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Copper deficiency is an independent risk factor for mortality in patients with advanced liver disease

Lei Yu[1] | Sarim Yousuf[2] | Shahrukh Yousuf[1] | Jeffrey Yeh[1] | Scott W. Biggins[1] | Chihiro Morishima[3] | Irene Shyu[3] | Galen O'Shea-Stone[4] | Brian Eilers[4] | Annie Waldum[4] | Valérie Copié[4] | Jason Burkhead[5]

[1]Department of Medicine, Division of Gastroenterology and Center for Liver Investigation Fostering Discovery, University of Washington, Seattle, Washington, USA

[2]College of Medicine, Ziauddin University, Karachi, Pakistan

[3]Department of Lab Medicine and Pathology, University of Washington, Seattle, Washington, USA

[4]Department of Chemistry, Montana State University, Bozeman, Montana, USA

[5]Department of Biological Sciences, University of Alaska Anchorage, Anchorage, Alaska, USA

Correspondence
Lei Yu, MD, Department of Medicine, Division of Gastroenterology, University of Washington, 1959 NE Pacific Street, Box 356175 Seattle, WA 98195-6175, USA.
E-mail: leiy@medicine.washington.edu

Jason Burkhead, Department of Biological Sciences, University of Alaska Anchorage, 3211 Providence Drive, Anchorage, AK 99508-4614, USA.
E-mail: jlburkhead@alaska.edu

Abstract

Background and Aim: Copper is an essential trace metal serving as a cofactor in innate immunity, metabolism, and iron transport. We hypothesize that copper deficiency may influence survival in patients with cirrhosis through these pathways.

Methods: We performed a retrospective cohort study involving 183 consecutive patients with cirrhosis or portal hypertension. Copper from blood and liver tissues was measured using inductively coupled plasma mass spectrometry. Polar metabolites were measured using nuclear magnetic resonance spectroscopy. Copper deficiency was defined by serum or plasma copper below 80 μg/dL for women or 70 μg/dL for men.

Results: The prevalence of copper deficiency was 17% (N = 31). Copper deficiency was associated with younger age, race, zinc and selenium deficiency, and higher infection rates (42% vs. 20%, $p = 0.01$). Serum copper correlated positively with albumin, ceruloplasmin, hepatic copper, and negatively with IL-1β. Levels of polar metabolites involved in amino acids catabolism, mitochondrial transport of fatty acids, and gut microbial metabolism differed significantly according to copper deficiency status. During a median follow-up of 396 days, mortality was 22.6% in patients with copper deficiency compared with 10.5% in patients without. Liver transplantation rates were similar (32% vs. 30%). Cause-specific competing risk analysis showed that copper deficiency was associated with a significantly higher risk of death before transplantation after adjusting for age, sex, MELD-Na, and Karnofsky score (HR: 3.40, 95% CI, 1.18–9.82, $p = 0.023$).

Abbreviations: μg, micrograms; BMI, body mass index; CTP class, Child-Turcotte-Pugh Class; Cu, copper; IL-1β, IL-1-beta; IQR, interquartile range; MELD-Na, Model for end stage liver disease–sodium score; NMR, nuclear magnetic resonance; pg, picograms; PLS-DA, partial least squares discriminant analyses; TNF-α, TNF alpha.

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Conclusions: In advanced cirrhosis, copper deficiency is relatively common and is associated with an increased infection risk, a distinctive metabolic profile, and an increased risk of death before transplantation.

INTRODUCTION

In the US, ~41,000 people die annually of advanced liver disease or cirrhosis.[1] Often attributed to decreased oral intake, hypermetabolic state, malabsorption, and inflammation, malnutrition is not only common in cirrhosis but also associated with poor prognosis regardless of disease etiologies.[2,3] Current guidelines in the management of malnutrition focus on protein and calorie intake, with limited considerations for trace metals, which have wide-ranging physiological effects.[2,3]

The transition metal copper (Cu) has an essential role in mammalian physiology.[4] Although Cu's high redox potential requires it to be closely sequestered within cells to avoid oxidative injury, this property makes it an ideal cofactor for a large group of so-called cuproenzymes involved in mitochondrial aerobic metabolism (cytochrome-C oxidase), antioxidant defense (superoxide dismutase), cross-linking of collagen (lysyl oxidase), iron transport (ceruloplasmin), and inflammation (NLRP3 inflammasome).[4,5] Cu uptake, transport, and utilization are largely mediated by the liver. In hepatic parenchymal cells, excess Cu is either sequestered by metallothioneins or exported by means of ATP7B, which traffics Cu to endosome or lysosome-derived compartments and bile.[6,7] Cu enters the circulation as a cofactor of ceruloplasmin.[8] Circulating Cu are then acquired by other tissues, including the heart, brain, and muscle.[9] Cu metabolism and liver function are therefore integrally linked.

The role of Cu in liver disorders is best recognized in the autosomal recessive Wilson disease, in which excessive hepatic Cu accumulation is the proven cause of progressive liver injury.[10] The other end of the spectrum, Cu deficiency, is less well understood. In bile duct–ligated rats, chelator-induced Cu deficiency worsened hepatic fibrosis, likely mediated through iron overload and reduced capacity for antioxidant defense.[11] In the literature, among patients without known liver diseases, consequences of Cu deficiency include tissue fibrosis, cytopenia, iron overload, and susceptibility to infections,[12–15] all of which are prevalent in patients with cirrhosis.[16,17] To further our preliminary observation of a small series of Cu-deficient patients,[18] we undertook the following study to examine the prevalence, clinical and molecular phenotypes of Cu deficiency, and its relationship with patient mortality in a large cohort of patients with advanced liver disease.

METHODS

Study design and population

We performed a retrospective cohort study using clinical data, blood, and liver explant tissues from consecutive patients who presented to the University of Washington Hepatology clinic for liver transplant evaluation between April and July 2017. Among 192 patients, we excluded 8 cirrhosis patients who did not have available blood samples and 1 patient who had metastatic carcinoid tumor without cirrhosis or portal hypertension. The remaining 183 patients constituted the final study cohort. All research was conducted in accordance with both the Declarations of Helsinki and Istanbul. Written informed consent was waived because of the retrospective nature of the study. The study protocol was approved by the University of Washington Institutional Review Board.

