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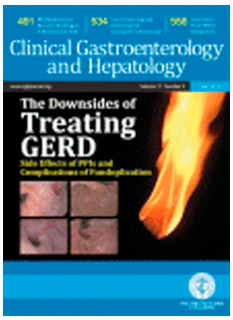

Immune Regulatory Mediators in Plasma from Patients with Acute Decompensation are Associated With 3-month Mortality

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Title: Immune Regulatory Mediators in Plasma from Patients with Acute Decompensation are Associated With 3-month Mortality

Short title: Inflammatory responses and mortality in AD/ACLF

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LIST OF ABBREVIATIONS

Acute decompensation: AD; Acute-on-chronic Liver Failure: ACLF; Cirrhosis-associated immune dysfunction: CAID; C-Reactive Protein: CRP; Docosahexaenoic acid: DHA; Docosapentaenoic acid: DPA; Eicosapentaenoic acid: EPA; Enzyme-linked immunosorbent sandwich assay: ELISA; Human albumin solution: HAS; Interleukin: IL; Intravenous: IV; Lipid mediator: LM; LPS-binding protein: LBP; Lipopolysaccharide: LPS; Model for End Stage Liver Disease: MELD; Monocyte-derived-macrophage: MDM; Peripheral blood mononuclear cells: PBMC; Prostaglandin E_2: PGE_2; Randomised controlled trial: RCT; Specialized pro-resolving mediators: SPMs; Tumor Necrosis Factor: TNF; White cell count: WCC

CONFLICT OF INTEREST

None of the authors have any conflicts of interest

AUTHOR CONTRIBUTIONS

AM and LC obtained and processed the samples used in this study. SV and LC performed clinical characteristic analysis. NB performed the majority of the laboratory work assisted by LC. JD and RAC performed the lipid metabolomic work. SS provided statistical oversight and SS and KB performed the statistical analyses for the clinical trial. ZS managed the clinical trial assisted by LC. NB, LC and AOB interpreted the data and NB prepared figures. NB and AOB designed the study and wrote the manuscript. LC, JD and SV helped with data interpretation. AOB supervised all aspects of the work, designed the clinical trial and obtained funding. All authors approved the final manuscript.

Abstract:

Background & Aims: Infection is a common cause of death in patients with cirrhosis. We investigated the association between the innate immune response and death within 3 months of hospitalization.

Methods: Plasma samples were collected on days 1, 5, 10, and 15 from participants recruited into the albumin to prevent infection in chronic liver failure feasibility study. Patients with acute decompensated cirrhosis were given albumin infusions at 10 hospitals in the United Kingdom. Data were obtained from 45 survivors and 27 non-survivors. We incubated monocyte-derived macrophages from healthy individuals with patients' plasma samples and measured activation following lipopolysaccharide administration, determined by secretion of tumor necrosis factor and soluble mediators of inflammation. Each analysis included samples from 4 to 14 patients.

Results: Plasma samples from survivors vs non-survivors had different inflammatory profiles. Levels of prostaglandin E2 were high at times of patient hospitalization and decreased with albumin infusions. Increased levels of interleukin 4 (IL4) in plasma collected at day 5 of treatment were associated with survival at 3 months. Incubation of monocyte-derived macrophages with day 5 plasma from survivors, pre-incubated with a neutralizing antibody against IL4, caused a significant increase in tumor necrosis factor production to the level of non-survivor plasma. Although baseline characteristics were similar, non-survivors had higher white cell counts and levels of C-reactive protein and renal dysfunction.

Conclusions: We identified profiles of inflammatory markers in plasma that are associated with 3-month mortality in patients with acute decompensated cirrhosis given albumin. Increases in prostaglandin E2 might promote inflammation within the first few days after hospitalization, and increased levels of plasma IL4 at day 5 are associated with increased survival. Clinicaltrialsregister.eu: EudraCT 2014-002300-24

KEY WORDS: TNF, MDM, death, immune response

Need to Know

Background: Infection is a common cause of death in patients with cirrhosis. We investigated the association between the innate immune response and death within 3 months of hospitalization.

Findings: In an analysis of plasma samples from patients given albumin infusions for acute decompensated cirrhosis or acute on chronic liver failure, we found differences in levels of cytokines in patients who survived 30 days vs patients who did not. Plasma from survivors had increased levels of interleukin 4, which reduced activation of monocyte-derived macrophages. Plasma levels of prostaglandin E2 decreased with albumin treatment.

Implications for Patient Care: Differences in plasma levels of cytokines, such as prostaglandin E2 and interleukin 4, might affect response to infections or treatment in patients with acute decompensated cirrhosis or acute on chronic liver failure.

INTRODUCTION

The most severe clinical presentation of liver cirrhosis is acute decompensation (AD) or Acute-on-Chronic-Liver Failure (ACLF)[1]. Patients are highly prone to bacterial infection secondary to immune dysregulation[2] termed 'cirrhosis-associated immune dysfunction' or CAID[2]. CAID causes a paradoxical phenotype in ACLF that combines exaggerated systemic inflammation with immune suppression. Potential immune restorative therapies should aim to improve immune function without worsening systemic inflammation; however, despite detailed work describing the ACLF phenotype[3,4] and its high clinical relevance, there are no licensed treatments to improve immune dysfunction.

We previously identified Prostaglandin E_2 (PGE_2) as a potential causative immune suppressive molecule [5,6]**.** Albumin has been reported to bind and catalyse PGE_2 inactivation [7] and we found that as albumin levels decreased in AD/ACLF, PGE_2 may be more bioavailable and injurious. We therefore proposed transfusing 20% human albumin solution (HAS) to antagonise the effects of PGE_2 [6] and prevent infection in our randomised controlled trial (RCT), ATTIRE (Albumin to prevent infection in chronic liver failure). In the single arm ATTIRE feasibility study of 79 patients at 10 sites, we demonstrated that 20% HAS infusions restored serum albumin levels to >30 g/dl and improved *ex vivo* immune function in AD/ACLF patients by day 3 of study participation through antagonism of PGE_2 [6,8]. However this study included samples from only the first few days of admission and was not linked with clinical outcome.

We therefore performed this follow-up study examining the inflammatory response throughout admission in albumin-treated patients and linked this to outcome. We selected mortality at 3 months following recruitment as our primary clinical outcome in order to study

whether the inflammatory response throughout admission differed between survivors and non-survivors and potential underlying molecular mechanisms.

Our study suggests that survivors and non-survivors exhibited distinct temporal profiles in immune function that corresponded with changes in white cell count and we propose a novel role for interleukin (IL)-4 in this process.

METHODS

Patient studies

Patients were recruited as part of the ATTIRE feasibility study, all were treated with daily intravenous (IV) 20% HAS if serum albumin <30g/L during the trial treatment period (up to 14 days post recruitment). All patients admitted to hospital with AD/severe worsening of liver cirrhosis complications, aged >18years, serum albumin <30g/L, predicted hospital admission by attending clinicians greater than 5 days and for full active management at admission were eligible. Patients were recruited within 72 hours of hospitalisation; full criteria are described elsewhere [8,9]. We sought written informed patients consent from patients or representatives if they lacked capacity. Research ethical approval was granted by London-Brent research ethics committee (ref:15/LO/0104). Plasma samples were randomly selected corresponding to days 1 (pre-treatment), 5, 10 and 15 (end of trial). Survivor and non-survivor groups were divided *a priori* based on death during 3 months follow up at local National Health Service sites. Data were obtained from a maximum 45 survivors and 27 non-survivors at baseline. Experimental studies were performed on samples available with n values in figure legends.

The trial is registered with European Medicines Agency (EudraCT 2014-002300-24) and adopted by NIHR (ISRCTN14174793). All authors had access to the study data and reviewed and approved the final manuscript.

Laboratory analysis are described in Supplemental Material. For multiple comparisons, significance was assessed by one-way ANOVA followed by Bonferroni adjusted pairwise t-tests. White cell count and CRP followed an approximately log-normal distribution each day

and values were therefore transformed (ln, natural logarithm) for statistical analyses. Number of tests were adjusted for the number of trial days. Differences in mean values between survivors and non-survivors were compared using two-tailed t-test allowing for unequal variances with Bonferroni correction.

