Cell death markers in cirrhotic patients with acute decompensation

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Abbreviations: AD, acute decompensation; ACLF, acute on chronic liver failure; AH, alcoholic hepatitis; ALD, alcoholic liver disease; K18, keratin 18; cK18, caspase-cleaved keratin 18; cK18:K18 ratio, ratio of caspase-cleaved keratin 18 to keratin 18 (apoptotic index); ELISA, enzyme-linked immunosorbent assay; DAMP, damage-associated molecular pattern; PAMP, pathogen-associated molecular pattern; IF, intermediate filament; CLIF, chronic liver failure consortium; PIRO concept, predisposition, injury, response, organ failures concept; IL-6, interleukin 6; IL-8, interleukin 8; IL-10,

interleukin 10; IL1-Ra, interleukin-1 receptor antagonist; NGAL, neutrophil gelatinase-associated lipocalin; sCD163, soluble cluster of differentiation 163; HNA-2, human non-mercaptalbumin-2; EDTA, ethylenediaminetetraacetic acid; ANOVA, analysis of variance; AUROC, area under receiver operating characteristic curve; HCV, hepatitis C virus; WCC, white cell count; CRP, c-reactive protein; INR, international normalised ratio; MELD, model for end-stage liver disease; HBV, hepatitis B virus; TUNEL stain, Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling stain.

ABSTRACT

The aims of this study were to determine the role of cell death in cirrhotic patients with acute decompensation (AD) and acute on chronic liver failure (ACLF) using plasma-based biomarkers. The patients studied were part of the CANONIC study (N=337; AD: 258; ACLF: 79); additional cohorts included healthy volunteers, stable cirrhotic patients and a group of 16 AD patients for histological studies. Caspase-cleaved keratin 18 (cK18) and keratin 18 (K18), which reflect apoptotic and total cell death respectively and cK18:K18 ratio (apoptotic index) were measured in the plasma by ELISA. The concentrations of cK18 and K18 increased and the cK18:K18 ratio decreased with increasing severity of AD and ACLF ($p<0.001$ respectively). Alcohol etiology, no previous decompensation and alcohol abuse were associated with increased cell death markers whereas underlying infection was not. Close correlation was observed between the cell death markers and, markers of systemic inflammation, hepatic failure, alanine amino transferase and bilirubin but not with markers of extra hepatic organ injury. TUNEL staining confirmed evidence of greater hepatic cell death in patients with ACLF as opposed to AD. Inclusion of cK18 and K18 improved the performance of the CLIF-C AD score in prediction of progression from AD to ACLF ($p<0.05$). *Conclusion*: Cell death, likely hepatic, is an important feature of AD and ACLF and its magnitude correlates with clinical severity. Non-apoptotic forms of cell death predominate with increasing severity of AD and ACLF. The data suggests that ACLF is a heterogeneous entity and shows that the importance of cell death in its pathophysiology is dependent on predisposing factors, precipitating illness, response to injury and the type of organ failure.

Introduction

An acute decompensating event (AD) is the most common hospital presentation of cirrhotic liver disease and can be successfully managed in most cases(1). However, 30% of patients present with or develop rapidly progressive hepatic and/or extra-hepatic organ failure, a condition referred to as acute on chronic liver failure (ACLF)(2). About 20% of these patients progress to multi-organ failure and death(2). The risk of death is closely related to the number of organ failures(2).

The pathophysiological basis of ACLF is not clearly understood and the care of patients is largely supportive. No targeted therapies are available. Current hypotheses describe ACLF as being driven by systemic inflammation induced by a cytokine storm, oxidative stress, immune dysfunction and increased risk of infection(3)(4)(5). As the syndrome is defined by the failure of hepatic and extra-hepatic organs(6), cell death is likely to be important(7) but the site, role, type and extent has not been fully defined. Cell death may result in a release of damage-associated molecular patterns (DAMPs) that could drive inflammasome activation, directly perpetuate further cell death and mediate additional organ failures.

Markers of cell death in particular, caspase-cleaved keratin 18 (cK18) and keratin 18 (K18), have been previously demonstrated to be clinically relevant in the diagnosis, assessment of disease severity and prognosis of a wide range of acute and chronic liver diseases including chronic and acute-on-chronic hepatitis B(8)(9), chronic hepatitis C(10), drug-induced liver injury(11),

non-alcoholic fatty liver disease(12)(13), alcoholic hepatitis(14), acute liver failure(15), and primary biliary cirrhosis(16) (reviewed in Supplementary Table 1), as well as in non-hepatological diseases such as breast and gastrointestinal cancer(17) (18) and sepsis(19). Keratins are the main epithelial subgroup of intermediate filament proteins (IFs)(20). K18 is expressed by both hepatocytes and cholangiocytes(21) as well as other non-hepatic tissues including kidney, intestine and lung (22) and after initiation of apoptosis, K18 is cleaved by activated caspases at two points. Firstly, early in apoptosis, at K18-Asp396 which is unique to K18 and then later at a common caspase cleavage site found in members of the linker L1-2 region of the central rod domain which is present in other members of the IF family(23)(24)(25). It is the neoepitope generated in the first cleavage that is recognized by the M30 antibody that is the basis for the most frequently used measurement of cK18 and widely taken to reflect hepatic apoptosis(26). Intact K18 can be measured using the M6 and M5 monoclonal antibodies. These are termed the M65 antibodies and they recognise protein epitopes of K18 and therefore, detect intact K18, its non-apoptotic fragments but also the apoptotic fragment. M65 values are widely taken to reflect necrotic cell death; however, should probably be regarded as a measure of total cell death. Whilst the measurement of circulating levels of cK18 and K18 have been widely interpreted as reflecting hepatic cell death caution in the interpretation of these data is required due to the potential for circulating cK18/K18 to be derived from non-hepatic tissues. Small studies, including liver histology, have suggested the importance of hepatic cell death in the pathogenesis of ACLF(7) but its importance in the pathophysiology of acute decompensation

of cirrhosis is unknown.

The aims of this study were to determine the changes in cK18 and K18 levels as measures of apoptotic and total cell death in the plasma of 337 patients with acute decompensation of liver disease who were enrolled in the prospective, multi-centre CANONIC study(6).

Materials and Methods

Patients

The samples and data of patients with AD and ACLF in the current study were obtained from the patients in the CANONIC study (6) which was prospective enrolled and was designed specifically to define the clinical and prognostic features of ACLF. Samples and data from healthy volunteers and those with stable cirrhosis were obtained from archived bio-banked material at the Royal Free hospital. Liver sections from patients with alcoholic hepatitis were obtained from the histology department of the Royal Free Hospital in London (UCL Biobank Ethical Review Committee approval number NC.2017.10) and from patients with HBV from the Third Affiliated Hospital of Sun Yat-Sen University, China [Human Ethics Committee of the Third Affiliated Hospital, approval number ZSSYME(2016)2-72]. All the samples were collected with informed consent from the patients and the principles of good clinical practice and the Declaration of Helsinki, 1951 were followed closely throughout.

The cohort of the CANONIC study included 1343 patients who were hospitalized with an acute decompensation of cirrhosis (bacterial infection, large volume ascites, gastrointestinal haemorrhage, hepatic encephalopathy, alone or in combination) in 29 Hepatology centres across 8 countries(6), 337 patients with plasma samples were available for analysis and they comprise the study population. The characteristics of the patients included in this study closely reflect the patients described in the CANONIC study. Additionally,

samples from 34 healthy volunteers and 44 patients with stable cirrhosis were used as controls.

Definitions

Definitions used in this study were as described in the CANONIC study(6).

Acute on Chronic Liver Failure: ACLF was defined in terms of organ failures according to the CLIF-Organ Failure Score(6) and diagnosis required: 1) single kidney failure; 2) single liver, coagulation, circulatory or respiratory failure and serum creatinine levels between ⩾1.5 and <2 mg/dl and/or hepatic encephalopathy grades I or II; 3) single cerebral failure (hepatic encephalopathy grades III or IV) associated with a serum creatinine between ⩾1.5 and <2 mg/dl; or 4) two or more organ failures.

Acute Decompensation: AD was defined as the acute development of ascites(27), hepatic encephalopathy(28), gastrointestinal haemorrhage(29), or bacterial infection(30) alone or in combination in patients who did not fulfill the criteria for the diagnosis of ACLF.

Study design

Baseline cK18 and K18 levels were measured and cK18:K18 ratio calculated. The collected data were analysed blindly by the data management centre of the European Foundation for the study of Chronic Liver Failure [(EF-CLIF) Barcelona]. The pre-defined end points of the analysis were to perform a descriptive analysis of cell death markers according to factors associated with

acute decompensation of cirrhosis using the PIRO concept; *P*redisposition (underlying factors such as age, etiology, etc.), *I*njury (precipitating factors), markers of *R*esponse (inflammation and infection) and, *O*rgan failures (presence, type and number). The prognostic value of the cell-death markers in patients with AD and ACLF and their relationship to 28-day and 90-mortality was then assessed.

Correlation analyses for cK18 and K18 with inflammatory markers and markers of macrophage activation that are known to be increased in ACLF (IL-6, IL-8, IL-10, IL-Ra, NGAL, sCD163) and marker of oxidative stress [human non-mercaptalbumin-2 (HNA-2)] were then performed. Data regarding these analytes partly overlap with previous publications (Claria et al.(5); Ariza et al.(31); Gronbaek et al.(32)). Correlation analyses for cK18 and K18 with markers of individual organ dysfunction were then performed (bilirubin, alanine aminotransferase, prothrombin time, creatinine, hepatic encephalopathy grade, mean arterial pressure and heart rate

Measurement of cK18 and K18 and calculation of cK18:K18 ratio

All blood samples were centrifuged at 2000 rpm for 10 minutes and the supernatants were stored at -80 within 4 hours of collection. Serum cK18 and K18 levels were then measured in baseline EDTA samples by ELISA [M30 Apoptosense (Peviva, UK) and M65 EpiDeath (Peviva, UK) respectively]. The cK18:K18 ratio (apoptotic index) was then calculated.

Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) staining of liver sections

Liver sections of patients with alcoholic hepatitis with and without ACLF and HBV infection with and without ACLF were prepared and stained for TUNEL positivity according to the kit protocol (In situ cell death detection kit, colorimetric, Roche, UK).

Statistical Analysis

Results are presented as frequencies and percentages for categorical variables, means and standard deviations for normally distributed continuous variables and median and interquartile range for not normally distributed continuous variables. Not normally distributed variables were log-transformed for some statistical analyses and for graphical comparisons. In univariate analyses, Chi-square test was used for categorical variables, Student's t-test or ANOVA for normal continuous variables and Mann-Whitney or Kruskal Wallis test for not normally distributed continuous variables. To assess the prediction of occurrence of ACLF in AD patients, logistic regression models were carried out. Factors showing a clinically and statistically significant association to the outcome in univariate analyses were selected for the initial model. The final models were fitted by using a step-wise forward method based on Likelihood Ratios with the same significance level ($p<0.05$) for entering and removing variables. To assess the strength of the association between cK18 and K18 levels and current scores for the prediction of ACLF and its outcome, we estimated the Area Under the ROC curve (AUROC). The

proportional-hazards model for Competing-Risks proposed by Fine and Gray(33) was used to assess the presence of independent factors of mortality. This model was chosen in order to account for liver transplantation as an event ‘competing’ with mortality. Harrell’s concordance index (C-index) was used to estimate the variables discrimination ability(34)(35). Statistical comparisons of the C-index with the current scores were carried out using the integrated discriminating improvement statistic(36). In all statistical analyses, significances was set at $p<0.05$.

Results

Patient characteristics

Three hundred and thirty seven patients with decompensated cirrhosis were studied of whom 258 (76.6%) presented with AD and 79 (23.4%) with ACLF. At 28 and 90 days, 41 (12.7%) and 68 (22.4%) of all patients had died respectively. Thirty-nine (15.1%) patients who presented with AD progressed to ACLF following admission. Eight patients (10.1%) who presented with ACLF regressed to AD. The baseline characteristics of the patient group are shown in Table 2. A further 16 patients with available liver biopsies were studied, 8 with ACLF and 8 without. The cause of cirrhosis in 8 patients was Hepatitis B virus infection and alcohol related cirrhosis with superimposed alcoholic hepatitis in 8. The baseline characteristics of the patient group are shown in Supplementary Table 2.

There were similar distributions of age and sex in the AD and ACLF groups. An alcohol-related liver disease etiology, presentation with recent alcohol use, bacterial infection, presence of ascites or its surrogates was associated with increased risk of ACLF. According to the definitions, patients with ACLF presented with higher rates of organ failure, significantly worse biochemical and hematological parameters and clinical scores. The markers of systemic inflammation, oxidative stress (HNA-2) and macrophage activation (sCD163) were also significantly higher in ACLF patients. This pattern of patient characteristics and inflammatory markers closely reflected those of the original CANONIC study (Supplementary Table 3).

