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Immune dysfunction in cirrhosis: Distinct cytokines phenotypes according to cirrhosis severity

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ABSTRACT

Background/objectives: Cirrhosis associated immune dysfunction has been proposed to switch from a pro-inflammatory phenotype in stable cirrhosis to an immunodeficient one in patients with decompensated cirrhosis and acute-on-chronic liver failure. The aim of the present study was to compare serum cytokine levels between healthy patients, stable cirrhosis, and decompensated cirrhotic patients with and without development of acute-on-chronic liver failure (ACLF); and to explore whether any of the measured cytokines is associated with cirrhosis severity and prognosis in ACLF patients.
Methods: Patients were enrolled from October 2013 to May 2014 in two hospitals located in Buenos Aires. Cirrhotic patients with an acute decompensating event were enrolled accordingly to the development of ACLF defined by the CANONIC study group. There were two control groups: healthy subjects ($n = 14$) and stable cirrhotic patients ($n = 14$). Demographic, clinical and biochemical data were obtained. Seventeen cytokines were measured using Bio-Plex Pro Human Cytokine 17-plex Assay.
Results: Of the 49 decompensated cirrhotic patients enrolled, 18 (36.7%) developed ACLF. Leukocyte count, MELD score at admission, Clif-SOFA at admission and day 7 were significantly higher in the ACLF group ($p = 0.046$, $p < 0.001$, $p < 0.001$, $p < 0.001$ respectively) as well as short-term mortality ($p < 0.001$) compared to stable and decompensated cirrhotic patients. In comparison with healthy controls, stable cirrhotic and decompensated cirrhotic patients showed increased levels of pro-inflammatory and anti-inflammatory cytokines: IL-6, IL-7, IL-8, IL-10, IL 12, and TNF-α. Decompensated cirrhotic patients with the development of ACLF showed a significant decrease of IL-7, IL-10, IL-12, TNF-α, MCP-1 and IFN-γ, but a sustained response of IL-6 and IL-8. When evaluating cirrhosis severity, IL-6 and IL-8 correlated positively with MELD score, whereas only IL-6 correlated positively with Clif-SOFA score at day 7; IL-2 correlated negatively with Clif-SOFA at admission. In comparison with all scores, leukocyte count showed positive correlation and IFN-γ negative correlation with disease severity. When evaluating survival, only MELD and Clif-SOFA scores had a significant association with mortality.

Abbreviations: ACLF, acute-on-chronic liver failure; AD, acute decompensating event; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HIV, human immunodeficiency virus; HLA-DR, human leukocyte antigen; IL-1β, interleukin 1 beta; IL-2, interleukin 2; IL-4, interleukin 4; IL-5, interleukin 5; IL-6, interleukin 6; IL-7, interleukin 7; IL-8, interleukin 8; IL-10, interleukin 10; IL-12, interleukin 12; IL-13, interleukin 13; IL-17, interleukin 17; IFN-γ, interferon gamma; MCP-1, monocyte chemo-attractant protein-1; MFI, mean fluorescence intensity; MIP-1β, macrophage inflammatory protein 1 beta; Gro, growth-regulated oncogene alpha; RANTES, regulated on activation, normal T cell expressed and secreted; SC, stable cirrhosis control group; TIPS, transjugular intrahepatic portosystemic shunt; TNF-α, tumoral necrosis factor alpha.

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Conclusions: Pro-inflammatory cytokines and chemo-attractant elements are increased in cirrhosis in comparison with healthy subjects, and display higher values concomitantly with cirrhosis progression. However, in acute-on-chronic liver failure an opposite cytokine pattern that can be resumed as a combination of immune paresis and excessive inflammatory response was observed. Several pro-inflammatory cytokines (IL-2, IL-6, IL-8 and IFN-γ) showed correlation with disease severity; their utility as prognostic biomarkers needs to be further studied.

1. Main content/introduction

Liver cirrhosis is the final stage of all progressive and chronic liver diseases. The natural history of cirrhosis was traditionally divided in two phases: a long-lasting initial phase denominated compensated cirrhosis, characterized by the absence of symptoms and excellent survival, followed by an advanced stage (i.e. decompensated cirrhosis), marked by the appearance of complications related to the presence of portal hypertension or liver dysfunction and associated with an elevated mortality rate [1]. Recently, a new clinical entity denominated acute-on-chronic liver failure (ACLF) has been identified as an alternative path in cirrhosis progression, characterized by its acute onset and poor prognosis [2]. Although several definitions were proposed, most of them had theoretical basis; currently, the only description based on observational data derives from a European prospective multicenter study named CANONIC (Clif Acute-On-Chronic Liver Failure in Cirrhosis). This consortium prospectively enrolled and studied 1343 hospitalized cirrhotic patients, defining ACLF as an acute deterioration of liver function in patients with cirrhosis, either secondary to superimposed liver injury or due to extra hepatic precipitating factors, which leads to end-organ dysfunction. The severity of this syndrome was objectively quantified by an *ad hoc* score and divided in three stages, according to the number or organ failures and increasing mortality observed [3].

Unraveling the pathophysiological pathway in advanced cirrhosis and ACLF is a major priority, since it would allow developing specific treatment strategies against factors related to poor prognosis and improve detection of sicker patients in order to modify liver allocation algorithms. In the CANONIC study, patients with ACLF had higher white cell count and plasma C-reactive protein levels than decompensated cirrhotic patients who did not developed ACLF; furthermore, both of these markers increased concomitantly with the number of organ failures, and white cell count was found to be an independent predictor of post-enrollment development of ACLF and ACLF-related mortality [3]. Based on these findings, an excessive systemic inflammatory response was proposed to be the causal factor for the morbidity and mortality observed in ACLF. Interestingly, systemic inflammation in response to bacterial infections was observed in only 30% of patients with ACLF, suggesting other "sterile" triggers may be involved [4]. An excessive inflammatory response has also been linked to the induction of ACLF by other authors. In a study by Mehta et al. that analyzed systemic hemodynamics and inflammation parameters in stable, decompensated and ACLF alcohol-related cirrhotic patients, the latter cohort portrayed significantly higher intrahepatic resistance and pro-inflammatory cytokines levels (TNF-α, IL-8 and IL-6) compared to the other groups [5]. In an ex-vivo study that evaluated cytokine production under baseline conditions and after stimulation by lipopolysaccharide in peripheral blood monocytes of advanced alcoholic cirrhotic patients and normal subjects, monocytes from cirrhotic patients were found to spontaneously produce six cytokines (TNF-alpha, IL-6, IL-8, MCP-1, RANTES and Gro) whereas normal monocytes only secreted small amounts of IL-8 and RANTES. These findings suggested an underlying pro-inflammatory phenotype in cirrhosis; importantly, none of the cirrhotic patients included had overt signs of infection [6].

However, systemic inflammation is not the only proposed pathophysiological pathway. An acquired alteration of the innate immune system in advanced cirrhosis and ACLF has been suggested to account for an inadequate response to pathogens, which ultimately derives into multiple organ failure and elevated mortality. This theory is based in alterations observed at different levels of the immune system; in a study that compared functional immune parameters between ACLF, compensated cirrhotic and septic patients, TNF-alpha production and monocyte HLA-DR levels were found to be severely decreased in sepsis and ACLF compared to stable cirrhosis, whereas IL-6 were highest in septic patients followed by ACLF subjects. Due to the similarities in the degree of cellular immune depression between ACLF and sepsis, the authors defined ACLF innate immune system alterations as a "sepsis like" immune paralysis [7]. In a cohort of alcoholic cirrhotic patients, a reversible neutrophil activation indicated by an increased resting burst and reduced phagocytic function was found to be associated with higher morbidity and mortality [8]. The migration capacity of this cellular subset was also proven to be altered in advanced cirrhosis when compared to stable cirrhosis [9]; and this was similarly portrayed by the detection of a functional exhaustion of monocytes leading to impaired pathogen clearance [10].

Systemic inflammation and an impaired immune response may not be mutually exclusive but operate simultaneously in decompensated cirrhosis and ACLF. Recently, a theory encompassing both features denominated cirrhosis-associated immune dysfunction (CAID) has been proposed. This syndrome describes excessive systemic inflammation as an initial phenotype in compensated cirrhosis, caused by persistent immune cell stimulation by bacterial and bacterial products translocation; under this constant stimulus, the immune response system eventually becomes exhausted and switches to a "immunodeficient" phenotype in late stages of decompensated cirrhosis, such as ACLF [11].

Despite the fact that there is plentiful evidence of the role of immune dysfunction in the pathogenesis in cirrhosis, the identification of objective, reproducible and readily-available surrogate biomarkers of this syndrome is still a work in progress. Several features have been analyzed, such as leukocyte count, procalcitonine and C-reactive protein; however, no clear cut-off points or extensive validation has been achieved so far [12]. Cytokines have also been proposed as prognostic tools in different stages of cirrhosis. In stable cirrhotic patients, they have been evaluated as tools in the detection of clinically-significant portal hypertension [13] and even as independent factors related to mortality in decompensated cirrhosis [14].

