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From stable disease to acute-on-chronic liver failure: Circulating cytokines are related to prognosis in different stages of cirrhosis

Josiane Fischer [a], Telma Erotides Silva [a], Pedro Eduardo Soares e Silva [a], Bruno Silveira Colombo [a], Mariana Costa Silva [a], Letícia Muraro Wildner [b], Maria Luiza Bazzo [b], Elayne Cristina Morais Rateke [b], Tania Silvia Frode [b], Silvana Vigil de Mello [b], Júlia S. Rosa [b], Esther Buzaglo Dantas-Correa [a], Janaína Luz Narciso-Schiavon [a], Leonardo Lucca Schiavon [a,*]

[a] Division of Gastroenterology, Federal University of Santa Catarina, Brazil
[b] Department of Clinical Analysis, Federal University of Santa Catarina, Brazil

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ABSTRACT

Introduction: Although both pro- and anti-inflammatory circulating cytokines are known to be elevated in liver cirrhosis, its clinical significance is not completely recognized. Our aim was to evaluate the prognostic significance of circulating cytokines interleukin (IL)-6, IL-17 and IL-10 in different stages of cirrhosis.

Methods: This prospective study included two cohorts: (1) stable cirrhosis attended in the Outpatient Clinic (n = 118), and (2) subjects hospitalized for acute decompensation (AD) (n = 130). Thirty healthy subjects served as control group.

Results: Patients with cirrhosis exhibited higher levels of cytokines as compared to controls. In stable cirrhosis, during a median follow-up of 17 months, liver-related events occurred in 26 patients. Higher IL-10 levels and Child-Pugh B/C were independently associated with reduced event-free survival. In AD cohort, death after 90 days of follow-up occurred in 39 patients and was independently associated with ascites, higher IL-6 and model for end-stage liver disease. IL-6 levels also showed higher AUROC than CRP for predicting bacterial infection in the AD cohort (0.831 ± 0.043 vs. 0.763 ± 0.048, respectively). IL-17 decreased at third day of hospitalization only in patients who progressed to death. Higher IL-6 levels were observed in acute-on-chronic liver failure (ACLF) patients even in the absence of bacterial infection whereas IL-10 was higher only in subjects with infection-related ACLF. Higher IL-10 and IL-17 levels were associated with progression to death in ACLF.

Conclusions: The pattern of immune response seems to vary according to the phase of cirrhosis and is related to prognosis, from stable disease to ACLF.

1. Introduction

Cirrhosis-associated immune dysfunction (CAID) is characterized by both immunodeficiency and systemic inflammation due to persistent and inappropriate activation of immune cells [1]. In compensated cirrhosis, even in the absence of intestinal bacterial translocation, damage-associated molecular patterns (DAMPs) released from necrotic hepatocytes can activate the immune system and lead to systemic inflammation. In the decompensated stage, bacterial products from intestinal bacterial translocation enhance immune activation, increasing circulating pro-inflammatory cytokines and immune cells expression of activation antigens [2]. Moreover, in parallel with the progression of the

Abbreviations: CAID, Cirrhosis-associated immune dysfunction; DAMPs, damage-associated molecular patterns; IL-6, interleukin-6; IL-10, interleukin-10; IL-17, interleukin-17; AD, acute decompensation; MELD, Model for End-Stage Liver Disease; ACLF, Acute-on-chronic liver failure; CBA, cytometric Bead Array; ROC, receiver operating characteristics; IQR, interquartile range; CRP, C-reactive protein; AUROC, area under the receiver operating characteristic; LPS, lipopolysaccharide; PRRs, Pattern Recognition Receptors; CARS, compensatory anti-inflammatory response syndrome.

* Corresponding author at: Rua Deputado Antônio Edu Vieira, 1310, Casa 217, 88.040-001 Florianópolis, SC, Brazil.
E-mail address: leo-jf@uol.com.br (L.L. Schiavon).

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disease, a state of immunosuppression occurs as a result of distinct mechanisms, including loss of function of immune surveillance by the liver and decreasing function of immune cells [3].

Cytokines are key components of the immune system, with multiple properties and biological functions. Systemic inflammation associated with CAID is related to the increase in circulating levels of pro-inflammatory cytokines such as interleukin (IL)-6 and IL-17 [4,5]. In contrast, increased concentrations of cytokines with anti-inflammatory properties such as IL-10 have also been described, especially in cases of advanced disease, and can be related to the "immunological paralysis" observed in these patients [6]. Evidence indicates a link between the magnitude of the immune disorder, mainly manifested by changes in the levels of pro-inflammatory cytokines and prognosis in liver cirrhosis. However, *in vivo* studies evaluating the patterns of this disorder in different stages of cirrhosis are still scarce. Our primary aim was to evaluate the prognostic significance of IL-6, IL-17, and IL-10 circulating levels in different stages of liver cirrhosis.

2. Materials and methods

2.1. Patients

This study is part of a project that aims to follow two cohorts of adult patients ($\geqslant$18 years of age) with liver cirrhosis followed at the University Hospital of the Federal University of Santa Catarina. Details about those cohorts were previously published [7] and are briefly presented below. Diagnosis of cirrhosis histologically defined (when available) or by combining clinical, imaging, and laboratory findings in the presence of portal hypertension. The first cohort included patients with stable cirrhosis attended in the Outpatient Clinic. In this group, the following exclusion criteria were applied: diagnosis of hepatocellular carcinoma, interferon-based therapy during the last 30 days, diagnosis of bacterial infections during the last 7 days, or refusal or inability of the patient to understand the terms of the informed consent. The second cohort comprised patients admitted to the emergency room for acute decompensation (AD) of cirrhosis. For these subjects, the following exclusion criteria were applied: hospitalization for elective procedures, admissions not related to complications of liver cirrhosis, or hepatocellular carcinoma outside the Milan criteria. Thirty subjects evaluated during routine laboratory tests served as healthy controls.