RESULTS

Distinct plasma-mediated inflammatory response phenotypes during hospitalisation in albumin-treated patients differentiated between survivors and non-survivors

We investigated the temporal profile of inflammatory responses in participants throughout the trial period, by examining the effect of plasma from days 1, 5, 10 and 15 on healthy monocyte derived macrophages (MDMs)[5,6]. MDMs were incubated with plasma from AD/ACLF patients or healthy volunteers and subsequent activation in response to LPS quantified by assessing levels of secreted TNF-α. As previously[6], plasma from AD/ACLF patients at day 1 reduced MDM TNF-α production compared to healthy volunteer plasma (Figure 1A). This immune suppressive effect waned during hospitalisation and, following targeted HAS infusions, reached healthy volunteer levels by end of trial treatment period (day 15) (Fig 1A). MDM production of the anti-inflammatory cytokine IL-10 at 24 hours post-stimulation mirrored this pattern with levels higher at day 1 and returning to those produced by cells incubated with healthy plasma by day 15 (Fig 1B).

When samples were divided into survivor and non-survivors, this observed pattern of immune activation differed between groups. MDMs sensitised with day 1 non-survivor plasma demonstrated a slightly lower production of TNF-α compared to survivors (Fig 1C). However, MDM TNF-α production in this group was rapidly and significantly restored to healthy levels by day 5 and maintained throughout the rest of the trial. In contrast, MDM TNF-α production following treatment with survivor plasma did not reach healthy plasma levels until day 15 of the trial (Fig 1C). Indeed, there was a 2.6 fold-increase in TNF-α levels between day 1 and day 5 in cells incubated with non-survivor plasma ($p < 0.001$) compared to 1.26 fold-increase in those treated with survivor plasma ($p > 0.05$). Neither patient group's

plasma elicited a *greater* TNF-α production than healthy volunteer plasma by day 15 of trial (Fig 1C). These distinct patterns of inflammatory response between survivors and non-survivors were mirrored by MDM production of IL-10 (Fig. 1B). Survivor plasma elicited a gradual decrease in MDM IL-10 production to reach the same level as healthy volunteer plasma by day 15, whereas non-survivor plasma elicited a more rapid fall in production by day 5, although differences did not reach significance (Fig 1D). These two inflammatory response phenotypes (rapid in non-survivors compared to gradual in survivors), appeared distinct by day 5 of the trial, marking this as a potential crucial time-frame that determines outcome. We therefore focused further analyses between baseline (day 1) and day 5.

Both groups of patients demonstrated similar baseline clinical characteristics but non-survivors developed increased serum white cell counts, C-reactive protein and renal dysfunction

Overall baseline patient data has been presented previously in this cohort with the majority having alcohol-related cirrhosis [8]. Non-survivors displayed non-significantly increased Model for End Stage Liver Disease (MELD) scores, ACLF scores, diagnosis of infection at baseline and antibiotic prescription (Table 1). The number of days in trial, baseline white cell count (WCC) and C-Reactive protein (CRP) were similar in both groups (Table 2). Serum creatinine was higher in non-survivors at baseline and renal dysfunction developed solely in the non-survivor group (30.8%, Table 1). As the differences in laboratory inflammatory response appeared during the first half of the trial, we compared the WCC and CRP values between groups during this period (up to day 7). On day 6 where the difference in WCC levels between groups reached its peak (Fig. 2A) the absolute WCC levels were significantly higher in non-survivors (p=0.029). Relative change from baseline WCC also significantly differed on day 6 between survivors and non-survivors (adjusted p=0.033, corrected for 4 tests, raw p=0.008) when values were compared. CRP followed a similar trend with higher levels in non-survivors, but differences in absolute levels did not reach statistical

significance, however the difference in relative change from baseline CRP significantly differed between groups on day 4 (adjusted p=0.032, corrected for 2 tests, raw p=0.016) (Fig 2B). Nosocomial infections developed in 10/26 non-survivors compared to 9/45 survivors following 48 hours of albumin treatment (p=0.19, chi-squared test).

Plasma levels of albumin, LBP and albumin-PGE_2 binding capacity during hospitalisation did not differ between survivors and non-survivors

Volume of HAS administered during the trial did not differ between survivor and non-survivor groups (Table 1) and levels of circulating albumin and its binding capacity to PGE_2 improved similarly in both groups throughout the trial (Fig 2C). Levels of plasma LPS-binding protein (LPB), used as a marker of endotoxin presence [10], did not differ between groups (Fig 2D).

Plasma lipid mediator profiles at day 1, 5, 10 and 15 differed between survivors and non-survivors

Lipid mediator (LM) analysis revealed differing trends between inflammation-initiating and resolution pathways in survivors and non-survivors according to day of sampling. We observed differences between DHA-derived Pro-resolving Lipid Mediators (SPMs) - D-series Resolvins (RvD1, RvD2, RvD3, RvD4, RvD5, RvD6, 17R-RvD1and 17R-RvD3), Protectins (PD1 and 17R-PD1) and Maresins (MaR1 and MaR2); n-3 DPA- derived SPMs - Resolvins (RvT1, RvT3, RvT4, RvD1n-3 DPA, RvD2n-3 DPA and RvD5n-3 DPA), Protectins (PD1n-3 DPA) and Maresins (MaR1n-3 DPA); EPA-derived SPMs - E-series Resolvins (RvE1, RvE2 and RvE3); and differences were observed in Arachidonic acid-derived Lipoxins (LXA4, LXB4, 15-epi-LXA4and 15-epi-LXB4) (Fig 3A, Supplemental Figure 1A-D).

Using orthogonal Partial Least Square Discriminant Analysis, a regression model that identifies variables contributing to separation of experimental groups, we found LM profiles of survivors were distinct from non-survivors, demonstrated by divergent clustering of LM profiles for patients from each group for all intervals tested (Suppl Fig 1 A-D). Assessment of

the variable in importance scores, which identify the contribution of each mediator in the observed separation between each of the groups, demonstrated an increased concentration of several SPM mediators associated with non-survivors (Suppl Fig 1 A-D). Members of the D-series resolvin family were linked with non-survivors, although increases in absolute amounts of these molecules did not reach statistical significance (Suppl Table 1).

Plasma LM profiles demonstrated PGE_2 concentrations were similarly elevated levels at day 1 (Fig 3A,B) (p=0.47) and fell substantially by day 5 in both groups (p=0.09). When cells incubated with day 5 plasma from both survivors and non-survivors were treated with pan-PGE_2 receptor antagonists, there was a similar increase in MDM TNF-α production (1.2-fold change for both groups) (Fig 3C).

Elevated plasma IL-4 concentration at day 5 was associated with 3-month survival and was able to switch plasma-mediated inflammatory response

We measured 30 cytokines in survivor and non-survivor plasma from days 1, 5, 10 and 15 by multiplex analysis (Suppl Table 2). No differences were observed between groups of the classical inflammatory cytokines linked to ACLF prognosis [11,12], such as TNF-α, IL-6, IL-8 and IL-10 (Suppl Figure 2A). A significant increase in IL-4 was found in survivor day 5 plasma (Fig 3D), the period at which survivor and non-*survivor* plasma-mediated inflammatory response phenotypes appeared distinct. Western blot analysis revealed that peripheral blood mononuclear cells (PBMCs) from AD/ACLF patients contained detectable levels of IL-4 protein (Suppl Figure 2B). Subsequently, we demonstrated that MDMs incubated with healthy volunteer plasma that had been supplemented with increasing concentrations of recombinant IL-4 caused decreased TNF-α production in response to LPS in a dose-dependent manner (Fig. 3E). MDM cells treated with day 5-survivor plasma that had been pre-incubated with neutralizing anti-IL-4 antibody (black and white squared box), significantly increased TNF-α production and this reached the same level as seen in cells incubated with non-survivor plasma (light grey box) (Fig. 3F). No effect was seen with neutralizing anti-IL-4 antibody in experiments using non-survivor plasma (Fig. 3F). Finally,

we observed that IL-4 plasma concentrations at day 5 correlated negatively with baseline MELD score (Suppl Fig 3).