Baseline patient characteristics were extracted manually from electronic medical records utilizing clinic notes and study reports. All patients had features of cirrhosis or portal hypertension based on history, physical exam, imaging, laboratory parameters, or hemodynamic measurements. No patient had Wilson disease. Two of the 183 patients had undergone liver transplantation 9 months and 20 years before their presentation. Both had cirrhosis in their liver graft based on clinical and histological features. One patient had ascites, varices, portal hypertension (HVPG = 14 mm Hg), and portal-based fibrosis in the setting of combined variable immune deficiency. Baseline history of infections was defined as any of the following infections within 1 year before blood sample collection: peritonitis, urinary tract infection, pneumonia, bacteremia with or without sepsis, clostridioides difficile, endocarditis, and skin infections. Nutritional risk was determined by trained dieticians using Subjective Global Assessment.[19] Functional assessment was determined by the physician team using the Karnofsky scoring system.[20]

Patients are defined as Cu deficient if their serum or plasma Cu concentrations measured from blood samples collected at baseline were below the normal range according to the University of Washington Laboratory (80–155 μg/dL for women and 70–140 μg/dL for men). Unused serum or plasma from routine clinical care were collected and frozen at −70 °C within 24 hours of blood draw. Special metal-free collection tubes were not used. Concentrations of Cu and other metals were measured using inductively coupled plasma mass spectrometry at

Oregon Health & Science University Elemental Analysis Core. TNF-α and IL-1-beta (IL-1β) were determined by ELISA (ELISAMax Deluxe Set, Biolegend Inc., San Diego, CA). Serum polar metabolites from a subgroup of patients (N = 63) were identified and quantitated using a Bruker 600 MHz (^{1}H Larmor frequency). AVANCE III solution nuclear magnetic resonance (NMR) spectrometer at Montana State University Department of Chemistry. Details of sample preparation and analysis are presented in the Supporting Information (http://links.lww.com/HC9/A168). For hepatic Cu assessment (N = 50), excess paraffin was removed from formalin-fixed paraffin-embedded liver explant tissues, and hepatic Cu and other metals were measured using inductively coupled plasma mass spectrometry. Hepatic Cu concentration was expressed as microgram (μg) per gram (g) of the dry weight of the specimen. Because there is no well-accepted lower limit of normal for hepatic Cu concentration, the median hepatic Cu concentration (9.5 μg/g) was chosen to define 2 groups for comparison of clinical and pathological features. Two dedicated hepatopathologists who were blinded to the patients' Cu deficiency status examined the histology with results at consensus.

The primary outcome of the study is patient death before liver transplantation from the time of baseline blood sample collection until study closure (October 31, 2019). Patient deaths were ascertained by manually searching all linked electronic medical records within and outside the University of Washington Medical System.

Statistical analysis

Baseline characteristics according to patients' Cu deficiency status were compared using chi-square test, Student *t* test or the Wilcoxon Rank-sum test. Shapiro-Wilk test was used to test for the normality of continuous variables. Spearman correlation was used to assess the relationship between serum Cu concentrations and serum albumin, ceruloplasmin, IL-1β, TNF-α, and hepatic Cu. Logistic regression was used to assess the relationship between Cu deficiency and infection before and after adjusting for MELD-Na score. Cause-specific competing risk analysis was used to assess the relationship between Cu deficiency and death before liver transplantation, where transplantation is treated as the competing risk.[21,22] Time to event was defined as the number of days from baseline blood sample collection until death, transplantation, last known follow-up, or study closure (October 31, 2019). Patients who underwent transplantation were censored at the time of transplantation. The following variables were determined *a priori* to be included in the multivariate model because of their known relationship with patient survival: age, sex, Model for End Stage Liver Disease–Sodium Score (MELD-Na), and functional status, as determined by the Karnofsky score. MELD-Na and Karnofsky scores were modeled as continuous variables. Cumulative incidence curves for death before transplantation were compared using the log-rank test. Analyses of clinical data were performed using STATA SE (Version 11.0, College Station, TX) and R (Version 4.0.2). Nonparametric correlation analysis was performed using JMP Pro 15 (SAS Institute, Cary, NC). Processing of 1D ^{1}H NMR spectra and metabolite profile analyses were performed using Chenomx NMR Suite Software, Version 8.4, Edmonton, Canada, MetaboAnalyst and R with full details of analytical procedures presented in the Supporting Information[23] (http://links.lww.com/HC9/A168).

RESULTS

Baseline clinical features according to Cu deficiency status

The prevalence of Cu deficiency was 17% (N = 31) within the study cohort (Table 1). Among patients who were Cu deficient, the median serum/plasma Cu concentration was 62 μg/dL compared with 110 μg/dL among those who were not. Cu deficiency was associated with younger age ($p = 0.007$) and race ($p = 0.04$). None of the Cu-deficient patients had prior weight loss surgery, inflammatory bowel disease, celiac disease, or zinc supplementation. Cu deficiency was more common in patients with alcohol-associated liver disease and cryptogenic cirrhosis. One patient with primary biliary cholangitis who also used alcohol heavily belonged to the Cu-deficient group. Overall, there was no statistically significant association between Cu deficiency and liver disease etiologies.

In terms of disease severity, Cu deficiency was associated with a slightly higher international normalized ratio ($p = 0.01$) and a greater proportion of patients with prior infections ($p = 0.01$, Table 1). The association between Cu deficiency and infections remains statistically significant after adjusting for MELD-Na score ($p = 0.02$). Even though Cu-deficient patients had slightly higher MELD-Na score and the proportion of patients with Child-Turcotte-Pugh (CTP) class C, these differences were not statistically significant. Similarly, Karnofsky score and nutritional risk were not significantly different between the 2 groups. All Cu-deficient patients had moderate to high nutritional risk.