The study protocol complies with the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee on Human Research of the Federal University of Santa Catarina.

2.2. Methods

In the cohort with stable cirrhosis, all patients were initially evaluated from June 2013 to February 2014. Development of complications, mortality, or liver transplantation was assessed by periodical phone calls. The second cohort included individuals hospitalized for AD between January 2011 and August 2013. AD was defined by acute development of hepatic encephalopathy, large ascites, gastrointestinal bleeding, bacterial infection, or any combination of these [8]. Patients were monitored during hospitalization and 30- and 90-day mortality was assessed by a phone call. In the case of more than one hospital admission during the study period, only the most recent hospitalization was considered.

Patients were conserved active drinkers if they reported an average overall consumption of 21 or more drinks per week for men and 14 or more drinks per week for women during the 4 weeks before enrollment (one standard drink is equal to 12 g absolute alcohol) [9].

Hospitalized individuals with a suspected infection at admission were clinically and laboratory evaluated to confirm the diagnosis and to establish the primary source of infection. Patients were followed for 48 h to ensure the diagnosis or exclusion of bacterial infection.

Hepatic encephalopathy was graded according to the West-Haven criteria [10]. Child-Pugh classification system [11] and the Model for End-Stage Liver Disease (MELD) [12] were calculated based on clinical and laboratory parameters obtained at admission in the case of hospitalized patients. Acute-on-chronic liver failure (ACLF) was defined as proposed by the EASL-CLIF Consortium [8].

2.3. Circulating cytokine measurements

Serum samples were obtained at initial evaluation for patients with stable cirrhosis and on the first day of hospitalization in the AD cohort. A subset of 74 hospitalized subjects also had serum samples collected on the third day of hospitalization. The quantitative determination of IL-6, IL-10, and IL-17A in the serum was performed using a Cytometric Bead Array (CBA) kit (BD Biosciences; San Diego, CA). The fluorescence produced by the CBA beads was measured on a FASCSVerse Flow Cytometer (BD FACSVerseTM, San Jose, CA, USA) and analyzed using Software FCAP Array 3.0 (BD Biosciences). Detection limits were 2.4 pg/mL for IL-6, 4.5 pg/mL for IL-10, and 18.9 pg/mL for IL-17A.

2.4. Statistical analysis

Normality of the variable distribution was determined using the Kolmogorov-Smirnov test. The correlation between the numerical variables was evaluated using Spearman's correlation coefficient. Continuous variables were compared using Student's *t*-test in the case of a normal distribution or if not, using the Mann-Whitney test. Categorical variables were evaluated by the chi-square test or Fisher's exact test, as appropriate. Hospitalization for acute decompensation, death, and liver transplantation were considered liver-related events. Univariate Cox regression analysis was used to investigate the association between the cytokine levels and event-free survival in the cohort with stable cirrhosis. Subsequently, a multivariate Cox regression analysis was performed by including the cytokines with statistical significance in the univariate Cox regression, in addition to variables classically associated with event-free survival in cirrhosis. Kaplan-Meier curve was used to illustrate event-free survival of outpatients with cirrhosis according to two strata, defined by the cutoff of selected cytokines. Survival differences between groups were compared using the log-rank test. Multiple logistic regression analysis was used to investigate the factors independently associated with bacterial infection and 90-day death or liver transplantation in the hospitalized cohort. The best cutoffs of the variables of interest for predicting the endpoints were chosen based on the receiver operating characteristics (ROC) curves. All tests were performed using SPSS software, version 22.0 (SPSS, Chicago, IL, USA). A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of included patients and cytokine levels in the study groups

A total of 118 patients with stable cirrhosis receiving outpatient follow-up, 130 patients hospitalized due to AD cirrhosis, and 30 healthy controls were included in the study. Tables 1 and 2 show the main characteristics of all cohorts and cytokine levels results.

Table 1
Characteristics of included subjects and factors associated with progression to liver-related events in patients with stable cirrhosis.

	All (n = 118)	No liver-related events[a] (n = 90)	Liver-related Events[a] (n = 26)	P
Age (years), mean ± SD	56.05 ± 11.91	55.61 ± 12.06	57.46 ± 11.91	0.491
Male gender, n (%)	77 (65.3)	58 (64.4)	18 (69.2)	0.651
Etiology of cirrhosis, n (%)				
Alcohol	52 (44.1)	43 (47.8)	8 (30.8)	0.124
Hepatitis C	37 (31.4)	24 (26.7)	12 (46.2)	0.059
Hepatitis B	6 (5.1)	5 (5.6)	1 (3.8)	1.000
Cryptogenic	9 (7.6)	8 (8.9)	1 (3.8)	0.681
Previous decompensation, n (%)	90 (76.3)	67 (74.4)	22 (84.6)	0.280
Active alcoholism, n (%)	13 (11.0)	9 (10.0)	3 (11.5)	0.730
Complication at evaluation, n (%)				
Ascites	31 (26.3)	19 (21.1)	12 (46.2)	0.011
Hepatic encephalopathy	13 (11.0)	6 (6.7)	7 (26.9)	0.009
Previous ascites	59 (50.0)	40 (44.4)	19 (73.1)	0.010
Laboratory data				
Leucocyte count ($\times 10^9$), median (IQR)	4.52 (3.64–5.80)	4.60 (3.60–5.94)	4.45 (3.66–5.22)	0.462
Sodium (meq/L), median (IQR)	138.00 (136.00–140.00)	138.00 (136.00–140.00)	139.00 (136.00–140.25)	0.614
Creatinine (mg/dL), median (IQR)	0.90 (0.80–1.03)	0.90 (0.80–1.03)	0.90 (0.80–1.10)	0.445
INR, median (IQR)	1.20 (1.13–1.28)	1.19 (1.12–1.28)	1.21 (1.15–1.35)	0.315
Albumin (g/dL), mean ± SD	3.32 ± 0.49	3.43 ± 0.42	2.93 ± 0.55	<0.001
CRP (mg/L), median (IQR)	3.50 (3.20–5.50)	3.50 (3.20–4.70)	3.80 (3.20–6.65)	0.173
Total bilirubin (mg/dL), median (IQR)	1.10 (0.80–1.80)	1.10 (0.80–1.70)	1.40 (0.80–2.00)	0.296
IL-6 (pg/mL), median (IQR)	2.22 (1.10–5.55)	2.13 (0.77–4.54)	3.56 (1.54–17.00)	0.013
IL-10 (pg/mL), median (IQR)	0.30 (0.03–0.74)	0.22 (0.00–0.58)	0.54 (0.22–0.92)	0.021
IL-17 (pg/mL), median (IQR)	9.68 (4.60–18.85)	9.68 (3.08–18.85)	11.36 (6.04–20.95)	0.323
Child-Pugh B/C, n (%)	51 (43.2)	34 (37.8)	17 (65.4)	0.012
MELD score, median (IQR)	9.80 (8.26–11.66)	9.62 (8.17–11.30)	11.25 (8.94–13.22)	0.034