DISCUSSION

Immune function and inflammation in AD/ACLF patients has emerged as a critical area of research with the aim of developing treatments that improve mortality [13]. Due to challenges associated with collecting primary cells from multiple sites, we used our *ex-vivo* immune assay to investigate plasma-mediated immune responses and sought to determine whether results were associated with 3-month mortality. These data demonstrate that HAS infusions continued to restore the plasma-mediated inflammatory response as defined by macrophage TNFα and IL-10 production in AD/ACLF patients to healthy volunteer levels beyond day 3 as shown previously [6,8]. This is consistent with recent data identifying changes in plasma IL-10 reflecting disease severity and improving following albumin treatment [4,14]. Survivors and non-survivors demonstrated distinct and unexpected plasma-mediated inflammatory response patterns during the trial, with non-survivors exhibiting a rapid increase of MDM activation to healthy levels that associated with an elevated white cell count. Despite a significant overall effect on our immune assay, the HAS-PGE_2 interaction did not appear to differentiate between these inflammatory phenotypes. Intriguingly, IL-4 at day 5 was significantly upregulated in survivors and mechanistic analyses suggest this may also represent a potential therapeutic target. Finally, a failed resolution of inflammation lipid mediator phenotype was observed in non-survivors.

Our novel data support a mechanistic protective role for IL-4. This cytokine has not been previously linked to CAID, although studies in humans and animal models support a protective role for elevated IL-4 in sepsis[15 16] and acute lung injury [17]. We demonstrated that manipulation of IL-4 switched immune restoration-like phenotypes between survival and non-survival and suggest IL-4 agonism may represent a potential immune-restorative target in

AD/ACLF patients that develop increased circulatory inflammatory markers during hospitalisation.

Previous studies demonstrated an association between high circulatory inflammatory markers e.g. CRP [18,19] and inflammatory cytokines at baseline [12,20,21] and increased mortality in AD/ACLF. In our cohort baseline clinical characteristics of the survivors and non-survivors were quite similar aside from serum creatinine and a non-significant increase in MELD score and infection rate, which may be due to the relatively small sample size. However the development of an elevated WCC, CRP and renal dysfunction during admission was significantly associated with 3 month mortality. Our work supports elevated PGE_2 being crucial in this process early in admission and IL-4 at later timepoints, although it is likely that other molecules are involved.

We previously showed that albumin treatment may alter plasma LM profiles in a small cohort of patients from the ATTIRE feasibility study [6]. In this study Principal Component Analysis revealed several families of SPMs that associated with good or poor outcome using samples taken at different time points before and after albumin infusions. This work remains promising but greater numbers of patients will be required to identify specific LMs for potential drug development targets or to predict outcome or response to treatment. The observation that SPMs are also upregulated in non-survivors indicates that while SPM biosynthetic pathways are active in these patients, production does not limit the unbridled inflammatory response. This could be due to delayed engagement of biosynthetic pathways, dysregulated receptor expression or dysregulated downstream signalling pathways.

Many studies have demonstrated the potential immunomodulatory properties of albumin [5,6,22]. However differing results from recent well-conducted outpatient studies suggest patient response may not be uniform [23,24]. All patients in this single arm study were treated with intravenous HAS which appeared to have a beneficial immune effect throughout hospitalisation by antagonising PGE_2 effects. However it is possible that the differing

inflammatory responses observed associated with patients' 3 month outcome may represent albumin treatment "successes" and "failures". Further studies are required to determine whether PGE_2 levels early in admission, IL4 levels at day 5 or a LM failure to resolve phenotype represent biomarkers or targets for a future precision medicine approach to improve treatment in these patients. This was a single arm study and we were unable to compare findings with non-treated patients.

Our immune assay was developed to investigate effects of plasma from AD/ACLF patients on macrophage function rather than primary cells, as it was not possible to collect fresh monocytes from multiple UK sites. However, our separate single site study has demonstrated an identical phenotype of reduced TNF-α production in fresh whole blood from AD/ACLF patients stimulated with LPS, supporting this approach [25]. Furthermore our laboratory assay was potentially able to differentiate between survivors and non-survivors and mechanistic work has identified a potential novel mediator of CAID, suggesting that the approach has validity. The association of the inflammatory phenotype in those who died at 3 months with development of an elevated WCC and CRP strengthens the laboratory work and aids potential clinical approaches in the future. Our cohort predominantly included patients with alcohol-driven liver disease and the laboratory analyses used samples from only patients with alcohol-related cirrhosis. Therefore our findings may not be applicable to cohorts containing large numbers of non-alcoholic steatohepatitis or viral hepatitis patients. Moreover we did not have sufficient samples to extend studies to compare between AD and ACLF. ATTIRE stage 2, a large scale RCT at 30 sites that finishes in June 2019 [26] will provide samples taken from patients treated with and without HAS at day 1, 5 and 10 in order to validate these findings and guide future immune restorative approaches in AD/ACLF. This will allow us to discern the potential effect of 20% HAS infusion on the establishment of different patterns of inflammatory responses observed and their relationship to survival.

We present novel data that describes CAID as a dynamic process and propose that the inflammatory response trajectory may represent the critical determinant of clinical outcome. Distinct plasma-mediated inflammatory responses, as defined by our laboratory assay and changes in WCC and CRP from baseline during the first week of hospitalisation, were associated with organ dysfunction and death in albumin-treated advanced liver disease patients. Finally our results PGE_2 and IL-4 as pivotal mediators that underlie the inflammatory response in these patients at different time points during their hospital admission.

FIGURE LEGENDS

Figure 1. Distinct immune restoration phenotype associated with survival develops over time

Levels of TNF-α (A, C) and IL-10 (B, D) secreted by MDMs were quantified by ELISA. Cells were sensitised with plasma from ATTIRE survivors, non-survivors and healthy volunteers for 30 minutes and stimulated with 100 ng/ml LPS for 4 (A, C) and 24 (B, D) hours. Boxes represent median with upper and lower quartiles. Bars represent minimum and maximum observations. Number of samples used for each day are written in parentheses. * $p < 0.05$, ** < 0.01, *** $p < 0.001$. Significance determined using one-way ANOVA and Bonferroni adjusted pairwise t-tests.

Figure 2. Plasma levels of albumin, LBP and albumin-PGE_2 binding capacity did not differentiate between groups

A, B) Daily white cell counts (WCC) (A) and C-Reactive Protein (CRP) (B) in survivor (n= 10 -38) and non-survivor (n = 6-22) groups during ATTIRE Feasibility study. Data expressed as mean and standard error. C) Levels of circulating albumin (g/L) (n=5-7) and percentage of PGE_2/3H-PGE_2 bound to survivor and non-survivor patient plasma protein, day 1, 5, 10 and

15 using equilibrium dialysis (n=3-5). D) Plasma lipopolysaccharide-binding protein (LBP) by ELISA in day 1 and 5 samples from survivors (n = 5-8) and non-survivors (n=7-10).

Figure 3. Plasma molecule study reveals differential IL-4 levels that associate with survival

A) 2-dimentional loading plot of plasma samples at day 5 (C, D). Survivors (n = 7), non-survivors (n = 8).

B) Levels of PGE_2 in human plasma from acute decompensation patients collected at indicated intervals. Results expressed as pg / mL, mean ± SEM. Dotted line represents mean levels from healthy volunteer plasma (n = 5). p-values correspond to unpaired T-Test (between survivors and non-survivors at days 1 and 5).

C) Levels of secreted TNF-α by MDMs quantified by ELISA. Cells were pre-treated with 50μM AH6809 (EP1-3 antagonist) and 10μM MF498 (EP4 antagonist) and sensitised with day 5 plasma from ATTIRE survivors and non-survivors and healthy volunteers (HV) for 30 minutes and stimulated with 100 ng/ml LPS for 4 hours. Data represent median with 25^{th}/75^{th} percentiles (n = 4-6). Bars represent minimum and maximum observations.

D) Plasma IL-4 levels on day 1 and day 5 samples from survivors (n = 8-9) and non-survivors (n = 7-8). Results expressed as pg / mL, mean ± SEM. * $p < 0.05$ determined by Student's t-test. Dotted line represents mean levels from healthy volunteer plasma (n = 5).