However, the recognition of prognostic biomarkers would probably be of utmost utility in ACLF, the most severe stage of decompensated cirrhosis, to improve current treatment strategies. Although several cytokines have been studied as part of the pathophysiology of ACLF and even suggested as prognostic factors, only a few were randomly selected, not allowing analyzing thoroughly its

involvement. In addition, heterogeneous definitions of this syndrome have been used; some authors considered ACLF only when patients had an acute deterioration in liver function, encephalopathy or hemodynamic instability and required ICU disregarding the etiology of the acute decompensating event [7]; others solely included patients with ACLF after hepatitis B flares or acute hepatitis B infection [15]. Only recently an objective and simple score system has been developed to define this syndrome and thus allow proper external validation; but even in a study where the validated definition of ACLF was used, only alcohol-related cirrhotic patients were included [5]. Whether selecting exclusively severely ill patients, patients with acute viral infections or alcoholic disease may have an impact in inflammatory markers is unknown, hence these results are difficult to generalize. Furthermore, most previous studies considered stable cirrhosis or chronic viral hepatitis as control groups; since immune system alterations are present even in early stages of cirrhosis, a healthy control group becomes necessary to accurately assess the magnitude of cytokine involvement.

In the present study we aim to describe a broad spectrum of serum cytokine levels between healthy controls, stable cirrhosis, and cirrhotic patients with an acute decompensating event with or without the development of ACLF (according to the CANONIC definitions [3]). In addition, we will explore whether any of the measured cytokines is associated with cirrhosis severity and prognosis in ACLF patients.

2. Patients and methods

2.1. Study groups

Patients were enrolled from October 2013 to May 2014 in peripheral wards or the Intensive Care Unit of the Dr. Francisco J. Muñiz and Dr. Carlos B. Udaondo Hospitals, located in the city of Buenos Aires, Argentina.

Forty-nine decompensated cirrhotic patients were included. The diagnosis of cirrhosis was based on previous liver biopsy, transient elastography (Fibroscan®) results, or a composite of clinical signs and findings provided by laboratory test, endoscopy, and radiologic imaging. Informed written consent was obtained from patients or their legal surrogates before enrollment. The Clinical Research Committee of the Hospitals approved the study, in accordance with the Declaration of Helsinki.

2.2. Inclusion criteria

Patients hospitalized for at least 1 day who had an acute decompensating event (AD) of cirrhosis were screened; defined by the acute development (within the last two weeks) of a first episode or new episode of ascites grade II or III according to the International Ascites Club; hepatic encephalopathy (acute change in mental status in a patient with previous normal consciousness and no evidence of neurological disease); gastrointestinal hemorrhage (upper and/or lower gastrointestinal bleeding of any etiology); any type of acute bacterial infection or any combination of these. Patients who developed AD for the first time as well as those with a prior history of AD (one or more episodes) who had recovered after specific treatment were enrolled.

Patient's data regarding acute decompensating events, active alcohol consumption (more than 140 g/week in women and more than 210 g/week in men), physical examination and laboratory measurements were collected.

Enrolled decompensated cirrhotic patients were divided into two groups. The first group included patients who fulfilled the definition of acute on chronic liver failure (ACLF) at enrollment or during the following seven days. The second group included patients that did not develop organ failure during the same period of time.

The definition of ACLF was extracted from the CANONIC study [3] (detailed in the definitions section). The seven day cut off point to define ACLF was considerably shorter than the adopted in the CANONIC study; this time frame was arbitrarily selected to try to avoid alterations in cytokine values due to medical treatment/interventions.

2.3. Control groups

Two control groups were included. The fist was a healthy control group (HC) conformed by adult volunteers (ages between 18 and 65 years old) without known acute or chronic illnesses. The second was a stable cirrhosis control group (SC), conformed by Child – Pugh A cirrhotic patients that attended for a routine check-up (without any decompensating event reported in the last 6 months such as ascites, hepatic encephalopathy, bacterial infection, variceal bleeding, neoplastic or autoimmune comorbidities declared) and were asked to participate.

2.4. Exclusion criteria

Patients were excluded if they were pregnant, were younger than 18 years old, had acute liver failure without liver cirrhosis or were cirrhotic patients admitted in the hospital for a programmed procedure such as endoscopic ligation, a surgical procedure (TIPS) or therapeutic paracentesis for refractory ascites, cirrhotic patients that develop acute decompensation in the postoperative period after a partial hepatectomy, requiring hemodialysis, had hepatocarcinoma, severe extra-hepatic diseases (chronic renal insufficiency, decompensated cardiac insufficiency, severe obstructive pulmonary disease, psychiatric disorders); hepatic autoimmune conditions (primary biliary cirrhosis, autoimmune hepatitis, primary sclerosing cholangitis) immunosuppressive treatment with the exception of steroid therapy in alcoholic hepatitis, HIV infection, patients unable to give consent, medical team in disagreement to offer intensive care treatment if necessary.

The cut-off points for organ failure and the Clif-SOFA score used to define acute on chronic liver failure (ACLF) were extracted from the prospective multicenter study performed by the CANONIC study [3]; the following definitions were considered:

- Decompensated cirrhotic patients without the development of acute-on chronic liver failure (non-ACLF): patients that suffered an AD without organ failure, with a single organ failure (excluding kidney failure) with serum creatinine levels below 1.5 mg/dL and no hepatic encephalopathy; and patients with single cerebral failure with serum creatinine level below 1.5 mg/dL.
- Decompensated cirrhotic patients with acute on chronic liver failure (ACLF): patients that suffered an AD with latter development of single kidney failure; with a single organ failure other than kidney and serum creatinine levels higher than 1.5 mg/dL and/or mild to moderate hepatic encephalopathy; patients with single cerebral failure and serum creatinine levels higher than 1.5 mg/dL. Patients with two or more organ failures were also included in this group.

2.5. Blood extraction and sample management

A venous blood extraction was performed at admission for leukocyte count and cytokine measurement in cirrhotic patients with an AD. Extraction was performed in the cirrhotic control group patients when attending routine clinical checkup, and healthy control samples were selected from healthy volunteers. All decompensated cirrhotic patients were included during the first

72 h of hospital admission, and blood samples for cytokine analysis were collected at admission. The rest of laboratory parameters were collected at admission and at day 7. The Clif-SOFA score was calculated at admission and at day 7, and patients were divided according to their score results in the ACLF and non-ACLF group.

2.6. Cytokines analysis

The Bio-Plex 200 system with HTF (Bio-Rad, Hercules, CA) was used to evaluate the sera from a total of 77 individuals ($n = 18$ ACLF, $n = 31$ non-ACLF, $n = 14$ HC, $n = 14$ SC). The levels of the following cytokines were assessed: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, IFN-γ, G-CSF, GM-CSF, TNF-α, MIP-1β and MCP-1. Their main function and characteristics of each cytokine included are detailed in Table 1. The assays were repeated and our data was replicated. The cytokine analysis was performed using the Bio-Plex Pro Human Cytokine 17-plex Assay (Bio-Rad, Hercules, CA). Briefly, the assay was performed at a serum dilution of 1:4 and 50 μl were added to each well. Mixed micro-beads (50 μl) were added, and the plate was incubated and agitated for 30 min, washed and re-incubated with 25 μl of detection antibody for 30 min. The plate was washed again and incubated with 50 μl of Streptavidin–Phycoerythrin for 10 min. The plate was then washed twice and the beads were re-suspended in the plate with 125 μl assay buffer and analyzed using the Bio-Plex 200 system. The readout for the concentration of each cytokine was detected as mean fluorescence intensity (MFI) by the instrument. These values were subsequently converted to pg/ml of cytokine based upon the MFI values from a set of standards that were run simultaneously in the assay.

2.7. Statistical analysis

Data was collected using a standardized case report form. Results are showed in percentages, mean and standard deviation for normally distributed data and in median and Q1–Q3 intervals for non-normal distributed data. Univariate analysis was performed with: chi-square, *t* student test for paired and unpaired data and for non-parametric test Mann–Whitney or Kruskal Wallis was used depending on the number of groups evaluated. To analyze the correlation between quantitative variables with non-normal distribution, Spearman rank correlation was used. Statistical analysis was performed with Infostat, Universidad Nacional de Córdoba. In all analysis the significance levels were set at $p < 0.05$.

3. Results

During the study screening period, a total of 77 subjects were enrolled; 49 were cirrhotic patient with an AD, 18 (36.7%) developed ACLF (15 patients at admission and 3 patients during the following week). Of the remaining 28 included subjects, 14 were enrolled in the SC group and 14 in the HC group.

