Clinics and Research in Hepatology and Gastroenterology (2019) 43, 410—416

Available online at ScienceDirect www.sciencedirect.com

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ORIGINAL ARTICLE

Plasma claudin-3 is associated with tumor necrosis factor-alpha-induced intestinal endotoxemia in liver disease

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Available online 30 April 2019

KEYWORDS
Claudin-3;
D-lactate;
Intestinal endotoxemia;
Liver cirrhosis;
Tumor necrosis factor-alpha;
Interferon gamma

Summary
Objective: To investigate intestinal endotoxemia (IETM), intestinal permeability (IP) and cytokine activity in patients with liver cirrhosis (LC).
Materials and methods: Twenty-nine patients with chronic hepatitis B (CHB), 28 with compensated LC, 33 with decompensated LC, 24 with spontaneous bacterial peritonitis (SBP), 26 with acute-on-chronic liver failure (ACLF), and 24 with decompensated LC complicated by hepatocellular carcinoma (HCC) were recruited. Thirty-one healthy people were included as a control group. Plasma tumor necrosis factor (TNF)-α, interferon (IFN)-γ, D-lactate, endotoxin, and claudin-3 levels were assayed. Data were compared using Pearson correlation testing and analysis of variance, with $P < 0.05$ considered significant.
Results: TNF-α, claudin-3, and endotoxin levels were significantly increased ($P < 0.05$) in the plasma of all patients with liver disease compared with that of controls, particularly in patients with decompensated LC, SBP, ACLF, or HCC ($P < 0.01$). IFN-γ was significantly higher in HCC than in other liver diseases ($P < 0.01$). Plasma D-lactate was significantly decreased in all liver diseases, except SBP ($P < 0.01$). TNF-α, endotoxin, and claudin-3 levels were positively correlated ($P < 0.01$), but correlations of IFN-γ with endotoxin or claudin-3 were not significant. The plasma D-lactate level did not significantly correlate with either TNF-α, endotoxin, or claudin-3 levels.
Conclusion: Plasma claudin-3, but not D-lactate, was found to be a marker of IP in patients with liver diseases. Elevated plasma TNF-α in such patients was likely to have injured the intestinal barrier, leading to IETM, especially in end-stage LC.

Introduction

Intestinal endotoxemia (IETM) may occur in patients with liver disease to cause secondary liver injury (SLI); this

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https://doi.org/10.1016/j.clinre.2018.11.014
2210-7401/

may impact disease progression and its prognosis [1,2]. Cytokines, including tumor necrosis factor (TNF)-α and interferon (IFN)-γ, are increased in many liver diseases, including fulminant hepatic failure (FHF), while chronic liver diseases have been associated with increased intestinal permeability [3,4] as shown by elevations of D-lactate and claudin-3 [3,5,6]. Gut leakiness has been shown to promote IETM and to aggravate liver cirrhosis (LC) [2,7—9]. However, few studies have evaluated any correlations in changes of intestinal permeability (IP) and IETM in patients with liver disease. Plasma or serum markers for IP changes are lacking. Cytokines, including TNF-α and IFN-γ, which promote intercellular permeability in vitro, need further study to clarify their functions in patients with liver diseases.

In this study, TNF-α, IFN-γ, D-lactate, endotoxin, and claudin-3 were assayed in patients with chronic hepatitis B (CHB), compensated LC, decompensated LC, LC with spontaneous bacterial peritonitis (SBP), acute-on-chronic liver failure (ACLF), and decompensated LC complicated with hepatocellular carcinoma (HCC). The pathogenesis of liver disease in such patients probably involves cytokine activity impacting on the development of IETM.

Materials and methods

Ethics statement

The Ethics Committee of Jiangxi Provincial Hospital approved the study and all patients gave written consent to their participation before inclusion.