SD = Standard deviation; IQR = Interquartile range; INR = International normalised ratio; CRP = C-reactive protein; MELD = Model for End-stage Liver Disease.
[a] Two patients were lost to follow-up.

Table 2
Characteristics of included subjects and factors associated with 90-day mortality among patients hospitalized for acute decompensation of cirrhosis.

	All (n = 130)	Survivors (n = 91)	Deaths (n = 39)	P
Age (years), mean ± SD	53.74 ± 11.29	53.01 ± 11.09	55.47 ± 11.72	0.260
Male gender, n (%)	95 (73.1)	65 (71.4)	30 (76.9)	0.517
Etiology of cirrhosis, n (%)				
Alcohol	42 (32.3)	26 (28.6)	16 (41.0)	0.164
Hepatitis C	53 (40.8)	37 (40.7)	16 (41.0)	0.969
Hepatitis B	7 (5.4)	6 (6.6)	1 (2.6)	0.674
Cryptogenic	12 (9.2)	11 (12.1)	1 (2.6)	0.107
Previous decompensation, n (%)	80 (61.6)	54 (59.3)	26 (66.7)	0.431
Active alcoholism, n (%)	49 (37.7)	30 (33.0)	19 (48.7)	0.089
Complication at evaluation, n (%)				
Ascites	60 (46.2)	31 (34.1)	29 (74.4)	<0.001
Hepatic encephalopathy	70 (53.8)	41 (45.1)	29 (74.4)	0.002
Gastrointestinal bleeding	62 (47.7)	51 (56.0)	11 (28.2)	0.004
Bacterial infection	39 (30.0)	19 (20.9)	20 (51.3)	0.001
ACLF, n (%)	30 (23.1)	8 (8.8)	22 (56.4)	<0.001
Laboratory data				
Leucocyte count ($\times 10^9$), median (IQR)	7.24 (4.99–10.39)	6.55 (4.19–9.59)	8.46 (6.27–13.06)	0.003
Sodium (meq/L), median (IQR)	136.00 (132.50–139.00)	137.00 (133.00–140.00)	133.00 (128.50–137.00)	0.001
Creatinine (mg/dl), median (IQR)	1.10 (0.90–1.50)	1.00 (0.80–1.20)	1.80 (1.00–2.50)	<0.001
INR, median (IQR)	1.37 (1.25–1.57)	1.34 (1.22–1.45)	1.59 (1.34–1.81)	<0.001
Albumin (g/dL), mean ± SD	2.41 ± 0.69	2.58 ± 0.69	1.98 ± 0.51	<0.001
CRP (mg/L), median (IQR)	9.05 (3.80–34.70)	7.29 (3.50–26.68)	20.60 (6.04–68.80)	0.004
Total bilirubin (mg/dL), median (IQR)	2.10 (0.95–3.73)	1.30 (0.90–3.10)	3.80 (2.40–10.20)	<0.001
IL-6 (pg/mL), median (IQR)	19.30 (7.94–44.62)	11.43 (5.65–22.70)	42.60 (22.06–168.16)	<0.001
IL-10 (pg/mL), median (IQR)	2.24 (1.18–4.22)	1.73 (0.91–3.43)	3.63 (2.43–6.86)	<0.001
IL-17 (pg/mL), median (IQR)	3.42 (0.00–20.04)	0.00 (0.00–12.88)	14.71 (0.00–146.01)	<0.001
Child-Pugh C, n (%)	50 (38.5)	21 (23.1)	29 (74.4)	<0.001
MELD score, median (IQR)	14.43 (10.76–19.43)	12.65 (10.04–15.61)	22.55 (18.01–27.90)	<0.001

SD = Standard deviation; IQR = Interquartile range; ACFL = Acute-on-chronic liver failure; INR = International normalised ratio; CRP = C-reactive protein; MELD = Model for End-stage Liver Disease.

The median IL-6 levels in control group was 0.86 pg/mL (IQR 0.55–1.98 pg/mL). IL-6 levels were significantly higher in patients with stable cirrhosis than in controls (P = 0.002), in hospitalized patients than in controls (P < 0.001) and in hospitalized patients than stable patients (P < 0.001). The median IL-10 was 0.00 pg/mL (IQR 0.00–0.03 pg/mL) in the control group. Higher levels of IL-10 were observed in patients with stable cirrhosis than in controls (P < 0.001), in hospitalized patients than in con-

trols (P < 0.001) and in stable patients (P < 0.001). IL-17 median levels in the control group were 0.66 pg/mL (IQR 0.00–11.04 pg/mL). In patients with stable cirrhosis IL-17 concentrations were significantly higher than those in controls (P = 0.001) and in hospitalized patients (P = 0.022).

