serum Zn levels in patients with NASH and grade 2 steatosis tended to be higher than those in patients with NASH and grade 1 steatosis (78.1±9.6

Table II. Correlations between serum trace element levels and biochemical or nutritional factors in patients with CLD-C and NASH.

		CLD-C (n=66)		NASH (n=26)	
Factor	Element	r-value	P-value	r-value	P-value
ALT	Zn	0.122	0.3305	0.110	0.5911
	Se	0.101	0.4388	-0.116	0.5710
	Cu	0.022	0.8642	0.259	0.2331
	Ferritin	0.667	<0.0001	0.523	0.0061
Alb	Zn	0.475	<0.0001	0.669	0.0002
	Se	0.516	<0.0001	0.592	0.0015
	Cu	0.208	0.1131	0.446	0.0330
	Ferritin	0.163	0.2635	-0.090	0.6631
HOMA-IR	Zn	-0.290	0.0192	-0.451	0.0309
	Se	-0.275	0.0332	-0.061	0.7823
	Cu	0.032	0.8034	-0.080	0.7379
	Ferritin	0.419	0.0022	0.411	0.0423
BMI	Zn	-0.078	0.5677	-0.194	0.3419
	Se	0.012	0.9334	-0.182	0.3732
	Cu	0.094	0.4980	0.185	0.3993
	Ferritin	0.144	0.3383	-0.064	0.7565

CLD-C, hepatitis C virus-related chronic liver disease; NASH, nonalcoholic steatohepatitis; ALT, alanine aminotransferase; Alb, albumin; HOMA-IR, homeostasis model for the assessment of insulin resistance; BMI, body mass index; Zn, zinc; Se, selenium; Cu, copper.

vs. 68.1±10.1 μg/dl; P=0.0783). The serum Se levels in patients with NASH and grade 3 steatosis were significantly higher than those in patients with NASH and grade 1 steatosis (15.0±2.1 vs. 11.6±1.6 μg/dl; P=0.0199; Fig. 2B). The serum ferritin levels in patients with CLD-C were significantly increased in proportion to the grade of hepatic steatosis (Fig. 2D). However, no significant association was identified between serum Cu levels and the severity of hepatic steatosis in the NASH or CLD-C groups (Fig. 2C).

The association between serum trace element levels and the stages of hepatic fibrosis were also assessed in patients with CLD-C and NASH. In both groups, serum Zn levels were significantly reduced as the stage of hepatic fibrosis progressed (Fig. 3A). Similarly, serum Se levels were decreased in proportion to the severity of hepatic fibrosis in both groups (Fig. 3B). Serum Cu levels in patients with CLD-C and F_3 hepatic fibrosis were significantly higher than those in patients with CLD-C and F_1 hepatic fibrosis (114.8±15.1 vs. 100.1±18.5 μg/dl; P=0.0275; Fig. 3C). By contrast, no significant associations were observed between serum ferritin levels and the severity of hepatic fibrosis in either CLD-C or NASH groups (Fig. 3D).

Discussion

The results of the present study indicated that some essential trace elements that contribute to hepatic inflammation, hepatic fibrosis and hepatic reserve were common between patients with CLD-C and patients with NASH. However, other essential trace elements involved in hepatic steatosis and insulin resistance differed between the CLD-C and NASH groups, although their average levels were approximately equivalent in the two groups. To the best of our knowledge, the present study is the first to compare the involvement of these essential trace elements in the pathogenesis of CLD-C with the pathogenesis of NASH.

It is well established that iron deposition in the liver can initiate reactive oxygen species and subsequently lead to hepatic inflammation in patients with CLD-C or NASH (26). The attenuation of iron overload by phlebotomy thus results in improved serum transaminase levels in such patients (27). The present findings confirmed the strong correlation between serum ALT and ferritin levels in patients with CLD-C and those with NASH.

Hypoalbuminemia derived from an unfavorable hepatic reserve results in a relative increase in α2 macroglobulin, which more strongly binds to Zn, eventually causing a substantial increase in the urinary excretion of Zn (28). Thus, the present study suggests that serum Zn levels are associated with serum Alb levels in patients with CLD-C and NASH.

