Cell Death Markers in Patients With Cirrhosis and Acute Decompensation

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The aims of this study were to determine the role of cell death in patients with cirrhosis and acute decompensation (AD) and acute on chronic liver failure (ACLF) using plasma-based biomarkers. The patients studied were part of the CANONIC (CLIF Acute-on-Chronic Liver Failure in Cirrhosis) study (N = 337; AD, 258; ACLF, 79); additional cohorts included healthy volunteers, stable patients with cirrhosis, 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 plasma by enzyme-linked immunosorbent assay. 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 aminotransferase, and bilirubin, but not with markers of extrahepatic organ injury. Terminal deoxynucleotidyl transferase dUTP nick-end labeling 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. Nonapoptotic 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 type of organ failure. (HEPATOLOGY 2018;67:989-1002).

An acute decompensating event (acute decompensation; 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 extrahepatic organ failure, a condition referred to as acute on chronic liver failure (ACLF).(2) Approximately 20% of these patients progress to...
multigorgan 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.\(^{(3-5)}\) Given that the syndrome is defined by the failure of hepatic and extrahepatic organs,\(^{(6)}\) cell death is likely to be important,\(^{(7)}\) but the site, role, type, and extent have 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,\(^{(11)}\) nonalcoholic fatty liver disease,\(^{(12,13)}\) alcoholic hepatitis (AH),\(^{(14)}\) acute liver failure,\(^{(15)}\) and primary biliary cirrhosis\(^{(16)}\) (reviewed in Supporting Table S1), as well as in nonhepatological diseases such as breast and gastrointestinal (GI) cancer\(^{(17,18)}\) and sepsis.\(^{(19)}\) Keratins are the main epithelial subgroup of intermediate filament (IF) proteins.\(^{(20)}\) K18 is expressed by both hepatocytes and cholangiocytes\(^{(21)}\) as well as other nonhepatic tissues, including kidney, intestine, and lung,\(^{(22)}\) and, after initiation of apoptosis, K18 is cleaved by activated caspases at two points: first, early in apoptosis, at K18-Asp396, which is unique to K18, and then later at a common caspase cleavage site, the linker L1-2 region of the central rod domain, present in other members of the IF family.\(^{(23-25)}\) It is the neopitope 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 recognize protein epitopes of K18 and therefore detect intact K18, its nonapoptotic fragments, but also the apoptotic fragment. M65 values are widely taken to reflect necrotic cell death; however, they should probably be regarded as a measure of total cell death. Although 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 because of the potential for circulating cK18/K18 to be derived from nonhepatic 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 AD 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 AD of liver disease who were enrolled in the prospective, multicenter CANONIC (CLIF Acute-on-Chronic Liver Failure in Cirrhosis) study.\(^{(6)}\)

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Patients 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, 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 biobanked material at the Royal Free Hospital (London, UK). Liver sections from patients with AH 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 hepatitis B virus (HBV) related liver disease from the Third Affiliated Hospital of Sun Yat-Sen University (Guangzhou, 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 1,343 patients who were hospitalized with an AD of cirrhosis (bacterial infection, large-volume ascites, GI hemorrhage, or hepatic encephalopathy [HE], alone or in combination) in 29 hepatology centers across eight countries; 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.

ACLF

ACLF was defined in terms of organ failures according to the Chronic Liver Failure Consortium (CLIF)-Organ Failure (OF) Score 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 HE grades I or II; (3) single cerebral failure (HE grades III or IV) associated with a serum creatinine between >1.5 and <2 mg/dL; or (4) two or more organ failures.

AD

AD was defined as the acute development of ascites, HE, GI hemorrhage, or bacterial infection 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 analyzed blindly by the data management center of the European Foundation for the study of Chronic Liver Failure ([EF-CLIF] Barcelona). The predefined endpoints of the analysis were to perform a descriptive analysis of cell-death markers according to factors associated with AD of cirrhosis using the PIRO concept; Predisposition (underlying factors such as age, etiology, etc.), Injury (precipitating factors), markers of Response (inflammation and infection), and Organ 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-day 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 (interleukin-6 [IL-6], interleukin-8 [IL-8], interleukin-10 [IL-10], interleukin-1 receptor antagonist [IL-Ra], neutrophil gelatinase-associated lipocalin [NGAL], and soluble cluster of differentiation 163 [sCD163]) and marker of oxidative stress (human non-mercaptalbumin-2; HNA-2) were then performed. Data regarding these analytes partly overlap with past publications (Claria et al., Ariza et al., and Gronbaek et al.). Correlation analyses for cK18 and K18 with markers of individual organ dysfunction were then performed (bilirubin, alanine aminotransferase [ALT], prothrombin time, creatinine, HE grade, mean arterial pressure [MAP], and heart rate).

