SYSTEMIC INFLAMMATION IN DECOMPENSATED CIRRHOSIS. CHARACTERIZATION AND ROLE IN ACUTE-ON-CHRONIC LIVER FAILURE.

Joan Clària[1,*], Rudolf E Stauber[3,*], Minneke J Coenraad[4], Richard Moreau[5], Rajiv Jalan[6], Marco Pavesi[20], Àlex Amorós[20], Esther Titos[1], José Alcaraz-Quiles[1], Karl Oettl[7], Manuel Morales-Ruiz[1], Paolo Angeli[8], Marco Domenicali[9], Carlo Alessandria[10], Alexander Gerbes[11], Julia Wendon[12], Frederik Nevens[13], Jonel Trebicka[14], Wim Laleman[13], Faouzi Saliba[15], Tania M. Welzel[16], Agustin Albillos[17], Thierry Gustot[18], Daniel Benten[19], François Durand[5], Pere Ginès[2], Mauro Bernardi[9] and Vicente Arroyo[20], for the CANONIC Study Investigators of the EASL-CLIF Consortium and the European Foundation for the Study of Chronic Liver Failure (EF-CLIF)

[1]Department of Biochemistry and Molecular Genetics and [2]Liver Unit, Hospital Clínic, IDIBAPS and CIBERehd Barcelona, Spain; [3]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Medical University of Graz, Graz, Austria; [4]Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, The Netherlands; [5]Inserm, U1149, Centre de Recherche sur l'Inflammation (CRI), UMRS1149; Université Paris Diderot-Paris 7, Département Hospitalo-Universitaire (DHU) UNITY; Service d'Hépatologie, Hôpital Beaujon, Assistance Publique-Hôpitaux de Paris; Laboratoire d'Excellence Inflamex, ComUE Sorbonne Paris Cité, Paris, France; [6]Liver Failure Group, Institute for Liver Disease Health, University College London, Royal Free Hospital, London, UK; [7]Institute of Physiological Chemistry, Medical University of Graz, Graz, Austria; [8]Unit of Internal Medicine and Hepatology, Dept. of Medicine, DIMED, University of Padova, Italy; [9]Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy; [10]Division of Gastroenterology and Hepatology, San Giovanni Battista Hospital, Torino, Italy; [11]Department of Medicine II, University Hospital LMU Munich, Liver Center Munich, Munich, Germany; [12]Kings College London, London, UK; [13]University Hospital Gasthuisberg, KU Leuven, Belgium; [14]Department of Internal Medicine I, University of Bonn, Germany; [15]Hôpital Paul Brousse, Université Paris-Sud, Villejuif, France; [16]J.W. Goethe University Hospital, Frankfurt, Germany; [17]Hospital Ramón y Cajal, Madrid, Spain; [18]Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium; [19]University Hospital Hamburg-Eppendorf, Germany; [20]EF-CLIF and EASL-CLIF Consortium, Barcelona, Spain

*****These authors contributed equally to this work

Keywords: Cirrhosis, acute-on-chronic liver failure, hepatorenal syndrome, systemic inflammation, cytokines.

Contact Information: Pere Ginès, MD. Scientific secretary of the EF-CLIF. Liver Unit, Hospital Clínic, Barcelona, Spain, pgines@clinic.ub.es

List of Abbreviations: AAH: acute alcoholic hepatitis, ACLF: acute-on-chronic liver failure, ACLF-RF: acute-on-chronic liver failure with renal failure, AD: Acute decompensation, ADH: antidiuretic hormone, CLIF: chronic liver failure, CRP: C-reactive protein, DAMPs: damage associated molecular patterns, HMA: human mercaptalbumin, HNA1: reversibly oxidized human non-mercaptalbumin-1, HNA2: irreversibly oxidized human non-mercaptalbumin-2, IL: interleukin, PAMPs: pathogen associated molecular patterns, PE: precipitating event, PCC: plasma copeptin concentration, PRC: plasma renin concentration, SCD: systemic circulatory dysfunction, SI: systemic inflammation, SIRS: systemic inflammatory response syndrome, WBC: white blood cell count.

Financial Support: The Study was supported by the European Foundation for the Study of Chronic Liver Failure (EF-CLIF).

Abstract

Acute-on-Chronic Liver Failure (ACLF) in cirrhosis is characterized by acute decompensation (AD), organ failure(s) and high short-term mortality. Recently, we have proposed [Systemic Inflammation (SI) Hypothesis] that ACLF is the expression of an acute exacerbation of the SI already present in decompensated cirrhosis. This study was aimed at testing this hypothesis and included 522 patients with decompensated cirrhosis (237 with ACLF) and 40 healthy subjects. SI was assessed by measuring 29 cytokines and the redox state of circulating albumin (HNA2), a marker of systemic oxidative stress. Systemic circulatory dysfunction (SCD) was estimated by plasma renin (PRC) and copeptin (PCC) concentrations. Measurements were performed at enrolment (baseline) in all patients and sequentially during hospitalization in 255. The main findings of this study were: (1) Patients with AD without ACLF showed very high baseline levels of inflammatory cytokines, HNA2, PRC and PCC. Patients with ACLF showed significantly higher levels of these markers than patients without ACLF; (2) Different cytokine profiles were identified according to the type of ACLF precipitating event (active alcoholism/acute alcoholic hepatitis, bacterial infection, others); (3) Severity of SI and frequency and severity of ACLF at enrolment were strongly associated. The course of SI and the course of ACLF (improvement, no change or worsening) during hospitalization and short-term mortality were also strongly associated; (4) The strength of association of ACLF with SI was higher than with SCD. **Conclusion:** Our data support SI as the primary driver of ACLF in cirrhosis.

Acute-on-Chronic Liver Failure (ACLF) in cirrhosis is a highly prevalent syndrome characterized by acute decompensation (AD), organ/system failure(s) and high 28-day mortality (32%) (1). ACLF is classified in 3 grades of severity (ACLF-I, II and III) according to the number of organ failures and may follow four different clinical courses during hospitalization: resolution, improvement (reduction in ACLF-grade), steady course, or worsening (2).

The Systemic Inflammation (SI) Hypothesis (3) proposes that ACLF is due to aggravation of the SI and associated systemic circulatory dysfunction (SCD) already present in AD, which leads to organ failure(s) as a consequence of organ hypo-perfusion and direct deleterious effects of inflammatory mediators on the organ microcirculation and cell physiology homeostasis. According with this hypothesis, AD would occurs in the setting of chronic SI due to translocation of pro-inflammatory molecules (Pathogen Associated Molecular Patterns, PAMPs) from the intestinal lumen to the systemic circulation and/or to the release or Damage Associated Molecular Patterns (DAMPs) from the diseased liver. ACLF would be the result of further increase of SI in the context of precipitating events [PE: mainly active alcoholism/Acute alcoholic hepatitis (AAH), bacterial infections, or other PE]. The SI Hypothesis was based on cytokine studies in small series of patients (4-7) and on the CANONIC study, in which a close relationship between blood leukocytes (WBC) and C-reactive protein (CRP) levels and the presence and severity of ACLF was observed (1).

In the current study we assessed the SI Hypothesis by two approaches. The first was aimed at examining the relationship between markers of SI, measured at enrolment and sequentially during hospitalization, and the presence, severity and clinical course

of ACLF and associated mortality in 552 patients hospitalized for AD. The second approach was aimed at assessing whether, as proposed by the SI hypothesis, ACLF in cirrhosis is not only due to an accentuation of the SCD and organ hypo-perfusion related to SI, but also to direct deleterious effects of SI in organ function. This assessment was performed by assessing the strength of the association of SCD and SI with ACLF and mortality.

Experimental Procedures

Patient selection, blood sampling, data collection and diagnostic criteria of organ failure and ACLF.

Two-hundred-thirty-seven patients with ACLF, either present at enrolment (n=180) or developed during hospitalization (n=57), and 285 patients without ACLF derived from the CANONIC investigation (1) were studied. Selection criteria were: 1. Availability of blood samples at enrollment; 2. Prospective intensive surveillance during hospitalization. Follow-up plasma samples obtained at pre-specified visits during hospitalization (2, 3-7, 8-14, 15-21 and 22-28 days after enrolment) were available in 255 patients, 157 with ACLF. Sequential blood samples prior to ACLF, at ACLF diagnosis and during post-ACLF follow-up were available in 20 out of the 57 patients developing ACLF during hospitalization. In the remaining patients with ACLF there were only samples at ACLF diagnosis and during follow-up. Samples were obtained in 2011 in Vacutainer EDTA tubes, centrifuged at 4ºC and the plasma frozen at -90ºC. Measurements were performed during 2015.

Clinical, laboratory and follow-up data were obtained from individual electronic-CRF. There was no patient with HIV infection. Diagnosis of organ failure, ACLF and ACLF grades are based on the CLIF-Consortium Organ Failure score and the Canonic

study criteria (1,8) (see **Supporting Information**). Clinical diagnosis of AAH was based on the NIAAA Alcoholic Hepatitis Consortia criteria (9): active alcoholism, serum bilirubin>3 mg/dL, AST>50 IU/mL and AST/ALT ratio> 1.5. SIRS was diagnosed using WBC count, heart and respiratory rates and body temperature (10).

Characterization of SI in AD. Relationship with ACLF.