E, F) Levels of secreted TNF-α by MDMs quantified by ELISA. Cells were sensitised with healthy volunteer plasma supplemented with several concentrations of recombinant human (rh) IL-4 (E) or day 5 plasma from survivors and non-survivors incubated with neutralizing anti-IL4 antibody (F) for 30 minutes and stimulated with 100 ng/ml LPS for 4 hours (n = 4-5). *$p < 0.05$ determined by paired T-test.

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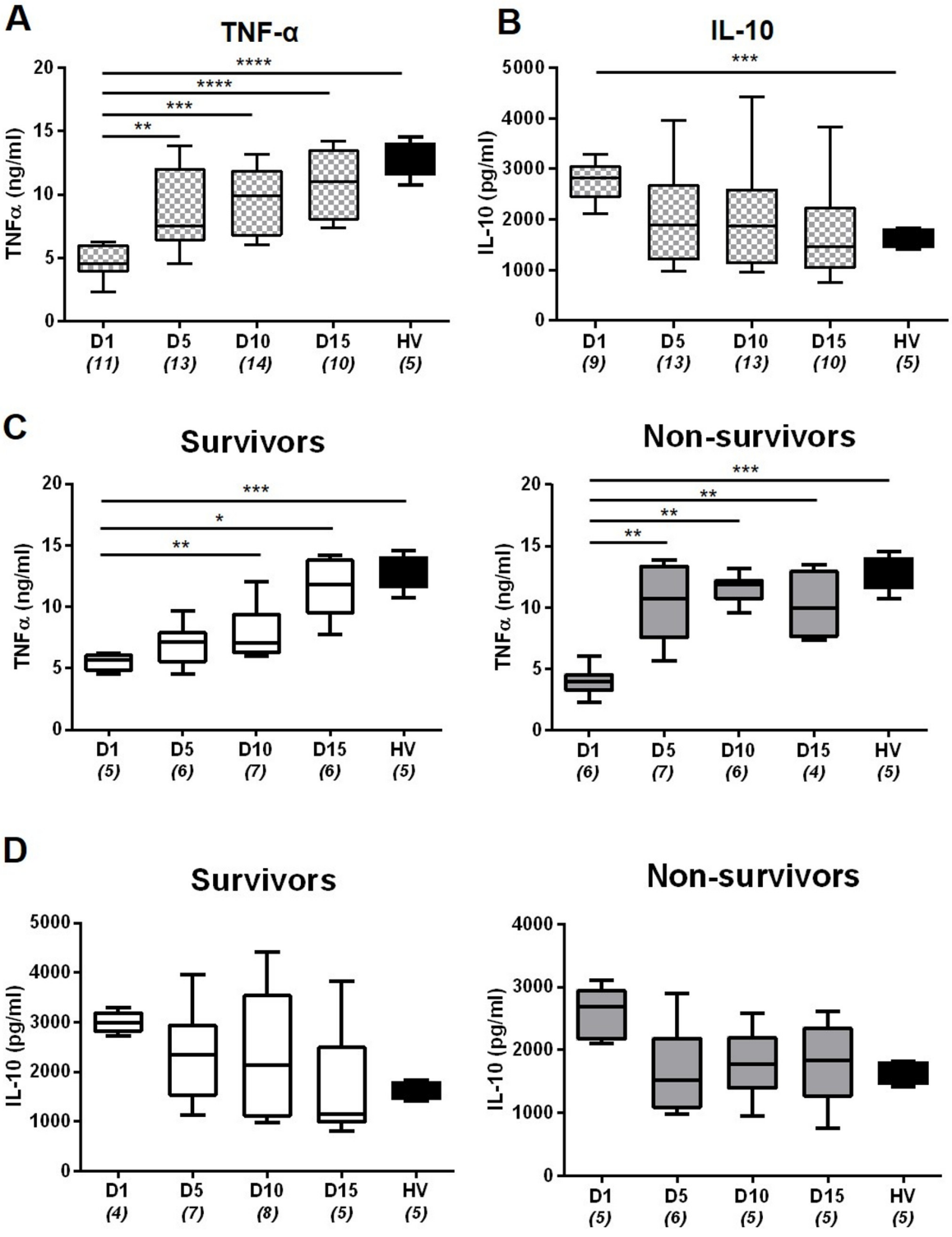
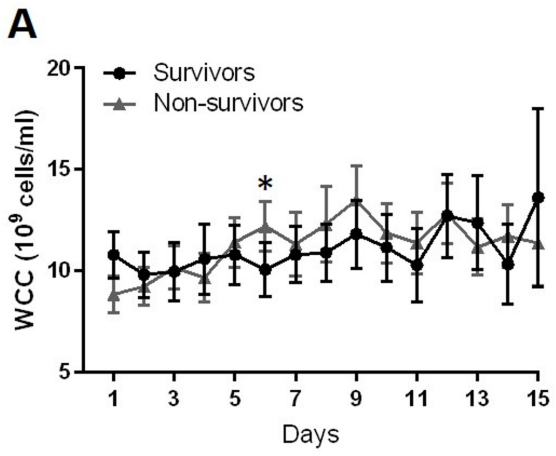
Table 1. Patient group characteristics

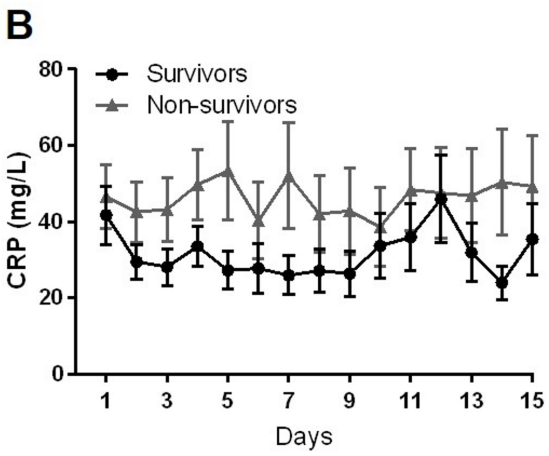
	Survivors		**Non survivors**	
Median Age, range (n=number of patients with data)	54.6, 30-75 (45)		55.3, 24-81 (27)	
Male Sex, number (%)	32/45 (71.1%)		15/27 (55.5%)	
Baseline median MELD score, IQR (n)	19.8, 16.0-23.1 (38)		23.6, 17.4-28.4 (20)	
Total HAS given Median, IQR (n)	850, 700-1500 (44)		1000, 400-1600 (26)	
Median days in trial, IQR (n)	14, 8-15 (44)		12, 4-15 (26)	
Number with infection diagnosed at recruitment (%)	16/45 (35.5%)		12/26 (48%)	
Prescribed antibiotics at recruitment, number (%)	22/45 (49%)		17/26 (65%)	
Number with new infection during trial after HAS treatment for >48h (%)	9/45 (20%)		10/26 (37%)	
Renal dysfunction diagnosis during trial (%)	0/44 (0.0%)		8/26 (30.8%)	
Alcohol consumption as aetiology (%)	43 (91%)		27 (100%)	
Active alcohol consumption at admission (%)	20 (43%)		5 (19%)	
ACLF scores, n (%)	0	35 (77.8%)	0	17 (63%)
	1	6 (13.3%)	1	4 (14.8%)
	2	3 (6.7%)	2	3 (11.1%)
	3	1 (2.2%)	3	3 (11.1%)

Table 2. Baseline blood tests of survivors and non-survivors

Median (IQR)	**Survivors (n = 45)**	**Non-survivors (n = 27)**
Albumin (g/L)	24.0 (21.5-26.5)	25.0 (22.0-27.0)
Bilirubin (µmol/L)	98.5 (50.5-244.5)	107.0 (64.0-275.0)
White cell count (10^6 cells/ml)	9.1 (6.2-12.6)	8.5 (5.7-11.5)
C-reactive Protein (mg/L)	29.5 (11.0-55.0)	37.5 (16.0-67.0)
Creatinine (mmol/L)	65.0 (52.0-81.0)	80.0 (63.0-118.0)
INR	1.7 (1.4-1.8)	1.7 (1.4-2.2)
Temperature (℃)	36.8 (36.5-37.5)	36.5 (36.1-36.8)