3.2. Cytokine concentrations in patients with stable cirrhosis

Circulating levels of the three evaluated cytokines were significantly higher in Child-Pugh B/C patients as compared to Child-Pugh A ones. The median IL-6 was 1.52 pg/mL (IQR 0.42–2.97 pg/mL) in Child-Pugh A patients and 5.54 pg/mL (IQR 2.06–12.09 pg/mL) in Child-Pugh B/C subjects (P < 0.001). Similarly, median IL-10 was 0.19 pg/mL (IQR 0.00–0.54 pg/mL) in Child-Pugh A patients and 0.49 pg/mL (IQR 0.15–0.78 pg/mL) in Child-Pugh B/C subjects (P = 0.006). IL-17 median values were 6.45 pg/mL (IQR 3.08–17.24 pg/mL) in Child-Pugh A and 11.27 pg/mL (IQR 6.45–19.71 pg/mL) in Child-Pugh B/C (P = 0.053). Correlation analysis between cytokine levels and other numerical variables is shown in Supplementary Table 1.

Stable patients were followed for a median of 16 months. During the follow-up, hepatic events occurred in 26 patients (22.0%), 11 hospitalizations due to AD cirrhosis, 13 deaths, and 2 liver transplants. Two patients were lost to follow-up and therefore were not included in the survival analysis. Table 1 shows the bivariate analysis of factors associated with the progression to hepatic events during follow-up. The univariate Cox regression analysis revealed that IL-10 levels (P = 0.002) were associated with event-free survival, unlike IL-6 and IL-17. Subsequently, we conducted a multiple Cox regression analysis including IL-10 and other variables classically associated with reduced survival (Child-Pugh classification, MELD, prior ascites, and encephalopathy). Higher IL-10 levels (HR 1.534, 95% confidence interval CI 1.155–2.038; P = 0.003) and Child-Pugh B/C (HR 2.648, 95% CI 1.179–5.948; P = 0.018) were independently associated with reduced event-free survival. The AUROC of IL-10 to predict event-free survival was 0.648 ± 0.062 and the best cutoff chosen based on ROC curve was 0.42 pg/mL. The Kaplan-Meier survival probability was 86.6% in patients with IL-10 < 0.42 pg/mL and 65.3% in those with IL-10 ⩾ 0.42 pg/mL (P = 0.003) (Fig. 1).

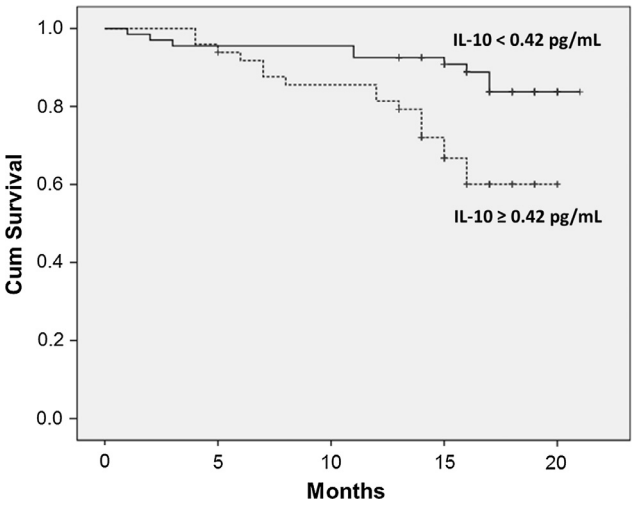

Fig. 1. Kaplan-Meier event-free survival of 116 outpatients with cirrhosis stratified according to the IL-10 cut-off level of 0.42 pg/mL. The survival probability after a median follow-up of 16 months was 86.6% for patients with IL-10 < 0.42 pg/mL and 65.3% for those with IL-10 ⩾ 0.42 pg/mL (P = 0.003).

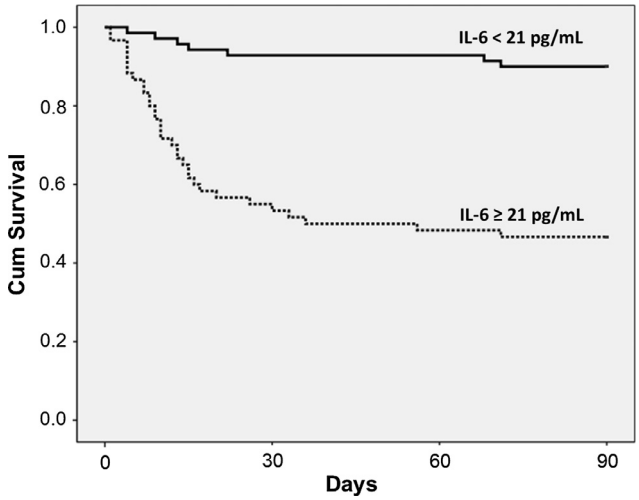

Fig. 2. Cumulative 90-day transplant-free survival of hospitalized patients with cirrhosis according to IL-6 levels. The Kaplan-Meier survival probability was 90.0% in patients with IL-6 < 21 pg/mL and 46.7% for subjects IL-6 ⩾ 21 pg/mL (P < 0.001).

3.3. Cytokine concentrations in patients hospitalized for acute decompensation of cirrhosis

Correlation analysis between cytokine levels and other numerical variables in the hospitalized cohort is shown in Supplementary Table 2.