To characterize SI, we measured the plasma levels of 29 cytokines involved in innate and adaptive immune responses and the redox state of circulating albumin (a marker of systemic oxidative stress (11,12)). Baseline measurements were obtained at enrolment or at ACLF onset in the 20 out of the 57 patients developing ACLF during hospitalization. To assess the role of SI in the pathogenesis of ACLF, we compared baseline plasma cytokine levels of patients with and without ACLF at enrolment and examined the relationship between these measurements and ACLF severity. Also we assessed the chronological relationship between changes in SI and the course of ACLF during hospitalization by comparing the levels of SI markers at ACLF diagnosis with those at the last measurement during hospitalization (prior to discharge or liver transplantation or death). Median time from enrolment to last assessment: 15 days; percentile 25-75:7-28). Differences in baseline cytokine profiles according to the etiology (PE) were also assessed to identify potential differences in SI mechanisms. Finally, pro-inflammatory/anti-inflammatory cytokine ratios were determined in healthy subjects and patients with and without ACLF to assess the profile of pro- and anti-inflammatory cytokine response in these patients. Cytokines were measured using a multiplexed bead-based immunoassay on a Luminex 100 Bioanalyzer (**Supporting Information** and **Supplementary Table 1**).

Systemic oxidative stress was estimated by measuring the percentage of albumin oxidized at the Cysteine-34 residue (Cys34) over the total albumin concentration.

The free Cys34 accounts for about 80% of the antioxidant capacity of human plasma (12,13). In the presence of systemic oxidative stress, Cys34 is converted from the reduced form with Cys34 into the free sulphydryl form (human mercaptalbumin, HMA) to mixed disulfides (human nonmercaptalbumin 1, HNA1). This process is reversible (14-16). A smaller fraction of Cys34, however, is highly oxidized to sulfenic, sulfinic and finally sulfonic forms (human nonmercaptalbumin 2, HNA2). Oxidation of albumin to HNA2 is irreversible and causes intense modifications of the protein structure (15,16). HNA1+HNA2 as well as HNA2 were used as markers of systemic oxidative stress(17-19). Reasons for the selection of the redox state of albumin over other markers of systemic oxidative stress and the methodology of measurement are detailed in **Supporting Information**.

Strength of the association of markers of SI and of SCD with ACLF or ACLF with renal failure (ACLF-RF).

Systemic circulatory function defines a series of physiological processes influencing the cardiac output and the activity of endogenous vasoactive systems that regulate arterial pressure and global organ perfusion. Micro-circulatory function (which is mainly dependent of endothelial and tissue factors) regulates the blood and oxygen delivery to the cells. Since plasma renin concentration (PRC) and the plasma concentration of copeptin (PCC) are very sensitive to changes in effective circulating blood volume (20,21), they were selected as markers of systemic circulatory dysfunction for this study. Copeptin is a stable cleavage product of the C-terminal part of the antidiuretic hormone (ADH) precursor that is produced in a 1:1 fashion with ADH. It is widely used to estimate vasopressin release because it allows an easier and more accurate immunological testing than ADH (21). Other criteria used for the selection of PRC and PCC as markers of systemic circulatory dysfunction and

the methodology of measurements are detailed in **Supporting Information**. Copeptin (as ADH) is filtered by the glomeruli and therefore may overestimate ADH secretion in patients with ACLF-RF.

The strength of the association of ACLF with PRC and with IL-8, IL-6 or HNA2 was estimated to assess whether ACLF is related not only to SCD but also to other effects of SI in organ function. IL-8, IL-6 and HNA2 were selected because they were the inflammatory markers more strongly associated with ACLF in our patients (see Results). PRC was selected because it was not influenced, as copeptin, by GFR in our patients (see Results) The strength of the association of ACLF-RF with markers of SI and PRC were also compared as an outcome, since renal failure is a paradigmatic organ failure in cirrhosis thought to be caused by severe impairment in cardiovascular function and organ hypo-perfusion (3,20,22). Four different approaches to analysis were used.

First, we visually assessed the distribution of baseline IL-8, IL-6, HNA2, and PRC according to the presence of ACLF and ACLF-RF by means of a scatter-plot of patients' values. The strength of association between SI or SCD and ACLF or ACLF-RF was estimated by comparing (Chi-square tests) the progression of ACLF or ACLF-RF frequency at three different levels (tertiles) of each SI markers versus PRC.

Second, we assessed the percentage of patients with ACLF or ACLF-RF showing normal levels of IL-8, IL-6, HNA2 and PRC.

The third approach included two different analyses: 1. A logistic regression model to assess which markers (IL-8, IL-6, HNA2 and/or PRC) were independently associated with the presence of ACLF or ACLF-RF; 2. A linear regression model assessing the

correlation of IL-8, IL-6, HNA2 and PRC with CLIF-Consortium Organ Failure score and serum creatinine as markers of ACLF and renal impairment severity.

Finally we assessed the association of changes in markers of SI and PRC during hospitalization with changes in the clinical course of patients with and without ACLF.

SI, SCD and mortality.

We also assessed the strength of the association of baseline levels of SI and SCD markers and of changes in these markers during hospitalization with short-term (28-day and 90-day) mortality.

Data analysis and statistical details

Among the 29 cytokines determined, 12 were undetectable in a high percentage (more than 30%) of the cirrhotic patients studied (**Supplementary Table 2**). Due to this feature, two independent analyses on the association of cytokines with ACLF and mortality were performed. The main analysis, which included the 17 cytokines showing detectable plasma levels in most patients, is detailed in the main body of the article. Undetectable levels of healthy subjects in this analysis (the majority of values for some cytokines) were assigned a value equal to the lower limit of detection. The second analysis was performed with the 12 cytokines with more than 30% of undetectable values. Results with this group are briefly mentioned in the text of the article and detailed in the **Supporting Information.**

Discrete variables are shown as counts (percentage) and continuous variables as mean (standard deviation). Non-normally distributed variables are summarized by the median (interquartile range) and were log-transformed for some statistical analyses and for graphical comparisons. In univariate statistical comparisons, the Chi-square test was used for categorical variables, while the Student's t-test or ANOVA were used for normal continuous variables and the Wilcoxon signed rank test or the

Kruskall-Wallis test for continuous variables not normally distributed. In all statistical analyses, significance was set at $p<0.05$.

Results

Characterization of SI in patients with AD. Relationship with ACLF.

The patient characteristics were similar to those of the whole CANONIC cohort (1) (**Supplementary Table 3**). Active alcoholism and bacterial infections were the most frequent PEs of ACLF, although in approximately 40% of patients no PE was identified. The presence and severity of ACLF and prognosis correlated closely with WBC and CRP levels. The prevalence of AAH (47 patients) among the 522 patients studied was only of 9% (65% among the 72 patients with active alcoholism). AAH was significantly more frequent in patients with ACLF (12.2%) than in those without ACLF (6.3%) ($p<0.05$). Only 78 patients (14.9%) showed SIRS. SIRS was significantly more frequent ($p<0.05$) in patients with bacterial infection (23.6% vs 11.9%), AAH (25.5% vs 13.9%) or ACLF (19.9% vs 10.9%).

Tables 1 and 2 show the results obtained with the 17 cytokines with detectable values in more than 70% of the patients. They were categorized as either cytokines with predominant inflammatory properties (TNFα, IL-6, IL-8, MCP-1, IP-10, MIP-1β, G-CSF and GM-CSF) in the innate immune system; cytokines with predominant anti-inflammatory properties (IL-10 and IL-1ra) or cytokines with other actions including cytokines with predominant activity in the adaptive immune system (IFNγ, IFNα2, IL-17a and IL-7), cytokines with predominant chemotactic activity on eosinophils (eotaxin) and cytokines that function as growth factors (EGF and VEGF) (**Supplementary Table 4**).

As compared to healthy controls, patients with cirrhosis showed markedly increased levels of all cytokines (**Table 1**). Patients presenting ACLF exhibited significantly higher levels of pro-inflammatory cytokines than patients without ACLF. The anti-inflammatory cytokines IL-10 and IL-1ra followed a similar pattern. In contrast, differences in the plasma levels of the remaining cytokines were slight or not significant. No significant differences in plasma cytokine levels at ACLF diagnosis were observed between patients with ACLF at enrolment and those who developed ACLF after enrolment (data not shown).

There was a clear direct relationship between the intensity of SI and severity of ACLF as indicated by a parallel and significant increase in IL-6, IL-8 and IL-1ra from ACLF grade I to ACLF grades II and III (**Table 2**).

The IL-8/IL-10 and IL-6/IL-10 ratios were determined as markers of the pro-inflammatory/anti-inflammatory cytokine response (**Supplementary Figure 1**). The two ratios were significantly higher in patients with cirrhosis than in healthy subjects. No significant differences between patients with and without ACLF were observed.

Supplementary Tables 5 and 6 show the results obtained with the cytokines showing detectable values in less than 70% of patients. All cytokines included in this analysis were also significantly higher in patients with decompensated cirrhosis with and without ACLF than in healthy controls (**Supplementary Table 5)**. However, significant differences between patients with and without ACLF were observed only in the inflammatory cytokine MIP-1α (higher in patients with ACLF). There were no significant differences in any of the cytokines included in this analysis across the three grades of severity of ACLF **(Supplementary Table 6).**

The profile of the 17 cytokines included in the main analysis varied according to the PE in patients with ACLF (**Table 3**). ACLF precipitated by bacterial infection showed

higher levels of TNFα, IL-6 and IL-1ra than ACLF precipitated by active alcoholism (p=0.004; p=0.02; and p=0.0269, respectively) or ACLF associated with other PEs or without PE (p=0.04; p=0.0002; and p=0.02, respectively). ACLF precipitated by active alcoholism showed higher levels of IL-8 than ACLF precipitated by bacterial infection (p=0.0001) or ACLF associated with other PEs or without PE (p=0.001). The cytokine profile in ACLF precipitated by both bacterial infections and active alcoholism shared the characteristics of ACLF precipitated by infection and active alcoholism alone. In ACLF patients with other PEs or without PE no cytokine showed significantly increased levels in comparison to the other groups of patients with ACLF.