Baseline biochemical and metabolomics analyses according to Cu deficiency status

Trace metal analyses indicated that zinc and selenium concentrations were lower in Cu-deficient patients. Using standard laboratory definitions, both zinc and selenium

TABLE 1 Baseline characteristics of 183 patients with cirrhosis or portal hypertension according to copper deficiency status

	Serum copper concentration (μg/dL, median, IQR)	Copper deficiency status, N (%)		
		Yes	No	*p*
Study subjects	103 (80, 123)	31 (17)	152 (83)	
Age (years, median, IQR)	—	54 (47, 60)	60 (52, 64)	0.007
Sex	—	—	—	0.8
Male	99 (80, 123)	16 (52)	92 (61)	—
Female	108 (84, 123)	15 (48)	60(39)	—
Race	—	—	—	0.04
White	104 (82, 123)	20 (64)	109 (72)	—
Black	140 (113, 157)	0	5 (3.3)	—
Hispanic	76 (61, 114)	7 (22)	9 (5.9)	—
Asian	95 (87, 107)	1 (3.2)	11 (7.2)	—
Other	116 (85, 142)	3 (9.7)	18 (12)	—
Underlying liver diseases	—	—	—	0.2
Alcohol	94 (76, 111)	11 (35)	39 (26)	—
NASH	106 (87, 131)	2 (6.4)	23 (15)	—
HCV	98 (82, 120)	8 (26)	50 (33)	—
HBV	83 (80, 98)	1 (3.2)	4 (2.6)	—
PBC	123 (90, 136)	1 (3.2)	3 (2.0)	—
PSC	157 (126, 182)	0	13 (8.6)	—
Autoimmune	94 (68, 131)	2 (6.4)	2 (1.3)	—
Cryptogenic	109 (63, 114)	6 (19)	15 (9.9)	—
Other	159 (119, 208)	0	3 (2.0%)	
HCC	—	—	—	0.1
No	100 (80, 123)	28 (90)	121 (80)	—
Yes	104 (82, 121)	3 (9.7)	31 (20)	—
Ascites	—	—	—	0.7
No	103 (76, 126)	11 (36)	48 (32)	
Yes	103 (83, 122)	20 (64)	104 (68)	
Variceal bleeding	—	10 (32)	37 (24)	0.3
No	103 (82, 123)	18 (58)	120 (80)	—
Yes	106 (65, 132)	13 (42)	31 (20)	—
Encephalopathy	—	—	—	0.1
No	110 (87, 132)	8 (26)	62 (41)	—
Yes	94 (77, 116)	23 (74)	90 (59)	—
History of infections[a]	—	—	—	0.01
No	103 (82, 123)	18 (58)	120 (80)	—
Yes	106 (65, 132)	13 (42)	31 (20)	—
BMI (median, IQR)	—	29 (23, 33)	30 (25, 34)	0.3
Karnofsky score (median, IQR)[b]	—	80 (70, 90)	90 (70, 90)	0.4
Nutritional risk[c]	—	—	—	0.4
Low	109 (83, 122)	0	6 (5.9)	—
Moderate	105 (76, 116)	10 (38)	40 (40)	—
High	96 (79, 121)	16 (62)	55 (54)	—

TABLE 1. (*continued*)

	Serum copper concentration (μg/dL, median, IQR)	Copper deficiency status, N (%)		
		Yes	No	*p*
Child-Turcotte-Pugh Class	—	—	—	0.2
A	111 (84, 124)	3 (9.7)	36 (24)	—
B	103 (82, 128)	17 (55)	76 (50)	—
C	92 (76, 114)	11 (35)	40 (26)	—
MELD-Na (median, IQR)	—	18 (13, 22)	16 (11, 20)	0.2
Sodium (meq/L, median, IQR)	—	137 (135, 139)	137 (134, 139)	0.8
Creatinine (median, IQR)	—	0.8 (0.6, 1.0)	0.9 (0.7, 1.2)	0.06
Total bilirubin (mg/dL, median, IQR)	—	2.2 (1.4, 3.8)	1.8 (1.1, 2.8)	0.08
Albumin (g/dL, median, IQR)	—	3.0 (2.5, 3.5)	3.2 (2.8, 3.7)	0.06
INR (median, IQR)	—	1.5 (1.4, 1.8)	1.4 (1.2, 1.6)	0.02
White blood cells (10^3/μL , median, IQR)	—	4.7 (3.8, 6.0)	4.4 (3.4, 5.9)	0.2
Hemoglobin (g/dL, median, IQR)	—	11.7 (9.7, 13.4)	11.9 (10.2, 13.6)	0.4
Platelet (10^3/μL, median, IQR)	—	76 (57, 121)	85 (65, 117)	0.3
Ceruloplamsin (mg/dL, median, IQR)[d]	—	17.1 (15.4, 21.2)	23.9 (20.8, 30.0)	0.001
Serum iron (μg/dL, median, IQR)	—	113 (81.3, 166)	122 (86.9, 177)	0.3
Serum zinc (μg/dL, median, IQR)	—	47.7 (36.7, 61.1)	56.4 (44.7, 74.2)	0.002
Zinc deficiency, N (%)[e]	—	—	—	0.04
No	110 (86, 131)	8 (26)	70 (46)	—
Yes	96 (77, 119)	23 (74)	82 (54)	—
Serum selenium (μg/dL, median, IQR)	—	10.7 (8.9, 12.3)	13.9 (11.8, 16.9)	0.001
Selenium deficiency, N (%)[f]	—	—	—	0.002
No	104 (82, 123)	29 (94)	152 (100)	—
Yes	33 (30, 36)	2 (6.4)	0	—
Follow-up length (days since initial blood collection, median, IQR)	—	269 (80, 538)	420 (179, 609)	0.04

[a]Infection history include any of the following: peritonitis, urinary tract infection, pneumonia, bacteremia with or without sepsis, clostridium difficile, endocarditis, and skin infections.
[b]Karnofsky score was available in 25/31 Cu-deficient patients and 139/152 normal Cu patients.
[c]Nutritional risk assessment was available in 26/31 Cu-deficient patients and 101/152 normal Cu patients.
[d]Ceruloplasmin was available in 17/31 Cu-deficient patients and 60/152 normal Cu patients.
[e]Normal range of zinc according to University of Washington Laboratory: 60–120 μg/dL.
[f]Normal range of selenium according to Mayo Clinic Laboratory: 7–15 μg/dL.

deficiencies were more common in Cu-deficient patients (Table 1). Overall, Cu concentrations correlated more closely with ceruloplasmin (Spearman $\rho = 0.618$, $p < 0.0001$) than albumin (Spearman $\rho = 0.217$, $p = 0.003$, Table 2). Cytokine analyses showed Cu correlated negatively with IL-1β (Spearman $\rho = -0.228$, $p = 0.004$, Table 2).