Relationship of cK18, K18 and cK18/K18 ratio to AD and ACLF

The median values of cK18 and K18 for both the AD and ACLF groups were significantly higher than healthy volunteers and stable cirrhosis patients (Table 2). A statistically significant stepwise increase in cK18 and K18 level was observed with increasing clinical severity from AD to ACLF and within ACLF grades (Table 2). In addition, patients who presented with AD but subsequently developed ACLF during hospitalisation had significantly higher levels of cK18 and K18 than those who remained in AD throughout their admission. Conversely, those patients who presented with ACLF but improved to AD during admission had lower baseline levels of cK18 and K18 (Table 2). Furthermore, a significant reduction in the ratio of cK18 level to K18 level (referred to as apoptotic index) was observed with increasing severity of AD and ACLF. Whilst overall both cK18 and K18 levels were markedly increased with clinical severity the reduction in the apoptotic index reflects that the relative magnitude of the increase in K18 was greater. In AD patients the apoptotic index was high indicating that apoptotic cell death predominated whereas in ACLF patients, the index was low suggesting that other non-apoptotic modes of cell death, were more significant.

Relationship of cK18 and K18 levels to Predisposition, Injury, Response and Organ failures.

Predisposition (Figure 1A. and Supplementary Table 4): No significant differences were observed in cK18 and K18 levels when patients were stratified by age or sex. Patients with underlying alcohol-related cirrhosis demonstrated a significant elevation in cK18 level and a non-significant

elevation in K18 level in comparison to non-alcohol-related etiologies whereas, patients with HCV-related liver disease demonstrated significantly reduced cK18 level and a trend toward reduced K18 levels in comparison to non-HCV etiologies. A previous episode of decompensation was strongly associated with a reduction of both cK18 and K18 levels.

Precipitating injury (Figure 1B. and Supplementary Table 5): Consumption of alcohol within the 3-months prior to admission, indicating likely alcoholic hepatitis as a precipitating cause of decompensation, was associated with a significantly higher cK18 and K18 level and a significant reduction in cK18:K18 ratio in comparison to those who were not abusing alcohol. In contrast, the presence of bacterial infection was not associated with a significant increase in cK18 or K18 level (figure 1B) but was clearly associated with evidence of systemic inflammation and cytokinemia (Table 3) Additionally, patients presenting without a clear precipitating event demonstrated a significant lower K18 but not cK18 level.

Response to injury (Figure 1C. and Supplementary Table 6): WCC was significantly associated with both cK18 and K18 levels and with a non-significant reduction in cK18:K18 ratio. CRP was significantly associated with K18 level. IL8, IL1Ra and sCD163 as well as NGAL were associated with both cK18 and K18 level and a reduction in cK18:K18 ratio. Furthermore IL10 and HNA2 were both associated with elevation of K18 but not cK18 level and a reduction in cK18:K18 ratio. IL8 correlated strongly with both cK18 and K18 and IL6, sCD163 and HNA2 correlated with K18. (Supplementary Figure 1).

Organ failures (Figure 1D. and Supplementary Table 7): Liver failure in isolation or in a combination with other organ failures was associated with significant elevation in both cK18 and K18 level and a reduction in cK18:K18 ratio. The liver was demonstrated as a possible source of the elevated cK18 and K18 levels as both bilirubin and alanine aminotransferase positively correlated to cK18 and K18 levels (Supplementary Figure 2). In contrast, cK18 or K18 levels did not correlate with with creatinine, prothrombin time, grade of hepatic encephalopathy or mean arterial pressure indicating that the source of increased cK18 and K18 was unlikely to be these extra hepatic organs. Heart rate, which is another component of systemic inflammatory response, was positively correlated with both cK18 and K18 level (Supplementary Figure 2). Kidney failure in isolation was not associated with elevation of either cK18 or K18 level; however, when kidney failure was associated with liver failure, a trend towards elevated cK18 and K18 level was demonstrated and a reduced cK18:K18 ratio observed (Supplementary Table 7). Isolated cardiac failure was not associated with elevation of cK18 and K18 level but only when cardiac failure was associated with liver failure (Supplementary Table 7).

Relationship of cK18, K18 and cK18:K18 ratio to progression from AD to ACLF and mortality

Prediction of Progression from AD to ACLF: Progression from AD to ACLF was not associated with age, sex, underlying etiology or precipitating event. Progression was associated with presence of ascites, significantly poorer

indices of hepatic function (bilirubin, albumin and INR), increased markers of systemic inflammation (WCC and CRP) and clinical prognostic scores. Both cK18 and K18 levels were significantly higher in those patients who progressed from AD to ACLF. (Supplementary Table 8). Both cK18 and K18 levels were independent predictors of progression from AD to ACLF in univariate and multivariate analysis. The addition of cK18 to the CLIF-C AD score led to a significant increase in its predictive accuracy (Table 4).

Prediction of mortality: In univariate analysis 28-day and 90-day transplant free mortality was strongly associated with cK18, K18 and cK18:K18 ratio in addition to a number of clinical parameters, liver and kidney biochemistry and clinical scores (Supplementary Table 9). cK18 and K18 remained significant in multivariate analysis in addition to age, presence of bacterial infection, INR, sodium and WCC. For prediction of mortality at 28 and 90 days in the AD population, K18 demonstrated a better predictive accuracy than the MELD score. The most accurate predictive score was the CLIF-C AD score and addition of cK18:K18 ratio non-significantly improved its accuracy (Table 5). Additionally, cK18, K18 and cK18:K18 ratio were highly significant when modeling cumulative incidence of death in 90 days in both the AD population and ACLF populations (Figure 2).

Histology – TUNEL staining:

TUNEL staining of liver tissue from patients with alcoholic hepatitis or HBV demonstrated that the presence of ACLF was associated with a marked elevation in end stage hepatic cell death as demonstrated by increased levels

of TUNEL positive cytoplasmic/extracellular staining [Figure 3A and 3B (40x magnification) and Supplementary Figure 3A and 3B (10x magnification). Clinical characteristics of these patients are described in Supplementary Table 2.

Discussion

This study demonstrates that markers of cell death, both apoptotic and non-apoptotic, are elevated in patients with AD and ACLF in comparison to stable cirrhosis or health and that they increase with the clinical severity of the syndrome. Additionally, the more immunogenic, non-apoptotic forms of cell death(37) predominate as clinical severity increases with progression from AD to ACLF. The demonstration that the only single organ failure associated with significant elevation of K18 markers and the positive correlation of K18 markers to markers of hepatic injury, bilirubin and alanine aminotransferase, and not markers of non-hepatic organ dysfunction suggest that the elevation of K18 markers demonstrated is likely to be predominantly derived from the liver. This interpretation is supported by the marked increase in TUNEL positive staining demonstrated in the liver biopsies of patient with ACLF as opposed to those without in patients with a background of HBV infection and those with alcoholic hepatitis. The data suggests that ACLF is associated with increased hepatic cell death independent of the underlying etiology and furthermore, that although ACLF is defined by multiple organ failure, products of cell death, are likely to be important in its pathogenesis. Whether there is additional contribution from cell death affecting other organs is not known and cannot be ruled out from the results of this study. The variability in the magnitude of increases in these markers highlights the heterogeneity of ACLF indicating that other associated factors also contribute to its pathogenesis.

From the pathophysiological perspective, a strong correlation with markers of systemic inflammation, oxidative stress and macrophage activation was observed indicating that cell death is an important feature of AD and ACLF. Additionally, the significant reduction in the cK18:K18 ratio seen in patients with ACLF as compared to those with AD suggests that whilst levels of both apoptotic and non-apoptotic modes of cell death markedly increase with clinical severity, it is non-apoptotic, and potentially more immunogenic, modes of cell death that dominate in ACLF. Zheng et al. observed a relative increase in K18 in relation to cK18 with increasing clinical severity in patients with acute deterioration of liver function in the context of chronic HBV-related liver disease(9) and the data presented here confirms and broadens this observation to ACLF. The shift in the dominant mode of cell death from apoptosis to non-apoptotic forms with increasing clinical severity also possibly explains the limited effect of the pan-caspase inhibitor, Emricasan when used in ACLF patients(38).

Current hypotheses describes ACLF as a syndrome driven by systemic inflammation(3)(4)(5). In keeping with previous studies, both WCC count and CRP were elevated in patients with ACLF. The profile of the correlations of cK18 and K18 to the cytokines tested suggests that with increasing clinical severity of ACLF, there is greater tissue injury and cell death with concomitant activation of mechanisms that increase neutrophil recruitment (IL8) and the activation of anti-inflammatory strategies to limit the immunological consequences of cell death (IL10, IL1RA, sCD163). Whilst it is likely that

elevation of DAMPs as a result of elevated rates of cell death would lead to exacerbation of inflammasome activation driving ongoing inflammation, it is possible that products of cell death have a direct cytotoxic effect and could therefore propagate liver injury independent of the inflammasome. This would account for the wide variation in cytokine profiles that have been demonstrated in ACLF patients(5).

Although the levels of cK18 and K18 were appropriately elevated in the patient population studied according to the severity of AD and ACLF, infection as a precipitating event was not associated with a significant difference in cK18 or K18 level but was associated with substantial increase in the markers of systemic inflammation and cytokinemia (Table 3). This suggests that pathogen-associated molecular patterns rather than DAMPs are likely to be more important in mediating organ injury in this context. In contrast, recent alcohol use as a precipitating event of AD or ACLF was associated with marked elevations in these markers indicating distinct pathophysiological mechanisms of decompensation. These data are supported by observations in liver biopsies of patients with alcohol related ACLF, where the predominant feature of infection was cholestasis(39) whereas balloon degeneration and cell death were the predominant features of severe alcoholic hepatitis(40) and necrosis predominates in patients with HBV related ACLF(41). The data presented confirms the recent observation by Bissonnette et al. that patients with alcoholic hepatitis have elevated levels of K18 and its fragments, however argues for caution in using elevation of cK18 and K18 levels as diagnostic of alcoholic hepatitis without considering the clinical severity of the

presentation. Patients with a clinically severe presentation of etiologies other than alcoholic hepatitis can also demonstrate marked elevations of cK18 and K18 levels, especially if they have ACLF. The absence of a clear precipitating event as a cause of AD and ACLF is observed in about 30-40% patients(6). The mechanisms underlying this are not clear but the data from the present study is against the idea that cell death is the defining mechanism.

The data also describes distinct patterns of severity of cell death in different sub-populations of patients with AD and ACLF suggesting that therapeutic approaches may need to be different depending upon the predisposing factors, the precipitating illness and the type of organ injury. Despite age being an independent predictor of mortality, no significant difference in cell death markers were demonstrated between younger and older patients. Patients who had not suffered a previous decompensating event demonstrated significantly higher levels of cell death markers, possibly explaining the previous observation that for a given severity of ACLF and WCC, the mortality of those with no previous decompensation was significantly higher(6). This observation may have several explanations. First, hepatic injury is known to induce hepatic cellular senescence(42)(43) and senescent hepatocytes have been demonstrated to be resistant to apoptosis(44). Second, the process of decompensation itself may induce organ tolerance(45)(46) through an as yet un-described mechanism. The data also shows lower levels of markers of cell death in patients with Hepatitis C infection compared with other etiologies. This may well represent a further effect of senescent hepatocyte resistance to apoptosis as increased numbers

of senescent hepatocytes have been demonstrated in Hepatitis C infected patients (47).

The addition of cK18 and K18 enhanced the prognostic power of all clinical scores both in terms of progression from AD to ACLF and short-term mortality and allowed stratification of risk of death by 90 days. As described previously, CLIF-C AD score performed best in predicting which patients would progress from AD to ACLF(48). Its prognostic value was significantly enhanced by inclusion of cK18 suggesting that this may be a useful biomarker to guide targeting of patients for enhanced monitoring and intensive therapy. However, from the analysis of the sub-groups outlined above, it is clear that, whilst there is a clear overall rise in markers of cell death with clinical severity, there is considerable variation in the mechanism and severity of cell death according to the etiology, precipitating events and type of organ failure. The clinical utility of cK18 and K18 as biomarkers in AD and ACLF may therefore be as a companion to define which patients may benefit from specific interventions, such as inhibitors of apoptosis, rather than provide prognostic information about groups of patients.