Patients and clinical samples

A series of 164 patients treated for liver diseases at our hospital between January 2016 and November 2017, as well as 31 healthy control participants, were recruited. Liver diseases included chronic hepatitis B (CHB, $n = 29$), compensated LC ($n = 28$), decompensated LC without severe complications ($n = 33$), LC complicated with SBP ($n = 24$), ACLF ($n = 26$), and decompensated LC complicated with HCC ($n = 24$) [10]. Patients were diagnosed on physical examination according to their clinical manifestations. The liver disease etiology for all patients was hepatitis B viral infection. A diagnosis of CHB was based on a history of serum hepatitis B surface antigen positivity for > 6 months, serum alanine aminotransferase levels greater than the upper limit of the normal level, serum total bilirubin < 10 times the normal level (171 μmol/L), and prothrombin time activity > 40% [11]. Liver cirrhosis was diagnosed on the basis of clinical, biochemical, and instrument results [12]. Decompensated LC was marked by the development of overt clinical signs, the most frequent of which were ascites, bleeding, encephalopathy, and jaundice [13]. Patients with LC and ascites but without other complications were recruited into the decompensated LC group. SBP was diagnosed when:

- the ascitic fluid polymorphonuclear leucocyte count was ≥ 250 cells/μL;
- the ascitic fluid culture was positive;
- and an intra-abdominal surgically treatable source for infection was not evident [14].

Patients were diagnosed with ACLF on admission according to the following diagnostic criteria: acute hepatic insult manifesting as jaundice (serum bilirubin ≥ 85 μmol/L) and coagulopathy (international normalized ratio ≥ 1.5 or prothrombin activity ≤ 40%), complicated within 2 weeks by ascites and/or encephalopathy in patients with previously diagnosed or undiagnosed chronic liver diseases [15]. Histological diagnoses of HCC tissue samples were made by experienced pathologists. Patients with the following diseases were excluded from the study:

- creatinine ≥ 1.5 mg/dL or hepatorenal syndrome;
- extra-hepatic organ failure;
- gastrointestinal bleeding within 6 months.

Blood samples were collected in EDTA-coated tubes when anticoagulation was required for assays of blood cytokines, D-lactate, endotoxins, and claudin-3. Plasma samples were separated by centrifugation at 3000 ×*g* for 10 min at 4 °C and stored at −80 °C until assayed. Serum TNF-α and IFN-γ were measured by ABC enzyme-linked immunosorbent assay (ELISA) kits (Research & Diagnostics, Minneapolis, MN, USA). Plasma D-lactate was assayed spectrophotometrically and endotoxin was detected with a limulus amebocyte lysate assay (Research & Diagnostics, Boyebio, Shanghai, China) [3,16]. The plasma claudin-3 concentration (0.312—20 ng/mL) was evaluated by ELISA using the manufacturer's standard kit (USCN Life Science Inc, Wuhan, China) [17].

Limulus amebocyte lysate endotoxin assay

One hundred microliters of plasma or standard were added to each well of a 96-well plate and then immediately incubated with 100 μL of biotinylated antibody (1×) for 1 h at 37 °C. After washing, this incubation was followed by an incubation with 100 μL of horse radish peroxidase-avidin (1×) for 30 min at 37 °C. After washing, 90 μL substrate solution per well was added and incubated for 15—25 minutes at 37 °C. Following this, 50 μL of stop solution was added to each well and the plate immediately read at 450 nm. Values were expressed as pg/mL. Intra-assay and inter-assay coefficients of variation were less than 8% and 10%, respectively [16].

Statistical analysis

SPSS version 19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Differences in the means of continuous variables were compared by analysis of variance when the data were normally distributed. Correlations were assessed for significance with Pearson's test. *P*-values < 0.05 were considered statistically significant.

Results

Participant characteristics

The disease groups did not differ significantly in terms of age or sex ratio. Model for end-stage liver disease

Table 1 Characteristics of study patients and controls.