TABLE 2 Correlation between concentrations of serum copper and albumin, ceruloplasmin, IL-1β, TNF-α, and hepatic copper.

	N	Spearman ρ	*p*
Albumin (g/dL)	183	0.217	0.003
Ceruloplasmin (mg/dL)	77	0.618	< 0.0001
IL-1β (pg/mL)	160	−0.228	0.004
TNF-α (pg/mL)	130	0.037	o.661
Hepatic copper (μg/g)	50	0.34	0.02

Multivariate analyses using supervised partial least squares discriminant analyses indicated significant differences in polar metabolite profiles between patient groups with and without Cu deficiency (Figure 1). Validation metrics including Q2, R2, and permutation tests, classification error rates (CER) and area under receiver operator curve (AUROC) confirmed the group separation from the partial least squares discriminant analyses model (Supporting Information, Figure S1, http://links.lww.com/HC9/A168). A subset of 15 metabolites that contributed the most to the separation as assessed by the variable of importance in projection (VIP score > 1.2) is depicted in Figure 2. Notably, Cu-deficient patients had lower

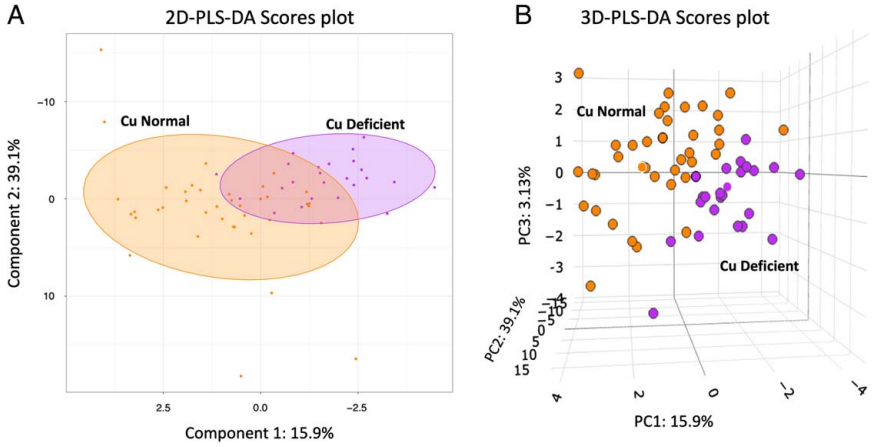

FIGURE 1 Two-dimensional (A) and 3-dimensional (B) partial least square-discriminant analysis (PLS-DA) scores plots of serum polar metabolite profiles from cirrhosis patients with (purple) and without (orange) copper deficiency. Abbreviations: Cu, copper

concentrations of metabolites related to trace metal (iron, zinc, and selenium), branched-chain amino acids (leucine, 2-hydroxyisobutyrate, 2-oxoisocaproate) and ketone (3-hydroxybutyrate) metabolism, and higher concentrations of metabolites involved in fatty acid (o-acetylcarnitine) and gut microbial (butyrate, glycolate) metabolism. Additional details from the metabolomics analyses are included in the Supporting Information (http://links.lww.com/HC9/A168).

Cu deficiency and patient outcomes

During a median follow-up of 396 days (interquartile range: 168, 602 d), the proportion of patients who required inpatient admissions (≥ 1 hospital admission) was significantly higher among Cu-deficient patients (77% vs. 47%, $p = 0.002$). In the entire cohort, 55 patients (30%) underwent liver transplantation and 23 patients (13%) died. Rates of transplantation were similar between those who were Cu deficient and those who were not (32% vs. 30%, respectively, $p = 0.8$).

Rates of patient death before transplantation were 22.6% (N = 7) in Cu-deficient patients compared with 10.5% (N = 16) in patients with normal Cu, with a significantly higher incidence of death before transplantation among patients with Cu deficiency (0.614 per 1000 person-days) compared with patients without Cu deficiency (0.257 per 1000 person-days, log-rank test $p = 0.04$, Figure 3). Cause-specific competing risk analysis showed that Cu deficiency was associated with a significantly higher risk of death before transplantation before and after adjusting for age, sex,

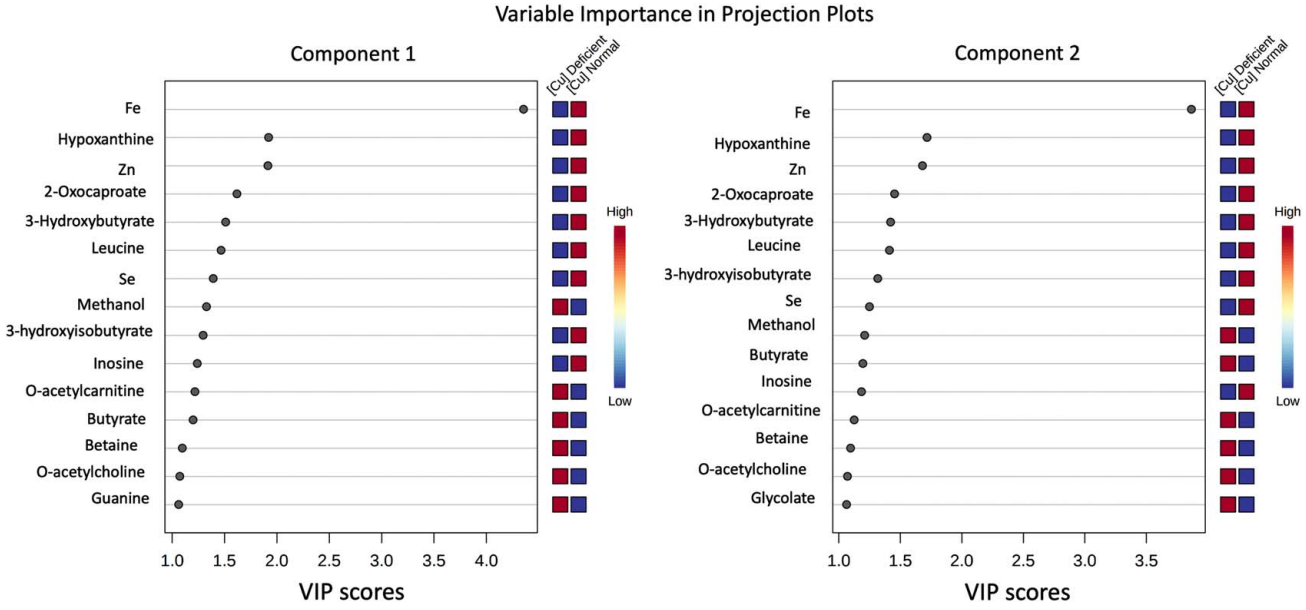

FIGURE 2 VIP Scores plots corresponding to the 15 most significant metabolites (VIP > 1.2) contributing to the separation of study patients with and without copper deficiency. PLS-DA model component 1 (A) and component 2 (B). Abbreviation: Cu, copper; VIP, variable of importance in projection.