There are limitations to this study that need to be acknowledged. The patient samples available for analysis were less than the total number of patients enrolled in the original CANONIC study(6) therefore there is potential for the introduction of a selection bias. However, the samples used for the analyses were obtained at random and the demographic, clinical and biochemical data for the analysed group were not statistically different to that of the original

study (Supplementary Table 2). Additionally, K18 is not specific to the liver and is found in other epithelial tissues including the GI tract, lung and kidney(26). Therefore, elevated circulating levels of K18 cannot be directly attributed solely to liver injury. However, from the data presented it can be seen that liver failure is associated with a marked elevation in K18 and its fragments and such elevations are not seen with other isolated organ failures. Additionally, cK18 and K18 correlated to markers of hepatic injury such as ALT and bilirubin and not markers of other organ dysfunction. Furthermore, TUNEL staining of liver biopsies from patients with two different etiologies have both demonstrated that the presence of ACLF is associated with a marked increase in hepatic cell death and so it seems likely that the elevation in plasma K18 markers is likely to be hepatic in origin. cK18 level, as measured by m30 antibody ELISA, reflects only the first cleavage of K18 occurring in early apoptosis and does not take account of the second caspase-cleaved K18 fragment produced at a later stage of apoptosis(25). Additionally, K18 level as measured by the M65 antibodies, reflects not only intact K18 and non-apoptosis derived fragments but also an apoptotic fragment and so does not exclusively reflect non-apoptotic cell death, rather is more a measure of total cell death(49) and further studies will be required to delineate the relative importance of other modes of cell death in ACLF.

In conclusion, the results of this study demonstrate that cK18 and K18 levels, reflecting apoptotic and total cell death, closely reflect the severity of an episode of acute decompensation of cirrhosis and this elevation is likely hepatic in origin. This supports the hypothesis that liver cell death is an

important feature of AD and ACLF. The data presented suggests that whilst there is a dramatic increase in levels of both apoptotic and non-apoptotic cell death with increasing clinical severity of decompensation, progression from AD to ACLF is associated with a relatively greater rise in non-apoptotic cell death. The severity of cell death is also closely related to the predisposing factors, precipitating illness, severity of systemic inflammation and the type and number of organ failures. Although, these markers of cell death do not add substantially to the CLIF-ACLF score in determining prognosis, it improves the performance of the CLIF-AD score suggesting that it could serve as a potential biomarker to select patients for treatment with new agents targeting cell death.

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Legends to Figures.

Figure 1. Caspase-cleaved Keratin (cK18), keratin 18 (K18), and cK18/K18 ratio in the cirrhotic patients with acute deterioration according to (A) Predisposing factors (B) Injury – Precipitating factor (C) Response and (D) Type of Organ failure (single organ failure). (*$p<0.05$).

Figure 2. Kaplan Meier analysis defining cumulative mortality according to measurements of Caspase-cleaved Keratin (cK18), Keratin 18 (K18), and cK18/K18 ratio in patients with (A) acute decompensation (no ACLF) and (B) ACLF.

Figure 3. A) TUNEL staining of liver biopsies of patients with alcoholic hepatitis without and with ACLF (40x magnification). B) TUNEL staining of liver biopsies of patients with HBV without and with ACLF (40x magnification). Numbering of images reflects the patient number as given in Supplementary Table 2.

Supplementary Materials

Prognostic and pathophysiological role of liver cell death markers in cirrhotic patients with acute decompensation

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No of Supplementary Tables: 9

Supplementary Figure 1.

This figure describes the correlations between markers of systemic inflammation, macrophage activation and oxidative stress with markers of cell death, Keratin 18 (K18) and Caspase Cleaved Keratin 18 (cK18).

Supplementary Figure 2.

This figure describes the correlations between markers of organ failures: liver; kidney; coagulation; brain; cardiac and markers of cell death, Keratin 18 (K18) and Caspase Cleaved Keratin 18 (cK18).

Supplementary Figure 3A+B.

Figure 3. A) TUNEL staining of liver biopsies of patients with alcoholic hepatitis without and with ACLF (10x magnification). B) TUNEL staining of liver biopsies of patients with HBV without and with ACLF (10x magnification). Numbering of images reflects the patient number as given in supplementary table 2.

Supplementary Table 1. selection of published studies using cK18 or K18 in acute and chronic liver diseases.

Authors	Year	Liver Disease	Findings
Bantel H. et al.[1]	2004	HCV	Detection of plasma cK18 is a more sensitive method of detecting early liver injury in HCV than measurement of transaminases.
Volkmann X. et al.[2]	2006	HCV	cK18 level is predictive of patient response to anti-HCV therapy.
Rutherford AE. et al. [3]	2007	ALF	Elevation of cK18 is associated with poor outcome in ALF.
Papatheodoridis G V. et al.[4]	2008	HBV	Plasma cK18 level can differentiation between the inactive HBV carrier state and HBeAg-negative chronic hepatitis B.
Diab DL. et al.[5]	2008	NAFLD/NASH	cK18 level can predict NASH in NALFD patients.
Volkmann X. et al.[6]	2008	ALF	cK18 levels in plasma are predictive of survival in ALF.
Feldstein A. et al.[7]	2009	NALFD/NASH	cK18 level can predict NASH in NALFD patients.
Tsutsui M et al.[8]	2010	NAFLD/NASH	cK18 and K18 plasma level reflect the NAFLD histological activity score score in patients with NAFLD.
Papatheodoridis G V. et al.[9]	2010	HCV/NAFLD	Serum cK18 level correlates to the severity of the liver histological lesion in HCV and in NALFD.
Farnik H, et al. [10]	2011	HBV	Circulating cK18 and K18 levels reduce with viral DNA levels in response to nucleos(t)ide analogue treatment of HBV, suggesting a reduction in necro-inflammation.
Shen J. et al.[11]	2012	NAFLD/NASH	cK18 level can predict NASH in NALFD patients.
Possamai LA. et al.[12]	2013	ALF	Hepatocellular apoptosis, as measured by cK18, peaks on day 1 of hospital admission for paracetamol-induced ALF and its level correlates strongly with poor outcome.

Zheng S-J et al.[13]	2014	HBV	cK18 and K18 levels are independent predictors of mortality in patients with HBV-ACLF.
Aida Y. et al.[14]	2014	NAFLD/NASH	cK18 level can predict NASH in NAFLD patients.
Thulin P. et al.[15]	2014	DILI	K18 level as measure by M65 is a potential biomarker of DILI.
Sekiguchi T. et al.[16]	2015	PBC	K18 biomarkers can predict the level of fibrosis in PBC and may predict poor patient outcomes.
Bissonnette J. et al.[17]	2017	AH	cK18 and K18 levels can be used to diagnose AH avoiding the need for biopsy.

Supplementary Table 2. Clinical characteristics of those patients whose liver biopsies were stained for TUNEL positivity.

Patient Number	ACLF no / yes	Age	Sex	Aetiology of liver disease	Precipitating event	
1	no	55	M	ALD	AH	
2	no	52	F	ALD	AH	
3	no	50	M	ALD	AH	
4	no	44	F	ALD	AH	
5	yes	34	M	ALD	AH	
6	yes	56	M	ALD	AH	
7	yes	54	F	ALD	AH	
8	yes	30	F	ALD	AH	
						viral load
9	no	23	M	HBV	none	2.34E+03
10	no	33	M	HBV	none	4.80E+08
11	no	49	F	HBV	none	1.33E+05
12	no	51	M	HBV	none	2.78E+03
13	yes	52	M	HBV	HBV reactivation	3.35E+03
14	yes	50	M	HBV	HBV reactivation	1.54E+07
15	yes	35	M	HBV	HBV reactivation	2.70E+06
16	yes	36	F	HBV	HBV reactivation	2.96E+04

Supplementary Table 3. Comparison of patient characteristics at baseline between current study cohort and whole CANONIC study cohort.

Baseline characteristic	Current study (n=337)	CANONIC study (n=1349)	P value
Age (years)	57±12	57±12	0.864
Male (n, %)	210(62.3)	854(63.3)	0.736
ACLF at enrolment (n, %)	79(23.4)	302(22.4)	0.679
Etiology (n, %)			
Alcohol	162(50.5)	664(52.1)	0.606
HCV	75(23.4)	248(19.5)	0.119
Alcohol + HCV	29(9.0)	122(9.6)	0.770
Other	55(17.1)	241(18.9)	0.466
Previous Decompensation (n, %)	247(75.8)	948(73.3)	0.369
Alcohol in last 3 months (n, %)	45(14.2)	218(17.1)	0.217
Ascites or its surrogates (n, %)	301(89.3)	1209(89.6)	0.870
GI bleeding (n, %)	54(16.0)	222(16.5)	0.848
Bacterial infection (n, %)	88(26.3)	325(24.2)	0.423
Organ Failures (n, %)			
Liver	56(16.6)	205(15.2)	0.519
Kidney	38(11.3)	169(12.5)	0.531
Brain	29(8.6)	99(7.3)	0.432
Coagulation	29(8.6)	106(7.9)	0.651
Cardiac	19(5.6)	64(4.7)	0.498
Respiratory	7(2.1)	33(2.5)	0.690
Markers inflammation and stress			
WBC ($x10^9$/L)	6.1(4.4-8.8)	6.2(4.3-9.2)	0.980
CRP (mg/L)	18(7-36)	18(7-43)	0.534
IL8 (pg/mL)	61(31-121)	52(26-113)	0.149
IL6 (pg/mL)	33(17-94)	27(13-65)	0.020
IL10 (pg/mL)	4.8(1.6-15.3)	4.8(1.5-15.6)	0.754
ILRA (pg/mL)	15(7-45)	14(6-40)	0.384
NGAL (ng/mL)	37(13-98)	35(13-95)	0.686
sCD163 (mg/L)	9.3(5.8-14.2)	8.7(5.2-13.5)	0.116

HNA-2 (%)	7.6(4.2-11.3)	7.0(3.3-11.7)	0.165
Laboratory values			
Bilirubin (mg/dL)	3.2(1.6-7.5)	3.0(1.6-7.4)	0.440
INR	1.5(1.3-1.9)	1.5(1.3-1.9)	0.201
Albumin (g/dL)	2.9(2.4-3.2)	2.9(2.4-3.2)	0.834
Creatinine (mg/dL)	1.0(0.8-1.4)	0.9(0.7-1.4)	0.388
Sodium (mmol/L)	135±6	135±6	0.973
Platelets ($x10^9$/L)	87(52-128)	91(57-136)	0.154
MELD	19±8	19±7	0.325
MELD Na	22±7	21±7	0.473
CP score	9.7±2.1	9.7±2.1	0.797
CLIF – OFs	8±2	8±2	0.373
28-day mortality (%)	41(12.2)	143(10.6)	0.700
3-month mortality (%)	68(20.2)	165(19.6)	0.675

Data are mean ± SD or median (Q1-Q3)

Supplementary Table 4. Keratin 18, Caspase cleaved Keratin 18 and cK18:K18 ratio stratified by predisposition in all patients.

	N	**K18** (U/L) **Median** (IQR)	**cK18** (U/L) **Median** (IQR)	**cK18:K18 Ratio Median** (IQR)
Age <57	160	985(496-2529)	1144(752-2198)	1.1(0.7-1.8)
Age ≥57	177	943(328-1930)	971(751-1505)	1.3(0.7-2.6)
Sex – Male	210	872(401-2060)	1012(725-1552)	1.2(0.8-2.4)
Sex – Female	127	1192(398-3065)	1050(793-1793)	1.1(0.6-2.2)
Etiology				
Alcohol (No)	159	922(398-1908)	948(719-1359)	1.2(0.7-2.3)
Alcohol (Yes)	162	1011(376-2542)	1156(818-2394)*	1.2(0.8-2.4)
HCV (No)	246	985(424-2510)	1073(779-2180)	1.2(0.8-2.3)
HCV (Yes)	75	752(327-1854)	967(712-1335)*	1.3(0.7-2.4)
Alcohol + HCV (No)	292	939(359-2347)	1032(779-1700)	1.2(0.7-2.5)
Alcohol + HCV (Yes)	29	989(648-1754)	949(671-1303)	1.0(0.8-1.6)
Other (No)	266	961(378-2343)	1050(761-1665)	1.2(0.8-2.3)
Other (Yes)	55	898(413-2318)	926(751-2171)	1.3(0.7-2.7)
Previous Decomp. (No)	79	1316(537-3413)	1170(893-2346)	1.1(0.7-1.8)
Previous Decomp. (Yes)	247	938(347-1908)*	978(713-1534)*	1.2(0.8-2.5)
Ascites with subrogates (No)	36	673(332-1713)	1061(632-1378)	1.5(0.9-2.8)
Ascites with surrogates (Yes)	301	978(406-2352)	1025(775-1750)	1.2(0.7-2.2)

*P value < 0.05
Keratin 18 (K18), Caspase Cleaved Keratin 18 (cK18)

Supplementary Table 5. Keratin 18, Caspase cleaved Keratin 18 and cK18:K18 ratio stratified by precipitating injury in all patients.