	Control group ($n = 31$)	CHB group ($n = 29$)	Compensated LC group ($n = 28$)	Decompensated LC group ($n = 33$)	SBP group ($n = 24$)	ACLF group ($n = 26$)	HCC group ($n = 24$)
Age (year)	43 (26—52)	49 (39—65)	59 (45—76)	53 (41—69)	54 (38—76)	56 (36—73)	57 (33—79)
Sex (M/F)	27/4	25/4	26/2	29/4	22/2	23/3	23/1
MELD score			6 (3—14)	12 (8—16)	17 (14—29)	20 (16—25)	11 (7—14)
Child—Pugh score			5 (5—6)	11 (9—13)	12 (10—13)	13 (12—14)	9 (8—11)
TNF-α	55.0 (0.8—105.9)	124.8 (86.0—194.7)	147.1 (88.5—237.0)	215.0 (125.5—280.1)	214.7 (161.8—288.8)	199.3 (144.2—245.4)	202.9 (128.6—351.8)
IFN-γ	9.3 (0.7—29.4)	9.6 (1.4—24.5)	5.0 (0.6—16.8)	9.9 (1.4—51.6)	5.8 (1.7—13.4)	7.9 (3.2—17.4)	24.8 (0.7—76.5)
Claudin-3	4.8 (0.5—16.5)	43.5 (9.7—98.5)	44.6 (4.0—97.7)	76.2 (14.7—129.4)	62.6 (23.2—129.3)	102.8 (51.4—177.3)	77.5 (10.1—162.0)
D-lactate	2506.9 (1143.4—4735.8)	1415.0 (569.7—2827.1)	1483.1 (734.2—2643.8)	1587.5 (768.3—3980.5)	2514.5 (753.3—5689.7)	832.4 (553.7—1287.3)	1025.6 (550.2—1431.6)
Endotoxin	57.2 (21.8—87.4)	143.1 (101.3—214.7)	160.2 (99.1—271.4)	273.4 (118.2—406.0)	263.3 (158.3—487.3)	266.7 (193.9—334.7)	234.0 (116.1—369.3)
Bilirubin (mg/dL)	0.72 (0.41—1.1)	1.16 (0.59—1.82)	1.29 (0.78—1.81)	4.68 (1.92—13.73)	7.23 (1.1—21.7)	5.62 (4.07—9.67)	1.70 (0.87—3.10)
Albumin (g/L)	45.6 (42.5—53.2)	43.4 (41.9—47.9)	38.2 (36.3—42.6)	31.5 (27.7—34.7)	27.5 (24.6—31.5)	25.5 (19.8—29.7)	30.3 (26.9—37.5)
Creatinine (mg/dL)	0.53 (0.27—0.71)	0.57 (0.29—0.87)	0.65 (0.37—0.92)	0.72 (0.41—1.18)	1.15 (0.72—1.48)	1.05 (0.67—1.28)	0.87 (0.74—1.22)
INR	0.98 (0.81—1.19)	1.01 (0.87—1.25)	1.17 (1.07—1.41)	1.38 (1.12—1.68)	1.46 (1.08—1.98)	1.93 (1.51—2.61)	1.42 (1.26—1.61)
Ascites (yes/no)	0/31	0/29	0/28	33/0	24/0	26/0	24/0
Encephalopathy (yes/no)	0/31	0/29	0/28	0/33	0/24	23/3	0/24
Hepatocellular carcinoma (yes/no)	0/31	0/29	0/28	0/33	0/24	0/26	24/0
Spontaneous bacterial peritonitis (yes/no)	0/31	0/29	0/28	0/33	24/0	0/26	0/24

Data are shown as the median (range); INR: international normalized ratio; MELD: model for end-stage liver disease; TNF-α: tumor necrosis factor-alpha; IFN-γ: interferon gamma; CHB: chronic hepatitis B; compensated LC: compensated liver cirrhosis; decompensated LC: decompensated liver cirrhosis; SBP: spontaneous bacterial peritonitis; ACLF: acute-on-chronic liver failure; HCC: hepatocellular carcinoma.