Supplementary Table 7 shows that the profile associated with active alcoholism was due to a great extent to the extremely high values of IL-8 observed in patients with AAH. However, the plasma levels of IL-8 were also higher in patients with active alcoholism without AAH than in those without active alcoholism (**Supplementary Table 7**). This increase in the plasma levels of IL-8 observed in patients with alcoholic cirrhosis, active alcoholism but no AAH was not detected in alcoholic cirrhotic patients who stopped drinking (**Supplementary Table 7**).

Supplementary Table 8 shows the cytokine profile in patients with and without SIRS. Patients with SIRS showed significantly higher plasma levels of the pro-inflammatory cytokines IL-6, IL-8, MCP-1, MIP-1β and G-CSF and of the anti-inflammatory cytokines IL-10 and IL-1ra than patients without SIRS.

HNA1+HNA2 and HNA2 were markedly increased (p<0.001) in cirrhotic patients with and without ACLF as compared to healthy controls (**Table 1**). Values for both HNA1+HNA2 and HNA2 were significantly higher in patients with ACLF than in those without (**Table 1**). There were no significant changes in the redox state of albumin across the three ACLF grades (**Table 2**).

SCD in patients with and without ACLF.

As shown in **Table 1**, PRC and PCC were markedly above normal range in patients with and without ACLF but values of these markers were significantly higher in the former than in the latter group. PCC was significantly higher in patients with ACLF-RF [48.7 (19.8-81.1)] than in patients with ACLF without renal failure [25.1(10.2-44.8) pmol/L; p<0.001]. In contrast, no significant differences in PRC were observed between these two groups [112 (35-276) vs 179 (33-460) microIU/ml; p=0.285]. These results indicate that PCC is a poor marker of SCD in patients with ACLF-RF since it reflects not only an increased release of the ADH precursor by the neurohypophysis as a consequence of SCD but also an impaired renal clearance of copeptin. PCC values in patients with ACLF without renal failure were significantly higher than those observed in the group of patients without ACLF [25.1(10.2-44.8 vs 9 (3-23) pmol/L; p<0.001)] indicating that ADH secretion is markedly increased in ACLF. No significant changes in PRC and PCC were observed across the three ACLF grades (**Table 2**).

Strength of the association of markers of SI and of SCD with the frequency and severity of ACLF or ACLF-RF at enrolment and the course of ACLF during hospitalization.

IL-8, IL-6 and HNA2, the SI markers more closely associated with the presence and severity of ACLF at enrolment (**Tables 1 and 2**), and PRC were analyzed for this purpose. PCC was not included in this analysis due to its low ability for the assessment of SCD in patients with ACLF-RF.

Figure 1 shows the distribution of patients with (red dots) and without (green dots) ACLF according to baseline values of PRC and IL-8 (left) or HNA2 (right) in the whole series. Patients with ACLF were predominantly distributed in the right part of the

figures (higher degree of SI, upper panels) and the strength of association of ACLF with both SI markers was greater than that with PRC (lower panels). Similar pattern of distribution was obtained with IL-6 (**Supplementary Figure 2**), with IL-8, IL-6 (not shown) and HNA2 after excluding patients with active alcoholism **(Supplementary Figure 3)** or with IL-8, IL-6 (not shown) and HNA2 when only patients with ACLF-RF were considered (**Supplementary Figure 4).**

Among the patients with ACLF, only 2 (1.7%) showed normal values (below the 95th percentile of the distribution in healthy subjects) of IL-6 and none had normal values of IL-8. In contrast, 17.7% showed normal PRC values (p<0.001) and 8.0% presented normal HNA2 values (p<0.001) (**Figure 2A**). The corresponding percentages in relation to patients with ACLF-RF were 0%, 0.8%, 16.4% and 8%, respectively (p<0.001) (**Figure 2B**). The corresponding values after excluding patients with active alcoholism are represented in **Supplementary Figure 5**.

The logistic regression model showed that the presence of ACLF was independently associated with IL-6, IL-8 and HNA2 but not with PRC (log PRC: RR 1.05, CI: 0.94-1.16, p=0.413; log IL-8: RR: 1.73, CI: 1.40-2.14, p<0.001; log IL-6: RR: 1.33, CI: 1.12-1.57, p<0.001; log HNA2: RR: 2.78, CI: 2.07-3.71, p<0.001). Only IL-8 and HNA2 were found to be significantly associated with the presence of ACLF-RF (RR: 1.45, 95%CI: 1.17-1.80, p<0.00 and RR: 2.80, 95%CI: 1.98-3.95, p<0.001, respectively).

The stepwise linear regression model fitted for CLIF-C Organ Failure Score based on the significance of the increase in model R-Square allowed to select HNA2 (p<0.0001) and both cytokines (IL-8, p<0.0001; IL-6, p=0.0008) as the markers most strongly related to the severity of ACLF. Similarly the stepwise model fitted for serum creatinine allowed to select HNA2 (p<0.0001) and IL-6 (p=0.0114) as the markers

more strongly related to the severity of renal failure. In both models, PRC did not contribute significantly to improve the final R-Square.

There was a significant association between changes in markers of SI during hospitalization and the course of ACLF **(Table 4)**. Improvement of ACLF was associated with decrease whereas worsening of ACLF was associated with increase in IL-6, IL-8 and HNA2. Inflammatory markers did not change in the steady form of ACLF or in patients with no ACLF throughout the study. In contrast, no relationship was observed between changes in PRC and the clinical course of ACLF

SI and mortality.

The strength of association between SI, SCD and short-term mortality were also assessed considering IL-8, IL-6, HNA2 and PRC values. Baseline values of IL-8, IL-6 (not shown) and HNA2 but not of PRC were strongly associated with 28-day mortality (**Figure 3**) and 90-day mortality (**Supplementary Figure 6**). Similarly, there was a significant association between the course of SI during hospitalization and the 28-day and 90-day mortality (**Table 5**). One hundred and three patients had CRP data available at enrolment and at the last follow-up assessment. Changes in CRP from enrolment in those patients who survived at 28 (median -4.0, IQR -13.1-1.6) and 90 days (-2.4, -11.1-1.9) were not significantly different from those corresponding to patients who died (-1.3, -19.6-3.7 and -7.1, -20.7-0; p-values 0.4918 and 0.2044, respectively). Twenty-eight-day and 90-day mortality rates were also significantly associated ($p<0.0001$) with SIRS at enrolment (23 out of 78 patients with SIRS, 29.5%, and 47 out of 433 without SIRS, 10.6%, died within 28-day after enrolment; at 90 days; the mortality rates were 42.9% and 21.6%, respectively).

Eighteen patients showed extremely high levels of IL-8 (>800 UI/ml) and/or IL-6 (>1000 UI/ml) at enrolment (**Figure 2**). The prevalence of severe forms of ACLF

(grades 2 or 3) and the 28-day mortality rate in this group of patients (39% and 28%%, respectively) was higher (p= 0.0833 and p=0.0774) than in the rest of the patients (21% and 13%, respectively).

Discussion

The discussion of the article is divided in four parts: 1. Role of SI in the pathogenesis and clinical course of AD and ACLF; 2. Cytokine response; 3. Mechanisms of organ failure in ACLF; 4. Relationship between SI and mortality.

The first part of the analysis (Tables 1-3) strongly suggests that SI is a major mechanism of AD and ACLF and supports most of the proposals of the SI Hypothesis: 1. AD of cirrhosis occurs in the setting of severe SI and oxidative stress; 2. SI is significantly more severe in patients with ACLF than in patients without ACLF. Interestingly, the reported levels of IL-6, IL-8 and TNFα in patients with severe sepsis, a condition also associated with organ failure, are similar to those found in our patients with ACLF (23-25); 3. Severity of SI correlates closely with severity of ACLF; 4. Finally, the course of SI during hospitalization is strongly associated with the course of AD and ACLF (development of ACLF during hospitalization in patients without ACLF at enrolment or improvement, steady course or worsening of ACLF in patients with ACLF at enrolment).

The characterization of cytokine response in our patients (second part of the analysis; Tables 1-3, Supplementary Table 7 and Supplementary Figure 1) disclosed interesting features. Whereas all the 17 cytokines and chemokines included in the main analysis were significantly increased in patients with AD with respect to healthy controls, only those involved in innate immune response were clearly ‘upregulated’ in patients with ACLF with respect to patients without ACLF. Indeed, we found that patients with ACLF had a predominance of pro-inflammatory cytokines (i.e., TNFα

and IL-6) and chemokines (IL-8, MCP-1, IP-10 and MIP-1β). These patients also had increased levels of G-CSF and GM-CSF, key regulatory cytokines that target committed progenitors to promote differentiation and activation of monocytes and neutrophils (26). In contrast, among other cytokines that are involved in the activation and shaping of the adaptive immune system (IFNγ, IL-17a and IL-7), IFNγ was only slightly higher in patients with ACLF than in those without. These findings suggest that whereas activation of both the innate and adaptive immune systems participates in the SI associated with AD, a dysregulated innate immune response might be the predominant mechanism in the progression of AD to ACLF.

The assessment of the overall cytokine profile showed that not only pro-inflammatory molecules but also major anti-inflammatory cytokines such as IL-10 and IL-1ra were increased in patients with AD with and without ACLF indicating a generalized activation of the inflammatory cytokines. There was, however, a clear unbalance in favor of pro-inflammatory cytokines as indicated by the significantly higher IL-6/IL-10 and IL-8/IL-10 ratios observed in patients with and without ACLF as compared to healthy subjects.