A decline in serum Se levels is frequently observed in patients with decompensated liver cirrhosis (29). However, lower serum Se levels do not indicate Se deficiency; rather, they imply an unfavorable hepatic reserve in these patients (29). The present study revealed a positive correlation between serum Se and Alb levels in both the CLD-C and NASH groups, suggesting that lower serum Se levels reflect an unfavorable hepatic reserve in these patients.

Our previous study revealed a close correlation between serum ferritin levels and HOMA-IR values in patients with CLD-C (9). The present study demonstrated a positive

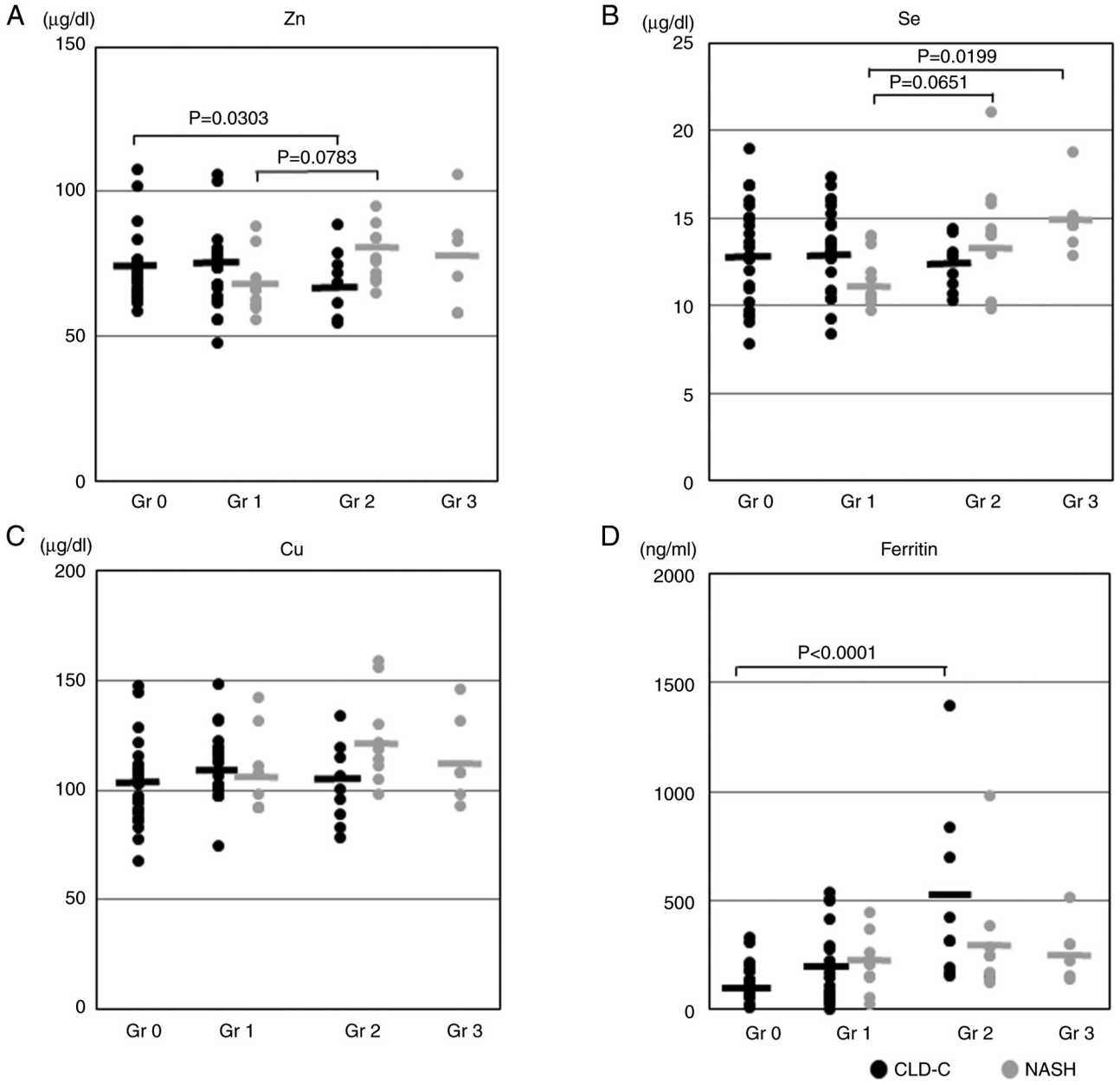

Figure 2. Association between the serum trace element levels and the grades of hepatic steatosis in the CLD-C and NASH groups. The horizontal bars represent the mean range of each serum trace element level. (A) Zn, (B) Se, (C) Cu and (D) ferritin. CLD-C, hepatitis C virus-related chronic liver disease; Cu, copper; NASH, nonalcoholic steatohepatitis; Se, selenium; Zn, zinc.

correlation between serum ferritin levels and HOMA-IR values in patients with NASH patients as well as those with CLD-C. Iron is likely to affect hepatic insulin sensitivity. It was thus hypothesized that the hepatic extraction and metabolism of insulin may be attenuated as the deposition of iron in the liver becomes more severe, leading to hyperinsulinemia in CLD-C and NASH (30,31).

The present study revealed that serum Zn levels were inversely correlated with HOMA-IR values in patients with CLD-C (9). Zn has been demonstrated to serve a crucial role in the stabilization of the insulin-like growth factor-1 (IGF-1) transcript (32). Zn deficiency can lead to an impairment of IGF-1 synthesis and subsequently an increase in insulin release from β cells in patients with CLD-C (33). The present analyses also elucidated an inverse correlation between serum Zn levels and HOMA-IR values in patients with NASH, which supports the inverse correlation between serum Zn levels and HOMA-IR values (10). The present study hypothesized that the putative mechanism by which Zn deficiency causes insulin resistance in patients with NASH is probably equivalent to that in patients with CLD-C. Notably, several studies have revealed a decrease in IGF-1 release in patients with NASH (34). The present study also confirmed that a significant correlation existed between serum Zn and IGF-1 levels in patients with NASH (data not shown).

Our previous study revealed an inverse correlation between serum Se concentrations and HOMA-IR values in patients with CLD-C (8). Lower serum Se levels may impair the activation of mitogen-activated protein kinase, leading to insulin resistance in such patients (35). Unexpectedly, the present study did not