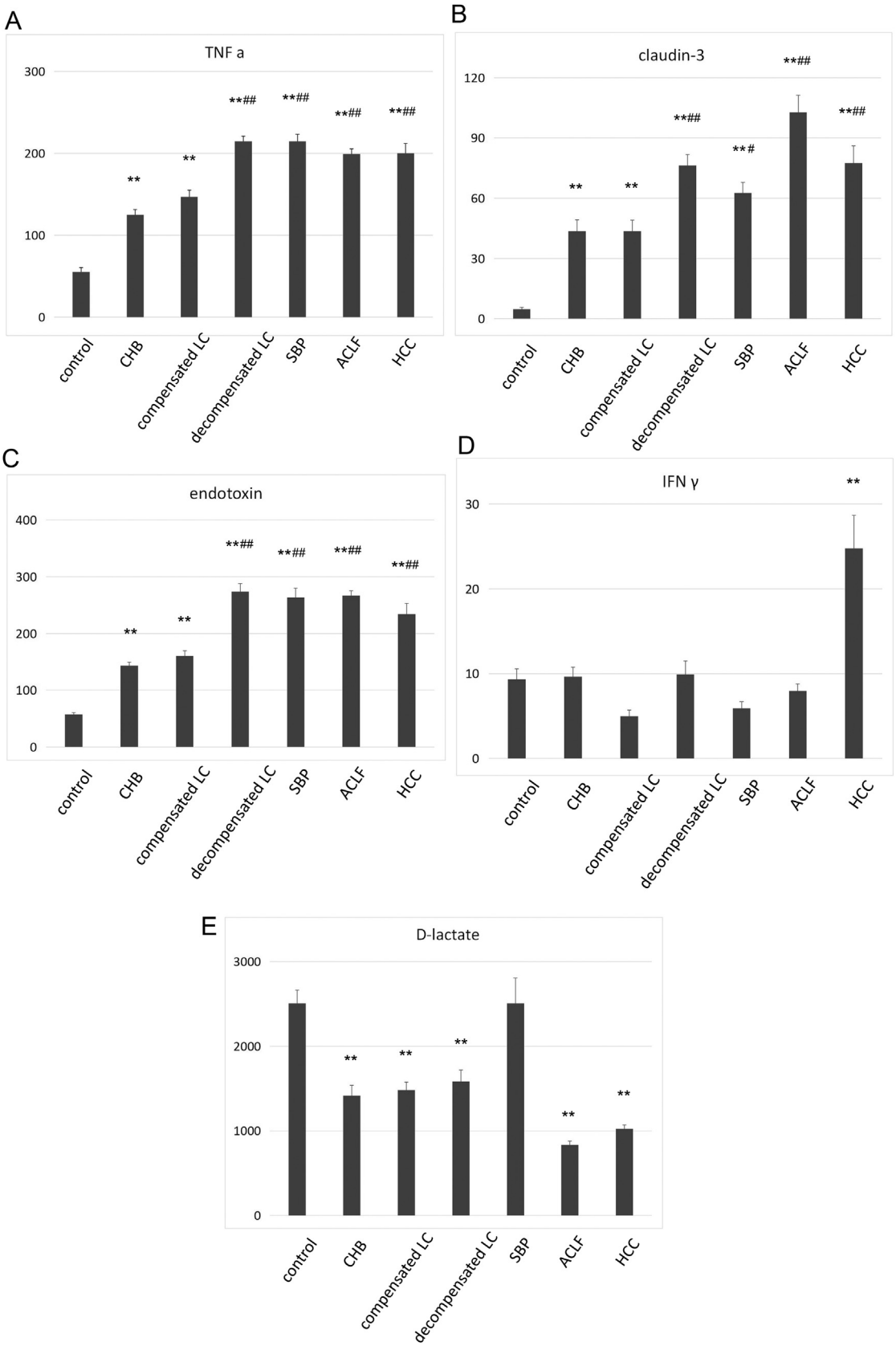

Figure 1 Column graphs illustrating TNF-α (pg/mL), claudin-3 (pg/mL), endotoxin (pg/mL), IFN-γ (pg/mL), and D-lactate (μg/L) concentrations in the plasma of patients with liver disease and controls as determined by enzyme-linked immunosorbent assay. $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. CHB and compensated LC. TNF-α: tumor necrosis factor-alpha; IFN-γ: interferon