Another outstanding feature was the observation of clear differences in cytokine profile of patients with ACLF according to the type of PE. Patients with active alcoholism as PE exhibited very high IL-8 levels, as described previously (27,28). Such increase in IL-8 was observed both in patients with and without AAH, although the grade of increase was greater in the former group than in the second. Patients with bacterial infection had a characteristic increase of a set of inflammatory cytokines. Finally, patients with other or without PE had no differential increase in any cytokine with respect to patients with active alcoholism or bacterial infection as PE. These data suggest specific mechanisms for SI depending on the underlying trigger

of ACLF. They also suggest that ACLF in patients with unidentifiable PE is unlikely related to unrecognized bacterial infection. Finally, they support a cause-to-effect relationship between PE, SI and ACLF development. This suggestion, however, does not exclude other mechanisms. In fact, dysfunctional or damaged organs by itself may stimulate cytokine and reactive oxygen species production (29) and, therefore, contribute to the development and/or progression of ACLF.

It is important to note that increased plasma levels of G-CSF, GM-CSF, TNFα, IL-6 and IFNγ have been shown to participate in the process called 'emergency hematopoiesis' which develops in the context of SI (30,31). Therefore, the activation of these cytokines found in patients with ACLF probably contributes to the leukocytosis associated with this syndrome and explain the close relationship between WBC, ACLF severity and patient prognosis (1,3).

The mechanism by which SI induces ACLF was explored in the third part of the analysis (Figures 1 and 2, Supplementary Figures 2, 3, 4 and 5 and logistic regression and stepwise linear regression models). Renal failure, is a well-recognized complication in patients with cirrhosis and bacterial infections (32), and evidences have been presented suggesting that it may be related to a sequence of events initiated by an exaggerated systemic inflammatory response that induces aggravation of splanchnic arterial vasodilation and impairment in cardiac output, homeostatic activation of endogenous neuro-hormonal systems to maintain arterial pressure, renal vasoconstriction and vasoconstriction in other organs (i.e. liver and brain), renal hypo-perfusion and renal failure (20,22). Our results showing that ACLF develops in the setting of an accentuation in the SI and SCD already present in patients with AD support that this sequence of events is also important in the pathogenesis of the syndrome.

However, several other findings obtained in the four analysis assessing the strength of association of SI and SCD with ACLF strongly suggest that the mechanism of ACLF is far more complex. 1. Whereas SI at enrolment (estimated by IL-6, IL-8 and HNA2) was strongly related with the frequency and severity of ACLF, this was not true for SCD (estimated by PRC); 2. The clinical course of ACLF was associated with parallel changes in the plasma levels of SI markers but not with PRC. 3. PRC was normal or slightly increased in many patients with ACLF or with ACLF-RF. In contrast, IL-6 and IL-8 were markedly increased in almost all these patients. 4. The logistic regression model assessing those markers (IL-8, IL-6, HNA2 and/or PRC) independently associated with the presence of ACLF or ACLF-RF and the linear regression model assessing the correlation of IL-8, IL-6, HNA2 and PRC with the severity of ACLF and of ACLF-RF showed significant relationships with IL-6, IL-8 and HNA2 but not with PRC. These results strongly suggest that non-hemodynamic mechanisms mostly mediate the deleterious effects of SI in organ function and participate in the pathogenesis of ACLF.

Three lines of evidence support this contention (33-38). The first includes investigations showing that the extension of SI into the organs [(i.e. as a consequence of extremely high circulating plasma levels of inflammatory mediators and perhaps also of pro-inflammatory molecules (PAMPS and DAMPS)] may lead to organ failure through direct deleterious effects on microcirculatory homeostasis, mitochondrial function and cell survival. The second includes investigations in patients and experimental animals with severe sepsis, a condition with many similarities with ACLF, showing that renal failure may occur in the setting of hyper-dynamic circulation and normal renal perfusion. Finally, the third derives from a recent study on trans-jugular kidney biopsies in 65 cirrhotic patients awaiting liver

transplantation. Among the numerous glomerular, vascular and acute or chronic tubulo-interstitial lesions found in these patients, only cortical and medullary infiltration by mononuclear cells and polymorphonuclear leukocytes associated with tubular cell injury was independently associated with the presence of renal failure (39). Therefore, as in severe sepsis, single or multi-organ failure in cirrhosis is probably the result a complex process involving numerous mechanisms including impairment is systemic circulatory and local microcirculatory functions, microcirculatory thrombosis, mitochondrial and cell dysfunction and cell death.

A final important observation was that IL-8, IL-6 and HNA2 at enrolment and their follow-up changes during hospitalization were directly associated with 28-day and 90-day mortality (fourth part of the analysis, Figure 3, Supplementary Figure 6 and Table 5). Patients who died showed significantly higher baseline plasma levels of these mediators than those who survived. Moreover, the plasma concentrations of IL-6, IL-8 and HNA further increased during hospitalization in the former and decreased in the latter group of patients. Sequential measurements of inflammatory mediators could therefore be a sensitive tool to assess treatment effectiveness and to predict prognosis in patients with ACLF.

In summary, the current study supports the SI hypothesis of AD and ACLF in cirrhosis. 1. AD occurs in the setting of very high plasma levels of cytokines and oxidized albumin; 2. ACLF develops when there is a further increase in these inflammatory mediators; 3. Although ACLF frequently occurs in the setting of severe SCD, inflammatory mediators are more strongly associated with the frequency, severity and clinical course of ACLF than PRC, indicating that both hemodynamic and non-hemodynamic mechanisms are important in the pathogenesis of ACLF. Different profiles of cytokine response were observed. Whereas SI in AD was

associated with very high levels of cytokines involved in both the innate and adaptive immune systems, cytokines associated with ACLF were mainly cytokines involved in the innate immune response. Different profiles of cytokine response were also identified according to PEs of ACLF. Severity of SI at enrolment and progression or regression of SI during hospitalization were closely associated with short-term prognosis. Sequential measurement of inflammatory mediators may, therefore, be useful as predictors of treatment effectiveness and patient prognosis.

REFERENCES

1. Moreau R, Jalan R, Gines P, Pavesi M, Angeli P, Cordoba J, et al. Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis. Gastroenterology 2013;144:1426-1437.
2. Gustot T, Fernández J, García E, et al. Clinical course of acute-on-chronic liver failure syndrome and effects on prognosis. Hepatology; 2015:62:243-52.
3. Bernardi J, Moreau R, Angeli P, Schnable B, Arroyo V. Mechanism of decompensation and organ failure in cirrhosis. From peripheral arterial vasodilation to systemic inflammation hypothesis. J Hepatol 2015;63:1272-1284.
4. Byl B, Roucloux I, Crusiaux A, Dupont E, Devière J. Tumor necrosis factor alpha and interleukin 6 plasma levels in infected cirrhotic patients. Gastroenterology 1993;104:1492-7.
5. Albillos A, de la Hera A, González M, et al. Increased lipopolysaccharide binding protein in cirrhotic patients with marked immune and hemodynamic derangement. Hepatology 2003;37:208-17.
6. Wasmuth HE, Kunz D, Yagmur E, et al. Patients with acute on chronic liver failure display "sepsis-like" immune paralysis. J Hepatol. 2005;42:195-201.
7. Bernsmeier C, Pop OT, Singanayagam A, et al. Patients with acute-on-chronic liver failure have increased numbers of regulatory immune cells expressing the receptor tyrosine kinase MERTK. Gastroenterology 2015;148:603-615.
8. Jalan R, Saliba F, Pavesi M, et al. Development and validation of a prognostic score to predict mortality in patients with acute-on-chronic liver failure. J Hepatol 2014;61:1038-47.

9. Crabb DW, Bataller R, Chalanasi NP, Kamath PS, Lucey M, Mathurin P, McClain C et al Standard definitions and common data elements for clinical trials: recommendations from the NIAAA Alcoholic Hepatitis Consortia. Gastroenterology 2016;150:785-790.
10. Seymour CW, Liu VX, Iwashyna TJ et al. Assessment of Clinical criteria of sepsis: For the third International Consensus Definitions for Sepsis and Septic Shock (Spesis-3). JAMA 2016;315:762-774).
11. Bruschi M, Candiano G, Santucci L, et al. Oxidized albumin. The long way of a protein of uncertain function. Biochim Biophys Acta 2013;1830:5473-9.
12. Anraku M, Chuang VT, Maruyama T, et al. Redox properties of serum albumin. Biochim Biophys Acta 2013;1830:5465-72.
13. Halliwell B, Gutteridge JM. The antioxidants of human extracellular fluids. Arch Biochem Biophys. 1990;280:1-8.
14. Imai H, Hayashi T, Negawa T, et al. Strenuous exercise-induced change in redox state of human serum albumin during intensive kendo training. Jpn J Physiol. 2002;52:135-40.
15. Era S, Kuwata K, Imai H, et al. Age-related change in redox state of human serum albumin. Biochim Biophys Acta 1995;1247:12-6.
16. Oettl K, Marsche G. Redox state of human serum albumin in terms of cysteine-34 in health and disease. Methods Enzymol 2010;474:181-95.
17. Anraku M, Kitamura K, Shintomo R, et al. Effect of intravenous iron administration frequency on AOPP and inflammatory biomarkers in chronic hemodialysis patients: a pilot study. Clin Biochem. 2008;41:1168-74.
18. Kadota K, Yui Y, Hattori R, et al. Decreased sulfhydryl groups of serum albumin in coronary artery disease. Jpn Circ J. 1991;55:937-41.