During the 90-day follow-up, 39 patients died (30%). Factors associated with progression to death within 90 days are detailed in Table 2. A logistic regression analysis to investigate factors independently associated with 90-day mortality was performed including the covariates MELD, Child-Pugh C, ACLF on admission, ascites, bacterial infection, sodium, CRP, IL-6, IL -10, and IL-17. In this analysis, the presence of ascites (OR 6.286, 95% CI 1.826–21.635, P = 0.004), MELD (OR 1.300, 95% CI 1.175–1.439; P < 0.001), and IL -6 (OR 1.002, 95% CI 1.000–1.004, P = 0.029) were associated with 90-day mortality.

The area under the receiver operating characteristic (AUROC) curve for IL-6 to predict mortality within 90 days was 0.779 ± 0.046. Fig. 2 shows the Kaplan-Meier curve for mortality during follow-up, according to IL-6 concentrations categorized at a cut-off of 21 pg/mL. The Kaplan-Meier survival probability was 90.0% in subjects with IL-6 < 21 pg/mL and 46.7% in those with IL-6 ⩾ 21 pg/mL (P < 0.001, log-rank test). With this cutoff point, IL-6 levels reached a diagnostic accuracy of 73%, sensitivity of 82%, specificity of 69%, positive predictive value of 53%, and negative predictive value of 90% for predicting mortality within 90 days.

3.4. Serial cytokine measurements in hospitalized patients with cirrhosis

Cytokine levels were evaluated in two time points (on the first and third day of hospitalization) in 74 patients. IL-6 levels did not change significantly when the two days were compared, regardless of the outcome. A significant reduction of IL-10 on the third day was observed both in survivors (median 1.58 pg/mL [IQR 0.90–3.41 pg/mL] vs. 0.56 pg/mL [IQR 0.18–1.09 pg/mL]; P < 0.001) and in those who died (3.74 pg/mL [IQR 2.63–5.85 pg/mL] vs. 1.33 pg/mL [IQR 0.69–3.89 pg/mL]; P = 0.013). A trend towards increased IL-17 levels on the third day (0.62 pg/mL [IQR 0.00–22.17 pg/mL] vs. 3.87 pg/mL [IQR 0.00–12.00 pg/mL]; P = 0.088) was observed. When the outcome was evaluated, this tendency to increase was restricted to survivors (0.00 pg/mL [IQR 0.00–11.36 pg/mL] vs. 3.16 pg/mL [IQR 0.00–9.32 pg/mL]; P = 0.538). Conversely, a

decrease in IL-17 levels on the third day was noted in those who died during follow-up (31.67 pg/mL [IQR 0.00–416.26 pg/mL] vs. 19.17 pg/mL [IQR 0.00–152.88 pg/mL]; P = 0.055).

3.5. Cytokine concentrations for the diagnosis of bacterial infection in cirrhosis

Bacterial infections within the first 48 h of hospitalization were diagnosed in 39 patients (30%). The bivariate analysis of factors associated with the presence of bacterial infection is shown in Table 3. A logistic regression analysis was performed including MELD, Child-Pugh C, ACLF on admission, ascites, sodium, total leukocytes, CRP, IL-6, and IL-10 as covariates. Bacterial infection diagnosed during the first 48 h of hospitalization was independently associated with the presence of ascites (OR 4.809, 95% CI 1.808–12.787, P = 0.002), CRP (OR 1.015, 95% CI 1.005–1.025; P = 0.004), and IL-6 (OR 1.003, 95% CI 1.000–1.005, P = 0.022).

The AUROCs of IL-6 and CRP for the diagnosis of bacterial infection were 0.831 ± 0.043 and 0.763 ± 0.048, respectively. Cutoffs were chosen based on the ROC curves and the best overall performance for IL-6 was observed with a cutoff of 23 pg/mL. This value showed an accuracy of 76%, sensitivity of 74%, specificity of 77%, positive predictive value of 58%, and negative predictive value of 88%. The best results for CRP were observed with a cutoff of 15 pg/mL. This value showed an accuracy of 69%, sensitivity of 71%, specificity of 69%, positive predictive value of 49%, and negative predictive value of 85%.

Table 3
Factors associated with the presence of bacterial infection diagnosed within the first 48 h after hospitalization.

	Absence of infection (n = 91)	Presence of infection (n = 39)	P
Age (years), mean ± SD	53.56 ± 10.43	54.15 ± 13.20	0.803
Male gender, n (%)	65 (71.4)	30 (76.9)	0.517
Etiology of cirrhosis, n (%)			
Alcohol	26 (28.6)	16 (41.0)	0.164
Hepatitis C	41 (45.1)	12 (30.8)	0.129
Hepatitis B	4 (4.4)	3 (7.7)	0.428
Cryptogenic	11 (12.1)	1 (2.6)	0.107
Previous decompensation, n (%)	56 (61.5)	24 (61.5)	1.000
Active alcoholism, n (%)	33 (36.3)	18 (41.0)	0.608
Complication at evaluation, n (%)			
Ascites	34 (37.4)	26 (66.7)	0.002
Hepatic encephalopathy	45 (49.5)	25 (64.1)	0.125
Gastrointestinal bleeding	49 (53.8)	13 (33.3)	0.032
ACLF, n (%)	13 (14.3)	17 (43.6)	<0.001
Laboratory data			
Leucocyte count (× 10^9), median (IQR)	6.66 (4.54–9.38)	9.54 (5.78–13.06)	0.012
Sodium (meq/L), median (IQR)	137.00 (133.00–140.00)	133.00 (131.00–137.00)	0.005
Creatinine (mg/dl), median (IQR)	1.00 (0.80–1.30)	1.30 (1.00–2.10)	0.001
INR, median (IQR)	1.37 (1.24–1.49)	1.42 (1.30–1.69)	0.102
Albumin (g/dL), mean ± SD	2.56 ± 0.68	2.04 ± 0.60	<0.001
CRP (mg/L), median (IQR)	6.04 (3.50–20.90)	33.35 (9.02–119.75)	<0.001
Total bilirubin (mg/dL), median (IQR)	1.50 (0.90–3.60)	2.70 (1.20–4.80)	0.131
IL-6 (pg/mL), median (IQR)	11.64 (5.06–22.28)	72.23 (22.47–198.86)	<0.001
IL-10 (pg/mL), median (IQR)	2.00 (0.99–3.60)	3.48 (1.54–6.86)	0.002
IL-17 (pg/mL), median (IQR)	1.23 (0.00–16.89)	9.10 (0.00–22.02)	0.298
Child-Pugh C, n (%)	28 (30.8)	22 (56.4)	0.006
MELD score, median (IQR)	13.83 (10.19–18.01)	16.39 (12.75–26.25)	0.002