3.1. Demographic and clinical characteristics

Demographic characteristics of all studied groups (HC, SC, ACLF and non-ACLF) are detailed in Table 2; as well as clinical and biochemical characteristics of patients included in the SC, ACLF and non-ACLF groups. Patients in all groups were comparable regarding sex and age; they were predominantly male and middle-aged (despite the fact healthy control patients were younger; the difference did not reach statistical significance). Regarding cirrhosis etiology, the SC group was mainly composed by patients with chronic HCV infection (92.8%) whereas the majority of patients in the both the ACLF and non-ACLF group had alcohol-related cirrhosis (55.5% and 38.8% respectively). Although there was a significant difference in cirrhosis etiology between all three groups ($p < 0.001$), when only ACLF and non-ACLF patients were compared, the difference did not reach statistical significance ($p = 0.34$). The occurrence of previous decompensating events was similar in all three groups of cirrhotic patients included ($p = 0.31$).

Table 1
Main function and characteristics of evaluated cytokines.

Cytokines	Predominant function	Main characteristics
IL-1β	Pro-inflammatory	It is considered an acute phase reactant; as an endogenous pyrogen it is associated with fever, inflammation, tissue destruction and occasionally even shock and death [31]
IL-2	Pro-inflammatory	Involved in proliferation, survival and differentiation of CD4 and CD8 T cells; also related to proliferation and enhanced cytokine production of B cells, natural killers and neutrophils [32]
IL-4	Anti-inflammatory	Promotes Th2 lymphocyte development and inhibits LPS-induced pro-inflammatory cytokine synthesis [33]
IL-5	Pro-inflammatory	Involved in the regulation of eosinophil maturation, recruitment and survival [34]
IL-6	Pro-inflammatory	Involved in the regulation of the acute-phase response to injury and infection. In the initial phase of innate immune response it mediates chemo-attraction of neutrophils, in latter phases stimulates monocyte recruitment [35]
IL-7	Pro-inflammatory	Promotes T cell differentiation, survival, and homeostasis. In humans, IL-7 and its receptor (IL-7R) are increased in diseases characterized by inflammation such as psoriasis and multiple sclerosis [36]
IL-8	Pro-inflammatory	Produced under conditions of inflammatory stimulation; it induces migratory and phagocytic activity in neutrophils and promotes angiogenesis [30,37]
IL-10	Anti-inflammatory	Inhibits monocyte/macrophage and neutrophil cytokine production and also promotes a shift of Th1-phenotype to T2 in T lymphocytes [33]
IL-12	Pro-inflammatory	Induces the production of IFN-γ, favors the differentiation of T helper 1 cells and constitutes a link between innate and adaptive immunity [38]
IL-13	Anti-inflammatory	Potent suppressor of expression of cytokines in monocyte/macrophages [33]
IL-17	Pro-inflammatory	Promotes expansion and recruitment of innate immune cells such as neutrophils, and also cooperates with TLR ligands, IL-1 beta, and TNF alpha to enhance inflammatory reactions [39]
IFN-γ	Pro-inflammatory	Promotes NK cell activity and Th1 differentiation by upregulating the transcription factor T-bet [40]
G-CSF	Pro-inflammatory	Influences the survival, proliferation and differentiation of all cells in the neutrophil lineage and impacts the function of mature neutrophils [41]
GM-CSF	Pro-inflammatory	Induces granulocyte and macrophage populations from precursor cells. It is also capable of T-cell activation through various myeloid intermediaries [42]
TNF-α	Pro-inflammatory	It is considered an acute phase reactant; as an endogenous pyrogen it is able to induce fever, apoptotic cell death, cachexia, inflammation and to inhibit tumorigenesis and viral replication [43]
MIP-1β	Pro-inflammatory	Chemotactic for monocytes and lymphocytes; may have a role in regulating hematopoiesis and stimulating production of L-1, TNF alpha, and histamine [44]
MCP-1	Pro-inflammatory	Key chemotactic factor for monocytes, also involved in monocytes/macrophages infiltration [45]

Table 2
Clinical and biochemical characteristics of stable cirrhotic patients and decompensated cirrhotic patients with and without ACLF.

Characteristics	Healthy controls (n = 14)	Stable cirrhosis (n = 14)	With ACLF (n = 18)	Without ACLF (n = 31)	P value
Age (years)	38 (34–60)	54 (44–59)	54 (47–59)	50 (45–58)	*0.41*
Sex (male)	64.2%	78.6%	94.4%	90.3%	*0.08*
Ascites (present)	0%	0%	66.6%	77.4%	*<0.001*
Cause of cirrhosis					
Alcohol	–	7.2% (n = 1)	55.5% (n = 10)	38.8% (n = 12)	*<0.001*
HCV	–	92.8% (n = 13)	27.8% (n = 5)	35.5% (n = 11)	
Alcohol + HCV	–	0%	0	9.6% (n = 3)	
Other causes	–	0%	16.7% (n = 3)	16.1% (n = 5)	
Potential precipitating events					
Bacterial infection	–	–	41.1% (n = 7)	45.1% (n = 14)	*0.79*
Alcohol consumption	–	–	23.5% (n = 4)	12.9% (n = 4)	*0.34*
Gastrointestinal bleeding	–	–	5.88% (n = 1)	12.9% (n = 4)	*0.44*
Other precipitating event	–	–	11.7% (n = 2)	6.4% (n = 2)	*0.52*
Unknown precipitating event	–	–	17.6% (n = 3)	22.5% (n = 7)	*0.68*
Biochemical features					
Leukocyte count ($\times 10^9$/L)	–	–	9.8 (±4)	7.7 (±3.7)	*0.046*
Platelet count ($\times 10^9$/L)	–	–	160 (100–198)	138 (80–163)	*0.25*
Serum sodium (mg/dL)	–	–	128 ± 8.5	127 ± 24	*0.26*
C-reactive protein (ULN)	–	–	3.88 (1.2–9)[a]	1.37 (1–2)[b]	*0.23*
Other features					
Child Pugh A stage	–	100% (n = 14)	0%	0%	*<0.001*
Child Pugh B stage	–	0%	38.9% (n = 7)	54.8% (n = 17)	
Child–Pugh C stage	–	0%	61.1% (n = 11)	45.2% (n = 14)	
MELD score at admission (mean and SD)	–	–	24 ± 5	15 ± 5	*<0.001*
Clif-SOFA score at admission (mean and SD)	–	–	8 ± 3	4.5 ± 2	*<0.001*
Clif-SOFA score at day 7 (mean and SD)	–	–	8 ± 4.5	3 ± 2	*<0.001*
With previous decompensating event	–	42.8% (n = 6)	66.6% (n = 12)	64.5% (n = 20)	*0.31*
Short term mortality (three-months)	0% (n = 14)	0% (n = 14)	57,1% (n = 14)	0% (n = 19)	*<0.001*

Note: data is expressed in percentage, median and interval Q1–Q3, mean and standard deviation. C-reactive protein is expressed in times above the upper limit of normal (ULN).
[a] Data available in 9 patients.
[b] Data available in 18 patients.

Table 3
Cytokine distribution in healthy patients, stable cirrhotic and decompensated cirrhotic patients with and without development of acute-on-chronic liver failure.

Cytokine distribution		Healthy control group (n = 14)	Stable control group (n = 14)	Non-ACLF group (n = 31)	ACLF group (n = 18)
IL-1 (pg/ml)	Median (range)	0 (0–0.05)	0 (0–7.32)	0 (0–5.58)	0 (0–0.42)
IL-2 (pg/ml)	Median (range)	0 (0–0)	0 (0–101.61)	0 (0–352.8)	0 (0–4.51)
IL-4 (pg/ml)	Median (range)	0 (0–0)	0 (0–1.75)	0 (0–6.57)	0 (0–0.28)
IL-5 (pg/ml)	Median (range)	0 (0–0.27)	0.565 (0.36–4.61)	0.225 (0–17.03)	0 (0–2.76)
IL-6 (pg/ml)	Median (range)	0 (0–1.54)	1.3 (0–156.61)	14.2 (0–435.77)	18.51 (2.37–988.85)
IL-7 (pg/ml)	Median (range)	0 (0–2.13)	4.035 (0.32–58.47)	2.67 (0.57–30.75)	1.01 (0–10.13)
IL-8 (pg/ml)	Median (range)	2.92 (0–114.35)	8.48 (4.44–14.95)	23.24 (1.42–246.63)	54.95 (0–695.69)
IL-10 (pg/ml)	Median (range)	0 (0–0.19)	3.635 (0–329.82)	3.05 (0–87.17)	0 (0–13.58)
IL-12 (pg/ml)	Median (range)	0.12 (0–1.22)	6.12 (0–193.56)	6.4 (0–217.24)	0.31 (0–39.85)
IL-13 (pg/ml)	Median (range)	0 (0–0)	0 (0–19.22)	0.96 (0–37.05)	0 (0–2.06)
IL-17 (pg/ml)	Median (range)	0 (0–0)	0 (0–34.39)	0 (0–368.55)	0 (0–9.61)
MCP-1 (pg/ml)	Median (range)	9.605 (0–38.91)	19.61 (0–60.96)	19.35 (0–753.7)	2.195 (0–89.97)
IFN-γ (pg/ml)	Median (range)	0 (0–12.04)	0 (0–481.35)	2 (0–7741.1)	0 (0–101.8)
TNF-α (pg/ml)	Median (range)	0 (0–0.45)	2.18 (0–112.48)	1.36 (0–127.87)	0 (0–8.09)
G-CSF (pg/ml)	Median (range)	0 (0–4.44)	2.24 (0–21.67)	4.36 (0–580.38)	1.08 (0–18.14)
GM-CSF (pg/ml)	Median (range)	0 (0–0)	0 (0–41.34)	0 (0–292.19)	0 (0–0)
MIP-1β (pg/ml)	Median (range)	52.395 (21.75–135.51)	135.71 (98.17–285.42)	70.47 (0–604.88)	71.42 (0–291.47)

Note: Non-ACLF: decompensated cirrhotic patients without the development of acute-on-chronic liver failure. ACLF: acute-on-chronic liver failure.