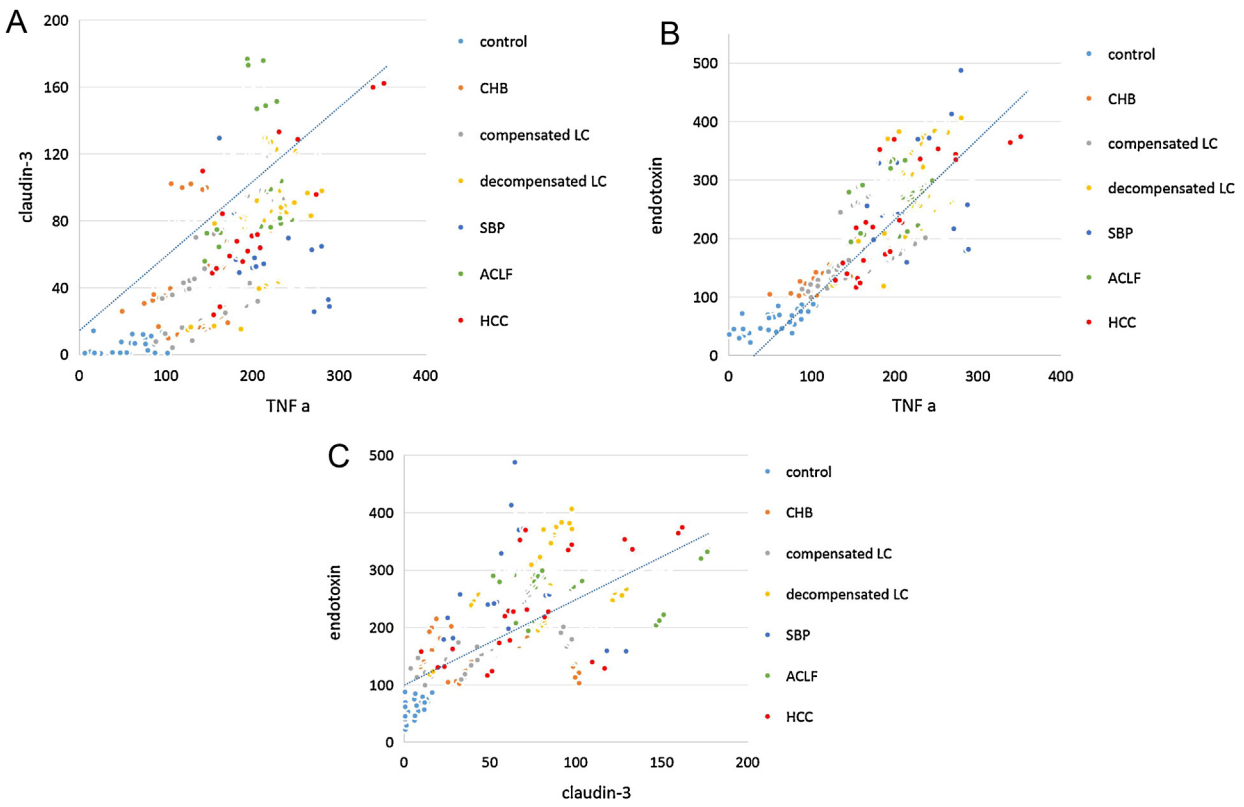

Figure 2 Correlations of clinical variables assessed by Pearson's test. $P < 0.05$ was considered significant. A. Correlation coefficient = 0.673, $p = 0.000$. B. Correlation coefficient = 0.842, $P = 0.000$. C. Correlation coefficient = 0.675, $P = 0.000$.