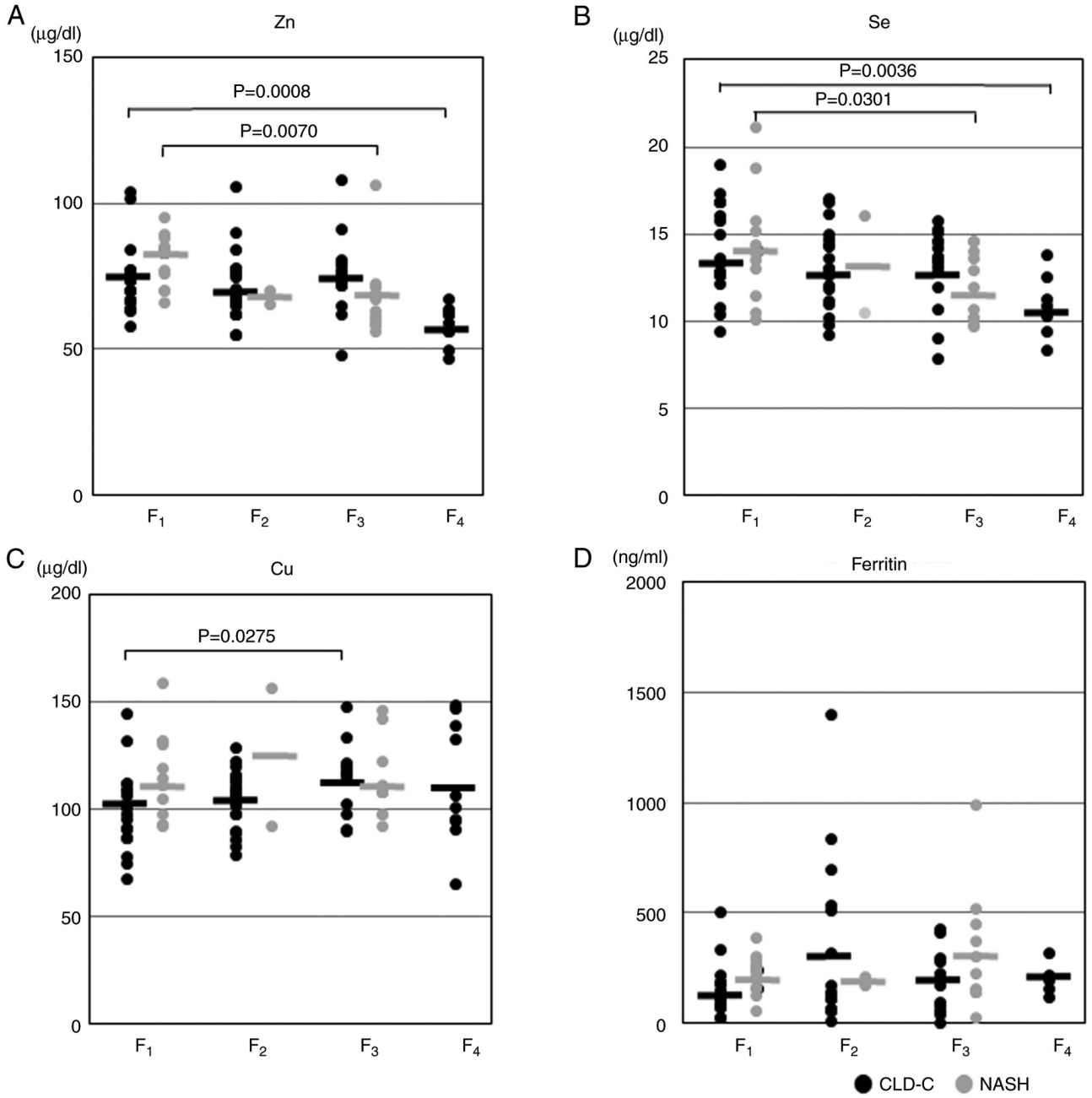

Figure 3. Association between the serum trace element levels and the stages of hepatic fibrosis in the CLD-C and NASH groups. The horizontal bars represent the mean range of each serum trace element level. (A) Zn, (B) Se, (C) Cu and (D) ferritin. CLD-C, hepatitis C virus-related chronic liver disease; Cu, copper; NASH, nonalcoholic steatohepatitis; Se, selenium; Zn, zinc.

show a significant correlation between serum Se levels and HOMA-IR values in patients with NASH. It is of interest that a high prevalence of type 2 diabetes mellitus (T2DM) is observed in individuals with relatively high levels of serum Se or relatively low Se levels (36). This result may explain the reason why we did not observe an inverse correlation between serum Se concentrations and HOMA-IR values in patients with NASH.

Selenoprotein P (SeP) has been suggested to serve an important role in insulin resistance (37). However, there are conflicting findings regarding circulating SeP levels in patients with NASH. A previous study detected higher serum SeP levels in patients with NASH compared with those in healthy controls or patients with NAFLD (38), whereas another study documented lower SeP levels in patients with NASH (39).

The grades of hepatic steatosis have been reported to be increased as serum Zn levels decrease in patients with CLD-C (9). Lower serum Zn levels may cause the inactivation of peroxisome proliferator-activated receptor-α, and a subsequent facilitation of lipid peroxidation in such patients (40). Nevertheless, the reason why no negative association was detected between the grade of hepatic steatosis and serum Zn levels in the patients with NASH in the present study remains uncertain. It is of interest that in a previous study the administration of zinc gluconate did not result in the improvement of hepatic steatosis in patients with NAFLD (41).