When evaluating AD, overt bacterial infections were more frequent in both ACLF and non-ACLF groups (41.1% and 45.1% respectively, p = 0.79). Biochemical variables were evaluated in ACLF and non-ACLF patients; only leukocyte count was significantly higher in the ACLF group when compared to the non-ACLF group (p = 0.046).

3.2. Prognostic scores and survival

Patients in the SC group were all included in stage A of the Child–Pugh score; decompensated cirrhotic patients were similarly distributed between stage B (ACLF group 38.9% and non-ACLF group 54.8%) and stage C of the Child Pugh score (ACLF group 61.1% and non-ACLF group 45.2%, p = 0.28). In the univariate analysis, mean values of prognostic scores (MELD at admission and Clif-SOFA at admission and at day 7) were significantly higher in the ACLF group (p < 0.001). Survival information was available in all the HC and SC patients, with no deaths registered in a three month period since enrollment. In the ACLF group, short term survival information was available in 78% of patients (n = 14) reaching 57% three-month mortality rate; whereas in the non-ACLF group (follow up information available in 61% of patients; n = 19), no deaths were registered in this follow-up period.

3.3. Comparison of cytokine values

The study compared the serum levels of 17 cytokines in the four groups of subjects included: decompensated cirrhotic patients with ACLF ($n = 18$), non-ACLF ($n = 31$), SC ($n = 14$) and HC ($n = 14$). Median and total range values of all studied cytokines in each study group are shown in Table 3, whereas its univariate analysis is presented in Fig. 1.

3.4. Stable cirrhotic control group

When compared to HC, patients in the SC group displayed significantly higher values of an anti-inflammatory cytokine: IL-10, ($p < 0.001$); and several pro-inflammatory cytokines: IL-6, ($p < 0.05$); IL-7, ($p < 0.001$); IL-12, ($p < 0.05$); TNF-α, ($p < 0.001$); G-CSF, ($p < 0.05$) and MIP-1β, ($p = 0.005$). In the remaining ten cytokines analyzed, no significant differences with the HC group were observed.

3.5. Decompensated cirrhotic patients without the development of ACLF

When compared to HC, patients in the non-ACLF group displayed significantly higher values of the following cytokines: IL-7, ($p < 0.001$); IL-10, ($p < 0.001$); IL-12, ($p = 0.001$); TNF-α, ($p < 0.001$) and G-CSF, ($p = 0.001$). Cytokines with significantly higher values when compared with SC were IL-6, ($p < 0.05$); IL-8, ($p < 0.01$) and MIP-1β ($p < 0.05$). Cytokines with significantly higher values only in comparison with the ACLF group were MCP-1 ($p = 0.04$) and IFN-γ ($p = 0.04$). Finally, in the remaining seven cytokines, no significant differences with other groups were observed.

3.6. Decompensated cirrhotic patients with the development of ACLF

When compared to the non-ACLF group, ACLF patients displayed significantly lower values of several cytokines: IL-7, ($p < 0.01$); IL-10, ($p < 0.01$); IL-12, ($p = 0.01$); TNF-α, ($p < 0.05$); MCP-1 ($p < 0.05$) and IFN-γ ($p = 0.04$). G-CSF is displayed in the same pattern with a trend towards significance ($p = 0.06$). All the aforementioned cytokines did not differ significantly with the values expressed by the HC group. Two pro-inflammatory cytokines, IL-6 and IL-8, showed a sustained response with significantly higher values in the ACLF group when compared to the SC group ($p = 0.01$ and $p = 0.001$ respectively). Finally, in the remaining seven cytokines, no significant differences with other groups were observed.

3.7. Cytokine distribution and cirrhosis etiology

In decompensated cirrhotic patients, cirrhosis etiology was mainly represented by hepatitis C and alcohol-related cirrhosis. In order to evaluate its influence in cytokine expression, cytokines with significant differences between at least two groups in the univariate analysis performed in the whole population studied (IL-6, IL-7, IL-8, IL-10, IL-12, G-CSF, MCP-1, IFN-γ and TNF-α) were divided according to the underlying etiology of cirrhosis, hepatitis C or alcohol-related, and analyzed in the non-ACLF and ACLF groups separately in Table 4. As shown, there were no significant differences in cytokine distribution when stratified by cirrhosis cause in these two groups.

3.8. Cytokine distribution and bacterial infections

In both groups of decompensated cirrhotic patients, bacterial infections were the most frequently reported acute decompensating events. When we compared cytokines with significant differences between at least two groups in the univariate analysis performed in the whole population studied (IL-6, IL-7, IL-8, IL-10, IL-12, G-CSF, MCP-1, IFN-γ and TNF-α) in non-ACLF and ACLF patients with and without bacterial infections, only IL-6 in the ACLF group was significantly higher in infected patients as opposed to other decompensating events (Table 5); in the remaining evaluated cytokines no statistical differences were observed.

3.9. Non-secretor phenotype

When evaluating each cytokine pattern individually, patients with similar values to healthy controls were detected distributed among all study groups (SC, non-ACLF and ACLF). In order to evaluate whether these patients shared similar features, we defined a subgroup denominated "non-secretors" for further analysis. The number of cytokines with similar values to healthy controls was quantified in every patient included in the SC, non-ACLF and ACLF groups. The median number of cytokines with concordance with healthy controls in the whole population of cirrhotic patients was 8; therefore we considered patients to be "non-secretors" if they had $\geqslant 8$ cytokines with similar values to HC. The analysis of patients according to their cytokine secretion phenotype and clinical characteristics is shown in Table 6. None of the demographic, clinical features or outcome were related to this "non-secretor" phenotype.

3.10. Cirrhosis severity and short-term survival

Cirrhosis severity was measured quantitatively by the MELD score at admission and the Clif-SOFA score at admission and at day 7 of hospitalization. When evaluating the correlation of cytokine values with MELD score, IL-6 and IL-8 showed a positive correlation (rho = 0.42; $p < 0.01$ and rho = 0.31; $p < 0.05$ respectively). Regarding Clif-SOFA at admission, IL-2 showed a negative correlation with this prognostic score (rho = −0.37). Finally, when considering Clif-SOFA score at day 7, IL-6 had a positive correlation (rho = 0.33; $p < 0.05$). When compared to all three prognostic scores, leukocyte count had a positive correlation (MELD score, rho = 0.46; $p < 0.01$, Clif-SOFA at admission, rho = 0.35; $p < 0.05$ and Clif-SOFA at day 7, rho = 0.36; $p < 0.05$) whereas IFN-γ depicted a negative correlation with severity (MELD score, rho = −0.33; $p < 0.05$; Clif-SOFA at admission, rho = −0.34; $p < 0.05$ and Clif-SOFA at day 7, rho = −0.32; $p < 0.05$).

Short term survival information was available in 74.6% of cirrhotic patients ($n = 47$); 8 deaths were reported (12.7%), all of them corresponding to patients in the ACLF group. In a univariate analysis that evaluated the association of short-term mortality with cytokine distribution and prognostic scores (Child–Pugh, MELD and Clif-SOFA score at admission and day 7), only MELD score at admission ($p = 0.03$), Clif-SOFA score at admission ($p = 0.001$) and at day 7 ($p = 0.003$) were found to be significantly higher in patients who died.