(MELD) and Child—Pugh scores were used to score liver disease severity (Table 1). Compared to the control group, serum TNF-α levels were significantly higher in all patients with liver disease (CHB, 124.8 ± 6.5 pg/mL; compensated LC 147.1 ± 7.9 pg/mL; decompensated LC, 215.0 ± 6.2 pg/mL; SBP, 214.7 ± 8.9 pg/mL; ACLF, 199.3 ± 6.2 pg/mL, and HCC, 202.9 ± 12.3 pg/mL) than in control participants (55.0 ± 5.4 pg/mL, $P < 0.01$; Fig. 1A). Serum TNF-α was significantly higher in patients with decompensated LC, SBP, ACLF, and HCC than those with CHB and compensated LC ($P < 0.01$). Plasma claudin-3 levels were significantly higher in all patients with liver disease (CHB, 43.5 ± 5.6 pg/mL; compensated LC, 44.6 ± 5.5 pg/mL; decompensated LC, 76.2 ± 5.4 pg/mL; SBP, 62.6 ± 5.2 pg/mL; ACLF, 102.8 ± 8.4 pg/mL; and HCC, 77.5 ± 8.3 pg/mL) than in control participants (4.8 ± 0.9 pg/mL; $P < 0.01$, Fig. 1B). The claudin-3 level was significantly higher in patients with decompensated LC, SBP, ACLF, and HCC than in those with CHB and compensated LC (all $P < 0.01$ except for SBP, $P < 0.05$). Plasma endotoxin was significantly higher in all patients with liver disease (CHB, 143.1 ± 6.2 pg/mL; compensated LC, 160.2 ± 9.1 pg/mL; decompensated LC, 273.4 ± 14.3 pg/mL; SBP, 263.3 ± 16.7 pg/mL; ACLF, 266.7 ± 8.6 pg/mL; and HCC, 234.0 ± 19.1 pg/mL) than in control participants (57.2 ± 3.3 pg/mL; $P < 0.01$, Fig. 1C). Plasma endotoxin was significantly higher in patients with decompensated LC, SBP, ACLF, and HCC than in those with CHB and compensated LC ($P < 0.01$). Serum IFN-γ in patients with HCC (24.8 ± 3.9 pg/mL) was significantly higher than in control participants (9.3 ± 1.2 pg/mL) and CHB (9.6 ± 1.2 pg/mL), compensated LC (5.0 ± 0.73 pg/mL), decompensated LC (9.9 ± 1.6 pg/mL), SBP (5.8 ± 0.7 pg/mL), and ACLF (7.9 ± 0.8 pg/mL) patients ($P < 0.01$, Fig. 1D). Plasma D-lactate was significantly lower in patients with CHB (1415.0 ± 122.6 pg/mL), compensated LC (1483.1 ± 92.4 pg/mL), decompensated LC (1587.5 ± 134.3 pg/mL), ACLF (832.4 ± 45.6 pg/mL), and HCC (1025.6 ± 43.0 pg/mL) than in control participants (2506.9 ± 155.6 pg/mL, $P < 0.01$, Fig. 1E). However, plasma D-lactate concentrations in patients with SBP (2514.5 ± 295.7 pg/mL) and controls were not significantly different (Fig. 1E). In summary, patients with liver disease showed significantly elevated plasma levels of TNF-α, claudin-3, and endotoxin levels and a significantly decreased D-lactate level.

gamma; CHB: chronic hepatitis B; compensated LC: compensated liver cirrhosis; decompensated LC: decompensated liver cirrhosis; SBP: spontaneous bacterial peritonitis; ACLF: acute-on-chronic liver failure; HCC: hepatocellular carcinoma.

Correlations of clinical variables

TNF-α, endotoxin and claudin-3 concentrations were positively correlated ($P < 0.01$ for all, Figs. 2A—C) in patients with liver diseases. Plasma IFN-γ did not significantly correlate with either endotoxin or claudin-3 ($P > 0.05$). Plasma D-lactate did not significantly correlate with TNF-α, endotoxin, or claudin-3 ($P > 0.05$). Thus, plasma levels of TNF-α, endotoxin and claudin-3 positively correlate with each other in patients with various liver diseases.