19. Kadowaki D, Anraku M, Tasaki Y, et al. Effect of olmesartan on oxidative stress in hemodialysis patients. Hypertens Res. 2007;30:395-402.
20. Schrier RW, Arroyo V, Bernardi M, et al. Peripheral arterial vasodilation hypothesis: a proposal for the initiation of renal sodium and water retention in cirrhosis. Hepatology 1988;8:1151-1157.
21. Bolignano D, Cabassi A, Fiaccadori E, et al. Copeptin (CTproAVP), a new tool for understanding the role of vasopressin in pathophysiology. Clin Chem Lab Med. 2014;52:1447-56.
22. Arroyo V, Fernandez J. Management of hepatorenal syndrome in patients with cirrhosis. Nat Rev Nephrol 2011;7:517-526.
23. Kragsbjerg P, Holmberg H, Vikerfors T. Dynamics of blood cytokine concentrations in patients with bacteremic infections. Scand J Infect Dis. 1996;28:391-8.
24. Casey LC1, Balk RA, Bone RC. Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. Ann Intern Med. 1993;119:771-8.
25. Pinsky MR1, Vincent JL, Deviere J, et al. Serum cytokine levels in human septic shock. Relation to multiple-system organ failure and mortality. Chest. 1993;103:565-75.
26. Bendall LJ, Bradstock KF. G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent. Cytokine Growth Factor Rev. 2014;25:355-67.
27. Huang YS1, Chan CY, Wu JC, et al. Serum levels of interleukin-8 in alcoholic liver disease: relationship with disease stage, biochemical parameters and survival. J Hepatol. 1996;24:377-84.

28. Dominguez M, Miquel R, Colmenero J, et al. Hepatic expression of CXC chemokines predicts portal hypertension and survival in patients with alcoholic hepatitis. Gastroenterology 2009;136:1639-50.
29. Abraham E., Singer M. Mechanisms of sepsis-induced organ dysfunction. Crit. Care Med. 2007;35:2408–2416.
30. Rickard JA, O'Donnell JA, Evans JM, et al. RIPK1 regulates RIPK3-MLKL-driven systemic inflammation and emergency hematopoiesis. Cell 2014;157:1175-88.
31. Croker BA, Silke J, Gerlic M. Fight or flight: regulation of emergency hematopoiesis by pyroptosis and necroptosis. Curr Opin Hematol. 2015;22:293-301.
32. Jalan R, Fernandez J, Wiest R, et al. Bacterial infections in cirrhosis: a position statement based on the EASL Special Conference 2013. J Hepatol. 2014;60:1310–1324.
33. Prowle JR, Bellomo R. Sepsis associated acute kidney injury: Macrohemodynamic and microhemodynamic alterations in the renal circulation. Semin Nephrol 2015;35:64-74
34. Zafrani L, Payen D, Azoulay E, Can I. The microcirculation of the Septic kidney. Semin Nephrol 2015; 35: 75.
35. Alobaidi R, Basu RK, Goldstein SL, Bagshaw SM. Sepsis associated acute kidney injury. Semin Nephrol 2015;35:2-11.
36. Parikh SM, Yang Y, He L, Tang C, Shan M, Dong Z. Mitochondrial function and disturbances in the septic kidney. Semin Nephrol 2015;35:108-119

37. Gomez H, Ince C, De Backer D, et al. A unified theory of sepsis-induced acute kidney injury: inflammation, microcirculatory dysfunction, bioenergetics, and the tubular cell adaptation to injury. Shock 2014;41:3-11.

38. Chovatiya R, Medzhitov R. Stress, inflammation, and defense of homeostasis. Mol Cell 2014;54:281-288.

39. Trawale JM, Paradis V, Rautou PE, et al. The spectrum of renal lesions in patients with cirrhosis: a clinicopathological study. Liver Int; 2010; 30:725-732.

Acknowledgements

The authors of the paper thank the nursing staff in the units of each center for their excellent support. They also thank the patients for their participation in the study, and Nicki van Berckel for her administrative support. We would like to thank the IDIBAPS Biobank for careful assistance in the handling of samples.

A list of CANONIC Study Investigators is provided in the Appendix.

Figure Legends

Figure 1. Upper Panels (A and B). Scatter plots of individual patients classified by the presence (red dots) or absence (green dots) of ACLF according to individual values of plasma renin concentration (PRC) and interleukin-8 (IL-8) (left) or human nonmercaptalbumin-2 (HNA2) (right). Two cut-off points for each marker divide each cohort into 3 tertiles (T1: lower values; T2: intermediate values; T3: higher values). Patients with ACLF are predominantly in the right part of the panels (higher degree of SI). Lower Panels (C and D) show the presence of ACLF across the three tertiles of both PRC and IL-8 and PRC and HNA2. Differences in the prevalence of ACLF within tertiles (T2 versus T1 and T3 vs T2) were more significant with IL-8 and HNA2 than with PRC.

Figure 2. Scatter plots of individual values of plasma renin concentration (PRC), interleukin-8 (IL-8), interleukin-6 (IL-6) and human nonmercaptalbumin-2 (HNA2) in patients with ACLF (A) and with ACLF associated with renal failure (B). Horizontal lines represent the upper normal limits (95% percentile of the distribution of values in healthy subjects).

Figure 3. Upper Panels (A and B). Scatter plots of patients who died (red dots) or survived (green dots) at 28-day after enrolment according to individual values of plasma renin concentration (PRC) and interleukin-8 (IL-8) (left) or human nonmercaptalbumin-2 (HNA2) (right). Two cut-off points for each marker divide each cohort into 3 tertiles (T1: lower values; T2: intermediate values; T3: higher values). Patients who died are predominantly in the right part of the panels (higher degree of SI). Lower Panels (C and D) show the mortality rates observed within each tertile of

both PRC and IL-8 and PRC and HNA2. Differences in mortality rates within tertiles (T2 vs T1 and T3 vs T2) were statistically significant only with IL-8 and HNA2.

Table 1. Plasma concentrations of renin (PRC), copeptin (PCC) and cytokines and albumin oxidation fractions in healthy subjects and in patients with and without ACLF.

	Healthy controls N=40	No ACLF N=285	ACLF N=237	p-value*
Markers of SCD				
PRC (micro IU/ml)	8(6-17)	65(17-242)	134(36-378)	<0.001
PCC (pmol/L)	0(0-10)	9(3-23)	31(13-61)	<0.0001
Pro-inflammatory Cytokines				
TNFα (pg/ml)	9(7-12)	20(14-27)	29(17-41)	<0.001
IL-6 (pg/ml)	0.3(0.3-0.3)	21(11-41)	39(17-115)	<0.001
IL-8 (pg/ml)	1.6(0.6-3.3)	37(20-76)	84(41-169)	<0.001
MCP-1 (pg/ml)	337(218-413)	318(228-436)	410(288-713)	<0.001
IP-10 (pg/ml)	328(234-428)	965(558-1676)	1184(665-2157)	0.004
MIP-1β (pg/ml)	13(6-17)	20(13-34)	28(19-50)	<0.001
G-CSF (pg/ml)	2.1(1.8-11)	23(11-50)	32(14-83)	0.001
GM-CSF (pg/ml)	7.5(7.5-7.5)	4.7(2.0-9.5)	7.3(3.5-16.8)	<0.001
Anti-inflammatory Cytokines				
IL-10 (pg/ml)	1.1(0.4-1.1)	3.4(1.1-9.2)	8.1(2.1-29.9)	<0.001
IL-1ra (pg/ml)	7(3-9)	10(5-22)	23(9-63)	<0.001
Other Cytokines				
IFNγ (pg/ml)	0.8(0.8-4.9)	6(2-18)	7(3-24)	0.044
IFNα2 (pg/ml)	3(3-3)	22 (8-56)	27 (11-63)	0.113
Eotaxin (pg/ml)	94(55-122)	110(81-155)	124(89-179)	0.018
IL-17a (pg/ml)	0.7(0.7-2.7)	3.7(1.6-10.3)	4.5(1.6-15.6)	0.128
IL-7 (pg/ml)	1.4(0.1-3.9)	2.6(1.0-8.5)	3.5(1.6-11.1)	0.012
EGF (pg/ml)	17(4-29)	26 (9-66)	19(8-41)	0.046
VEGF (pg/ml)	26(26-28)	85 (28-226)	91(29-252)	0.745
Albumin Oxidation Fractions*				
HMA (%)	71(68-74)	53(42-62)	45(33-56)	<0.001
HNA1+HNA2 (%)	28(25-30)	46.4 (37.5-56.9)	51.8 (42.2-65.6)	<0.001
HNA2 (%)	1.3(0.3-1.9)	4.5(2.5-8.8)	9.8(5.6-14.8)	<0.001

Data are median (Inter-Quartille Range, IQR)

*P value between ACLF and NO ACLF.

** According to the redox state at cysteine 34

Table 2. Plasma concentrations of renin (PRC), copeptin (PCC) and cytokines and albumin oxidation fractions according to the grade of ACLF.