4. Discussion

Our study is based on the recently published definition of ACLF by the CANONIC study [3], the first large multicenter, prospective study that offered an "evidence-based" definition of this syndrome. Similarly to previously reported, in the present study the prevalence of ACLF in decompensated cirrhotic patients admitted to the hospitals represented approximately one third of the total. Patients in this group were mainly males, active drinkers and had alcoholic cirrhosis, even though without statistical significance compared to those patients who did not developed ACLF. When

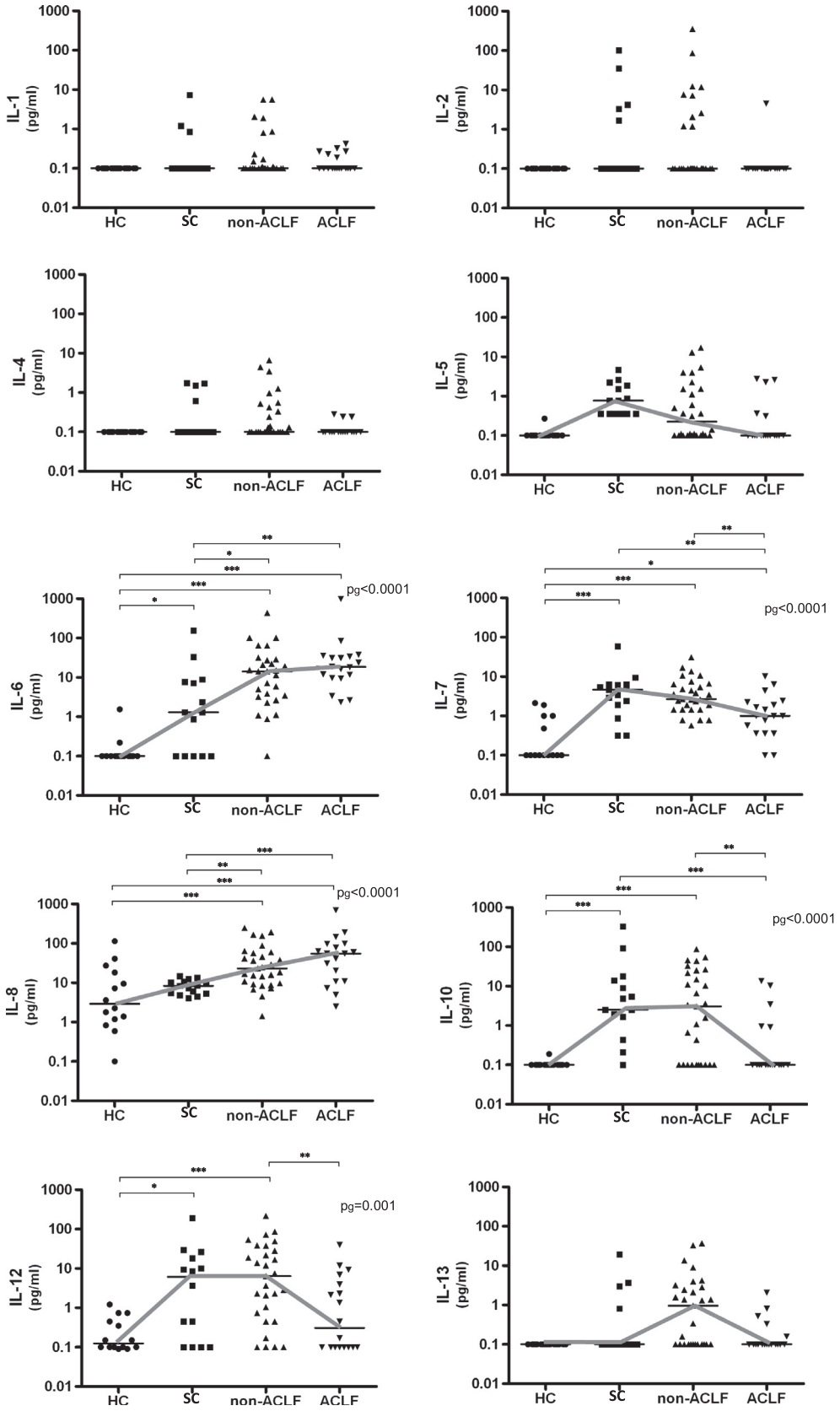

Fig. 1. Cytokine distribution in all study groups: healthy control group (HC), stable cirrhosis control group (SC), decompensated cirrhotic patients without the development of acute on chronic liver failure (non-ACLF) and decompensated cirrhotic patients with acute on chronic liver failure (ACLF). Note: in order to display the results in a logarithmic scale, the value 0 in the *y* axis is replaced by 0.1. *p* global (pg) Kruskal–Wallis are shown in graphics. *: $p < 0.05$; **: $p < 0.01$; ***$p < 0.001$.

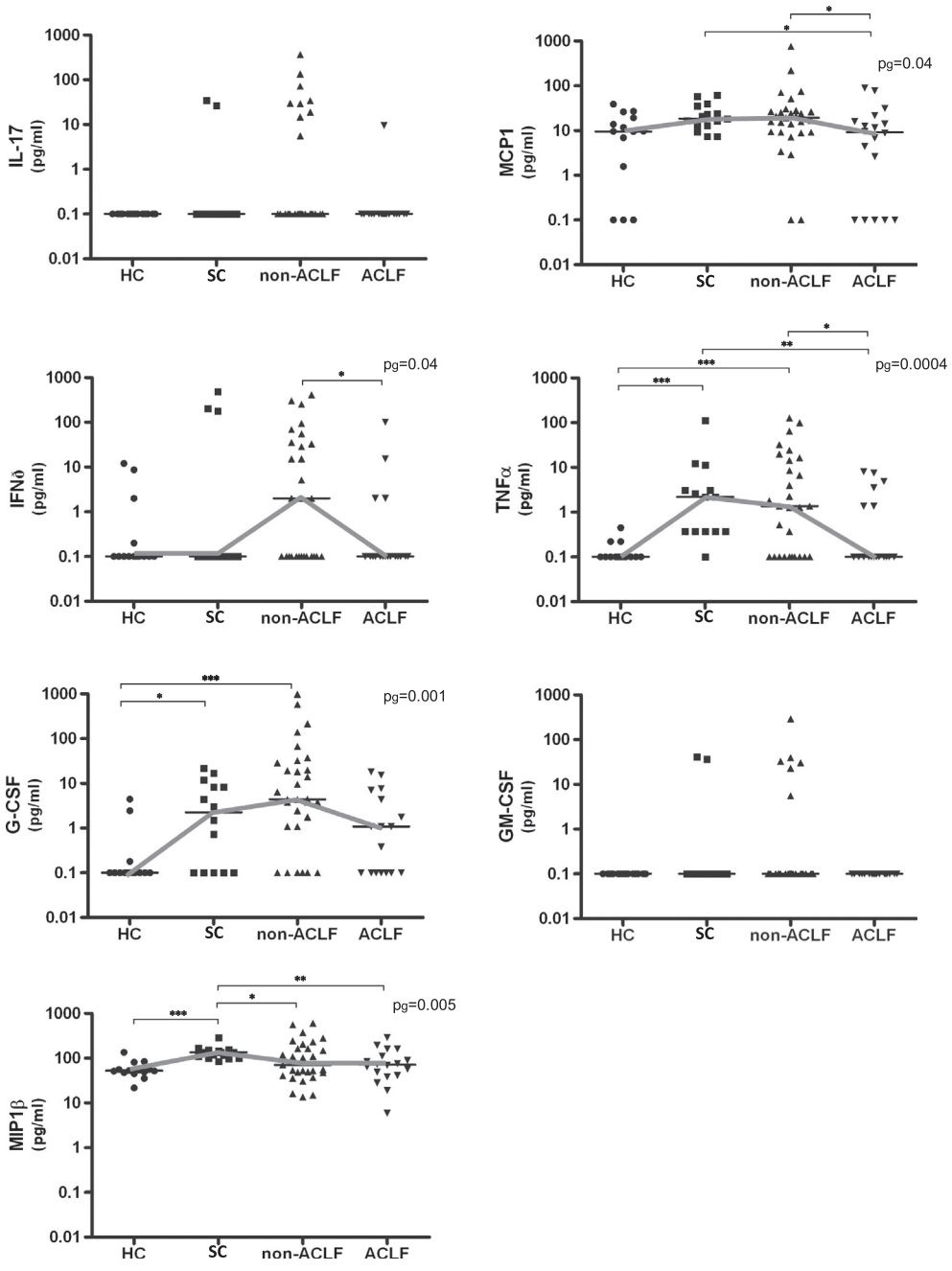

Fig. 1 (*continued*)

evaluating biochemical features, leukocyte count was significantly higher in ACLF patients, as previously reported. Regarding outcome, three-month mortality rate in the ACLF group reached 57%, significantly higher to other cirrhotic groups. This survival rate does not differ from the reported in the CANONIC study, with slightly lower mortality in the non-ACLF group (probably due to lost to follow up of almost 40% patients). These similarities in clinical features and short-term survival support the hypothesis that our study groups are comparable to those described in this larger cited study.

The key observations made in our paper are that pro-inflammatory cytokines and chemo-attractant elements are increased in cirrhosis in comparison with controls, and display higher values concomitantly with cirrhosis progression. Although ACLF patients seem to have preserved a chemo-attractant function for leukocytes and acute phase reactant protein synthesis, as suggested for sustained IL-6 and IL-8 values found, other leukocyte growth and attractant factors, pro and anti-inflammatory cytokines (IL-7, IL-10, IL-12, MCP-1, IFN-γ, TNF-α, G-CSF) displayed significantly decreased levels in this subset of patients, compatible with an inappropriate immune response.