MEASUREMENT OF cK18 AND K18 AND CALCULATION OF cK18:K18 RATIO

All blood samples were centrifuged at 2,000 rpm for 10 minutes, and the supernatants were stored at –80°C.
within 4 hours of collection. Serum cK18 and K18 levels were then measured in baseline ethylenediaminetetra-acetic acid samples by enzyme-linked immunosorbent assay (ELISA; M30 Apoptosense [Peviva, Tewkesbury, UK] and M65 EpiDeath [Peviva], respectively). The cK18:K18 ratio (apoptotic index) was then calculated.

**TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE dUTP NICK-END LABELING STAINING OF LIVER SECTIONS**

Liver sections of patients with AH with and without ACLF and HBV infection with and without ACLF were prepared and stained for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) positivity according to the kit protocol (in situ cell death detection kit, colorimetric; Roche, Welwyn Garden City, UK).

**STATISTICAL ANALYSIS**

Results are presented as frequencies and percentages for categorical variables, means and SDs for normally distributed continuous variables, and median and interquartile range (IQR) for not normally distributed continuous variables. Not normally distributed variables were log-transformed for some statistical analyses and for graphical comparisons. In univariate analyses, the chi-square test was used for categorical variables, the Student t test or analysis of variance for normal continuous variables, and Mann-Whitney U test 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 receiver operating characteristic curve (AUROC). The proportional hazards model for competing risks proposed by Fine and Gray 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.

**Results**

**PATIENT CHARACTERISTICS**

Three hundred 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%) 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 1. A further 16 patients with available liver biopsies were studied, 8 with ACLF and 8 without. The cause of cirrhosis in 8 patients was HBV infection and alcohol-related cirrhosis with superimposed AH in 8 patients. The baseline characteristics of the patient group are shown in Supporting Table S2.

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 as well as 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 (Supporting Table S3).

**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 step-wise 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
hospitalization 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. Whereas 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 nonapoptotic modes of cell death were more significant.

**RELATIONSHIP OF cK18 AND K18 LEVELS TO PREDISPOSITION, INJURY, RESPONSE AND ORGAN FAILURES**

**Predisposition**

No significant differences were observed in cK18 and K18 levels when patients were stratified by age or..

| 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)    | 0.906   |
| 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 (×10^9/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   |
| IL-8 (pg/mL)           | 48 (26-94)  | 110 (61-205) | <0.001  |
| IL-6 (pg/mL)           | 27 (16-60)  | 63 (20-130)  | 0.001   |
| IL-10 (pg/mL)          | 3.9 (1.4-9.9) | 9.1 (2.0-37.2) | 0.001   |
| IL-8A (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 (×10^9/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-Os                | 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).
sex (Fig. 1A; Supporting Table S4). Patients with underlying alcohol-related cirrhosis demonstrated a significant elevation in cK18 level and a nonsignificant elevation in K18 level in comparison to non-alcohol-related etiologies, whereas patients with hepatitis C virus (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

Consumption of alcohol within the 3 months preceding admission, indicating likely AH 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 (Fig. 1B; Supporting Table S5). In contrast, the presence of bacterial infection was not associated with a significant increase in cK18 or K18 level (Fig. 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

White cell count (WCC) was significantly associated with both cK18 and K18 levels and with a nonsignificant reduction in cK18:K18 ratio (Fig. 1C; Supporting Table S6). C-reactive protein (CRP) was significantly associated with K18 level. IL-8, IL1-Ra, and sCD163 as well as NGAL were associated with both cK18 and K18 level and a reduction in cK18:K18 ratio. Furthermore, IL-10 and HNA-2 were both associated with elevation of K18, but not cK18, level and a reduction in cK18:K18 ratio. IL-8 correlated strongly with both cK18 and K18, and IL-6, sCD163, and HNA-2 correlated with K18 (Supporting Fig. S1).