	ACLF – I N=126	ACLF – II N=86	ACLF – III N=25	p-value*
Markers of SCD				
PRC (micro IU/ml)	169(40-383)	114(28-352)	87(33-258)	0.771
PCC (pmol/L)	34(16.62)	27(13-45)	47(11.134)	0.224
Pro-inflammatory Cytokines				
TNFα (pg/ml)	30(21-43)	26(15-36)	32(17-43)	0.029
IL-6 (pg/ml)	34(18-96)	43(13-106)	111(32-355)	0.018
IL-8 (pg/ml)	62(37-112)	97(48-192)	144(80-292)	<0.001
MCP-1 (pg/ml)	412(299-633)	376(277-646)	660(322-1773)	0.089
IP-10 (pg/ml)	1218(717-2258)	1162(617-1946)	1689(899-2728)	0.267
MIP-1β (pg/ml)	27(18-43)	28(19-55)	46(20-61)	0.112
G-CSF (pg/ml)	32(15-70)	29(14-81)	39(15-209)	0.673
GM-CSF (pg/ml)	6.8(3.7-15.0)	7.5(2.7-20.1)	11.3(5.1-29.6)	0.512
Anti-inflammatory Cytokines				
IL-10 (pg/ml)	4.3(1.1-17.9)	15.3(5.5-41.5)	12.4(6.6-40.8)	<0.001
IL-1ra (pg/ml)	17(10-45)	26(8-63)	49(24-135)	0.019
Other Cytokines				
IFNγ (pg/ml)	7(3-24)	9(3-25)	7(2-16)	0.906
IFNα2 (pg/ml)	27(11-61)	25(12-74)	30(12-55)	0.968
Eotaxin (pg/ml)	124(90-178)	128(90-180)	103(87-176)	0.951
IL-17a (pg/ml)	3.4(1.6-13.3)	5.5(1.8-27.8)	8.1(1.5-15.2)	0.623
IL-7 (pg/ml)	3.5(1.7-8.0)	3.8(1.4-14.6)	3.5(2.1-6.9)	0.790
EGF (pg/ml)	20(9-51)	19(7-38)	14(7-25)	0.233
VEGF (pg/ml)	95(28-246)	77(27-278)	89(35-201)	0.950
Albumin oxidation fractions&				
HMA (%)	44(33-56)	45(32-58)	51(42-58)	0.621
HNA1+HNA2 (%)	52.9 (42.2-64.0)	51.3 (42.2-66.9)	48.7 (41.8-58.3)	0.781
HNA2 (%)	9.5(5.1-13.9)	9.8(5.6-15.1)	11.1(7.8-15.1)	0.205

Data are median (Inter-Quartille Range, IQR)

* Comparison between ACLF grades

& According to the redox state at cysteine 34

Table 3. Plasma concentrations of renin (PRC), copeptin (PCC) and cytokines and albumin oxidation fractions in patients with ACLF according to the presence and type of Precipitating Events (PEs).

	No PEs (n=94)	Bacterial infection-No active alcoholism (n=63)	Active alcoholism-No bacterial infection (n=28)	Bacterial infection-Active alcoholism (n=11)	Other Pes (n=19)	p-value*
Markers of SCD						
PRC (micro IU/ml)	151 (50-474)	164 (28-447)	92 (31-295)	205 (112-998)	69 (25-210)	0.3021
PCC (pmol/L)	31 (15-59)	38 (16-64)	24 (7-36)	33 (4-112)	34 (16-55)	0.4654
Pro-inflammatory Cytokines						
TNFα (pg/ml)	29 (17-41)	32 (26-47)	21 (14-32)	34 (18-53)	27 (20-35)	0.0256
IL-6 (pg/ml)	30 (14-69)	72 (28-358)	37 (13-122)	83 (34-466)	34 (21-104)	0.0002
IL-8 (pg/ml)	64 (38-104)	92 (47-167)	211 (141-351)	158 (99-310)	50 (28-92)	<0.0001
MCP-1 (pg/ml)	372 (279-484)	512 (299-1072)	515 (361-846)	832(294-1024)	342 (220-554)	0.0143
IP-10 (pg/ml)	1349 (717-2120)	1053 (631-2479)	882 (580-2265)	1596 (482-1996)	1153 (646-2215)	0.7356
MIP-1β (pg/ml)	24 (16-51)	30 (21-49)	28 (18-61)	43 (32-55)	26 (15-33)	0.2005
G-CSF (pg/ml)	29 (12-69)	39 (17-89)	32 (14-64)	35 (19-434)	31 (11-179)	0.4916
GM-CSF (pg/ml)	6.1 (3.2-14.4)	11.9 (3.3-32.5)	6.7 (4.2-14.4)	9.4 (3.8-17.4)	5.4 (2.6-12.5)	0.1754
Anti-inflammatory Cytokines						
IL-10 (pg/ml)	6.2 (1.9-25.8)	17.8 (4.7-55)	8.3 (1.0-22.9)	24.5 (5.9-40.2)	5.1 (1.9-12.3)	0.0790
IL-1ra (pg/ml)	19 (8-47)	41 (13-100)	16 (8-49)	25 (14-37)	16 (9-33)	0.1666
Other Cytokines						
IFNγ (pg/ml)	7 (2-27)	6 (3-16)	11 (3-24)	8 (4-17)	2 (1-28)	0.4394
IFNα2 (pg/ml)	23 (9-54)	41 (20-97)	19 (5-55)	30 (11-57)	17 (8-29)	0.0447
Eotaxin (pg/ml)	122 (91-176)	130 (88-180)	131 (89-214)	91 (67-178)	93 (79-116)	0.0782
IL-17a (pg/ml)	3.7 (1.3-22.7)	4.4 (1.9-13)	2.9 (1.6-14.4)	8.1 (5.4-28.7)	2.8 (1.5-11.8)	0.5340
IL-7 (pg/ml)	3.0 (1.6-8.2)	5.8 (2.1-11.1)	2.7 (1.2-7.5)	6.4 (2.2-18.5)	2.2 (0.7-11.9)	0.0966
EGF (pg/ml)	17 (9-48)	20 (9-32)	14 (6-33)	20 (9-26)	16 (3-62)	0.9015
VEGF (pg/ml)	97 (34-286)	109 (28-240)	84 (27-127)	91 (56-148)	62 (14-299)	0.7647
Albumin Oxidation Fractions&						
HMA (%)	44 (32-55)	44 (32-55)	49 (39-57)	32 (19-49)	48 (35-63)	0.2151
HNA1+HNA2 (%)	53.5 (42.6-65.7)	53.6 (41.8-66.7)	50.3 (42.8-59.6)	68.2 (50.6-80.9)	51.6 (37.4-64.7)	0.2489
HNA2 (%)	9.6 (5.1-14.8)	12.3 (8.0-15.3)	8.6 (7.0-13.3)	10.3 (6.5-14)	7.0 (3.9-10.7)	0.0682

Data are median (Inter-Quartile Range, IQR)

* p-value overall

& According to the redox state at cysteine 34

Table 4. Relationship between ACLF course, plasma concentrations of renin (PRC) and inflammation markers.

Parameter	n	Enrolment	Last assessment	p-value*
PRC (micro IU/ml)				
Improvement **	71	113.5 (27.5 – 294.3)	82.9 (23.3 – 330.4)	0.9187
No Change **	26	93.1 (57.6 – 362.5)	262.3 (87.0 – 1212)	0.8450
Worsening **	40	168.6 (81.7 – 1508.0)	259.7 (44.3 – 3023.0)	0.7415
Non-ACLF patients #	96	111.5 (29.0-284.1)	110.0 (59.5-251.4)	0.9188
IL-6 (pg/ml)				
Improvement	71	32.2 (13.1 – 104.4)	25.5 (12.2 – 46.7)	0.0002
No Change	26	66.8 (25.2 –125.5)	50.1 (19.8 –117.7)	0.8589
Worsening	41	34.3 (17.7 – 111.2)	75.9 (20.6 – 134.3)	0.0744
Non-ACLF patients	87	25.0 (13.0-42.0)	16.5 (9.6-33.5)	0.0012
IL-8 (pg/ml)				
Improvement	72	89.5 (36.7 – 174.3)	56.7 (35.6 – 119.1)	0.0003
No Change	26	118.8 (65.4 – 209.6)	186.8 (72.7 – 268.0)	0.8450
Worsening	41	82.7 (48.9 – 136.7)	116.0 (52.9 – 198.6)	0.1196
Non-ACLF patients	98	40.8 (22.7-75.4)	38.7 (22.4-68.7)	0.6137
HNA2 (%)				
Improvement	72	9.6 (5.3 – 14.1)	8.9 (5.0 – 13.4)	0.2215
No Change	25	15.1 (11.0 – 20.3)	15.8 (11.2- 20.5)	0.6383
Worsening	41	10.7 (8.1 – 13.3)	13.2 (9.6 – 18.4)	0.0020
Non-ACLF patients	98	6.3 (3.3-9.4)	6.1 (2.1-11.7)	0.3099

Data are median (Inter-Quartille Range, IQR)

** Wilcoxon Signed Rank Sum Test.*

*** "Improvement"/ "Worsening": reduction/ increase of ≥1 grade from enrolment in ACLF classification. "No change": same classification as at enrolment in ACLF patients.*

 # Non-ACLF patients: patients without ACLF throughout the whole study

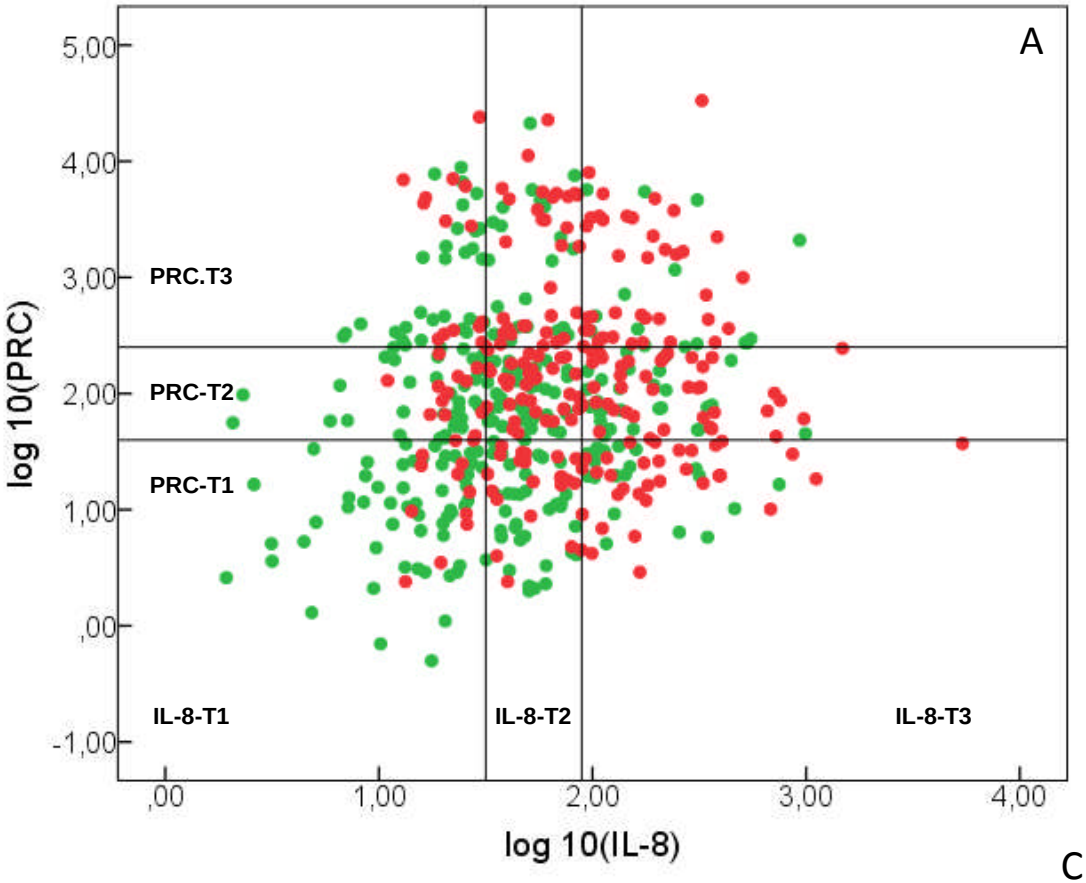
Table 5. Changes from enrolment (median and inter-quartile range) in plasma concentrations of renin (PRC) and inflammation markers according to mortality at 28 and 90 days after enrolment.