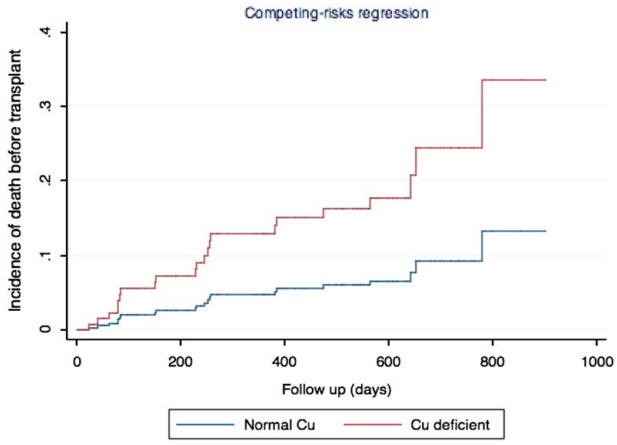

FIGURE 3 Cumulative incidence of death before liver transplantation according to copper deficiency status among 183 study patients with cirrhosis and portal hypertension. Abbreviation: Cu, copper.

MELD-Na, and Karnofsky scores (Table 3, adjusted HR for death before transplantation: 3.40, 95% CI, 1.18–9.82, $p = 0.023$).

In the Cu-deficient group, 1 patient died from liver failure likely related to alcohol recidivism. Five deaths were infection-associated multiorgan failure (septic emboli from endocarditis N = 1, pneumonia N = 2, and sepsis from peritonitis N = 2). One patient died 1 day after liver transplantation from shock of unclear etiology. His autopsy did not show signs of bleeding from anastomoses of the graft. In patients who were not Cu deficient, 1 person died of liver failure from alcohol recidivism. The rest of the deaths were related to infection (N = 8), bleeding (N = 1), cancer progression (N = 2), and multiorgan failure without associated infection (N = 4).

Analysis of liver explants according to hepatic Cu concentrations

Among 55 patients who underwent liver transplantation, 50 liver explants were available. The median hepatic Cu concentration was 9.5 μg/g. The interquartile range was 2.8 and 131.4 μg/g. The lowest and highest hepatic Cu concentrations were 1.8 and 277.6 μg/g. When these 50 explants were divided according to the median hepatic Cu concentration, patients with hepatic Cu below the median had lower serum/plasma Cu at baseline ($p = 0.05$, Table 4). There was a modest but significant positive correlation between serum/plasma Cu and hepatic Cu (Spearman $\rho = 0.34$, $p = 0.02$, Table 2). Hepatic iron concentration was higher in patients with hepatic Cu below the median, but this difference was not significant. In terms of liver disease etiologies, the most notable difference was that alcohol-associated liver disease was more common in patients whose hepatic Cu was below the median (36% vs. 12%), whereas PSC was only present among those with hepatic Cu above the median (12% vs. 0%). These differences were not statistically significant when other liver diseases were also included. Detailed histological assessment showed that nonparenchymal (Kupffer cell) iron deposition was significantly more common in patients with hepatic Cu below the median level ($p = 0.04$), whereas apoptosis was less common ($p = 0.05$, Table 4).

DISCUSSION

Using carefully collected data from 183 consecutive patients with advanced liver disease, the current study described detailed clinical features and longitudinal outcomes for those who were Cu deficient. We showed that Cu deficiency was present in most etiologies of cirrhosis, with the exception of cholestatic liver diseases. Although it did not correlate with traditional markers of disease severity, such as MELD-Na score or CTP class, it was independently associated with increased patient death before liver transplantation.

Given the liver's central role in maintaining Cu metabolism, it is not surprising that Cu deficiency exists in cirrhosis. In clinical nutrition, Cu deficiency is best defined by its serum or plasma concentration.[24,25] Established causes include excessive zinc intake, Menkes disease, gastrointestinal diseases, or surgeries

TABLE 3 Cause-specific hazard model accounting for liver transplantation as competing risk estimating the risk of death before transplantation associated with copper deficiency before and after adjusting for other variables

	Univariate			Multivariate[a]		
Variables	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***
Copper deficiency	2.67	1.01–7.04	0.047	3.40	1.18–9.82	0.023
Age	1.04	0.99–1.10	0.140	1.05	0.99–1.12	0.083
Sex	0.55	0.24–1.27	0.163	0.70	0.29–1.68	0.424
MELD-Na score	1.16	1.07–1.26	< 0.001	1.14	1.05–1.25	0.003
Karnofsky score	0.96	0.94–0.98	0.002	0.96	0.94–0.99	0.005

[a]Adjusted for age, sex (women as referent population), MELD-Na and Karnofsky score. Only patients with complete data (N = 164) were included in the multivariate regression model.

TABLE 4 Comparison of baseline characteristics, liver explant histological features, and hepatic trace metal concentrations according to hepatic copper concentration (above or below median, 9.5 μg/g dry weight)