	N	**K18** (U/L) **Median** (IQR)	**cK18** (U/L) **Median** (IQR)	**Ratio cK18:K18 Median** (IQR)
Alcohol in last 3 months (No)	271	818(338-1949)	959(719-1513)	1.3(0.8-2.4)
Alcohol in last 3 months (Yes)	45	2213(1062-4590)*	1591(1237-3609)*	1.0(0.6-1.5)*
GI bleeding (No)	283	958(416-2273)	1019(780-1643)	1.2(0.7-2.2)
GI bleeding (Yes)	54	954(359-2510)	1132(626-2435)	1.4(0.9-2.6)
Bacterial infection (No)	247	918(377-2060)	1019(769-1643)	1.2(0.8-2.3)
Bacterial infection (Yes)	88	1217(429-2476)	1032(739-1775)	1.4(0.9-2.6)
No precipitating event	151	778(376-1764)	961(751-1371)	1.3(0.8-2.3)
Precipitating event	170	1129(419-2530)*	1116(739-1912)	1.1(0.7-2.0)

*P value < 0.05
Keratin 18 (K18), Caspase Cleaved Keratin 18 (cK18)

Supplementary Table 6. Keratin 18, Caspase cleaved Keratin 18 and cK18:K18 ratio stratified by inflammatory markers, cytokines and markers of oxidative stress in all patients.

	N	**K18**(U/L) **Median**(IQR)	**cK18**(U/L) **Median**(IQR)	**cK18:K18 Ratio** **Median**(IQR)
WBC ($x10^9$/L) <8	227	838(339-1737)	950(696-1371)	1.2(0.7-2.5)
WBC ($x10^9$/L) 8-12	67	1139(453-2611)	1219(829-2290)	1.2(0.8-2.0)
WBC ($x10^9$/L) >12	42	2749(853-7016)*	1984(985-3995)*	1.0(0.6-1.4)
CRP (mg/L) <10	87	903(330-1462)	988(740-1373)	1.4(0.8-2.8)
CRP (mg/L) 10-20	60	1139(459-2542)	1114(810-1988)	1.0(0.6-1.6)
CRP (mg/L) >20	118	1218(453-2894)*	1156(783-2500)	1.3(0.8-2.0)
IL8 (pg/mL) <61	102	558(279-1217)	879(665-1214)	1.7(0.9-2.9)
IL8 (pg/mL) ≥61	101	2194(869-5138)*	1505(997-3517)*	1.0(0.6-1.5)*
IL6 (pg/mL) <33	99	842(327-2542)	1061(696-2180)	1.4(0.8-2.7)
IL6 (pg/mL) ≥33	99	1306(502-2529)	1183(838-2207)	1.0(0.7-1.8)*
IL10 (pg/mL) <4.8	96	820(332-2216)	1078(780-1702)	1.3(0.8-2.9)
IL10 (pg/mL) ≥4.8	94	1542(413-3577)*	1152(712-2500)	1.0(0.6-1.8)*
IL1Ra (pg/mL) <14.7	101	687(330-1908)	926(712-1524)	1.3(0.8-2.7)
IL1Ra (pg/mL) ≥14.7	101	1377(454-3765)*	1243(830-2611)*	1.1(0.6-2.9)
NGAL (ng/mL)) <37	140	668(331-1581)	939(713-1379)	1.4(0.8-2.9)
NGAL (ng/mL)) ≥37	143	1002(398-2273)*	1124(740-2041)*	1.1(0.7-2.2)
sCD163 (mg/L) <9.3	164	704(299-1542)	939(678-1371)	1.4(0.8-3.0)
sCD163 (mg/L) ≥9.3	164	1139(504-2894)*	1156(801-2346)*	1.1(0.6-1.8)*
HNA2 (%) <7.6	102	777(310-2311)	1064(685-2041)	1.5(0.9-2.9)
HNA2 (%) ≥7.6	101	1218(542-3272)*	1197(814-2239)	1.0(0.6-1.7)

*P value < 0.05
Keratin 18 (K18), Caspase Cleaved Keratin 18 (cK18)

Supplementary Table 7. Keratin 18 (K18), Caspase Cleaved Keratin 18 (cK18) and cK18:K18 ratio stratified by organ failures in all patients.

	N	K18(U/L) Median(IQR)	cK18(U/L) Median(IQR)	Ratio cK18:K18 Median(IQR)
Single organ failures				
Liver (No)	301	931(359-2213)	998(742-1571)	1.2(0.7-2.4)
Liver (Yes)	30	1809(694-8945)*	1375(801-9355)*	1.0(0.8-1.4)
Kidney (No)	312	948(378-2331)	1037(739-1665)	1.2(0.7-2.3)
Kidney (Yes)	19	985(708-2354)	997(879-1591)	0.9(0.6-1.4)
Brain (No)	320	963(406-2348)	1044(775-1700)	1.2(0.7-2.2)
Brain (Yes)	11	537(377-978)	706(675-982)	1.4(0.8-3.8)
Coagulation (No)	322	955(377-2318)	1022(742-4662)	1.2(0.7-2.3)
Coagulation (Yes)	9	1199(717-3737)	1232(998-1534)	1.0(0.7-1.1)
Cardiac (No)	329	961(401-2343)	1034(752-1662)	1.2(0.7-2.2)
Cardiac (Yes)	2	421(332-510)	1018(464-1571)	2.8(0.9-4.7)
Respiratory (No)	330	955(398-2318)	1037(752-1662)	1.2(0.7-2.3)
Respiratory (Yes)	1	3788(3788-3788)	638(638-638)	0.2(0.2-0.2)
Single and Multiple				
Liver (No)	281	859(331-1910)	978(726-1526)	1.3(0.8-2.7)
Liver (Yes)	56	2347(867-8339)*	1417(938-3639)*	0.9(0.6-1.3)*
Kidney (No)	299	898(347-1934)	998(726-1569)	1.3(0.8-2.5)
Kidney (Yes)	38	2349(954-7611)*	1407(971-3711)*	0.7(0.5-1.3)*
Brain (No)	308	941(378-2223)	1019(742-1645)	1.2(0.7-2.3)
Brain (Yes)	29	1034(407-4965)	1269(860-2822)	1.1(0.7-2.1)
Coagulation (No)	308	935(376-2085)	1016(731-1591)	1.2(0.8-2.4)
Coagulation (Yes)	29	2176(717-4843)*	1232(890-2528)	0.9(0.6-1.7)*
Cardiac (No)	318	941(398-2213)	1019(742-1572)	1.2(0.8-2.3)
Cardiac (Yes)	19	2458(510-5087)*	2171(921-3711)*	0.9(0.6-1.6)
Respiratory (No)	330	947(400-2288)	1030(746-1661)	1.2(0.7-2.2)
Respiratory (Yes)	7	2476(267-5087)	1264(921-2290)	0.6(0.2-3.4)

*P value < 0.05

Keratin 18 (K18), Caspase Cleaved Keratin 18 (cK18)

Supplementary Table 8. Univariate analysis factors associated with progression of AD patients to ACLF

	AD patients at enrolment		P value
	No progression to ACLF (N=195)	Progression to ACLF (N=39)	
Age[1]	58±11	58±11	0.951
Male	126(64.6)	25(64.1)	0.951
Etiology			
Alcohol	88(47.1)	19(51.4)	0.633
HCV	48(25.7)	7(18.9)	0.384
Alcohol + HCV	16(8.6)	3(8.1)	0.929
Other	35(18.7)	8(21.6)	0.682
Previous Decomp.	141(73.4)	26(70.3)	0.691
Alcohol in last 3 months	18(9.7)	4(11.4)	0.759
Ascites with subrogates[1]	162(83.1)	38(97.4)	0.020
HE	50(25.6)	13(33.3)	0.323
GI bleeding[1]	33(16.9)	3(7.7)	0.145
Bacterial infection	43(22.2)	11(29.0)	0.366
Laboratory values			
Bilirubin[1]	2.6(1.5-5.5)	4.5(2.4-9.1)	0.005
INR[1]	1.4(1.3-1.7)	1.8(1.4-2.2)	<0.001
Albumin[1]	2.9(2.6-3.2)	2.7(2.2-3.0)	0.007
Creatinine[1]	0.9(0.7-1.2)	1.1(0.8-1.4)	0.165
Sodium[1]	136±5	132±9	0.009
Leucocyte count[1]	5.4(4.0-8.0)	6.8(5.2-11.2)	0.001
Neutrophil count	191(50-3056)	220(30-4224)	0.993
Platelets	91(55-141)	101(65-124)	0.574
C-reactive protein[1]	15(6-30)	28(14-39)	0.012
MELD	16±5	20±6	<0.001
MELD Na	18±6	24±6	<0.001
CP score	9.0±1.9	10.4±1.8	<0.001
CLIF AD score	51±8	60±10	<0.001
CLIF – OF	6.9±1.0	7.7±1.3	<0.001
CLIF – SOFA	5.7±2.1	7.1±1.9	<0.001
M30	933(679-1363)	1456(998-2198)	<0.001
M65	716(319-1605)	1404(542-3788)	0.002
M30:M65 ratio	1.4(0.9-2.7)	1.1(0.7-1.8)	0.117

[1]Variables included in the multivariate model for the stepwise selection

Supplementary Table 9. Univariate analysis of factors associated with survival.

	28 days mortality^		P value*
	Alive (N=283)	**Dead (N=41)**	
Age[1]	58±12	57±9	0.891
Male	176(62.2)	24(58.5)	0.565
Etiology			
Alcohol	140(51.7)	21(52.5)	0.789
HCV	59(21.8)	13(32.5)	0.135
Alcohol + HCV	26(9.6)	2(5.0)	0.368
Other	46(17.0)	4(10.0)	0.512
Previous Decomp.	210(76.4)	25(65.8)	0.133
Alcohol in last 3 months[1]	36(13.5)	9(23.7)	0.074
Ascites with subrogates[1]	248(87.6)	40(97.6)	0.106
HE[1]	91(32.2)	20(48.8)	0.044
GI bleeding	47(16.6)	7(17.1)	0.792
Bacterial infection[1]	64(22.8)	19(46.3)	0.002
Organ Failures			
Liver	33(11.7)	17(41.5)	<0.001
Kidney	24(8.5)	12(29.3)	<0.001
Brain	20(7.1)	7(17.1)	0.038
Coagulation	18(6.4)	7(17.1)	0.033
Cardiac	10(3.5)	7(17.1)	<0.001
Respiratory	3(1.1)	3(7.3)	0.009
Laboratory values			
Bilirubin[1]	2.8(1.5-6.4)	6.9(2.9-25.0)	<0.001
INR[1]	1.5(1.3-1.8)	1.9(1.5-2.3)	<0.001
Albumin	2.9(2.5-3.2)	2.6(2.3-3.2)	0.211
Creatinine[1]	1.0(0.7-1.3)	1.3(0.9-2.8)	<0.001
Sodium[1]	136±5	131±8	<0.001
Leucocyte count[1]	5.8(4.2-8.2)	8.7(6.4-14.4)	<0.001
Neutrophil count	205(44-27000)	170(20-4900)	0.382
Platelets	91(57-137)	63(39-115)	0.079
C-reactive protein[1]	16(6-32)	37(18-61)	<0.001
MELD	18±6	26±8	<0.001
MELD Na	20±7	29±7	<0.001
CP score	9.4±2.0	11.1±1.8	<0.001
CLIF AD score	51±8	61±10	<0.001
CLIF ACLF score	47±9	55±7	<0.001
CLIF – OF	7.5±1.7	9.8±2.8	<0.001
CLIF - SOFA	6.6±2.7	9.6±3.8	<0.001
M30	981(729-1511)	1643(949-3609)	<0.001
M65	863(347-1917)	2334(929-5151)	<0.001
M30:M65 ratio	1.3(0.8-2.5)	0.9(0.5-1.3)	0.005

^13 patients were transplanted and considered as a secondary event
*p value is obtained with a competing risks model with variables log transformed when necessary.
[1]Variables included in the multivariate model for the stepwise selection

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Prognostic and pathophysiological role of liver cell death markers in cirrhotic patients with acute decompensation

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Tables: 5

Table 1. Patient characteristics at baseline stratified by presence or absence of ACLF at enrolment.