SD = Standard deviation; IQR = Interquartile range; ACFL = Acute-on-chronic liver failure; INR = International normalised ratio; CRP = C-reactive protein; MELD = -Model for End-stage Liver Disease.

3.6. Circulating IL-6, IL-10, and IL-17 in patients with acute-on-chronic liver failure

Thirty patients had ACLF on admission and another seven had developed the complication before the third day of hospitalization; they were evaluated together. The characteristics and factors associated with ACLF are presented in Table 4. The most common precipitating factor was bacterial infection, present in 21 patients (56.8%). IL-6 medians were significantly higher in patients with bacterial infection with or without ACLF as compared to individuals without both complications (P < 0.05). Interestingly, even in the absence of bacterial infection as a precipitating factor, patients with ACLF exhibited higher IL-6 levels when compared to individuals without ACLF or infection (21.97 pg/mL [IQR 8.65–3.30 pg/mL] vs. 11.01 pg/mL [IQR 4.67–21.94 pg/mL]; P = 0.039). Subjects only with bacterial infection showed similar IL-6 levels to those with ACLF only (22.14 pg/mL [IQR 10.47–52.45 pg/mL] vs. 21.97 pg/mL [IQR 8.65–3.30 pg/mL]; P = 0.506) (Fig. 3A). The IL-10 concentrations

Table 4
Factors associated with the presence of acute-on-chronic liver failure (ACLF) diagnosed within the first 48 h after hospitalization.

	No ACLF (n = 93)	ACLF (n = 39)	P
Age (years), mean ± SD	53.83 ± 11.46	54.81 ± 11.98	0.803
Male gender, n (%)	63 (67.7)	32 (86.5)	0.030
Etiology of cirrhosis, n (%)			
Alcohol	23 (24.7)	19 (51.4)	0.003
Hepatitis C	40 (43.0)	13 (35.1)	0.410
Hepatitis B	5 (5.4)	2 (5.4)	1.000
Cryptogenic	11 (11.8)	1 (2.7)	0.177
Previous decompensation, n (%)	56 (60.2)	24 (64.9)	0.623
Active alcoholism, n (%)	32 (34.4)	17 (45.9)	0.221
Complication at evaluation, n (%)			
Ascites	28 (30.1)	17 (45.9)	0.087
Hepatic encephalopathy	27 (29.0)	18 (48.6)	0.034
Gastrointestinal bleeding	48 (51.6)	14 (37.8)	0.156
Bacterial infection, n (%)	18 (19.4)	21 (56.8)	<0.001
Laboratory data			
Leucocyte count (× 10^9), median (IQR)	6.34 (4.349–9.08)	10.26 (7.06–15.14)	<0.001
Sodium (meq/L), median (IQR)	137.00 (133.30–140.00)	133.00 (129.00–136.00)	<0.001
Creatinine (mg/dl), median (IQR)	1.00 (0.80–1.20)	1.90 (1.45–2.85)	<0.001
INR, median (IQR)	1.34 (1.22–1.48)	1.48 (1.34–1.80)	<0.001
Albumin (g/dL), mean ± SD	2.51 ± 0.70	2.13 ± 0.56	0.004
CRP (mg/L), median (IQR)	7.29 (3.50–22.10)	32.40 (5.94–104.50)	0.001
Total bilirubin (mg/dL), median (IQR)	1.34 (0.90–3.30)	3.10 (1.55–8.15)	<0.001
IL-6 (pg/mL), median (IQR)	11.64 (5.36–22.90)	54.19 (22.11–209.48)	<0.001
IL-10 (pg/mL), median (IQR)	1.76 (1.00–3.54)	3.60 (2.22–9.20)	<0.001
IL-17 (pg/mL), median (IQR)	0.00 (0.00–14.25)	14.10 (0.00–41.13)	0.020
Child-Pugh C, n (%)	27 (29.0)	23 (62.2)	<0.001
MELD score, median (IQR)	12.75 (10.11–15.81)	22.81 (18.37–28.75)	<0.001

SD = Standard deviation; IQR = Interquartile range; INR = International normalised ratio; CRP = C-reactive protein; MELD = Model for End-stage Liver Disease.