Several studies have established that in advanced cirrhotic patients, especially when clinically significant portal hypertension is present, a state of permanent inflammatory activation exists and is associated with a negative outcome in patient prognosis [16,17]. This is thought to be secondary to translocation of bacteria; a phenomenon predisposed by malnutrition, altered enteric flora species and bacterial overgrowth (dysbiosis), increased bowel stasis, altered gut permeability and decreased mucosal defense mechanisms. But not only bacteria, also bacterial products

Table 4
Cytokine distribution according to cirrhosis etiology in non-ACLF and ACLF patients.

Non-ACLF group (n = 23)				ACLF group (n = 15)		
Cytokines	HCV (n = 11)	Alcohol (n = 12)	p	HCV (n = 5)	Alcohol (n = 10)	p
IL-6 (pg/ml)	14.2 (3.8–28)	4.9 (2.6–38)	1	12 (11.7–18)	17.5 (3.3–30)	1
IL-7 (pg/ml)	2.6 (0.8–3.8)	3.9 (2.3–10.6)	0.18	0.57 (0.3–1)	1 (0.3–1.9)	0.7
IL-8 (pg/ml)	24.8 (8.2–64.2)	18.6 (10.2–46)	0.9	31 (4.9–54.6)	57 (10.9–97)	0.28
IL-10 (pg/ml)	3.33 (0–25)	7 (0.6–21.7)	0.62	0 (0–3.4)	0 (0–0.9)	0.66
IL-12 (pg/ml)	2.3 (0.2–53.1)	11.5 (2.8–33)	0.62	0 (0–9.2)	0.3 (0–3.9)	0.9
G-CSF (pg/ml)	2.4 (0–31.9)	10 (4.3–22.8)	0.09	0.4 (0–1)	1 (0–4.3)	0.46
MCP-1 (pg/ml)	17.5 (8.7–26)	16.1 (9.1–23.8)	0.93	2.6 (0–31)	7.8 (0–11)	0.9
IFN-γ (pg/ml)	1 (0–15.3)	8.6 (0–69.1)	0.67	0	0	0.9
TNF-α (pg/ml)	0 (0–24.2)	2 (1.3–12.6)	0.16	0	0 (0–3.5)	0.7

Note: Only cytokines with significant differences between at least two groups in univariate analysis were included. Patients with cirrhosis due to hepatitis C and alcohol combined (n = 3) were excluded, as well as patients with other etiologies (n = 8). Values are expressed in median an Q1–Q3. Non-ACLF: decompensated cirrhotic patients without the development of acute-on-chronic liver failure. ACLF: acute-on-chronic liver failure.

(lipopolysaccharides) generate a continuous inflow of pathogen-associated molecular products (PAMPs) that sustain systemic inflammation [11,18–20]. However, this permanent inflammatory state has also been described in compensated cirrhosis, though in a lesser degree. In this scenario, damage associated molecular patterns (DAMPs) are thought to be originated from hepatocellular injury, thus causing sterile inflammation [11,21]. These theories could explain our findings in stable cirrhotic patients, who showed higher levels of pro-inflammatory cytokines (IL-6, TNF alpha, IL-7, IL-5, IL-12), other chemo-attractant elements such as MCP-1, IL-8 and anti-inflammatory cytokines such as IL-10 compared with healthy subjects, without any evidence of overt infection. It is important to note than almost half of them had clinical signs of portal hypertension (treatment for esophageal varices or previous history of hepatic encephalopathy and ascites) and therefore would qualify as advanced cirrhotic patients. Furthermore, the fact that bacterial infections as precipitating events had also no impact in cytokine levels when compared to non-infected patients could also be explain by the alternative sterile triggers or DAMPs theory as cause of systemic inflammation in compensated cirrhosis [11].

Regarding pathogenic theories of advanced cirrhosis, in a review by Albillos et al. it has been proposed that cirrhosis-associated immune dysfunction (CAID) encompasses acquired immunodeficiency and systemic inflammation phenotypes in a dynamic fashion; the expression of each depending on disease stage, extent of liver injury and presence of environmental stimulation [11]. Initially, due to the continuous activation of the immune system caused by bacteria and bacterial products translocation (PAMPs) originated in the gut, decompensated cirrhosis exhibits a predominantly "pro-inflammatory" phenotype, with augmented production and increased serum levels of pro- and anti-inflammatory cytokines [11,22]. Our findings are in concordance with this theory; in our cohort of non-ACLF patients, pro-inflammatory and anti-inflammatory cytokines IL-7, IL-10, IL-12, TNF-α and chemo-attractant markers MCP-1 and G-CSF were significantly higher than in HC and similar to SC. Greater inflammatory activation concomitant with cirrhosis progression could explain the significantly higher levels of IL-6, IL-8 and IFN-γ in non-ACLF patients when compared to SC. The non-ACLF group was the only one found capable of secreting IFN-γ, possibly depicting a still functioning response to excessive inflammatory mediators [11]. Elevated levels of IFN-γ have been extensively reported in different clinical scenarios of cirrhotic patients; higher levels of serum IFN-γ have been describe in hepatitis-B related cirrhosis [23] when compared to healthy controls, as well as in decompensated cirrhotic patients with massive ascites in comparison with cirrhotic patients without ascites [24], suggesting an association with disease severity. However, when considering septic patients, IFN-γ has revealed an inverse relationship with the severity of sepsis, both in human and murine subjects [25,26]. Since the immune system compromise present in ACLF has been compared to the "immune paresis" observed in sepsis, we believe this pathogenic scenario may explain the elevated levels of IFN-γ in non-ACLF patients followed by significantly lower levels in the ACLF group.

In ACLF, the final stage of advanced cirrhosis, a phenomenon known as endotoxin tolerance is proposed to occur. Endotoxin tolerance is defined as a reduced responsiveness to a lipopolysaccharide challenge following a first encounter with an endotoxin; it involves the participation of macrophages and subsequent down-regulation of several pro-inflammatory cytokines. Endotoxin tolerance occurs in order to protect against a lethal challenge of bacterial products and to prevent infection and ischemia–reperfusion damage and related mortality. Despite the fact that this immune alteration may have a physiological role in some settings and

Table 5
Cytokine distribution according to bacterial infections as acute decompensating event in non-ACLF and ACLF patients.

Non-ACLF group (n = 31)				ACLF group (n = 18)		
Cytokines	Bacterial Infection (n = 14)	Other decompensating event (n = 17)	p	Bacterial infection (n = 7)	Other decompensating event (n = 11)	p
IL-6 (pg/ml)	19.1 (5.9–64)	14.2 (3.5–24.6)	*0.34*	35.6 (12–84.8)	16.2 (3.3–23.7)	*0.02*
IL-7 (pg/ml)	2.4 (1.4–4.1)	3.7 (1.9–7.7)	*0.34*	1.45 (0.3–2.2)	1 (0.3–2.4)	*0.82*
IL-8 (pg/ml)	24 (10.2–56.4)	34.7 (10.2–73)	*0.86*	54.6 (4.9–59.9)	78 (10.9–145)	*0.16*
IL-10 (pg/ml)	3 (0–14.5)	5 (0–27.6)	*0.52*	0	0 (0–0.9)	*0.38*
IL-12 (pg/ml)	7.4 (0.4–16.7)	5 (0.89–35)	*0.72*	0 (0–2.1)	0.4 (0–6.9)	*0.49*
G-CSF (pg/ml)	3.7(1–31.9)	7.9 (0.88–19.6)	*0.93*	0.2 (0–15.3)	1 (0–4.3)	*0.91*
MCP-1 (pg/ml)	23.8 (15–26.2)	15.7 (9.1–21)	*0.12*	4.4 (0–12.6)	9.5 (0–31)	*0.29*
IFN-γ (pg/ml)	0 (0–2)	15.3 (0–69)	*0.25*	0	0 (0–15.3)	*0.58*
TNF-α (pg/ml)	0.3 (0–14.3)	2 (0.6–10.5)	*0.34*	1.36 (0–7.5)	0	*0.17*

Note: Only cytokines with significant differences between at least two groups in univariate analysis were included. Values are expressed in median and Q1–Q3. Non-ACLF: decompensated cirrhotic patients without the development of acute-on-chronic liver failure. ACLF: acute-on-chronic liver failure.

Table 6
Characteristics of cirrhotic patients according to their cytokine secretor phenotype.