	Hepatic copper concentration (μg/g dry weight)		
	≤9.5	>9.5	*p*
N	25	25	—
Age (median, IQR)	56 (52, 62)	60 (54, 62)	0.4
Female, N (%)	12 (48)	9 (36)	0.4
Serum copper concentration (μg/dL, mean, SD)	93.8 ± 29	110 ± 28	0.05
MELD-Na score (median, IQR)	18 (10, 22)	17 (12, 24)	0.7
Time from baseline blood sample collection to transplantation (days, median, IQR)	193 (107, 368)	173 (55, 537)	0.8
Explant histology, N (%)			
Portal inflammation	—	—	0.3
Grade 1	13 (52)	19 (76)	—
Grade 2 or 3	8 (32)	4 (16)	—
Piecemeal necrosis	—	—	0.06
Grade 1	6 (24)	12 (48)	—
Grade 2	6 (24)	1 (4)	—
Steatosis	13 (52)	9 (36)	0.3
Apoptosis	—	—	0.05
Grade 1	4 (16)	11 (44)	—
Grade 2	2 (8)	0	—
Ballooning	6 (24)	8 (32)	0.5
Iron			
Intrahepatocytic	10 (42)	11 (44)	0.9
Nonparenchymal (Kupffer cell)	12 (48)	5 (20)	0.04
Hepatic copper (μg/g, median, IQR)	5.5 (3.8, 7.2)	17.9 (12, 52.9)	<0.001
Hepatic iron (μg/g, median, IQR)	126 (54.4, 279)	66.6 (46.7, 263)	0.3
Hepatic zinc (μg/g, median, IQR)	26.5 (22.5, 29.2)	35.1 (24.6, 44.1)	0.2

Abbreviations: IQR, interquartile range.

leading to malabsorption and kidney disease.[25–27] Because none of the Cu-deficient patients had these conditions, cirrhosis is likely an independent and previously underrecognized risk factor for Cu deficiency. Furthermore, because nutritional status and serum albumin were not significantly different between the 2 study groups, we suspect Cu deficiency is not a mere epiphenomenon of protein malnutrition but rather a unique micronutrient deficiency with distinct functional consequences. The strong correlation between circulating Cu and ceruloplasmin, whose turnover rate depends on Cu availability,[28] corroborates this suggestion.

The most significant finding of the current study is the relationship between Cu deficiency and increased risk of patient death before transplantation. Among patients with alcoholic hepatitis or alcohol-associated cirrhosis, one previous study by Dhanda et al[29] did not find a relationship between Cu status and patient survival. The follow-up duration of 90 days, however, was significantly shorter than the current study. Although baseline cirrhosis severity (MELD-Na or CTP class) was not associated with Cu deficiency in the current cohort, history of bacterial infections was twice as prevalent among Cu-deficient patients compared with those with normal Cu. Because infections are not only important direct causes of death in cirrhosis but also indirect mediators of all major complications of the disease, such as bleeding, encephalopathy, and renal dysfunction,[30] the association between infection and Cu deficiency may be one explanation for the higher mortality in these patients.

How does Cu affect host immunity in relation to infections and liver injury? First, Cu deficiency causes reduced superoxide generation in neutrophils and macrophages, which directly compromises their bactericidal activity.[15,31] Although it is well established that these innate immune cells do not function normally in cirrhosis,[32,33] whether Cu deficiency contributes to this phenotype has not been investigated. Second, Cu deficiency reduces enzyme activity of ceruloplasmin, a ferroxidase—leading to iron overload,[12] which is associated with high

infection and mortality risk in cirrhosis.[34,35] Kupffer cell iron deposition has also been observed in a mouse model with impaired intestinal Cu absorption.[36] In the current study, the greater degree of Kupffer cell iron deposition, as well as higher overall hepatic iron in liver explants with lower Cu concentrations, may reflect functional impairment of ceruloplasmin. Finally, global differences in polar metabolites and transition metals in our patients indicate that Cu deficiency can influence metabolism on a systemic level. Cu-deficient patients exhibited reduced levels of metabolites involved in branched-chain amino acids catabolism, which are associated with not only histological activity and portal systemic shunting in cirrhosis but also sepsis.[37,38] In our cohort, Cu-deficient patients had significantly higher IL-1β, a cytokine produced during NLRP3 inflammasome activation, which is regulated by Cu.[5,39] This may explain the difference in serum levels of 3-hydroxybutyrate, a ketone body metabolite, which is closely linked to NLRP3 activity.[40,41] Detailed mechanistic studies, including more complete metabolomics profiling, will be required to understand Cu deficiency–related immune dysregulation in cirrhosis.

Although the current study was not designed to elucidate the mechanisms of Cu deficiency, 2 considerations warrant discussion based on our understanding of Cu homeostasis in the context of cirrhosis. First, because Cu must be obtained from the diet, and dietary Cu directly affects serum Cu and cuproenzyme activity,[42] insufficient oral intake is one potential cause of Cu deficiency in this population. Not only are cirrhosis patients well known to have decreased oral intake,[2] recent examination of the standard Western diet suggests decreased Cu intake over the past several decades.[43] It is possible that a combination of reduced appetite due to the disease state plus poor food choices make some cirrhosis patients susceptible to Cu deficiency. Second, because Cu forms cytoplasmic complexes with glutathione to mitigate its propensity for oxidative injury,[44] and that glutathione storage is often reduced in erythrocytes and hepatocytes of cirrhosis patients,[45,46] it is conceivable that Cu deficiency is an adaptive response when glutathione is depleted. Although this adaptation may protect hepatic parenchymal cells, reduced systemic Cu availability could, however, adversely affect the whole organism as suggested by the higher mortality rate among Cu-deficient patients in our study. Future investigations should carefully assess dietary intake, as well as hepatic and systemic markers of oxidative stress in cirrhosis patients with Cu deficiency.

Our study has several limitations that should be addressed in future work. The current cohort was comprised of patients who were undergoing liver transplant evaluation in a single academic center. To be more generalizable, our findings should be confirmed with a larger population and include cirrhosis patients who may not be transplant candidates, such as those with other medical comorbidities. The nutritional status of patients in the current study was assessed using Subjective Global Assessment based on a combination of history and physical parameters.[19] Although the method is valuable in quantifying nutritional risk in cirrhosis, it is less accurate compared with more objective techniques in terms of assessing muscle mass, which is a major site of Cu storage.[47–49] Ceruloplasmin in the current study was measured using nephelometry, the standard immunological assay in most clinical laboratories. Consequently, it is difficult to state unambiguously that Cu deficiency–associated reduction in ceruloplasmin concentration affected serum ferroxidase activity, which requires a specialized enzymatic method.[50] Because limited sample availability only allowed us to perform metabolomics and liver tissue analyses from a subset of patients, the findings warrant cautious interpretation and necessitate further validation. Finally, because Cu levels may change over time, future studies should measure Cu serially to determine whether such changes do occur and whether they correlate with nutritional status and disease progression.