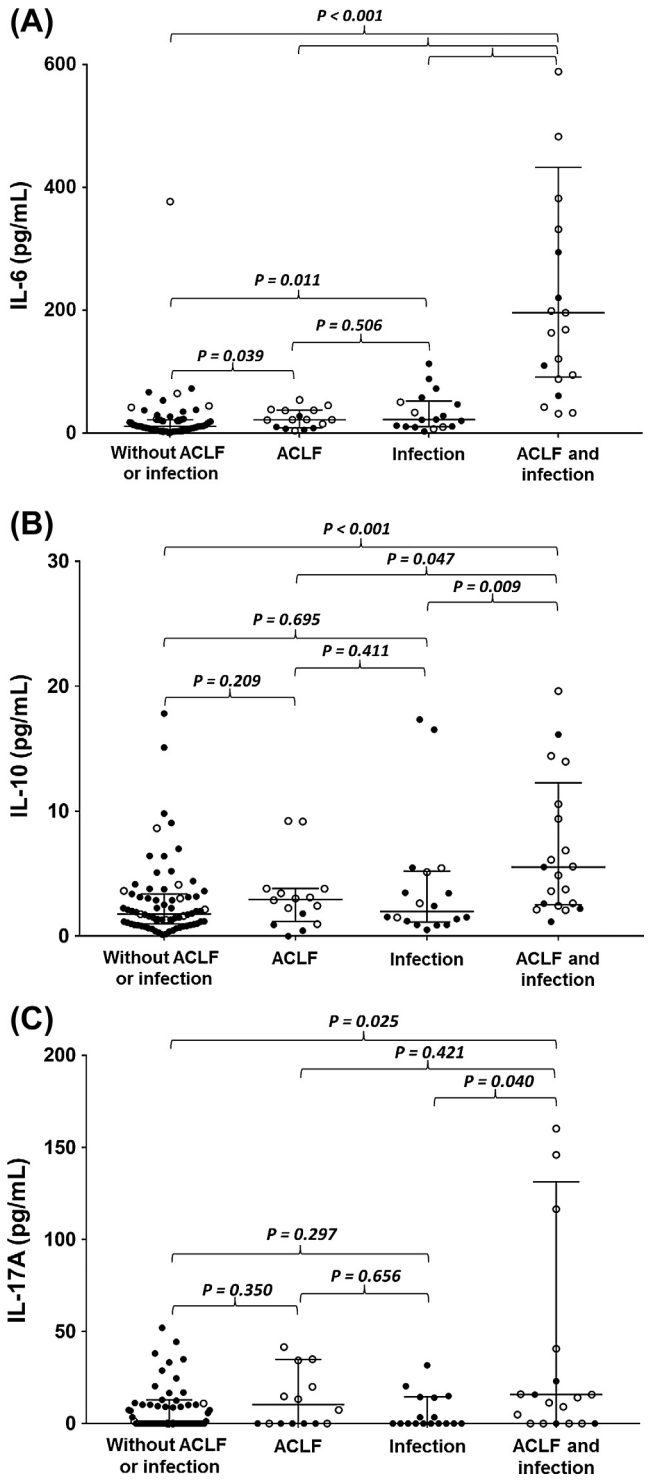

Fig. 3. Levels of IL-6 (Fig. 3A), IL-10 (Fig. 3B) and IL-17 (Fig. 3C) in hospitalized patients according to the presence of ACLF and bacterial infection during the first 48 h of hospitalization. Patients who survived at ninety day are represented by solid black dots and non-survivors are represented by white dots. The bars represent median and interquartile ranges. Extreme outliers are not shown for better visibility.

were significantly higher in patients with ACLF associated with infection than in all other groups ($P < 0.05$) (Fig. 3B). No differences were observed in IL-10 levels in further comparisons. Similarly, IL-17 levels were significantly higher in patients with ACLF associated with infection than in patients without these two complications ($P = 0.025$) or with infection only ($P = 0.040$) (Fig. 3C).

When we consider only the 37 patients with ACLF, IL-6 levels did not significantly differ for those who died as compared to survivors (54.19 pg/mL [IQR 31.82–198.86 pg/mL] vs. 44.24 pg/mL [IQR 7.90–238.67 pg/mL]; $P = 0.371$). Higher levels of IL-10 (3.80 pg/mL [IQR 2.63–9.22 pg/mL] vs. 2.00 pg/mL [IQR 0.79–8.18 pg/mL]; $P = 0.052$) and IL-17 (15.78 pg/mL [IQR 4.90–116.47 pg/mL] vs. 0.00 pg/mL [IQR 0.00–17.59 pg/mL]; $P = 0.034$) were associated with progression to death in patients with ACLF.

4. Discussion

In this prospective study including two cohorts of patients with cirrhosis, the intensity and significance of the immune derangement varied according to the severity of the cirrhosis and the clinical context. In patients with stable cirrhosis, the concentrations of IL-6, IL-10, and IL-17 were higher than those in healthy controls, and were related to various parameters related to the severity of liver disease. This immune disorder, characterized by elevated concentrations of both anti-inflammatory and proinflammatory cytokines, is probably related to chronic endotoxemia, common in patients with liver cirrhosis [13]. A recent study demonstrated a significant increase in IL-6 and IL-10 levels after stimulation with lipopolysaccharide (LPS), both in healthy volunteers and in patients with cirrhosis [14]. Human data on the impact of endotoxemia on IL-17 levels are scarce. However, a study in rats showed that this cytokine release might be stimulated after the induction of endotoxemia by injection of LPS [15].

Hepatic events occurred in 22% of the stable patients during follow-up, and were related to higher IL-6 and IL-10 medians. However, in the multiple Cox regression analysis, only IL-10 was independently related to decreased event-free survival. No studies investigating the relationship between IL-10 levels and prognosis in patients with stable cirrhosis were found. However, a study that included 64 patients with stable cirrhosis found higher levels in those with Child-Pugh C [6]. This same study demonstrated a decrease in HLA-DR expression in patients with advanced cirrhosis, possibly mediated by high IL-10 levels in response to endotoxemia [6]. These findings, characteristic of "immune paralysis" in patients with cirrhosis, may explain the relationship between higher IL-10 levels and the poor prognosis observed in this study.