Discussion

Bacterial endotoxin produced by Gram-negative bacteria along the entire intestine translocates through the intestinal mucosa and into the blood to cause IETM [3,18]. IETM can occur in 80%—100% of patients with severe hepatitis and contributes to the development of secondary liver injury in FHF. The activity of IETM in the pathogenesis of various stages of liver disease has been described [1]. In this study, elevated plasma endotoxin was present in all patients with various liver diseases, and was significantly increased in those with decompensated LC, SBP, ACLF, or HCC. IETM persisted in the progression of liver disease and was frequent in end-stage LC.

Previous studies of changes in IP in liver cirrhosis did not find a significant correlation of IP and endotoxemia [2,9]. A reliable IP marker is therefore needed. As L-lactate is the form primarily produced in humans, while D-lactate can enter the circulation from the colon, D-lactate levels may indicate a change in IP. Elevated plasma D-lactate has been associated with IP in mouse and rat models of liver disease [3,19]. However, D-lactate was found not to be elevated in most of the patients studied by us and did not correlate with changes of either TNF-α or endotoxin ($P > 0.05$ for both). Such results are consistent with previous studies that found no significant differences in plasma D-lactate in patients with non-alcoholic fatty liver disease and controls, but did observe increases in the plasma bacterial endotoxin level with IP [20,21]. The relationship between D-lactate and IP in liver disease may be dependent on species differences.

Claudin-3 is a transmembrane protein subunit of tight junctions, and its loss is thought to indicate tight junction rupture and change in IP [22,23]. Elevated plasma claudin-3 concentrations thus indicate intestinal epithelial barrier dysfunction [5,6,24,25]. In this study, the plasma claudin-3 level was elevated in all patients with liver disease and correlated with the plasma endotoxin level. Significantly increased plasma claudin-3 in decompensated patients with LC, SBP, ACLF, and HCC was consistent with increased IP in end-stage LC. Plasma claudin-3 was significantly lower in patients with SBP than ACLF, but plasma endotoxin levels in patients with SBP and ACLF were similar. In addition to endotoxin of enteric origin, circulating infection most likely contributed to the increased plasma endotoxin in SBP. The results indicate that the plasma claudin-3 level was elevated by impaired IP and was associated with IETM.

IFN-γ may be involved in increasing IP [4,26]; however, in this study, serum IFN-γ was elevated only in HCC and did not correlate with plasma claudin-3 or endotoxin levels. Consequently, it was probably not involved in any changes in IP in these patients. The increase in serum IFN-γ in patients with HCC may have resulted from endocrine activity to defend against carcinoma invasion and metastasis [27,28]. TNF-α, which is secreted by monocytes and macrophages in patients with FHF, can induce hepatocyte necrosis [29—31] and damage the intestinal cellular barrier in vitro or in vivo [3,32—34]. TNF-α has been shown to associate with aggravated IETM in cirrhotic patients [35,36]. Elevated serum TNF-α was seen in all patients with liver disease, especially in patients with end-stage liver cirrhosis (decompensated LC, SBP, ACLF, and HCC), and correlated with plasma claudin-3 and endotoxin levels.

In conclusion, plasma claudin-3, but not D-lactate, was a marker of IP in this series of patients with various liver diseases. TNF-α injured the intestinal barrier to induce IETM, and contributed to the pathogenesis of liver disease, particularly end-stage LC. The measurement of plasma claudin-3 may help in the detection of IP in liver diseases. Clinical interventions to decrease the TNF-α level and protect the intestinal mechanical barrier from damage may reduce injury caused by IETM and should be investigated as a treatment for liver disease.

Author contributions

Z.H. Wang and J.F. Hu designed the study and experiments. Z.H. Wang and A.Y. Wang evaluated the cases and collected data. Z.H. Wang and Z.B. Gong performed the experiments. Z.H. Wang and I. Biviano analyzed the data. Z.H. Wang and H. Liu wrote the paper.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgments

Supported by project of Department of Science and Technology of Jiangxi Province, China (No. 20142BBG70094, No. 20133BBG70047).

We thank the healthy volunteers for their participation in the study. We thank International Science Editing for editing this manuscript.

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