The present findings also demonstrated that the severity of hepatic steatosis was increased in proportion to serum Se levels in patients with NASH. Spaur *et al* (42) reported that

higher serum Se levels were associated with the severity of hepatic steatosis in participants of the National Health and Nutrition Examination Survey. In *db/db* rats (an animal model of T2DM), long-term supplementation with selenite, which is one of the inorganic selenium compounds, has been shown to result in the exacerbation of hepatic steatosis by reducing the antioxidant defense capacity, despite the improvement in hyperglycemia (43). By contrast, Bonnefont-Rousselot *et al* (44) documented that serum Se levels were independent of the grade of hepatic steatosis in patients with NAFLD. In an experimental animal model of NASH, the administration of selenoneine, an organoselenium compound, has been shown to alleviate the degree of hepatic steatosis (45). As aforementioned, the relationship between serum Se levels and the degree of hepatic steatosis remains controversial.

It is well established that a decrease in the activity of collagenase derived from Zn deficiency results in the progression to more advanced hepatic fibrosis (46). This explains why the stage of hepatic fibrosis became more severe as serum Zn levels gradually decreased in patients with CLD-C and NASH in the present study.

A previous report revealed that supplementation with Se can inhibit the procollagen synthesis in an animal model of hepatic fibrosis via a decrease in oxidative stress, and the subsequent reduced collagen formation and enhanced collagen degradation (47). These results may indicate that a decline in serum Se levels can lead to the development of hepatic fibrosis in patients with CLD-C (8) or NASH (11). Concerning the serum Se concentrations in the patients with NASH in the present study, it was observed that the serum Se levels were increased in proportion to the degree of hepatic steatosis, and that the serum Se levels were decreased in parallel with the degree of hepatic fibrosis.

In the present study, serum Cu levels were increased as the stage of hepatic fibrosis was enhanced in patients with CLD-C, whereas these levels did not affect the stage of hepatic fibrosis in patients with NASH. Cu is likely to serve a pivotal role in hepatic fibrosis as a cofactor (48). Nobil *et al* (14) revealed that serum Cu levels in patients with NAFLD with more severe activity were significantly lower compared with those in patients with NAFLD and no activity (14). By contrast, a recent study reported a strong correlation between higher serum Cu levels and the risk of NAFLD in women (49), although no significant difference in serum Cu concentrations was found in the present study between male and female patients with NASH. Lower serum ceruloplasmin levels may cause a high susceptibility to oxidative stress in hepatocytes, eventually leading to lower circulating Cu concentrations in patients with NAFLD with severe activity. Aigner *et al* (13) also observed that hepatic Cu content was lower in patients with NASH than in those with NAFLD because of lower Cu availability in the patients with NASH.

Serum ferritin levels may predict the severity of hepatic fibrosis in patients with NASH (12). Excessive iron activates hepatic stellate cells by increasing α-smooth muscle actin, collagen and transforming growth factor-β (22). However, this was not confirmed in patients with CLD-C or NASH in the present study.

There are several study limitations to consider. First, some of the results obtained did not reach statistical significance due to the small sample size. Therefore, it was not possible to analyze the data in the present study separately for male and female patients. A large-scale study is thus required to verify the present findings. Second, the present study could not confirm whether the female patients were in menopause, thus bias may have occurred with respect to serum ferritin levels. Third, the correlation between dietary intake and the circulating levels of each trace element was not investigated, and the results may be biased because of imbalanced dietary intake of these trace elements. Moreover, the correlations between serum Zn, Se, Cu or ferritin levels and HCV-RNA loads were not assessed. However, our previous study explored the correlation between serum Se levels and HCV-RNA load and no significant correlation was found (8).

In conclusion, the involvement of iron in the pathogenesis of hepatic inflammation was common to both patients with CLD-C and those with NASH. In addition, lower serum Zn and Se levels indicated an unfavorable hepatic reserve and advanced hepatic fibrosis in both of these groups. However, the roles of Zn and Se in the pathogenesis of hepatic steatosis and insulin resistance were distinct between the CLD-C and NASH groups. Further studies are required to clarify the mechanisms by which disordered essential trace element metabolism may evoke hepatic steatosis and insulin resistance in patients with NASH.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TH and SK designed the study. KF, SM, JT, AM and TM collected the samples and supported the study techniques. TH performed data analyses and wrote the original draft. TM edited the original draft. TM and AM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committees of both the Kagawa Prefectural University of Health Sciences (approval no. 305) and Kagawa University Faculty of Medicine (approval no. 2020-045). Written informed consent was obtained from each individual.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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