Baseline characteristic	AD (n=258)	ACLF (n=79)	P value
Age (years)	58±12	55±12	0.111
Male (n, %)	165(64.0)	45(57.0)	0.262
Etiology (n, %)			
Alcohol	115(46.9)	47(61.8)	0.023
HCV	61(24.9)	14(18.4)	0.244
Alcohol + HCV	22(9.0)	7(9.2)	0.951
Other	47(19.2)	8(10.5)	0.080
Previous Decompensation (n, %)	186(73.5)	61(83.6)	0.078
Alcohol in last 3 months (n, %)	25(10.3)	20(27.4)	<0.001
Ascites or its surrogates (n, %)	222(86.1)	79(100.0)	<0.001
GI bleeding (n, %)	41(15.9)	13(16.5)	0.905
Bacterial infection (n, %)	58(22.7)	30(38.0)	0.007
Organ Failures (n, %)			
Liver	20(7.8)	36(45.6)	<0.001
Kidney	-	38(48.1)	-
Brain	8(3.1)	21(26.6)	<0.001
Coagulation	5(1.9)	24(30.4)	<0.001
Cardiac	2(0.8)	17(21.5)	<0.001
Respiratory	1(0.4)	6(7.6)	<0.001
Inflammatory and oxidative stress markers			
WBC (x10^9/L)	5.7(4.2-8.2)	7.6(5.8-12.1)	<0.001
CRP (mg/L)	16(6-32)	23(10-54)	0.010
IL8 (pg/mL)	48(26-94)	110(61-205)	<0.001
IL6 (pg/mL)	27(16-60)	63(20-130)	0.001

IL10 (pg/mL)	3.9(1.4-9.9)	9.1(2.0-37.2)	0.001
ILRA (pg/mL)	13(6-30)	25(10-91)	<0.001
NGAL (ng/mL)	28(12-73)	95(28-384)	<0.001
sCD163 (mg/L)	8.7(5.1-12.5)	14.1(9.0-20.0)	<0.001
HNA-2 (%)	6.0(3.8-9.7)	10.2(6.6-14.0)	<0.001
Laboratory values			
Bilirubin (mg/dL)	2.8(1.5-5.9)	9.7(2.6-21.3)	<0.001
INR	1.5(1.3-1.7)	1.9(1.5-2.6)	<0.001
Albumin (g/dL)	2.9(2.5-3.2)	2.8(2.2-3.3)	0.249
Creatinine (mg/dL)	0.9(0.7-1.2)	1.8(0.9-3.0)	<0.001
Sodium (mmol/L)	135±6	134±6	0.110
Platelets (x10^9/L)	91(55-135)	66(48-111)	0.014
MELD	17±6	28±7	<0.001
MELD Na	19±6	30±6	<0.001
CP score	9.2±1.9	11±2	<0.001
CLIF – OFs	7±1	11±2	<0.001
28-day mortality (%)	16(6.2)	25(31.7)	<0.001
3-month mortality (%)	34(13.2)	34(43.0)	<0.001

Data are mean ± SD or median (Q1-Q3)

Table 2. cK18, K18 and cK18:K18 ratio stratified by patient group.

	cK18 (U/L) **Median** (IQR)	**K18** (U/L) **Median** (IQR)	**cK18:K18 ratio Median** (IQR)
Healthy Controls(n=34)	201(107-357)	11(11-11)	18,3(9,7-32,4)
Stable Cirrhotics (n=44)	182(103-275)	245(98-650)	0,7(0,4-1,5)
All decompensated (n=337)	1034(751-1662)	955(398-2343)	1,19(0,74-2,24)
P value	<0.001	<0.001	<0.001
No ACLF at enrolment (n=258)	975(712-1530)	818(330-1854)	1.3(0.8-2.7)
ACLF at enrolment (n=79)	1213(921-2719)	1766(708-4658)	0.9(0.6-1.6)
P value	<0.001	<0.001	<0.001
ACLF I at enrolment (n=36)	1103(849-1583)	1100(682-2283)	0.9(0.7-1.3)
ACLF 2 at enrolment (n=32)	1228(906-3164)	2082(508-4994)	1.0(0.6-2.1)
ACLF 3 at enrolment (n=11)	2701(1264-12736)	4994(2476-10826)	0.6(0.4-0.7)
P value	0.020	0.004	0.048
AD throughout (n=195)	933(679-1363)	716(319-1605)	1.4(0.9-2.7)
ACLF to AD (n=8)	1053(828-1954)	633(376-3141)	1.2(0.8-2.3)
AD to ACLF (n=39)	1456(998-2198)	1404(542-3788)	1.1(0.7-1.8)
ACLF throughout (n=71)	1232(921-2794)	1901(853-4843)	0.9(0.6-1.3)
P value	<0.001	<0.001	0.001

Table 3. Markers of inflammation, oxidative stress, macrophage activation and cell death stratified by the presence of absence of infection.

	No infection (n=247)	Infection (n=88)	P value
WBC (x10^9/L)	6.0(4.4-8.2)	6.7(4.7-11.2)	0.044
CRP (mg/L)	16(6-27)	34(11-69)	<0.001
IL8 (pg/mL)	56(27-112)	80(41-128)	0.017
IL6 (pg/mL)	26(15-57)	72(28-353)	<0.001
IL10 (pg/mL)	3.8(1.2-10.2)	9.4(3.6-26)	<0.001
ILRA (pg/mL)	14(6-31)	26(10-76)	<0.001
sCD163 (mg/L)	9.1(5.2-13.9)	9.5(7.0-16.5)	0.053
NGAL (ng/mL)	30(12-85)	49(18-140)	0.062
HNA-2 (%)	7.0(3.8-10.5)	9.4(5.6-12.9)	0.005
cK18 (U/L)	1019(769-1643)	1032(739-1775)	0.760
K18 (U/L)	918(377-2060)	1217(429-2476)	0.194
cK18:K18 ratio	1.2(0.8-2.3)	1.1(0.6-2.2)	0.185

Data are mean ± SD or median (Q1-Q3)

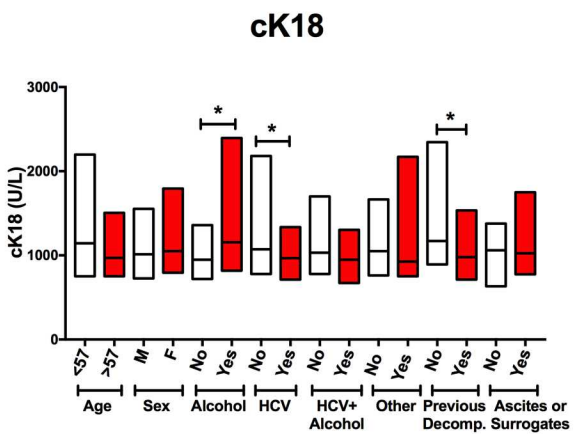
Table 4. Performance of cK18 and K18 level, cK18:K18 ratio and clinical scores in predicting AD patients who will progress to ACLF.

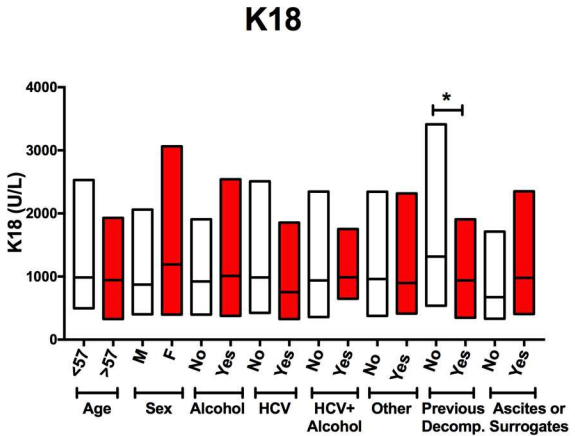
	Progression to ACLF AUROC (95% CI)	P value
Ln(cK18)	0.670(0.576-0.764)	
Ln(K18)	0.655(0.554-0.756	
Ln(cK18:K18)	0.581(0.479-0.682)	
MELD	0.710(0.618-0.802)	
MELD+Ln(cK18)	0.740(0.653-0.826)	ns
MELD+Ln(K18)	0.723(0.632-0.815)	ns
MELD+Ln(cK18:K18)	0.709(0.616-0.802)	ns
MELDna	0.729(0.637-0.820)	
MELDna+Ln(cK18)	0.745(0.658-0.833)	ns
MELDna+Ln(K18)	0.736(0.642-0.831)	ns
MELDna+Ln(cK18:K18)	0.728(0.633-0.822)	ns
CLIF-C AD	0.737(0.655-0.820)	
CLIF-C AD+Ln(cK18)	0.765(0.690-0.841)	<0.05
CLIF-C AD+Ln(K18)	0.760(0.679-0.841)	ns
CLIF-CAD+Ln (cK18:K18)	0.744(0.660-0.827)	ns

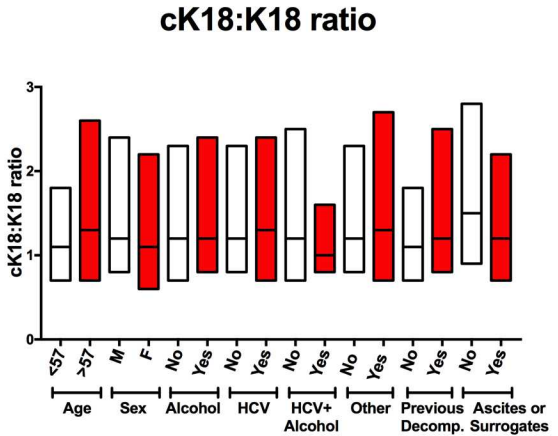
Table 5. Performance of cK18 and K18 levels, cK18:K18 ratio and clinical scores in predicting 28-day and 90-day mortality in AD patients.

	28 days mortality C-index (95% CI)	P value	90 days mortality C-index (95% CI)	P value
Ln(cK18)	0.571(0.408-0.733)		0.585(0.481-0.689)	
Ln(K18)	0.659(0.518-0.800)		0.640(0.543-0.737)	
Ln(cK18:K18)	0.634(0.501-0.767)		0.622(0.531-0.712)	
MELD	0.628(0.498-0.758)		0.721(0.637-0.804)	
MELD+(cK18)	0.654(0.524-0.783)	0.592	0.735(0.654-0.817)	0.401
MELD+(K18)	0.703(0.574-0.831)	0.273	0.743(0.662-0.824)	0.327
MELD+ (cK18:K18)	0.675(0.551-0.799)	0.385	0.733(0.652-0.814)	0.493
MELDna	0.695(0.566-0.823)		0.751(0.672-0.831)	
MELDna+(cK18)	0.698(0.567-0.830)	0.927	0.756(0.675-0.837)	0.912
MELDna+(K18)	0.737(0.609-0.866)	0.201	0.767(0.684-0.850)	0.368
MELDna+(cK18:K18)	0.733(0.613-0.854)	0.267	0.762(0.682-0.841)	0.459
CLIF-C AD	0.764(0.644-0.884)		0.752(0.675-0.828)	
CLIF-C AD+(cK18)	0.767(0.646-0.887)	0.897	0.755(0.678-0.832)	0.956
CLIF-C AD+(K18)	0.789(0.670-0.908)	0.320	0.771(0.692-0.850)	0.376
CLIF-C AD+(cK18:K18)	0.796(0.681-0.911)	0.213	0.770(0.692-0.848)	0.374