In summary, despite the limitations discussed above, our data confirmed the presence of Cu deficiency as an underrecognized micronutrient deficiency in a substantial proportion of patients with advanced cirrhosis. Cu deficiency was associated with a reduced level of ceruloplasmin and higher IL-1β, indicating NLRP3 activation, both of which are consistent with existing knowledge on Cu's role in mammalian physiology. Most importantly for clinicians, our data showed for the first time that Cu deficiency was associated with infections and an increased risk of death before liver transplantation independent of liver disease severity. Our data lend support to the current guideline that it would be reasonable to consider assessing Cu levels in advanced liver disease.[3] This may be especially relevant if zinc supplementation is being considered because it can induce Cu deficiency.[26] It is tempting to consider Cu, in the context of "precision nutrition," a potential therapeutic target where careful correction will improve not only body's Cu store, but also Cu-dependent immunity, enzyme function, and metabolism. A more concrete understanding of Cu deficiency's role in cirrhosis, especially in terms of the risks and benefits of supplementation, would require larger prospective studies with careful assessment of diet, anthropometry, biochemical analyses, and clinical endpoints.

AUTHOR CONTRIBUTIONS

Lei Yu: conceptualization, data curation and analysis, manuscript draft, review, and editing; Sarim Yousuf: manuscript draft, review and editing, and visualization; Shahrukh Yousuf: data curation, Jeffrey Yeh: data

curation; Scott W. Biggins: manuscript review and editing; Chihiro Morishima: data curation, manuscript review, and editing, Irene Shyu: data curation and analysis; Galen O'Shea-Stone: data curation, analysis, and manuscript editing; Brian Eilers: data curation and analysis, Annie Waldum: data curation and analysis, Valérie Copié: data curation, analysis, manuscript review, and editing; Jason Burkhead: conceptualization, data curation and analysis, manuscript draft, review, and editing.

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CONFLICT OF INTERESTS

The authors have no conflicts to report.

ORCID

Lei Yu https://orcid.org/0000–0002–4622–657X
Chihiro Morishima https://orcid.org/0000–0001–5330–9464
Valérie Copié https://orcid.org/0000–0002–2778–1463
Jason Burkhead https://orcid.org/0000–0001–5457–311X

REFERENCES

1. Asrani SK, Kouznetsova M, Ogola G, Taylor T, Masica A, Pope B, et al. Increasing health care burden of chronic liver disease compared with other chronic diseases, 2004-13. Gastroenterology. 2018;155:719–729.e4.
2. European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu, European Association for the Study of the Liver. EASL Clinical Practice Guidelines on nutrition in chronic liver disease. J Hepatol. 2019;70:172–93.
3. Lai JC, Tandon P, Bernal W, Tapper EB, Ekong U, Dasarathy S, et al. Malnutrition, frailty, and sarcopenia in patients with cirrhosis: 2021 Practice Guidance by the American Association for the Study of Liver Diseases. Hepatology. 2021;74:1611–44.
4. Uauy R, Olivares M, Gonzalez M. Essentiality of copper in humans. Am J Clin Nutr. 1998;67:952S–599S.
5. Deigendesch N, Zychlinsky A, Meissner F. Copper regulates the canonical NLRP3 inflammasome. J Immunol. 2018;200:1607–17.
6. Nyasae L, Bustos R, Braiterman L, Eipper B, Hubbard A. Dynamics of endogenous ATP7A (Menkes protein) in intestinal epithelial cells: copper-dependent redistribution between two intracellular sites. Am J Physiol Gastrointest Liver Physiol. 2007;292:G1181–94.
7. Polishchuk EV, Concilli M, Iacobacci S, Chesi G, Pastore N, Piccolo P, et al. Wilson disease protein ATP7B utilizes lysosomal exocytosis to maintain copper homeostasis. Developmental Cell. 2014;29:686–700.
8. Terada K, Kawarada Y, Miura N, Yasui O, Koyama K, Sugiyama T. Copper incorporation into ceruloplasmin in rat livers. Biochim Biophys Acta. 1995;1270:58–62.
9. Linder MC, Wooten L, Cerveza P, Cotton S, Shulze R, Lomeli N. Copper transport. Am J Clin Nutr. 1998;67:965S–71S.
10. Mansouri A, Gaou I, Fromenty B, Berson A, Letteron P, Degott C, et al. Premature oxidative aging of hepatic mitochondrial DNA in Wilson's disease. Gastroenterology. 1997;113:599–605.
11. Song M, Zhou Z, Chen T, Zhang J, McClain CJ. Copper deficiency exacerbates bile duct ligation-induced liver injury and fibrosis in rats. J Pharmacol Exp Ther. 2011;339:298–306.
12. Harris ZL, Durley AP, Man TK, Gitlin JD. Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux. Proc Natl Acad Sci U S A. 1999;96:10812–7.
13. Harless W, Crowell E, Abraham J. Anemia and neutropenia associated with copper deficiency of unclear etiology. Am J Hematol. 2006;81:546–9.
14. Achard MES, Stafford SL, Bokil NJ, Chartres J, Bernhardt PV, Schembri MA, et al. Copper redistribution in murine macrophages in response to Salmonella infection. Biochem J. 2012;444:51–7.
15. Babu U, Failla ML. Copper status and function of neutrophils are reversibly depressed in marginally and severely copper-deficient rats. J Nutr. 1990;120:1700–9.
16. Abu Rajab M, Guerin L, Lee P, Brown KE. Iron overload secondary to cirrhosis: a mimic of hereditary haemochromatosis? Histopathology. 2014;65:561–9.
17. Taylor NJ, Manakkat Vijay GK, Abeles RD, Auzinger G, Bernal W, Ma Y, et al. The severity of circulating neutrophil dysfunction in patients with cirrhosis is associated with 90-day and 1-year mortality. Aliment Pharmacol Ther. 2014;40:705–15.
18. Yu L, Liou IW, Biggins SW, Yeh M, Jalikis F, Chan L-N, et al. Copper deficiency in liver diseases: a case series and pathophysiological considerations. Hepatology Communications. 2019;3:1159–65.
19. Detsky AS, Baker JP, O'Rourke K, Johnston N, Whitwell J, Mendelson RA, et al. Predicting nutrition-associated complications for patients undergoing gastrointestinal surgery. JPEN J Parenter Enteral Nutr. 1987;11:440–6.
20. Orman ES, Ghabril M, Chalasani N. Poor performance status is associated with increased mortality in patients with cirrhosis. Clin Gastroenterol Hepatol. 2016;14:1189–95.e1.
21. Sapir-Pichhadze R, Pintilie M, Tinckam KJ, Laupacis A, Logan AG, Beyene J, et al. Survival analysis in the presence of