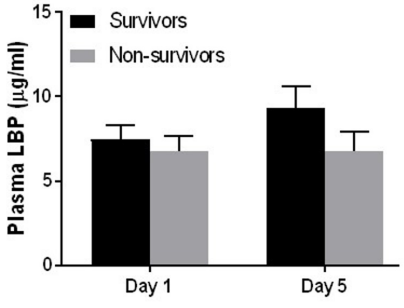

A
TNF-α
TNFα (ng/ml)
D1 (11)
D5 (13)
D10 (14)
D15 (10)
HV (5)
B
IL-10
IL-10 (pg/ml)
D1 (9)
D5 (13)
D10 (13)
D15 (10)
HV (5)
C
Survivors
Non-survivors
D1 (5)
D5 (6)
D10 (7)
D15 (6)
HV (5)
D1 (6)
D5 (7)
D10 (6)
D15 (4)
HV (5)
D
Survivors
Non-survivors
D1 (4)
D5 (7)
D10 (8)
D15 (5)
HV (5)
D1 (5)
D5 (6)
D10 (5)
D15 (5)
HV (5)

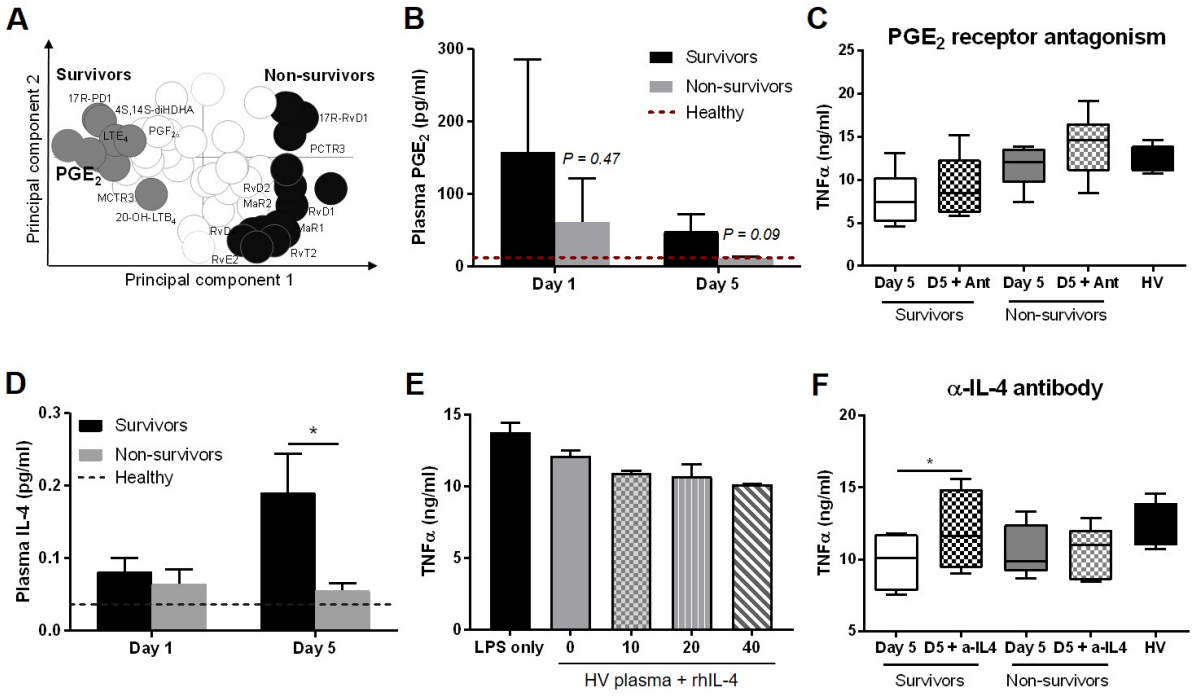




C

		Day 1	Day 5	Day 10	Day 15
Albumin levels (g/L)	**Survivors**	26.75 ± 2.86	30.67 ± 3.54	31.71 ± 2.05	33 ± 2.94
	Non-survivors	23.5 ± 5.72	28 ± 3.68	29.95 ± 3.89	30.66 ± 2.49
PGE_2 binding (%)	**Survivors**	28.12 ± 10.23	35.1 ± 9.01	33.99 ± 17.06	28.97 ± 7.83
	Non-survivors	24.34 ± 2.72	36.2 ± 16.93	33.28 ± 12.77	30.85 ± 3.25





SUPPLEMENTAL METHODS

Criteria for decompensation of liver cirrhosis

These were defined as acute onset or worsening of complications of cirrhosis including gastroesophageal haemorrhage, jaundice, alcoholic hepatitis, ascites, hepatic encephalopathy, sepsis or infection including spontaneous bacterial peritonitis, hepatorenal syndrome or renal dysfunction.

Renal dysfunction was defined as an increase in serum creatinine level by ≥50% compared to creatinine level at baseline. Infection was defined as when a patient was prescribed a new antibiotic(s) and clinical data was recorded to subsequently categorise infection according to standard international criteria [1]. Both of these endpoints were recorded no earlier than 48 hours after the onset of albumin treatment.

Monocyte-derived macrophages (MDM) *ex-vivo* immune function assay

MDMs obtained from healthy volunteers [2,3] were sensitized with plasma for 30 minutes at 37 °C and then stimulated with 100 ng/ml of lipopolysaccharide (LPS, *Salmonella abortus equi* S-form,[TLRgrade™], Enzo Life Science, United Kingdom). Cell supernatants were collected at 4 and 24 hours post-stimulation and stored at - 80℃. Tumor Necrosis Factor (TNF)-α (4 hour samples) and IL-10 levels (24 hour samples) in supernatants were quantified by enzyme-linked immunosorbent assay (DuoSet ELISA, R&D Systems, USA) following manufacturer protocol. For selected experiments pan-PGE_2 receptors were antagonised with 50µM AH6809 (EP1-3 antagonist) (Cayman Chemicals, MI, USA) and 10µM MF498 (EP4 antagonist) (Cayman Chemicals, MI, USA), prior to plasma sensitization and LPS stimulation.

For soluble IL-4 blocking experiments, plasma from healthy volunteers or ATTIRE patients was incubated with either 1 µg/ml human IL-4 antibody (clone #34019, R&D Systems, USA) or mouse IgG_{2B} isotype control (clone #20116, R&D Systems, USA) for 1 hour at 37 ℃ prior to

MDM sensitization. In other experiments recombinant human IL-4 (Peprotech, NJ, USA) at different concentrations were added to healthy volunteer plasma.

Plasma soluble mediator analysis

Circulating albumin levels were measured at local hospital laboratories as part of the study protocol. Plasma LPS-binding protein (LBP) levels were quantified by ELISA following manufacturer's protocol (R&D Systems, USA). Briefly, plasma was thoroughly thawed and diluted in reagent diluent (Phosphate Buffer Saline containing 5% bovine serum albumin) prior to being added to antibody-coated microplates. Quantification of immobilized antibody-enzyme conjugates was performed by monitoring horseradish peroxidase activities in the presence of the substrate 3, 3', 5, 5'-tetramethylbenzidine; which was measured spectrophotometrically by the increased absorbency at 450 nm.

Levels of multiple cytokine and chemokine levels were measured in patient and healthy volunteer plasma by electrochemiluminescence detection using the *V-Plex* Pro-inflammatory panel 1, Cytokine Panel 1 and Chemokine Panel 1 kits from Meso Scale Discovery (MSD, Meso Scale Discovery, Rockville, Maryland, USA). Data were acquired using a SECTOR S 6000 plate reader (MSD).

Plasma lipid mediators levels were quantified by High Performance Liquid Chromatography as described in [3].

Western Blotting

Peripheral blood mononuclear cells (PBMC) were obtained from acutely decompensated inpatients admitted to University College Hospital with complications of cirrhosis or healthy volunteers. Full NHS Research Ethics Committee ethical approval was obtained (REC Reference number 15/LO/0800).