Stable cirrhotic patients (n = 14), non-ACLF patients (n = 31) and ACLF patients (n = 18)

Characteristics	Cytokine secretor phenotype (n = 29)	Cytokine non-secretor phenotype (n = 34)	p
Sex (male)	25 (86%)	31 (91%)	0.53
Age (years)	49 ± 12	53 ± 9	0.13
Cirrhosis etiology[a]			
Hepatitis C	12 (41.3%)	17 (58.6%)	0.64
Alcohol	11 (47.8%)	12 (52%)	
Precipitating event			
Bacterial Infection (n = 21)	9 (31%)	12 (35.2%)	0.72
Other (n = 42)	20 (68.7%)	22 (64.7%)	
Study group distribution			
Stable cirrhosis (n = 14)	7 (24.2%)	7 (20.6%)	0.17
Non-ACLF (n = 31)	17 (58.6%)	14 (41.2%)	
ACLF (n = 18)	5 (17.2%)	13 (38.2%)	
Three-month mortality	2 (10.5%)	6 (21.4%)	0.44

Note: Patients with ⩾8 cytokines with similar values to healthy controls were considered non-secretors. Non-ACLF: decompensated cirrhotic patients without the development of acute-on-chronic liver failure. ACLF: acute-on-chronic liver failure.
[a] The combination of hepatitis C and alcohol (n = 3) or other etiologies (n = 8) were not included.

represent a protective response against an overwhelming dysregulation of the pro-inflammatory process, it has also been associated with an increased risk of infection and related mortality [27]. This "immune deficient phenotype" [11,28] has also been suggested by findings of Wasmuth et al., demonstrating a reduced ex vivo TNF-a secretion and monocyte HLA-DR expression with elevated levels of IL-6 and IL-10 in patients with acutely decompensated cirrhosis compared to subjects with stable cirrhosis, and comparable to patients with severe sepsis; therefore defining this immune alteration as a "sepsis-like" immune paralysis [7]. Other authors have shown monocyte functional exhaustion leading to impaired pathogen clearance in acute liver failure and ACLF patients, or severe functional failure of neutrophils in cirrhotic patients with alcoholic hepatitis [8,10]. In this latter paper, circulating neutrophils were shown not only to be primed in this cohort (which would suggest good bactericidal function) but also activated, causing an inability to generate further oxidative burst when presented to bacterial challenge. The authors speculate this reversible neutrophil dysfunction could be the explanation for the paradox of increased inflammation and simultaneous heightened risk of infection.

An excessive inflammatory profile has also been suggested as the main pathogenic pathway in studies such as the CANONIC [3] where the degree of inflammatory reaction estimated by the leukocyte count was an independent predictor of post-enrollment development of ACLF and ACLF-associated mortality. It is suggested that an excessive inflammatory response may induce tissue damage and organ failure, depending not only on the intensity of the inflammatory response per se but also on the intrinsic capacity of host organs to endure the effects of the inflammatory response. Mehta et al. [5] selected a cohort restricted to alcoholic cirrhosis, and also found that mean SIRS score, white cell count, C-reactive protein, IL-6, IL-8, TNF-a and IL-10 were significantly higher in the ACLF group compared with non-ACLF and stable cirrhotic patients, which correlated with increased portal pressure, elevated intrahepatic resistance and decreased liver blood flow. Although the present study did not include the evaluation of cellular function, one crucial aspect of the innate immune response (circulating cytokines) was evaluated in a more thorough approach. We found that not only TNF-α excretion was lower in ACLF patients compared to non-ACLF and SC and similar as healthy controls, but this pattern was also present in most of the studied cytokines. The compromise of cytokines with a predominant pro-inflammatory function (Th1 and Th2 pathways) such as IL-5, IL-7, IL-12, IFN-γ, chemo-attractant molecules such as MCP-1 and a diminished expression of anti-inflammatory cytokines such as IL-10 may reinforce the concept of immune paresis, showing initially increased levels in SC and non-ACLF patients compared to healthy controls, with latter exhaustion when analyzing the ACLF group.

However, some of the evaluated cytokines such as IL-6 and IL-8 are displayed in an in-crescendo pattern, with lower values in healthy controls and then escalating with higher values in the ACLF group. IL-6 is known to provoke neutrophil movement and acute phase reactant protein's production by the liver, and IL-8 is a strong chemo-attractant for neutrophils; therefore, these elevated values could explain the higher leukocyte count and C-reactive protein value detected in the ACLF group, despite the incapability of these patients to develop a proper immune response. This apparent opposite cytokine pattern could account for the contradictory findings of previous studies and shed some light in the pathophysiology of this syndrome.

When considering cirrhosis etiology, neither alcohol-related nor viral hepatitis modified cytokine levels; this lack of influence of cirrhosis etiology in inflammation markers has already been reported in the CANONIC study, where alcoholic versus non-alcoholic cirrhosis did not altered the leukocyte count or C-reactive protein values [3]. Regarding several patients depicting a "non-secretor" phenotype, defined as having ⩾8 cytokines with comparable values to healthy controls, no commonalities in demographic features, cirrhosis etiology, decompensating event or outcome was detected, thus discarding the theory of a patient subgroup based on cytokine secretion.

The clinical relevance of analyzing cytokine involvement in different stages of cirrhosis exceeds better understanding cirrhosis physiopathology; the identification of serum biomarkers also allows improving current treatment strategies and even liver allocation algorithms. As previously mentioned, cytokines have been suggested as prognostic tools. In the study performed by Girón-Gonzalez et al. that evaluated serum concentrations of IL-6, TNF-α and its soluble receptors I–II as prognostic markers of disease severity and mortality in cirrhotic patients, both IL-6 and TNF-α were more elevated in advanced cirrhotic patients (Child Pugh stage C vs Child A and B), whereas only TNF-R I was an independent prognostic marker of mortality [14]. TNF soluble receptor 55 and IL-2 soluble receptor have also been found to positively correlate with cirrhosis severity when patients were stratified in Child–Pugh stages [29]; and IL-8 has also been found to significantly increase in cirrhotic patients, particularly in end-stage liver cirrhosis [30]. It should be stressed out that none of this studies included patients with acute-on-chronic liver failure, the most severe stage of decompensated cirrhosis. In our study, we found a positive correlation of leukocyte count with cirrhosis severity, as previously described by the CANONIC study [3], but also with IL-6 and IL-8, similarly to previously mentioned studies in decompensated cirrhosis. However, we found a negative correlation of IL-2 and IFN-γ; although these findings have not been reported by other authors, low serum levels of IFN-γ have been described in severe sepsis, perhaps suggesting a parallelism with this syndrome.

Only MELD and Clif-SOFA score portrayed a significant association with short-term mortality; however, due to the small sample size and short follow-up period, these results should be further explored in larger prospective studies.

Our study has several limitations; since it had an exploratory design, the study size was small and although it was sufficient to stablish a correlation between several cytokines and disease severity, it was probably underpowered to assess survival. Also, no cellular functional analysis was performed, since we prioritized studying a broad number of easily available biomarkers that could be adopted in every day clinical practice.

In summary, when analyzing innate immune response in stable, non-ACLF and ACLF cirrhotic patients from a cytokine-involvement point of view, we found that systemic inflammation increases concomitantly with cirrhosis severity, portraying a pro-inflammatory phenotype as described in the CAID syndrome. In ACLF an opposite cytokine pattern was found, concordant with an acquired immunodeficient phenotype, but perhaps revealing a still conserved chemo-attractant function for neutrophils. In the current search for new biomarkers to improve prognostic scores, the identification of several cytokines that showed correlation with disease severity endorses the need for further research on its utility as prognostic biomarkers.

Further analysis of the remaining components of innate immune response, as well as validation of our findings in a larger cohort are needed, but our data provide an alternative explanation for the pathophysiology of this newly defined syndrome and may be useful in the future for the improvement of prognostic tools and therapeutic interventions.

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Conflict of interest

None to be declared.