competing risks: the example of waitlisted kidney transplant candidates. Am J Transplant. 2016;16:1958–66.
22. Lau B, Cole SR, Gange SJ. Competing risk regression models for epidemiologic data. Am J Epidemiol. 2009;170:244–56.
23. Chong J, Soufan O, Li C, Caraus I, Li S, Bourque G, et al. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. Nucleic Acids Res. 2018;46:W486–94.
24. Harvey LJ, Ashton K, Hooper L, Casgrain A, Fairweather-Tait SJ. Methods of assessment of copper status in humans: a systematic review. Am J Clin Nutr. 2009;89:2009S–24S.
25. Danks DM. Copper deficiency in humans. Annu Rev Nutr. 1988;8:235–57.
26. Duncan A, Yacoubian C, Watson N, Morrison I. The risk of copper deficiency in patients prescribed zinc supplements. J Clin Pathol. 2015;68:723–5.
27. Bledsoe AC, King KS, Larson JJ, Snyder M, Absah I, Choung RS, et al. Micronutrient deficiencies are common in contemporary celiac disease despite lack of overt malabsorption symptoms. Mayo Clin Proc. 2019;94:1253–60.
28. Broderius M, Mostad E, Wendroth K, Prohaska JR. Levels of plasma ceruloplasmin protein are markedly lower following dietary copper deficiency in rodents. Comp Biochem Physiol C Toxicol Pharmacol. 2010;151:473–9.
29. Dhanda A, Atkinson S, Vergis N, Enki D, Fisher A, Clough R, et al. Trace element deficiency is highly prevalent and associated with infection and mortality in patients with alcoholic hepatitis. Aliment Pharmacol Ther. 2020;52:537–44.
30. Arvaniti V, D'Amico G, Fede G, Manousou P, Tsochatzis E, Pleguezuelo M, et al. Infections in patients with cirrhosis increase mortality four-fold and should be used in determining prognosis. Gastroenterology. 2010;139:1246–56; 1256.e1–5.
31. White C, Lee J, Kambe T, Fritsche K, Petris MJ. A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. J Biol Chem. 2009;284:33949–56.
32. Rajkovic IA, Williams R. Abnormalities of neutrophil phagocytosis, intracellular killing and metabolic activity in alcoholic cirrhosis and hepatitis. Hepatology. 1986;6:252–62.
33. Tritto G, Bechlis Z, Stadlbauer V, Davies N, Francés R, Shah N, et al. Evidence of neutrophil functional defect despite inflammation in stable cirrhosis. J Hepatol. 2011;55:574–81.
34. Maiwall R, Kumar S, Chaudhary AK, Maras J, Wani Z, Kumar C, et al. Serum ferritin predicts early mortality in patients with decompensated cirrhosis. J Hepatol. 2014;61:43–50.
35. Maras JS, Maiwall R, Harsha HC, Das S, Hussain MS, Kumar C, et al. Dysregulated iron homeostasis is strongly associated with multiorgan failure and early mortality in acute-on-chronic liver failure. Hepatology. 2015;61:1306–20.
36. Nose Y, Kim BE, Thiele DJ. Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. Cell Metab. 2006;4:235–44.
37. Iwasaki Y, Sato H, Ohkubo A, Sanjo T, Futagawa S, Sugiura M, et al. Effect of spontaneous portal-systemic shunting on plasma insulin and amino acid concentrations. Gastroenterology. 1980;78:677–83.
38. Reisinger AC, Posch F, Hackl G, Marsche G, Sourij H, Bourgeois B, et al. Branched-chain amino acids can predict mortality in ICU sepsis patients. Nutrients. 2021;13:3106.
39. Wree A, Eguchi A, McGeough MD, Pena CA, Johnson CD, Canbay A, et al. NLRP3 inflammasome activation results in hepatocyte pyroptosis, liver inflammation, and fibrosis in mice. Hepatology. 2014;59:898–910.
40. Hughes MM, O'Neill LAJ. Metabolic regulation of NLRP3. Immunol Rev. 2018;281:88–98.
41. Youm Y-H, Nguyen KY, Grant RW, Goldberg EL, Bodogai M, Kim D, et al. The ketone metabolite β-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. Nat Med. 2015;21:263–9.
42. Turnlund JR, Scott KC, Peiffer GL, Jang AM, Keyes WR, Keen CL, et al. Copper status of young men consuming a low-copper diet. Am J Clin Nutr. 1997;65:72–8.
43. Klevay LM. Is the Western diet adequate in copper? J Trace Elem Med Biol. 2011;25:204–12.
44. Freedman JH, Ciriolo MR, Peisach J. The role of glutathione in copper metabolism and toxicity. J Biol Chem. 1989;264:5598–605.
45. Altomare E, Vendemiale G, Albano O. Hepatic glutathione content in patients with alcoholic and non alcoholic liver diseases. Life Sci. 1988;43:991–8.
46. Loguercio C, Taranto D, Vitale LM, Beneduce F, Del Vecchio Blanco C. Effect of liver cirrhosis and age on the glutathione concentration in the plasma, erythrocytes, and gastric mucosa of man. Free Radic Biol Med. 1996;20:483–8.
47. Owen CA. Distribution of copper in the rat. Am J Physiol. 1964;207:446–8.
48. Moctezuma-Velazquez C, Ebadi M, Bhanji RA, Stirnimann G, Tandon P, Montano-Loza AJ. Limited performance of subjective global assessment compared to computed tomography-determined sarcopenia in predicting adverse clinical outcomes in patients with cirrhosis. Clin Nutr. 2019;38:2696–703.
49. Figueiredo FAF, Perez RM, Freitas MM, Kondo M. Comparison of three methods of nutritional assessment in liver cirrhosis: subjective global assessment, traditional nutritional parameters, and body composition analysis. J Gastroenterol. 2006;41:476–82.
50. Schosinsky KH, Lehmann HP, Beeler MF. Measurement of ceruloplasmin from its oxidase activity in serum by use of o-dianisidine dihydrochloride. Clin Chem. 1974;20:1556–63.

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