Briefly, venous blood was collected in vacutainer tubes containing heparin and layered onto 15 mL Ficoll-Paque PLUS (GE Healthcare, United Kingdom). PBMCs were separated by density gradient centrifugation and residual erythrocyte contamination was removed using Ammonium-Chloride-Potassium (ACK) lysis buffer (Lonza, Switzerland). Cell pellets were then lysed in RIPA buffer (Sigma Aldrich, MO, USA) supplemented with protease inhibitors. 30 µg of cell lysates were then loaded onto a 4-15% Tris-Glycine gel (Bio-Rad, CA, USA), electrophoresed and transferred onto a PVDF membrane. Membrane was immunoblotted with rabbit monoclonal anti-IL4 antibody (ab62351, Abcam, United Kingdom) and mouse monoclonal anti-GAPDH (sc-32233, Santa Cruz Biotechnology, TX, USA). Anti-rabbit (sc-2301, Santa Cruz Biotechnology, TX, USA) or anti-mouse (NA931VS, GE Healthcare, United Kingdom) horseradish-peroxidase-tagged antibodies were used for secondary binding and chemiluminescence (Clarity Western ECL, Bio-Rad, CA, USA) was used to visualise proteins.

Statistical analysis

Unless stated otherwise, data are presented as mean ± Standard Deviation (SD). . Two-tailed (unpaired) t-tests were performed when comparing two independent groups of values where a normal distribution was reasonable to assume.

SUPPLEMENTAL REFERENCES

1. Bajaj JS, O'Leary JG, Reddy KR, et al. Second infections independently increase mortality in hospitalized patients with cirrhosis: the North American consortium for the study of end-stage liver disease (NACSELD) experience. Hepatology 2012;56:2328-2335.

2. O'Brien AJ, Fullerton JN, Massey KA, et al. Immunosuppression in acutely decompensated cirrhosis is mediated by prostaglandin E2. Nat Med 2014;20:518-523.

3. China L, Maini A, Skene SS, et al. Albumin Counteracts Immune-Suppressive Effects of Lipid Mediators in Patients With Advanced Liver Disease. Clin Gastroenterol Hepatol 2018;16:738-747.e7.

Supplemental Table 1. Plasma Lipid Mediator profiles

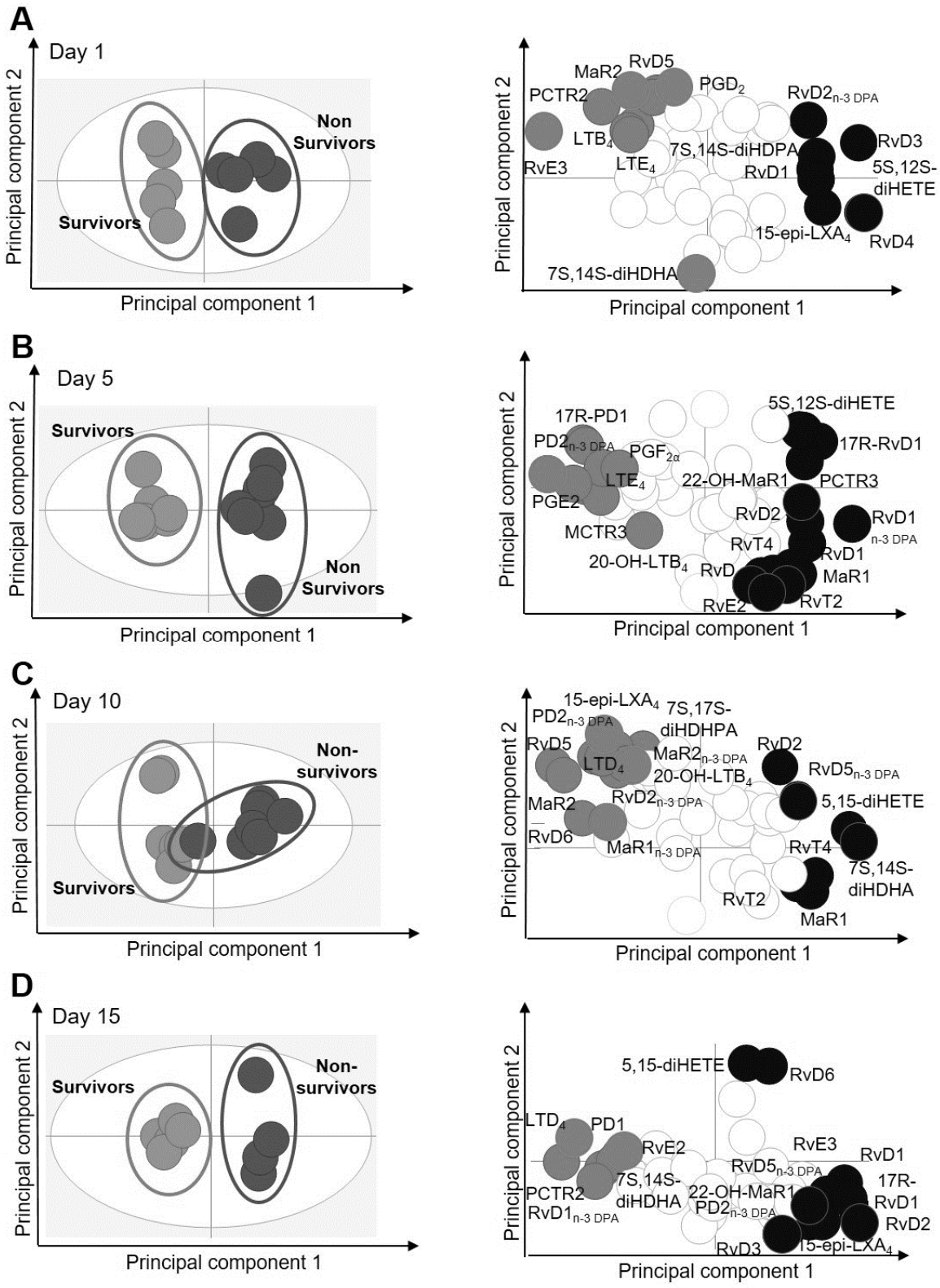
	Survivors				Non-survivors			
	Day 1		Day 5		Day 1		Day 5	
DHA Bioactive Metabolome (pg/ml)	Average	SEM	Average	SEM	Average	SEM	Average	SEM
RvD1	0.30	0.16	0.46	0.29	2.34	1.57	1.04	0.34
RvD2	0.68	0.20	2.98	2.16	0.63	0.29	13.46	7.22
RvD3	0.17	0.10	0.18	0.15	0.42	0.08	0.21	0.07
RvD4	0.98	0.55	1.31	0.63	3.81	1.34	2.24	0.94
RvD5	1.21	0.83	1.44	0.52	0.57	0.25	1.77	0.59
RvD6	0.72	0.40	0.73	0.25	0.54	0.17	0.40	0.22
17R-RvD1	1.00	0.81	0.11	0.06	0.21	0.17	0.38	0.15
17R-RvD3	0.12	0.05	0.10	0.09	0.08	0.06	0.26	0.20
PD1	0.84	0.46	4.74	3.98	0.80	0.52	3.39	2.05
10S,17SdiHDHA	5.94	4.21	9.35	8.39	7.75	4.49	3.60	1.78
17R-PD1	0.51	0.25	0.89	0.48	0.27	0.19	0.20	0.07
PCTR1	1.24	1.38	1.46	1.21	1.66	1.85	0.93	0.99
PCTR2	1.22	0.89	123.44	117.81	0.00	0.00	25.12	26.75
PCTR3	16.51	18.45	2.55	2.75	0.00	0.00	43.99	30.11
MaR1	0.00	0.00	1.44	1.01	0.00	0.00	3.81	1.65
MaR2	0.79	0.51	0.29	0.14	0.31	0.22	0.78	0.37
7S,14S-diHDHA	8.74	6.73	9.99	4.36	7.45	6.34	15.89	7.31
22-OH-MaR1	0.00	0.00	1.12	1.10	0.91	0.65	4.73	2.21
4S,14S-diHDHA	0.59	0.42	0.36	0.15	0.13	0.08	0.14	0.08
MCTR1	19.54	21.85	0.82	0.89	9.04	10.11	0.00	0.00
MCTR2	2.26	2.53	49.75	53.73	0.00	0.00	0.22	0.23
MCTR3	46.72	50.54	22.74	21.50	16.75	13.01	1.46	1.56
n-3 DPA Bioactive Metabolome (pg/ml)	**Survivors**				**Non-survivors**			
	Day 1		**Day 5**		**Day 1**		**Day 5**	
	Average	SEM	Average	SEM	Average	SEM	Average	SEM
RvT1	0.38	0.38	0.67	0.41	0.35	0.25	1.55	0.88
RvT2	0.12	0.07	0.41	0.32	1.48	1.40	1.40	1.05
RvT3	0.48	0.14	5.06	5.14	0.73	0.31	1.00	0.55
RvT4	0.44	0.24	0.42	0.21	0.60	0.40	0.74	0.39
$RvD1_{n3\ DPA}$	0.77	0.28	0.48	0.09	0.47	0.19	1.81	0.61
$RvD2_{n3\ DPA}$	0.38	0.27	1.37	0.87	1.09	0.50	3.32	2.15
$RvD5_{n3\ DPA}$	1.05	0.55	1.49	0.79	1.18	0.88	1.21	0.96
$PD1_{n3\ DPA}$	0.81	0.47	0.37	0.17	1.22	0.23	0.30	0.22
10S,17S-diHDPA	0.60	0.30	0.12	0.13	0.62	0.43	0.48	0.47
$PD2_{n3\ DPA}$	0.26	0.20	0.41	0.18	0.39	0.35	0.06	0.06