As expected, patients hospitalized for acute decompensation of cirrhosis showed significantly higher IL-6 and IL-10 levels than those with stable cirrhosis and healthy controls. Although the presence of infections partially explains these findings, even in the absence of clinically apparent bacterial infections, cirrhosis complications are related to an increase in bacterial translocation, also contributing to the increase of circulating cytokines in hospitalized patients [16]. In contrast, in this study IL-17 levels were higher in stable subjects than in hospitalized patients. Th17 cells have been recently discovered, and the significance of circulating IL-17 has not been completely elucidated, especially among patients with hepatic diseases [17]. One conceivable explanation for the relatively low levels of IL-17 among hospitalized patients would be a possible inhibitory effect of high IL-10 concentrations on Th-17 cells [18]. However, further studies are needed to investigate the factors related to stimulation/inhibition of IL-17 and its clinical significance in patients with advanced liver disease.

In patients hospitalized for acute decompensation of cirrhosis, cytokine concentrations were also associated with variables related to the intensity of hepatic dysfunction and mortality within 90 days in the bivariate analysis. However, in the logistic regression analysis, only IL-6 together with ascites and MELD were independently associated with death. A study including 58 patients with cirrhosis hospitalized with sepsis found higher IL-6 levels among those who died within 28 days [19]. Similar results were

reported in a more recent study that included 233 patients with cirrhosis who developed fever during hospitalization [20]. These two studies included selected groups of subjects with cirrhosis (septic or feverish) and did not control for other variables related to mortality in those patients. In the current study, individuals admitted in the emergency department were included consecutively, probably reflecting the usual clinical features of these patients and allowing a better extrapolation of the results.

When serial measurements of the cytokines were performed, IL-10 concentrations decreased in the first three days of hospitalization, regardless of the outcome. However, IL-17 levels decreased significantly only in patients who died. Data on serial measurements of interleukins in patients with cirrhosis, especially in relation to IL-17, are scarce. It is possible that the initially high concentrations of IL-17 reflect disease severity; however, maintaining circulating levels of IL-17 may be important for the preservation of the immunological balance during acute events.

In the present study, the serum levels of IL-6 and IL-10 were associated with the presence of bacterial infection in the bivariate analysis. However, only IL-6 remained associated with it in the logistic regression analysis. The overall performance of IL-6 was superior to CRP for bacterial infection diagnosis. The relationship between circulating IL-6 and infection in cirrhosis has been known for several years. A Belgian study from the 1990s investigated IL-6 in 32 patients with cirrhosis and demonstrated that values above 200 pg/mL had a sensitivity of 100% and specificity of 89% for sepsis [21]. These results were subsequently confirmed by Le Moine et al. in a prospective study with 57 patients with decompensation of cirrhosis [22], in which IL-6 was superior to CRP for infection diagnosis, using the same cutoff point as the Belgian study. Taken together, these findings indicate that IL-6 is a potential prognostic marker and has good performance in early diagnosis of bacterial infection in cirrhosis.

ACLF was associated with higher IL-6 levels, even in the absence of bacterial infection, when compared to patients without ACLF or infection. However, higher IL-10 and IL-17 levels were observed only in patients with ACLF and infection. A German study that investigated 27 patients with ACLF found higher circulating IL-6 and IL-10 in these subjects when compared to individuals with stable cirrhosis [23]. Bacterial components can cause an excessive inflammatory response resulting in tissue damage and organ failure [24]. However, a significant proportion of patients with ACLF do not have clinically apparent bacterial infections. Also in these cases, an excessive immune response is the most likely cause of tissue damage and organ failure. This response can be triggered by pathogen-associated molecular patterns (PAMPs) released by bacteria, which were eliminated after translocation from the intestinal lumen [25]. Furthermore, the excessive inflammatory response may also result from the activation of Pattern Recognition Receptors (PRRs) by endogenous molecules (non-bacterial) during the process of cell death [26]. These theories are supported by the findings of this study that indicate an *in vivo* relationship between a more intense inflammatory response and progression to organ dysfunction in cirrhosis, even in the absence of bacterial infection.

In patients with ACLF, higher IL-10 and IL-17 levels were associated with 90-day mortality. In a study that included 51 patients with acute liver failure and 39 patients with cirrhosis hospitalized in an intensive care unit, IL-10 has been shown to be associated with mortality in both groups [27]. These results can be explained by the central role of IL-10 in the compensatory anti-inflammatory response syndrome (CARS), a complex condition related to functional deactivation of monocytes, immunoparesis, and predisposition to infection [28]. There are very few data regarding IL-17 in ACLF. A study in patients with ACLF secondary to hepatitis B virus reactivation found increased circulating Th17 cells and IL-17 mRNA expression in individuals who died [29]. Although our results are limited by the relatively small number of patients, these findings suggest a role of both pro-inflammatory and anti-inflammatory responses in the outcome of ACLF. It is important to note that macrophage activation seems to play an important role in pathogenesis of cirrhosis [30]. Therefore, in addition to cytokines reported here, it would be of interest to address the significance of other cytokines mainly produced by macrophages, such as TNF, IL-1 and IL-18, not only in patients with ACLF, but also in other stages of cirrhosis.

In summary, IL-6, IL-10, and IL-17 levels are increased in liver cirrhosis, especially in its more severe forms. In stable patients, a decreased event-free survival was related to higher levels of the anti-inflammatory cytokine IL-10. In contrast, a robust pro-inflammatory response, represented by higher levels of IL-6, was independently related to the presence of bacterial infection and progression to death in patients hospitalized for acute decompensation. Although ACLF was characterized by a marked systemic inflammation even in the absence of infection, in patients with ACLF, a stronger anti-inflammatory response seems to be associated with a worse prognosis.

Conflict of interest

Nothing to report.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2016.12.017.

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