		28-day Survivors				28-day Deaths		
Marker	**N**	**Enrolment**	**Last assessment**	**Change**	**N**	**Enrolment**	**Last assessment**	**Change**
PRC (micro IU/ml)	138	116.7 (30.1-332.5)	119.8 (27.8-407)	-6.8 (-113.6-62.2)	37	99.0 (32.5-353.1)	201.9 (57.7-1156)	25.3 (-51.8-317.2)
IL-6 (pg/ml)	137	32.2 (15.0106.3)	26.3 (13.5-58.8)*	-5.3 (-36.9-10.5)	38	51.5 (21.2-173.1)	63.3 (31.4-168.9)	19.3 (-43.4-68.6) ##
IL-8 (pg/ml)	139	71.7 (35.6-153.7)	65.9 (36.0-125.0)*	-3.8 (-41.7-16.3)	38	107.8 (74.8-246.4)	147.3 (79.2-260.7)	6.7 (-42.1-62.2)
HNA2 (%)	139	9.5 (5.6-14.9)	10.7 (6.5-15.3)	-0.1 (-3.4-3.3)	36	12.9 (8.5-16.5)	14.1 (9.5-18.8) *	2.4 (-1.4-6.4) #
		90-day Survivors				**90-day Deaths**		
Marker	**N**	**Enrolment**	**Last assessment**	**Change**	**N**	**Enrolment**	**Last assessment**	**Change**
PRC (micro IU/ml)	105	108.7 (26.4-282.0)	89.6 (23.2-378.2)	-0.2 (-87.9-56.5)	66	171.3 (40.9-1576)	246.8 (64.1-1212)	19.7 (-171.2-204.8)
IL-6 (pg/ml)	104	40.3 (15.3-120.1)	23.6 (11.7-49.8) **	-8.4 (-57.4-3.0)	67	31.9 (17.3-117.8)	48.9 (25.5-129.2) *	14.7 (-12.2-60.9) ##
IL-8 (pg/ml)	106	83.2 (35.6-157.6)	56.4 (35.3-105.4) *	-7.2 (-50.7-9.2)	67	84.7 (50.5-204.6)	124.2 (64.8-227.1)	9.6 (-717.1-77.0) ##
HNA2 (%)	106	9.4 (5.6-13.6)	9.3 (5.3-15.0)	0.03 (-3.3-3.1)	65	12.5 (7.4-16.4)	13.4 (9.4-17.4) *	1.4 (-2.5-6.4)

Data are median (Inter-Quartille Range, IQR)

(*) p<0.05 vs enrolment; (**) p<0.01 vs enrolment.

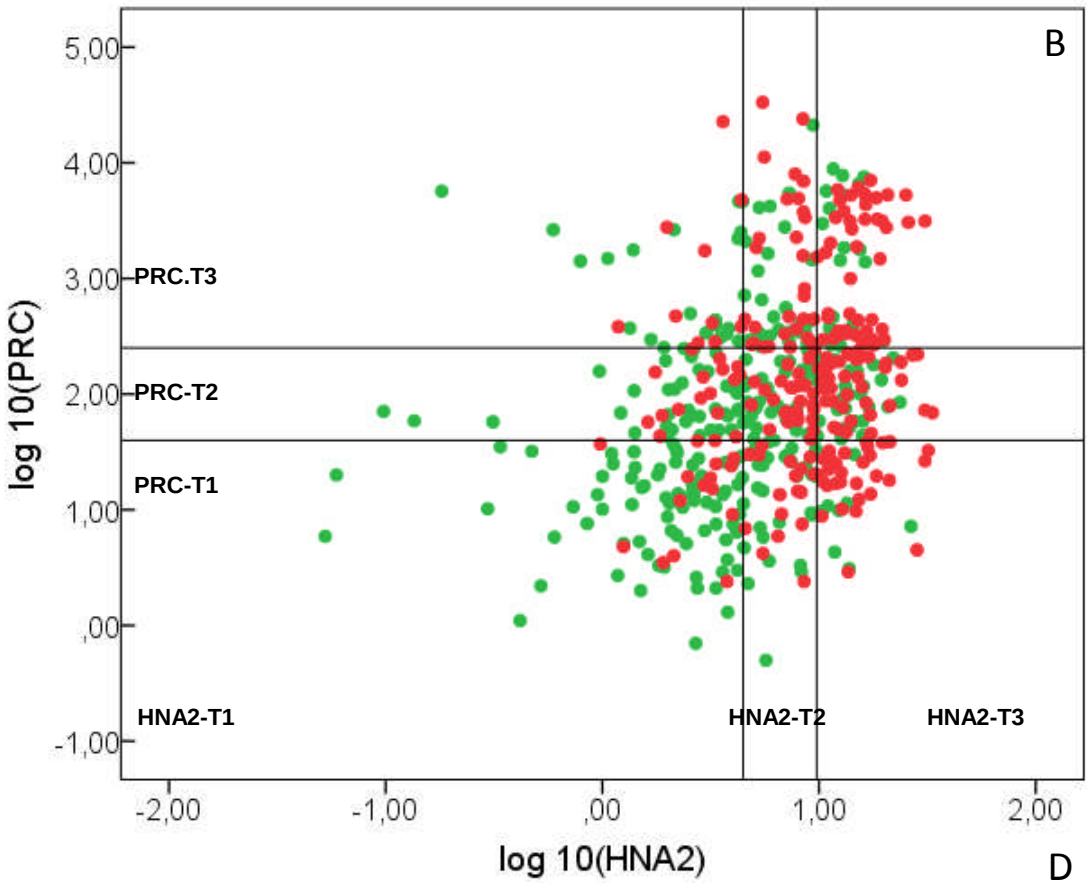
(#) p<0.05 vs change from baseline in survivors; (##) p<0.001 vs change from baseline in survivors.



C

	IL8-T1	IL8-T2	IL8-T3	Overall PRC
PRC-T3	17/53 (32%)	35/56 (62%)	47/64 (73%)	99/173 (57%)*
PRC-T2	14/47 (30%)	27/61 (44%)	34/60 (57%)	75/168 (45%)
PRC-T1	11/67 (16%)	20/53 (38%)	30/50 (60%)	61/170 (36%)
Overall IL-8	42/167 (25%)	82/170 (48%)#	111/174 (64%)#	n=511