$MaR1_{n3\ DPA}$	0.36	0.40	1.06	0.50	0.20	0.22	1.08	0.71
7S,14S-diHDPA	0.37	0.26	0.19	0.20	1.34	1.14	0.89	0.49
$MaR2_{n3\ DPA}$	5.58	1.92	5.36	1.90	6.62	3.72	8.67	5.95
EPA Bioactive metabolome (pg/ml)	**Survivors**				**Non-survivors**			
	Day 1		**Day 5**		**Day 1**		**Day 5**	
	Average	SEM	Average	SEM	Average	SEM	Average	SEM
RvE1	0.71	0.59	2.62	1.17	0.46	0.45	2.51	1.31
RvE2	0.46	0.36	0.59	0.28	0.00	0.00	3.36	3.30
RvE3	2.00	0.68	1.98	0.59	0.00	0.00	1.73	1.49
AA Bioactive Metabolome (pg/ml)	**Survivors**				**Non-survivors**			
	Day 1		**Day 5**		**Day 1**		**Day 5**	
	Average	SEM	Average	SEM	Average	SEM	Average	SEM
LXA_4	0.20	0.12	1.51	1.41	0.22	0.17	0.35	0.22
LXB_4	56.89	33.64	28.43	9.18	49.68	26.23	32.28	14.62
5S,15S-diHETE	0.67	0.75	0.70	0.55	0.94	0.51	0.46	0.24
15-epi-LXA_4	0.76	0.46	2.25	1.24	4.03	2.48	4.85	2.73
15-epi-LXB_4	0.00	0.00	5.99	3.66	0.93	0.80	1.39	1.04
LTB_4	59.61	39.43	19.08	10.53	13.32	7.91	7.38	2.79
5S,12S-diHETE	0.57	0.64	0.85	0.44	2.51	1.95	3.91	2.56
20-OH-LTB_4	39.32	24.93	41.82	25.07	7.22	4.76	19.70	15.52
LTC_4	104.49	42.32	245.07	58.23	140.27	60.57	380.26	212.29
LTD_4	2.28	1.08	2.57	2.48	2.46	2.32	5.54	3.88
LTE_4	99.42	75.69	44.22	22.49	17.65	5.42	15.99	2.57
PGD_2	47.06	32.13	13.55	5.12	26.30	15.98	10.14	4.67
PGE_2	158.05	127.58	49.01	23.36	62.15	60.14	11.75	2.49
PGF_{2a}	92.10	66.27	32.92	16.63	129.55	135.36	15.65	4.06
TXB_2	760.53	562.20	280.12	158.57	441.14	291.99	761.01	632.45

Supplemental Table 2.

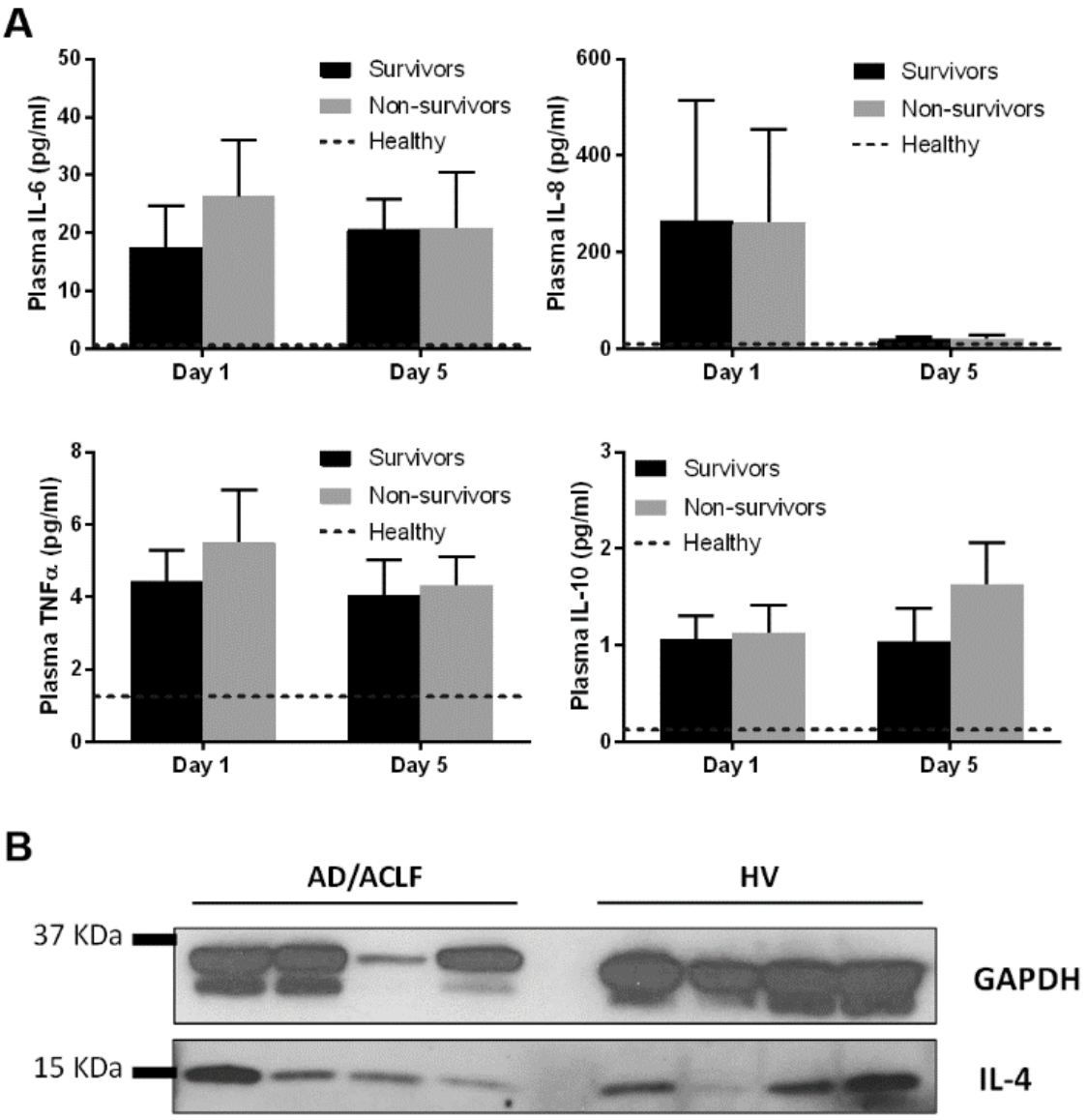
	Survivors				Non-survivors				HV	
	Day 1		Day 5		Day 1		Day 5			
Plasma analyte	pg/ml	SD	pg/ml	SD	pg/ml	SD	pg/ml	SD	pg/ml	SD
CCL2	378.4	174.6	323.7	131.1	266.1	192.9	319.4	194.3	159.4	51.9
CCL3	41.8	17.8	31.6	46.4	38.9	16.4	27.4	12.9	13.9	2.8
CCL4	159	114.1	135.1	100.8	99.5	19.8	92.1	27.6	34.9	5.8
CCL17	80.3	12.2	126	34.2	157.3	30.5	86.9	17.1	50.3	21.3
CCL22	378.9	74	764.5	164.6	932	136.8	408	62.4	838.7	200.3
Eotaxin	914.8	480.8	885.1	315.4	842.2	459.4	1047.8	429.6	647.4	126.4
Eotaxin-3	138.5	66.1	150.7	59.3	117.4	43.2	142.7	60.7	647.4	126.4
GM-CSF	0.18	0.04	0.17	0.04	0.18	0.04	0.10	0.03	0.06	0.03
IFN-γ	8.54	2.53	9.14	2.18	3.29	1.05	6.16	2.24	1.78	0.99
IL-1α	2.05	3.21	2.67	1.82	4.85	5.2	4.52	3.70	n/a	n/a
IL-1β	0.59	0.14	1.7	0.42	0.41	0.12	1.34	0.95	n/a	n/a
IL-2	0.62	0.27	0.89	0.35	0.36	0.23	0.58	0.33	1.17	1.06
IL-4	0.082	0.02	0.19	0.05	0.065	0.02	0.056	0.01	0.03	0.01
IL-5	0.73	0.5	1.17	1	0.67	0.26	1.04	0.74	0.56	0.20
IL-6	17.5	7.16	20.68	5.08	26.3	9.66	20.94	9.5	0.13	0.04
IL-7	6.43	1.49	4.95	0.84	5.20	1.07	5.32	0.91	2.93	0.63
IL-8	265.4	35	359.3	202.4	261.1	68.7	156.8	34.9	3.6	1.14
IL-10	1.06	0.24	1.03	0.34	1.13	0.28	1.63	0.43	0.13	0.07
IL-12p70	0.45	0.37	0.58	0.60	0.29	0.07	0.58	0.60	0.18	0.07
IL-13	2.12	1.53	2.82	2.13	1.59	0.85	2.19	2.85	0.72	0.39
IL-15	7.28	1.7	5.73	1.9	7.36	4.13	6.0	1.58	2.68	0.51
IL-16	358.9	218.6	341.3	250.2	472.3	543.05	511.2	344.1	142.8	35.17
IL-17	5.97	1.86	6.01	1.78	3.26	0.39	2.59	0.28	2.18	0.74
IL-23	99.23	124.6	90.1	61.54	94.68	79.16	64.9	54.9	105.7	52.3
IP-10	1104.1	1040.9	1085.2	568.5	729.3	499.3	1085.2	568.5	220.4	63.3
MCP-4	334.5	81.8	443.4	51	344.3	45.6	636.6	80.9	243.4	96.8
TNF-α	4.45	0.83	4.05	0.97	5.52	1.44	4.33	0.78	1.26	0.11
TNF-β	0.53	0.17	0.42	0.10	0.52	0.14	0.48	0.11	0.29	0.06
VEGF	130.9	163.3	125.7	157.1	32.71	20	103.4	172.8	12.38	2.04