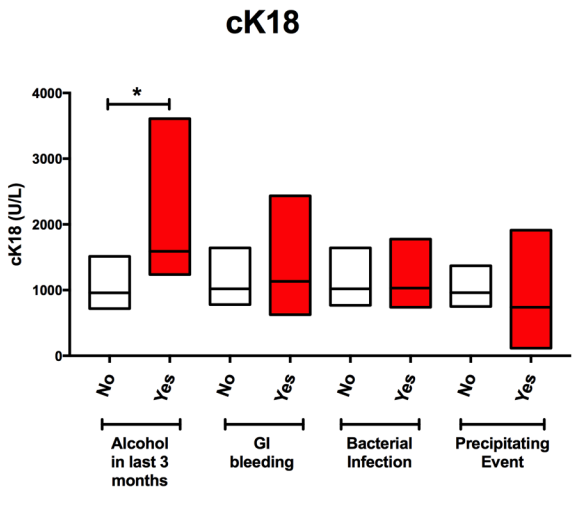
A - Predisposition

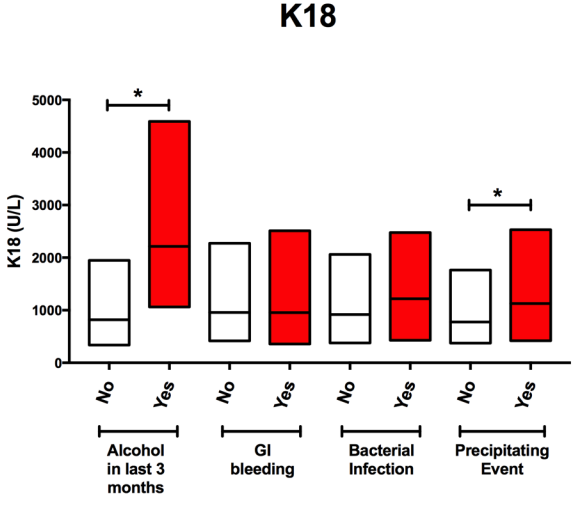


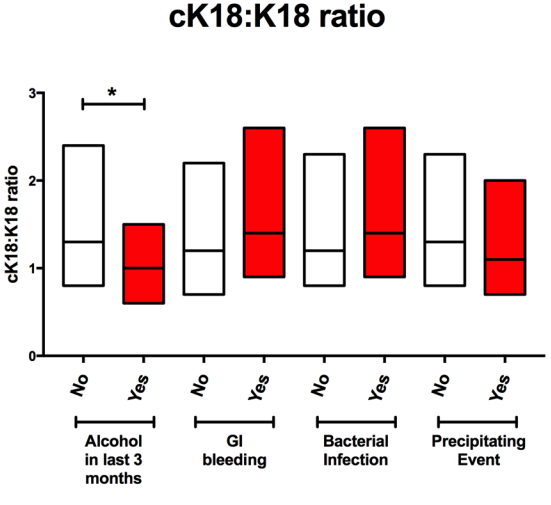




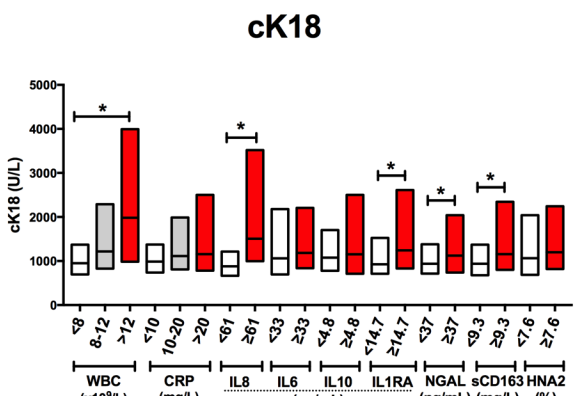
B - Injury - precipitating event

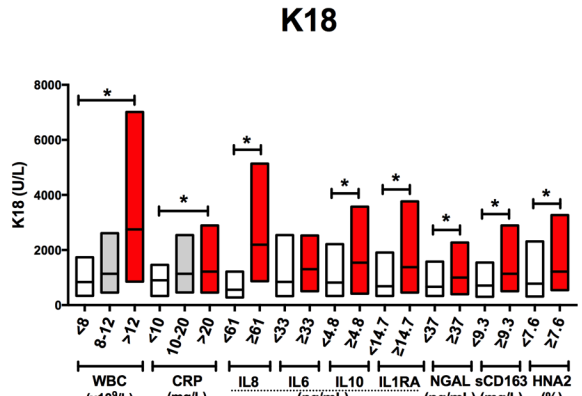


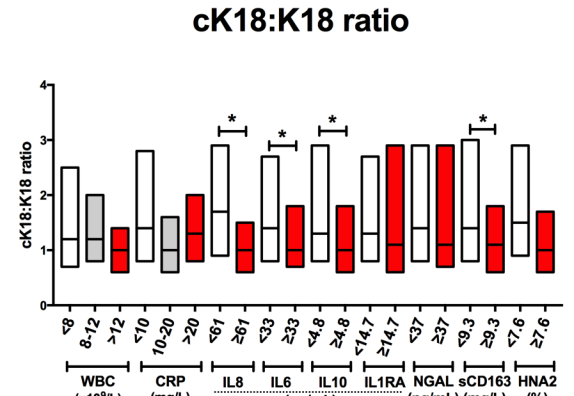




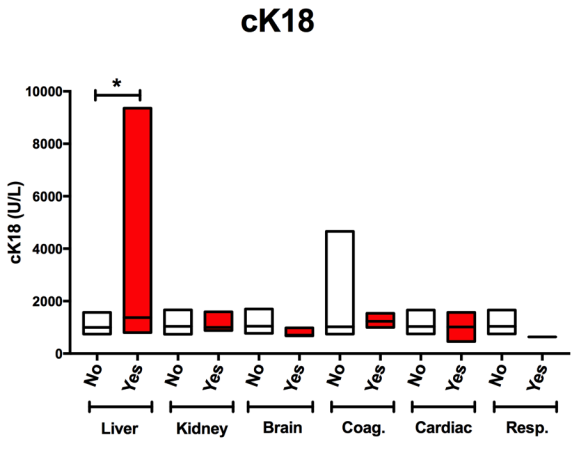
C - Response

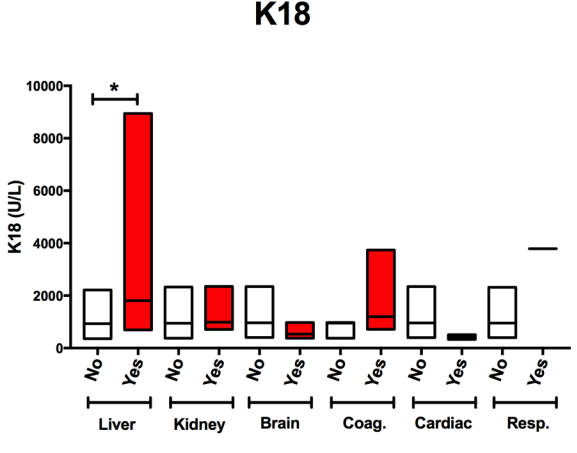

cK18
cK18 (U/L)
5000
4000
3000
2000
1000
0
<8
8-12
>12
<10
10-20
>20
<61
≥61
<33
≥33
<4.8
≥4.8
<14.7
≥14.7
<37
≥37
<9.3
≥9.3
<7.6
≥7.6
WBC (x10⁹/L)
CRP (mg/L)
IL8
IL6
IL10
IL1RA
(pg/mL)
NGAL (ng/mL)
sCD163 (mg/L)
HNA2 (%)


K18
K18 (U/L)
8000
6000
4000
2000
0
<8
8-12
>12
<10
10-20
>20
<61
≥61
<33
≥33
<4.8
≥4.8
<14.7
≥14.7
<37
≥37
<9.3
≥9.3
<7.6
≥7.6
WBC (x10⁹/L)
CRP (mg/L)
IL8
IL6
IL10
IL1RA
(pg/mL)
NGAL (ng/mL)
sCD163 (mg/L)
HNA2 (%)


cK18:K18 ratio
cK18:K18 ratio
4
3
2
1
0
<8
8-12
>12
<10
10-20
>20
<61
≥61
<33
≥33
<4.8
≥4.8
<14.7
≥14.7
<37
≥37
<9.3
≥9.3
<7.6
≥7.6
WBC (x10⁹/L)
CRP (mg/L)
IL8
IL6
IL10
IL1RA
(pg/mL)
NGAL (ng/mL)
sCD163 (mg/L)
HNA2 (%)

D - Organ Failures - single





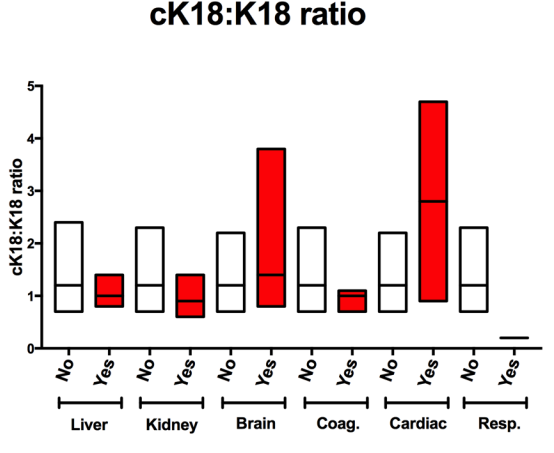
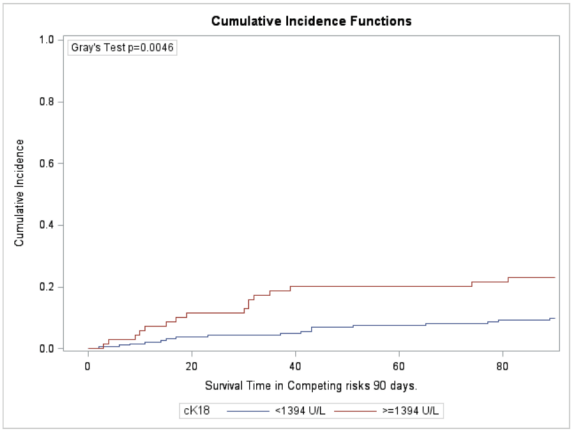
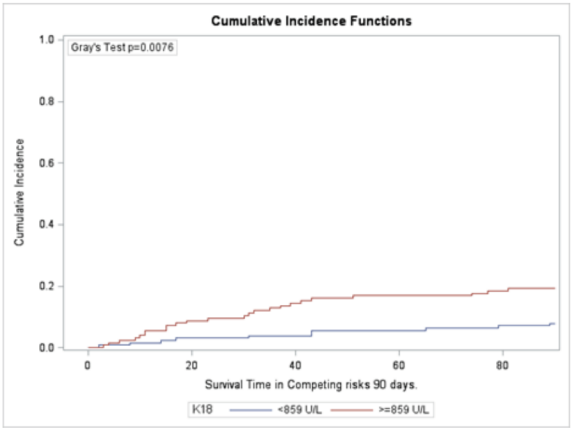