***p<0.05 vs previous tertile; #p<0.01 vs previous tertile**



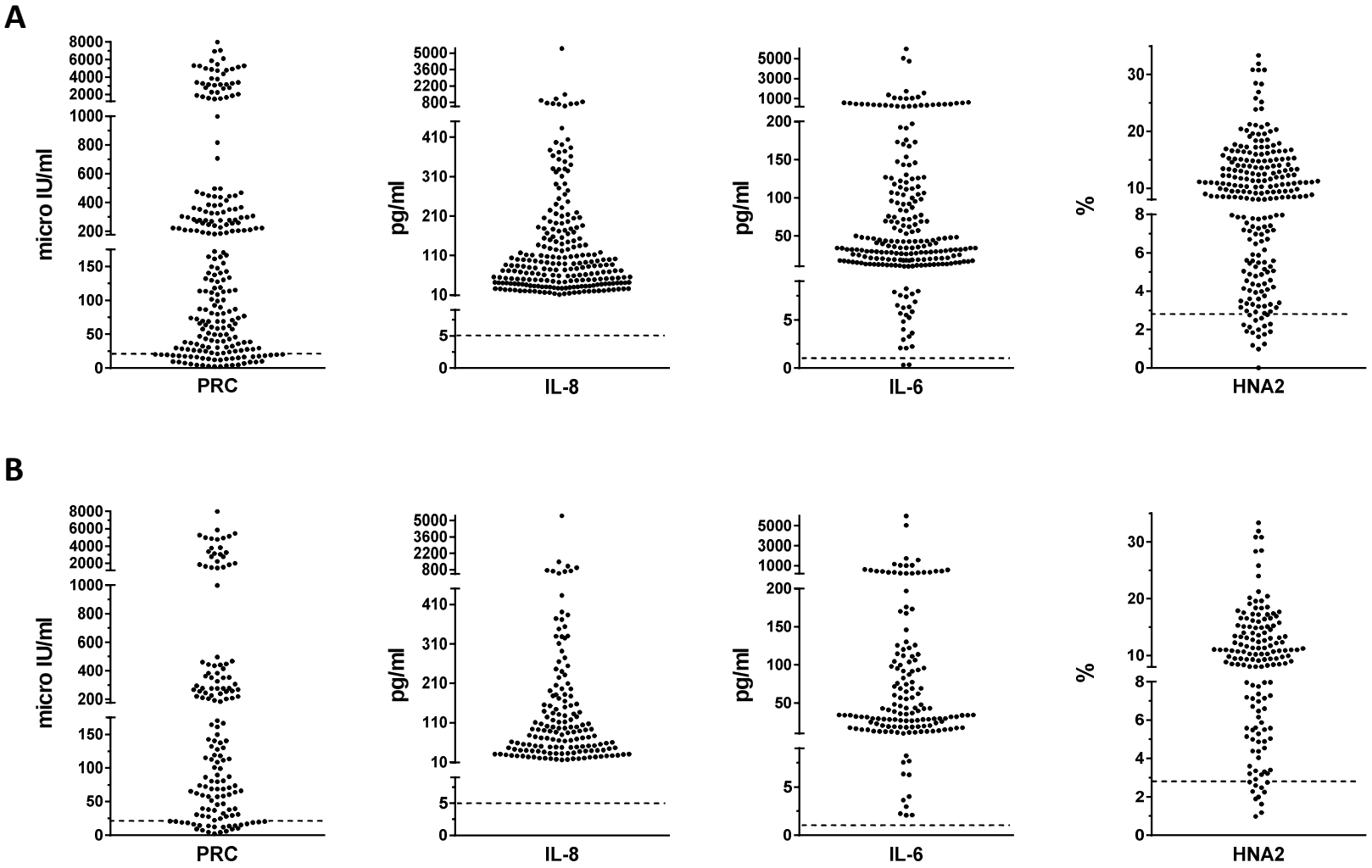
D

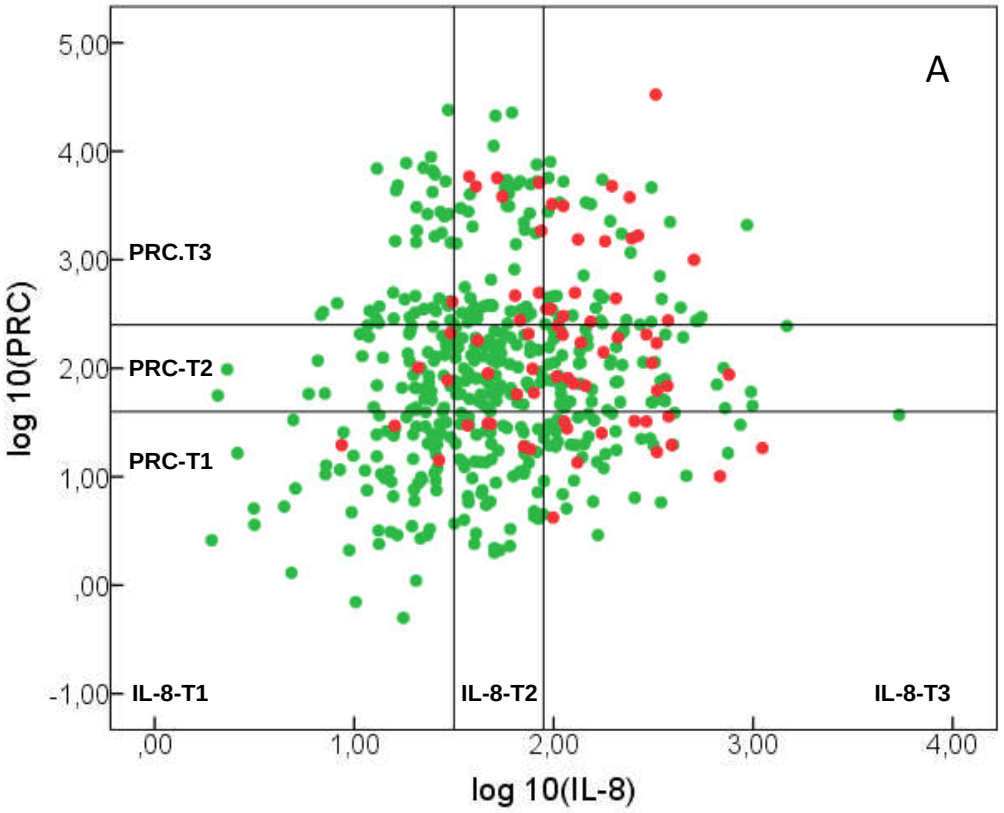
	HNA2-T1	HNA2-T2	HNA2-T3	Overall PRC
PRC-T3	10/33 (30%)	31/60 (52%)	56/76 (74%)	97/169 (57%)
PRC-T2	16/51 (31%)	26/59 (44%)	34/58 (59%)	76/168 (45%)
PRC-T1	14/84 (17%)	17/46 (37%)	28/36 (78%)	59/166 (36%)
Overall HNA2	40/168 (24%)	74/165 (45%)#	118/170 (69%)#	n=503

***p<0.05 vs previous tertile; #p<0.01 vs previous tertile**

Figure 1

Figure 2

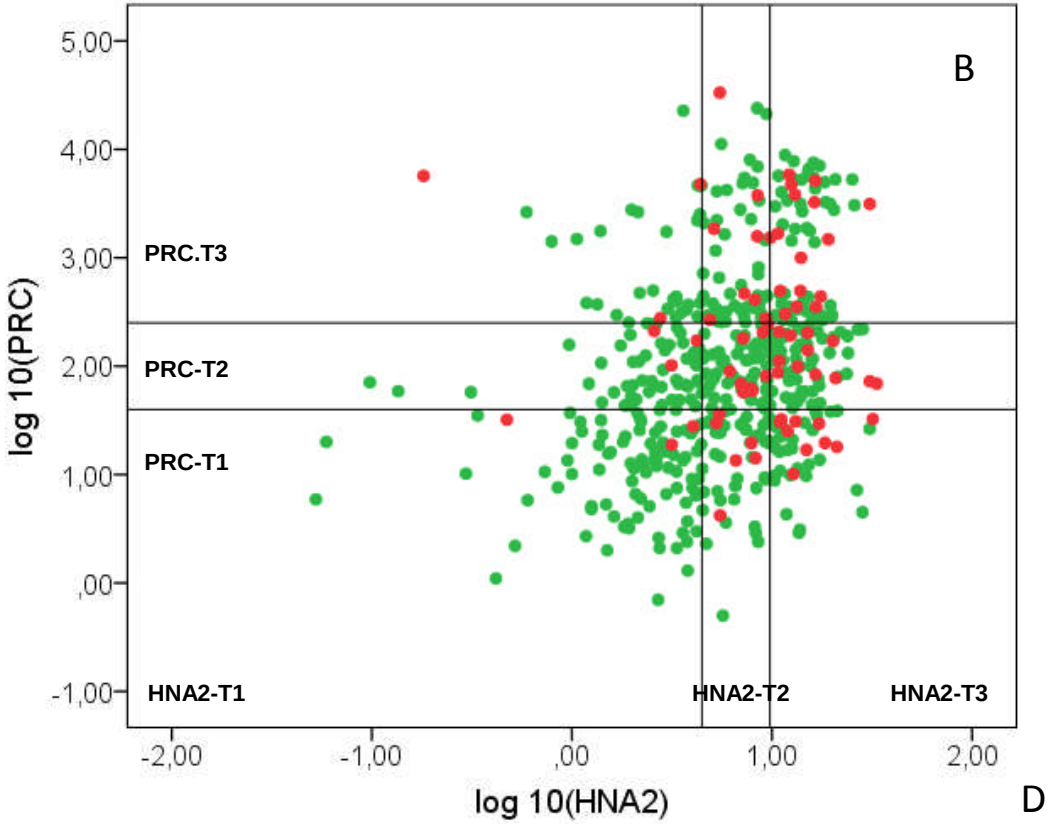

A
micro IU/ml
8000
6000
4000
2000
1000
800
600
400
200
150
100
50
0
PRC
pg/ml
5000
3600
2200
800
410
310
210
110
10
5
0
IL-8
pg/ml
5000
3000
1000
200
150
100
50
5
0
IL-6
%
30
20
10
8
6
4
2
0
HNA2
B
micro IU/ml
8000
6000
4000
2000
1000
800
600
400
200
150
100
50
0
PRC
pg/ml
5000
3600
2200
800
410
310
210
110
10
5
0
IL-8
pg/ml
5000
3000
1000
200
150
100
50
5
0
IL-6
%
30
20
10
8
6
4
2
0
HNA2



C

	IL8-T1	IL8-T2	IL8-T3	Overall PRC
PRC-T3	3/67 (5%)	5/53 (9%)	12/50 (24%)	20/170 (12%)
PRC-T2	2/47 (4%)	5/61 (8%)	12/60 (20%)	19/168 (11%)
PRC-T1	2/53 (4%)	10/56 (18%)	20/64 (31%)	32/173 (19%)
Overall IL-8	7/167 (4%)	20/170 (12%)*	44/174 (25%)#	N=511

***p<0.05 vs previous tertile; #p<0.01 vs previous tertile**



D

	HNA2-T1	HNA2-T2	HNA2-T3	Overall PRC
PRC-T3	3/84 (54%)	6/46 (13%)	10/36 (28%)	19/166 (11%)
PRC-T2	2/51 (4%)	7/59 (12%)	10/58 (17%)	19/168 (11%)
PRC-T1	3/33 (9%)	10/60 (17%)	18/76 (24%)	31/169 (18%)
Overall HNA2	8/168 (5%)	23/165 (14%)#	38/170 (22%)*	N=503

***p<0.05 vs previous tertile; #p<0.01 vs previous tertile**

Figure 3