Supplemental Figure 1



A-C) 2-dimentional score plot (Left) and loading plot (Right) of samples at Day 1 (A), Day 5 (B), Day 10 (C) and Day 15 (D). Gray ellipse in the score plot denotes 95% confidence regions. Day 1: survivors n = 5, non-survivors n = 5; Day 10: survivors n = 6, non-survivors n = 7; Day 15: survivors n = 5, non-survivors = 4.

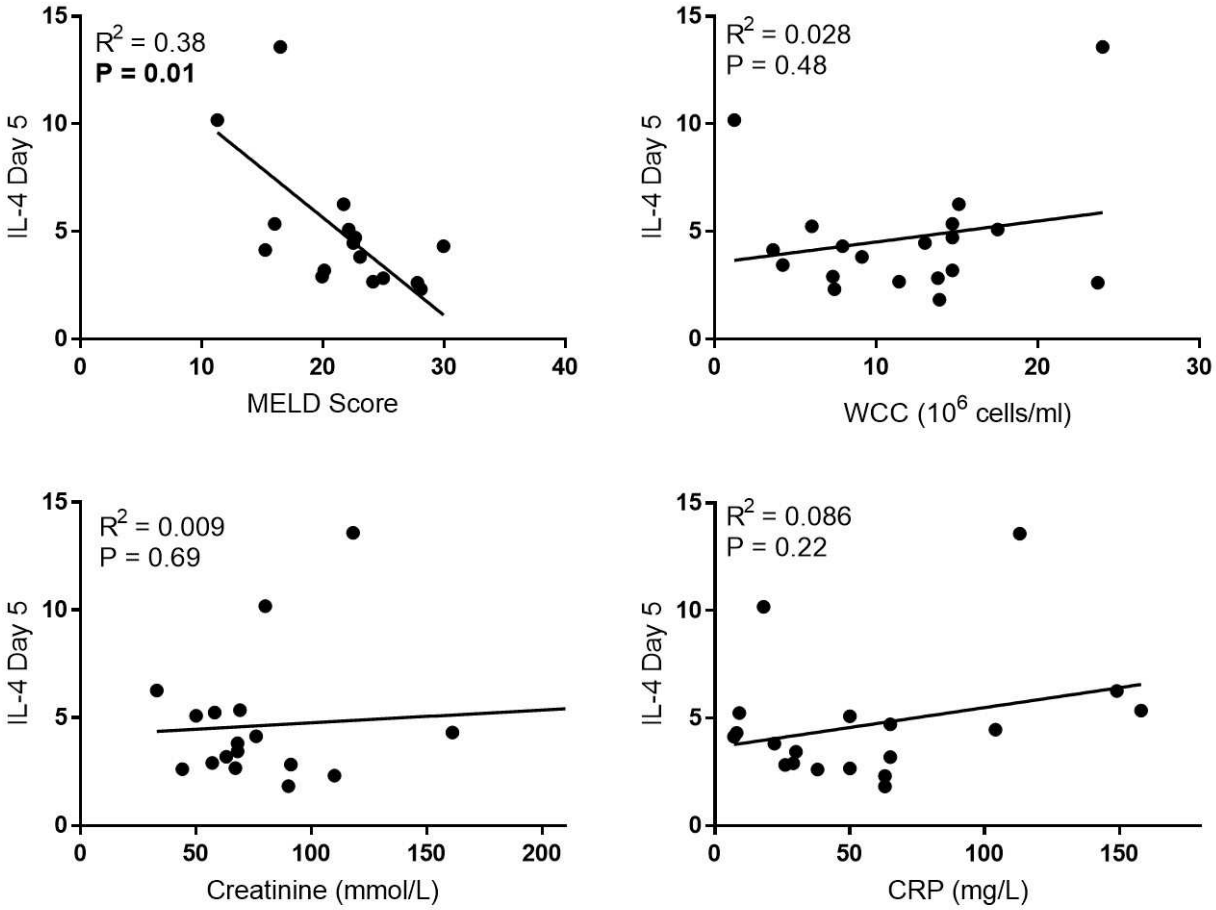
Supplemental Figure 2



A) Plasma cytokine levels on day 1 and day 5 samples from survivors (n = 8-9) and non-survivors (n = 7-8). Results are expressed as pg / mL, mean ± SEM. Dotted line represents mean levels on healthy volunteer plasma (n = 5).

B) IL-4 was detected by immunoblotting on peripheral blood mononuclear cells of acutely decompensated (AD) patients and healthy volunteers (HV). Expression of GAPDH was used as input loading control.

Supplemental Figure 3



Day 5 plasma IL-4 levels correlations with baseline clinical characteristics. Data represents product of Pearson's Correlation Coefficient (n = 13-16). Best-fit linear slope is also included.