Figure 2A. AD patients





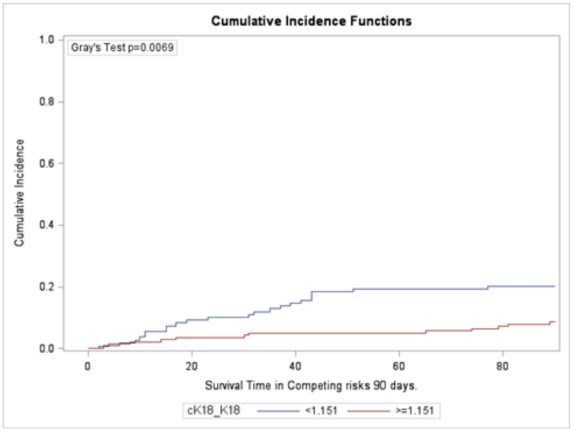
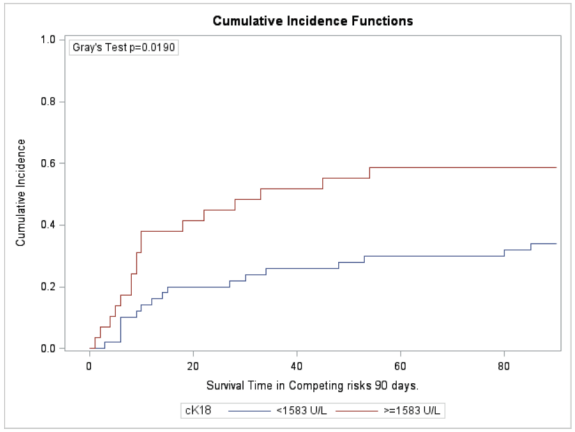
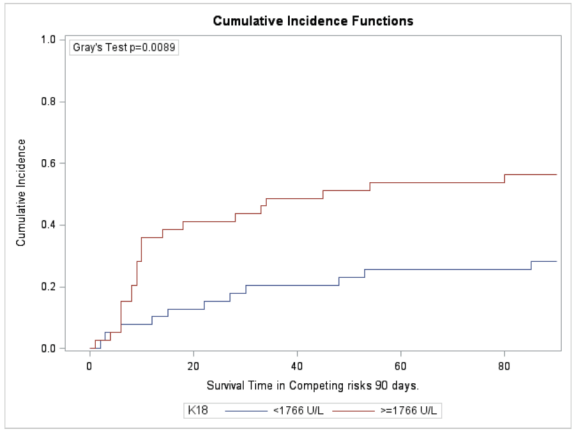
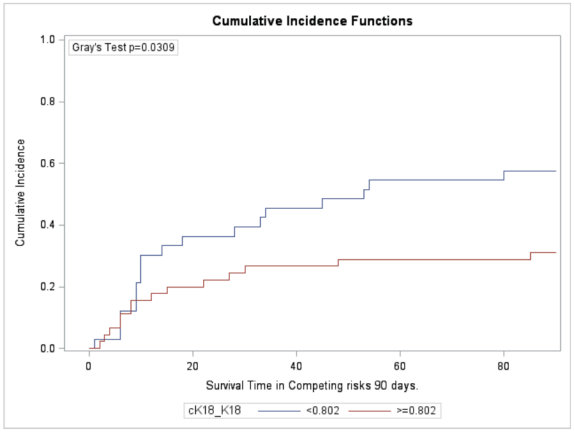
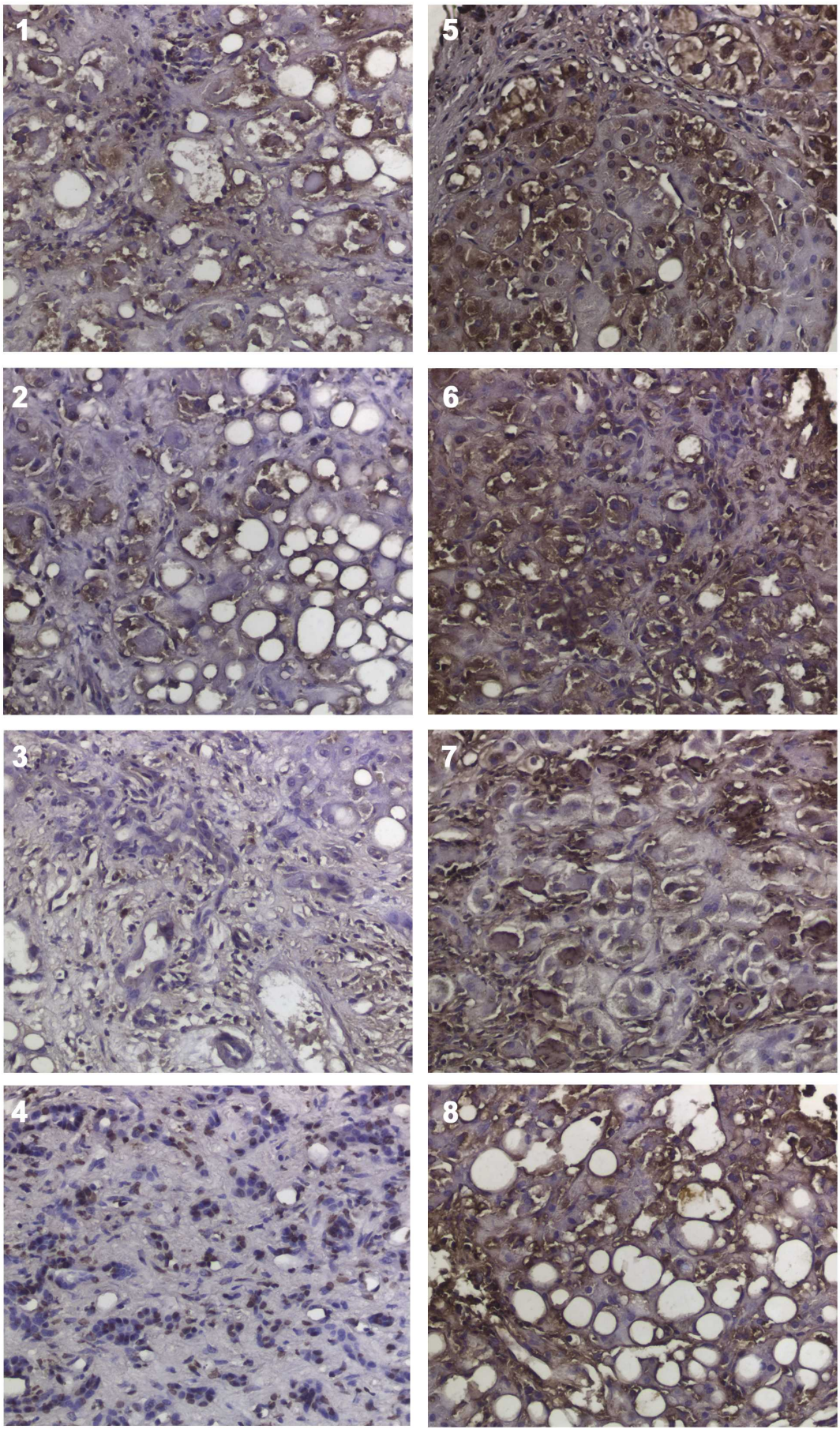