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References

[1] G. D'Amico, G. Garcia-Tsao, L. Pagliaro, Natural history and prognostic indicators of survival in cirrhosis: a systematic review of 118 studies, J. Hepatol. 44 (2006) 217–231.
[2] S.K. Asrani, J.G. O'Leary, Acute-on-chronic liver failure, Clin. Liver Dis. 18 (2014) 561–574.
[3] R. Moreau, R. Jalan, P. Gines, M. Pavesi, P. Angeli, J. Cordoba, et al., Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis, Gastroenterology 144 (2013) 1426–1437. 37 e1-9.
[4] V. Arroyo, R. Moreau, R. Jalan, P. Gines, Acute-on-chronic liver failure: a new syndrome that will re-classify cirrhosis, J. Hepatol. 62 (2015) S131–S143.
[5] G. Mehta, R.P. Mookerjee, V. Sharma, R. Jalan, Systemic inflammation is associated with increased intrahepatic resistance and mortality in alcohol-related acute-on-chronic liver failure, Liver Int. 35 (2015) 724–734.
[6] K.A. Tazi, J.J. Quioc, W. Abdel-Razek, Z. Tellier, C. Guichard, E. Ogier-Denis, et al., Protein array technology to investigate cytokine production by monocytes from patients with advanced alcoholic cirrhosis: an ex vivo pilot study, Hepatol. Res. 39 (2009) 706–715.
[7] H.E. Wasmuth, D. Kunz, E. Yagmur, A. Timmer-Stranghoner, D. Vidacek, E. Siewert, et al., Patients with acute on chronic liver failure display "sepsis-like" immune paralysis, J. Hepatol. 42 (2005) 195–201.
[8] R.P. Mookerjee, V. Stadlbauer, S. Lidder, G.A. Wright, S.J. Hodges, N.A. Davies, et al., Neutrophil dysfunction in alcoholic hepatitis superimposed on cirrhosis is reversible and predicts the outcome, Hepatology (Baltimore, MD) 46 (2007) 831–840.
[9] C. Fiuza, M. Salcedo, G. Clemente, J.M. Tellado, In vivo neutrophil dysfunction in cirrhotic patients with advanced liver disease, J. Infect. Dis. 182 (2000) 526–533.
[10] C. Bernsmeier, V. Patel, R. Tidswell, E. Triantafyllou, A. Singanayagam, W. Khamri, et al., P164 monocyte exhaustion: a mechanistic explanation for susceptibility to infection in liver failure, J. Hepatol. (60) (2014) S120–S121.
[11] A. Albillos, M. Lario, M. Alvarez-Mon, Cirrhosis-associated immune dysfunction: distinctive features and clinical relevance, J. Hepatol. 61 (2014) 1385–1396.
[12] M. Dirchwolf, A.E. Ruf, Role of systemic inflammation in cirrhosis: from pathogenesis to prognosis, World J. Hepatol. 7 (16) (2015) 1974–1981.
[13] M. Buck, G. Garcia-Tsao, R.J. Groszmann, C. Stalling, N.D. Grace, A.K. Burroughs, et al., Novel inflammatory biomarkers of portal pressure in compensated cirrhosis patients, Hepatology (Baltimore, MD) 59 (2014) 1052–1059.
[14] J.A. Giron-Gonzalez, C. Martinez-Sierra, C. Rodriguez-Ramos, M.A. Macias, P. Rendon, F. Diaz, et al., Implication of inflammation-related cytokines in the natural history of liver cirrhosis, Liver Int. 24 (2004) 437–445.
[15] X. Hu, S. Ma, X. Huang, X. Jiang, X. Zhu, H. Gao, et al., Interleukin-21 is upregulated in hepatitis B-related acute-on-chronic liver failure and associated with severity of liver disease, J. Viral Hepat. 18 (2011) 458–467.
[16] M. Cazzaniga, E. Dionigi, G. Gobbo, A. Fioretti, V. Monti, F. Salerno, The systemic inflammatory response syndrome in cirrhotic patients: relationship with their in-hospital outcome, J. Hepatol. 51 (2009) 475–482.
[17] R. Behroozian, M. Bayazidchi, J. Rasooli, Systemic Inflammatory response syndrome and MELD score in hospital outcome of patients with liver cirrhosis, Middle East J. Digestive Diseases 4 (2012) 168–172.
[18] V. Arroyo, R. Garcia-Martinez, X. Salvatella, Human serum albumin, systemic inflammation, and cirrhosis, J. Hepatol. 61 (2014) 396–407.
[19] N. Sipeki, P. Antal-Szalmas, P.L. Lakatos, M. Papp, Immune dysfunction in cirrhosis, World J. Gastroenterol. 20 (2014) 2564–2577.
[20] R. Wiest, M. Lawson, M. Geuking, Pathological bacterial translocation in liver cirrhosis, J. Hepatol. 60 (2014) 197–209.
[21] P. Kubes, W.Z. Mehal, Sterile inflammation in the liver, Gastroenterology 143 (2012) 1158–1172.
[22] R. Jalan, J. Fernandez, R. Wiest, B. Schnabl, R. Moreau, P. Angeli, et al., Bacterial infections in cirrhosis: a position statement based on the EASL special conference 2013, J. Hepatol. 60 (2014) 1310–1324.
[23] H. Chen, D. Zhang, S. Wang, X. Wang, C. Yang, Significance of correlation between interferon-gamma and soluble intercellular adhesion molecule-1 and interleukin-17 in hepatitis B virus-related cirrhosis, Clinics Res. Hepatol. Gastroenterol. 37 (2013) 608–613.
[24] B.M. Attar, C.M. Moore, M. George, N. Ion-Nedelcu, R. Turbay, A. Zachariah, et al., Procalcitonin, and cytokines document a dynamic inflammatory state in non-infected cirrhotic patients with ascites, World J. Gastroenterol. 20 (2014) 2374–2382.
[25] D.W. Jekarl, J.Y. Kim, S. Lee, M. Kim, Y. Kim, K. Han, et al., Diagnosis and evaluation of severity of sepsis via the use of biomarkers and profiles of 13 cytokines: a multiplex analysis, Clin. Chem. Lab. Med.: CCLM/FESCC 53 (2015) 575–581.
[26] S. Ono, C. Ueno, S. Aosasa, H. Tsujimoto, S. Seki, H. Mochizuki, Severe sepsis induces deficient interferon-gamma and interleukin-12 production, but interleukin-12 therapy improves survival in peritonitis, Am. J. Surg. 182 (2001) 491–497.
[27] J.M. Cavaillon, M. Adib-Conquy, Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis, Crit. Care (London, England) 10 (2006) 233.
[28] G. Tiegs, A.W. Lohse, Immune tolerance: what is unique about the liver, J. Autoimmun. 34 (2010) 1–6.
[29] P. Garcia Ruiz, J. Canora Lebrato, A. Diez Ruiz, D. Fuchs, H. Wachter, [Soluble interleukin-2 and tumor necrosis factor receptor in liver cirrhosis. Relationship with clinical severity and prognosis], Med. Clin. 122 (2004) 441–443.
[30] H.W. Zimmermann, S. Seidler, N. Gassler, J. Nattermann, T. Luedde, C. Trautwein, et al., Interleukin-8 is activated in patients with chronic liver diseases and associated with hepatic macrophage accumulation in human liver fibrosis, PLoS ONE 6 (2011) e21381.
[31] S. Giraldo, J. Sanchez, Q. Felty, D. Roy, IL1B (interleukin 1, beta), Atlas Genet. Cytogenet. Oncol. Haematol. 13 (2009) 273–275.
[32] W. Liao, J.X. Lin, W.J. Leonard, IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation, Curr. Opin. Immunol. 23 (2011) 598–604.
[33] A.A. Cuneo, M.V. Autieri, Expression and function of anti-inflammatory interleukins: the other side of the vascular response to injury, Curr. Vasc. Pharmacol. 7 (2009) 267–276.
[34] C.J. Sanderson, Interleukin-5, eosinophils, and disease, Blood 79 (1992) 3101–3109.
[35] J. Scheller, A. Chalaris, D. Schmidt-Arras, S. Rose-John, The pro- and anti-inflammatory properties of the cytokine interleukin-6, Biochim. Biophys. Acta 1813 (2011) 878–888.
[36] C.R. Willis, A. Seamons, J. Maxwell, P.M. Treuting, L. Nelson, G. Chen, et al., Interleukin-7 receptor blockade suppresses adaptive and innate inflammatory responses in experimental colitis, J. Inflammation (London, England) 9 (2012) 39.
[37] R. Dong, S. Zheng, Interleukin-8: a critical chemokine in biliary atresia, J. Gastroenterol. Hepatol. 30 (2015) 970–976.
[38] G. Trinchieri, Interleukin-12 and the regulation of innate resistance and adaptive immunity, Nat. Rev. Immunol. 3 (2003) 133–146.
[39] J.J. Yu, S.L. Gaffen, Interleukin-17: a novel inflammatory cytokine that bridges innate and adaptive immunity, Front. Biosci.: J. Virtual Lib. 13 (2008) 170–177.

[40] J.R. Schoenborn, C.B. Wilson, Regulation of interferon-gamma during innate and adaptive immune responses, Adv. Immunol. 96 (2007) 41–101.
[41] A.W. Roberts, G-CSF: a key regulator of neutrophil production, but that's not all!, Growth Factors (Chur, Switzerland) 23 (2005) 33–41
[42] P. Bhattacharya, M. Thiruppathi, H.A. Elshabrawy, K. Alharshawi, P. Kumar, B.S. Prabhakar, GM-CSF: an immune modulatory cytokine that can suppress autoimmunity, Cytokine (2015).
[43] J.R. Bradley, TNF-mediated inflammatory disease, J. Pathol. 214 (2008) 149–160.
[44] K.E. Driscoll, Macrophage inflammatory proteins: biology and role in pulmonary inflammation, Exp. Lung Res. 20 (1994) 473–490.
[45] S.L. Deshmane, S. Kremlev, S. Amini, B.E. Sawaya, Monocyte chemoattractant protein-1 (MCP-1): an overview, J. Interferon Cytokine Res.: Official J. Int. Soc. Interferon Cytokine Res. 29 (2009) 313–326.