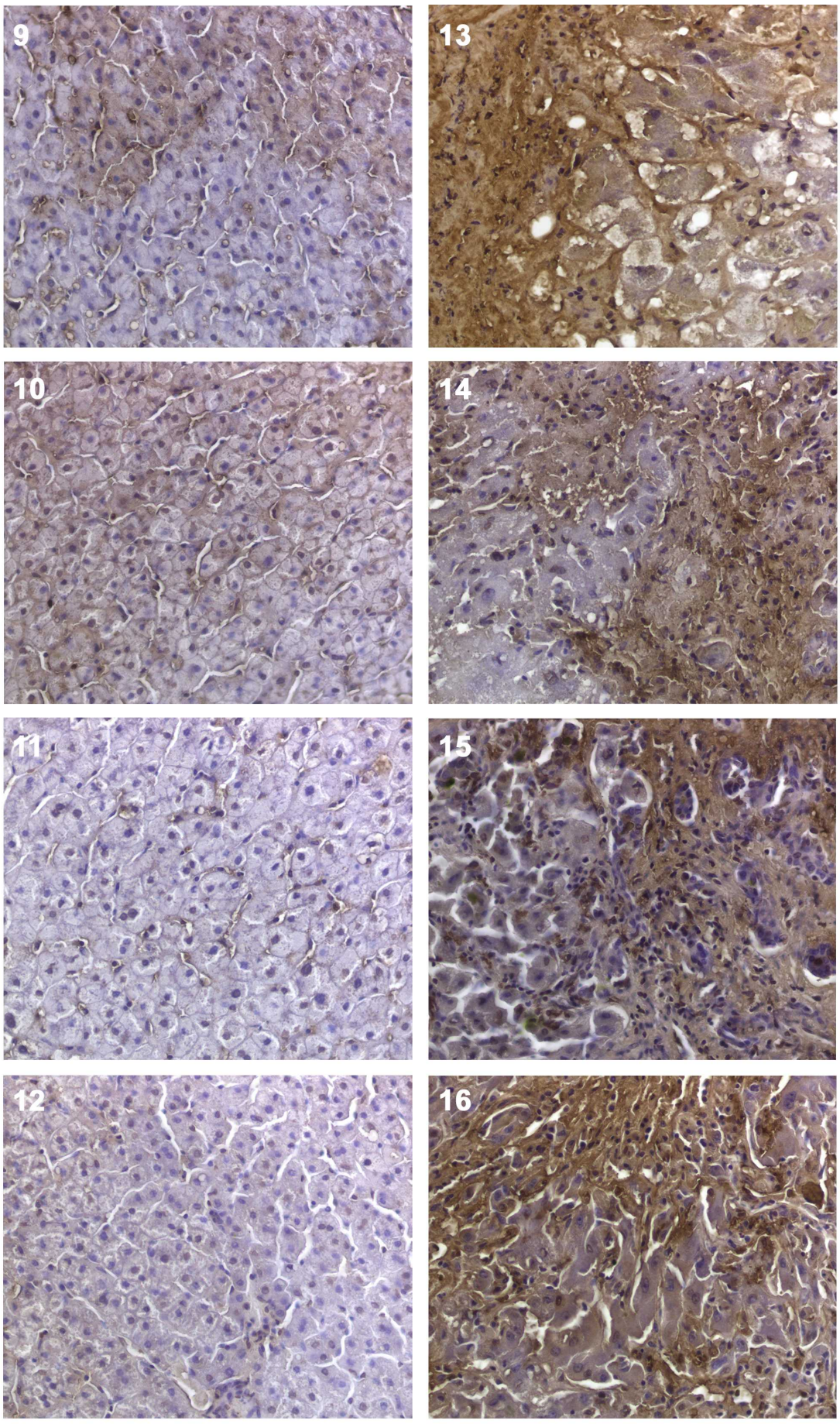
Figure 2B. ACLF patients

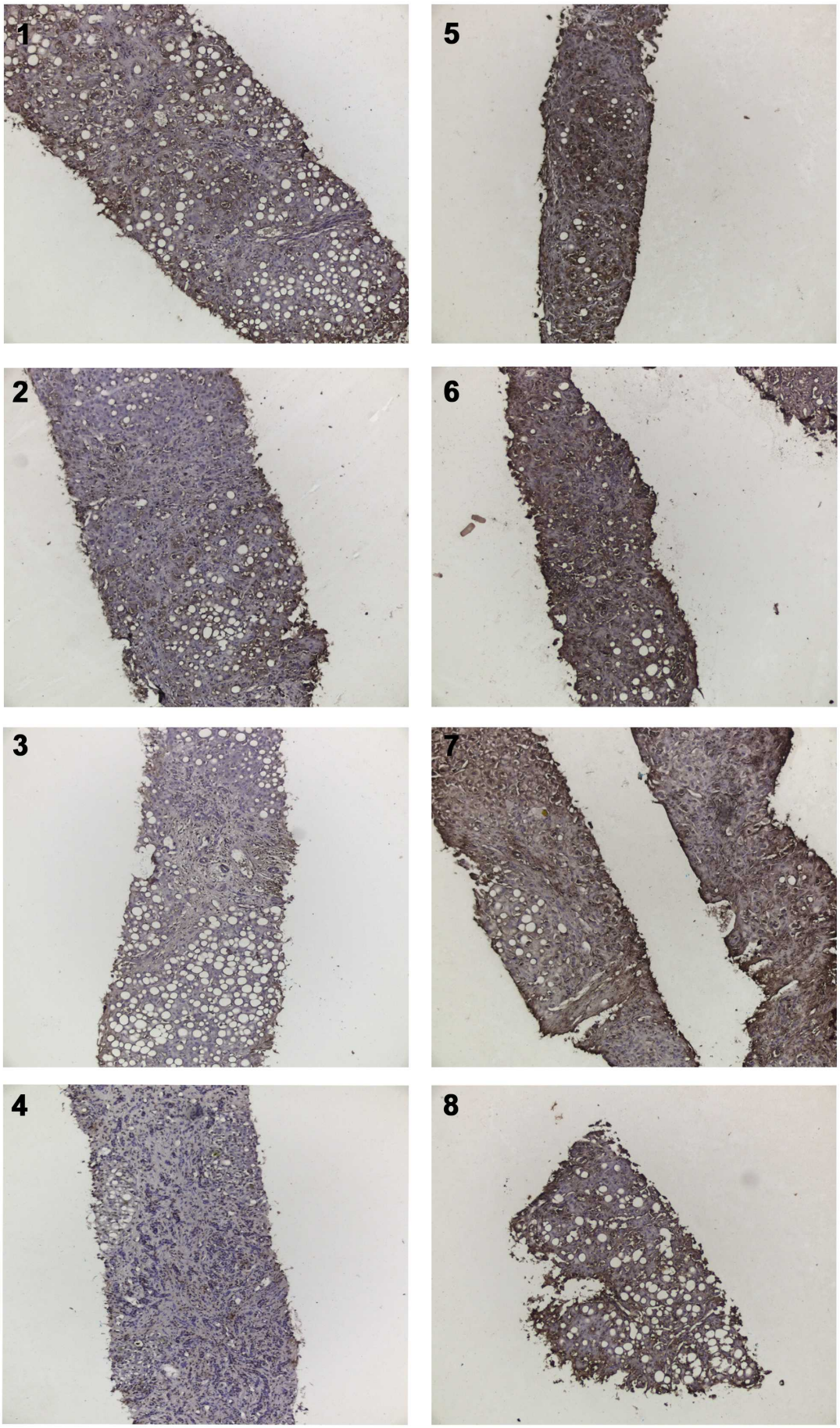







no ACLF
ACLF
1
5
2
6
3
7
4
8


no ACLF
ACLF
9
13
10
14
11
15
12
16


no ACLF
ACLF
1
5
2
6
3
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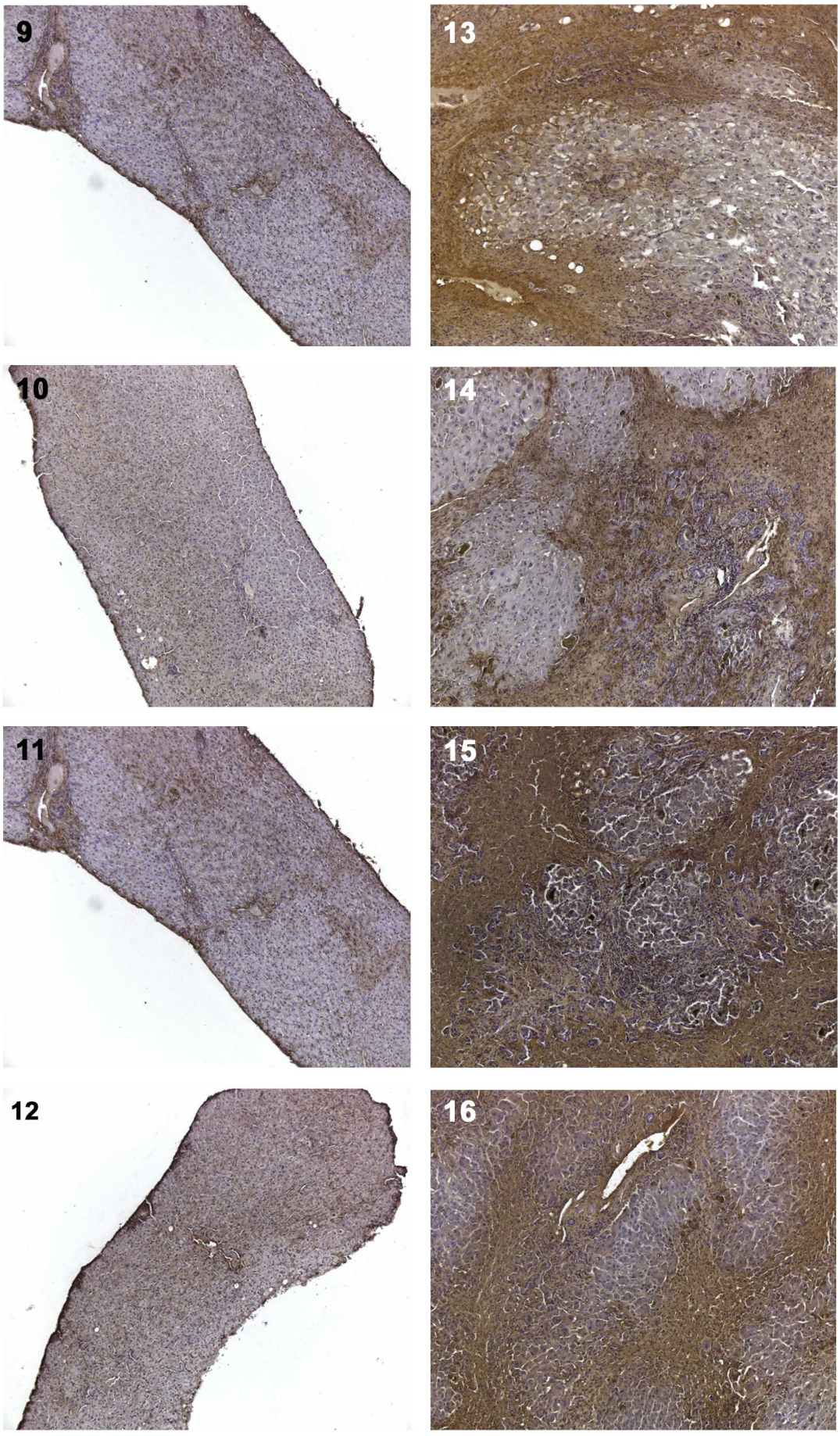
4
8


no ACLF
ACLF
9
13
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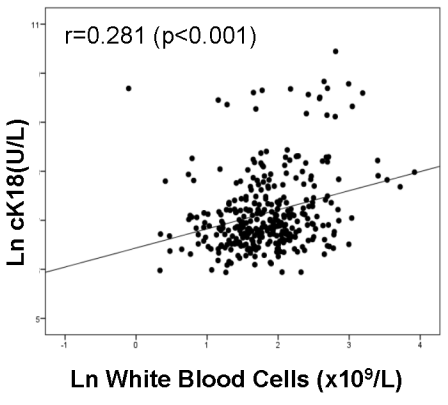
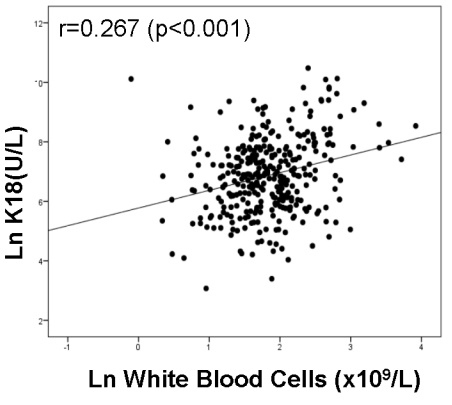
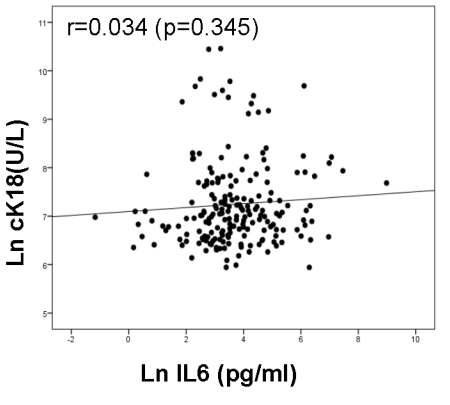
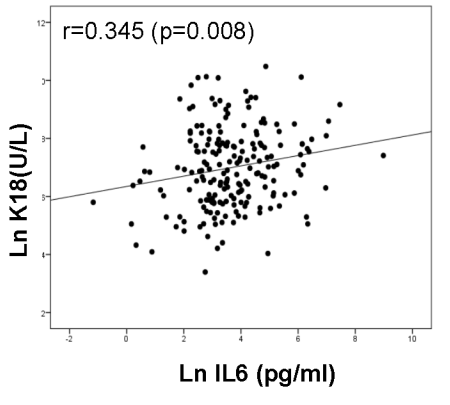
14
11
15
12
16

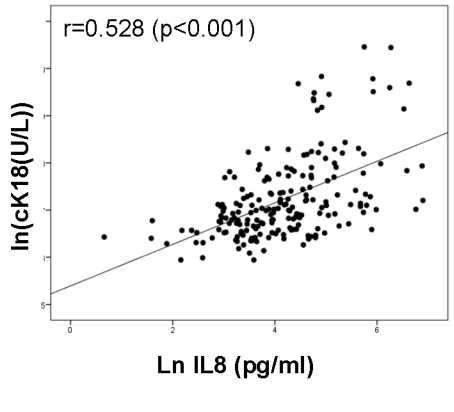
Supplementary Figure 1. correlation of cK18 and K18 to markers of inflammation, oxidative stress and macrophage activation

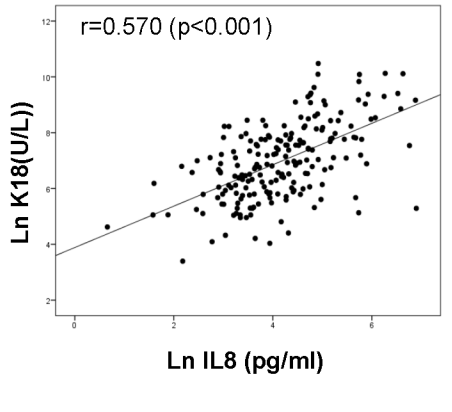


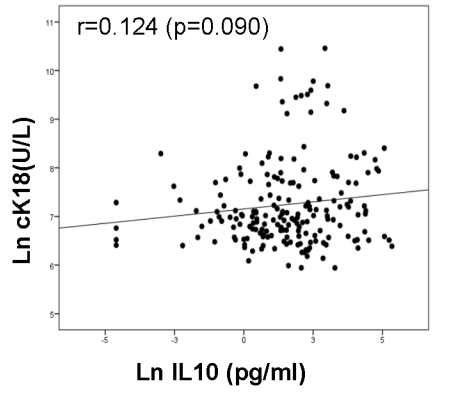


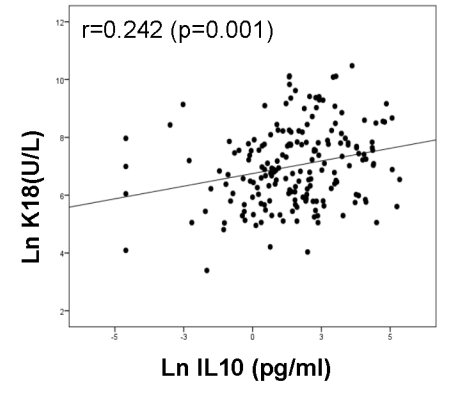


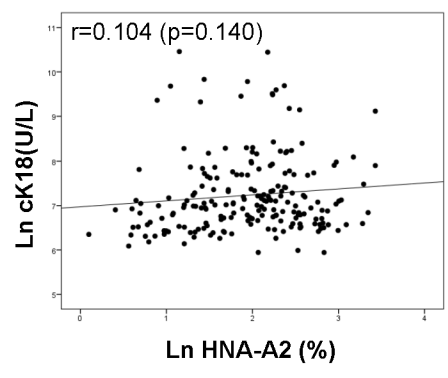


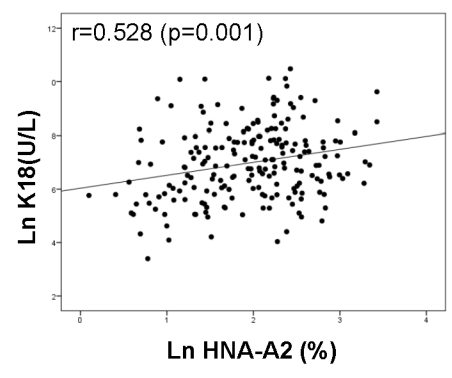


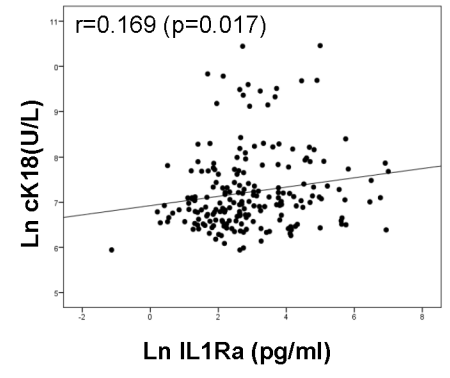


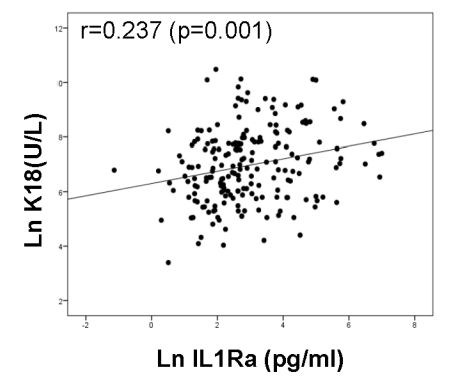


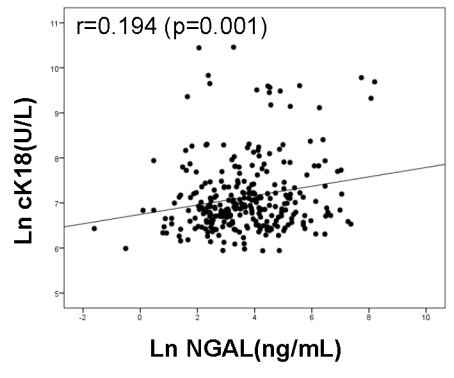


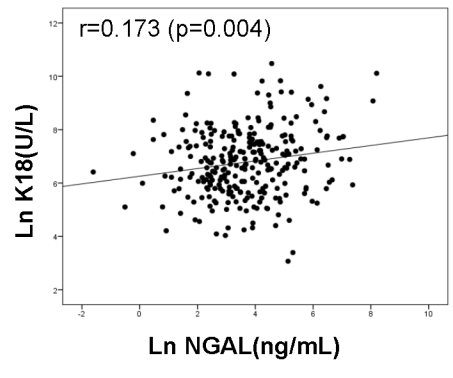


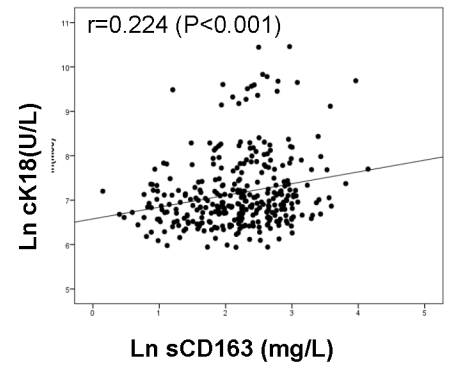


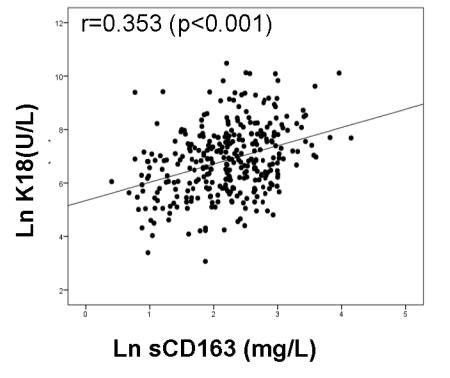












Supplementary Figure 2. correlation of cK18 and K18 to markers of hepatic and extra hepatic organ dysfunction