Organ Failures

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 (Fig. 1D; Supporting Table S7). The liver was demonstrated as a possible source of the elevated cK18 and K18 levels because both bilirubin and ALT positively correlated to cK18 and K18 levels (Supporting Fig. S2). In contrast, cK18 or K18 levels did not correlate with creatinine, prothrombin time, grade of HE, or MAP indicating that the source of increased cK18 and K18 was unlikely to be these extrahepatic organs. Heart rate, which is another component of systemic inflammatory response, was positively correlated with both cK18 and K18 levels (Supporting Fig. S2). 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 toward elevated cK18 and K18 level was demonstrated and a reduced cK18:K18 ratio observed (Supporting Table S7). Isolated cardiac failure was not associated with elevation of cK18 and K18 level, but only when cardiac failure was associated with liver failure (Supporting Table S7).

| TABLE 2. cK18, K18, and cK18:K18 Ratio Stratified by Patient Group |
|---------------------------------------------------------------|
| **cK18 (U/L)** | **K18 (U/L)** | **cK18:K18 Ratio** |
| **Median (IQR)** | **Median (IQR)** | **Median (IQR)** |
| Healthy controls (n = 34) | 201 (107-357) | 11 (11-11) | 18.3 (9.7-32.4) |
| Stable patients with cirrhosis (n = 44) | 182 (103-275) | 245 (98-650) | 0.7 (0.4-1.5) |
| All decompensated (n = 337) | 1,034 (751-1,662) | 955 (398-2,343) | 1.19 (0.74-2.24) |
| **P value** | <0.001 | <0.001 | <0.001 |
| No ACLF at enrollment (n = 258) | 975 (712-1,530) | 818 (330-1,854) | 1.3 (0.8-2.7) |
| ACLF at enrollment (n = 79) | 1,213 (921-2,719) | 1,766 (708-4,658) | 0.9 (0.6-1.6) |
| **P value** | <0.001 | <0.001 | <0.001 |
| ACLF 1 at enrollment (n = 36) | 1,103 (849-1,583) | 1,100 (882-2,283) | 0.9 (0.7-1.3) |
| ACLF 2 at enrollment (n = 32) | 1,228 (906-3,164) | 2,082 (508-4,994) | 1.0 (0.6-2.1) |
| ACLF 3 at enrollment (n = 11) | 2,701 (1,264-12,736) | 4,994 (2,476-10,026) | 0.6 (0.4-0.7) |
| **P value** | 0.020 | 0.004 | 0.048 |
| AD throughout (n = 195) | 933 (679-1,363) | 716 (319-1,605) | 1.4 (0.9-2.7) |
| ACLF to AD (n = 8) | 1,053 (828-1,954) | 633 (376-3,141) | 1.2 (0.8-2.3) |
| AD to ACLF (n = 39) | 1,456 (998-2,198) | 1,404 (542-3,788) | 1.1 (0.7-1.8) |
| ACLF throughout (n = 71) | 1,232 (921-2,794) | 1,901 (853-4,843) | 0.9 (0.6-1.3) |
| **P value** | <0.001 | <0.001 | 0.001 |
FIG. 1. cK18, K18, and cK18/K18 ratio in patients with cirrhosis and acute decompensation according to (A) predisposing factors, (B) injury-precipitating factor, (C) response, and (D) type of organ failure (single organ failure). *P < 0.05.
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 international normalized ratio [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 (Supporting Table S8). 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 (Supporting Table S9). 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 Model for End-Stage Liver Disease (MELD) score. The most accurate predictive score was the CLIF-C AD score, and addition of cK18:K18 ratio nonsignificantly 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 (Fig. 2).

Histology: TUNEL Staining

TUNEL staining of liver tissue from patients with AH 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 (Fig. 3A,B [magnification, 40×] and Supporting Fig. 3A,B [magnification, 10×]). Clinical characteristics of these patients are described in Supporting Table S2.

Discussion

This study demonstrates that markers of cell death, both apoptotic and nonapoptotic, 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, nonapoptotic forms of cell death(37) predominate as the 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

| 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 (<10⁹/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 |
| IL-8 (pg/mL)                                      | 56 (27-112)     | 80 (41-128)      | 0.017 |
| IL-6 (pg/mL)                                      | 26 (15-57)      | 72 (28-353)      | <0.001 |
| IL-10 (pg/mL)                                     | 3.8 (1.2-10.2)  | 9.4 (3.6-26)     | <0.001 |
| IL-RA (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 |
| NSAL (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)                                         | 1,019 (769-1,643) | 1,032 (739-1,775) | 0.760 |
| K18 (U/L)                                          | 918 (377-2,060) | 1,217 (429-2,476) | 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 (0.618-0.802) | ns |
| MELDna                  | 0.728 (0.637-0.820) |
| MELDna +Ln(cK18)        | 0.745 (0.658-0.833) |
| MELDna +Ln(K18)         | 0.736 (0.642-0.831) |
| MELDna +Ln(cK18+K18)    | 0.728 (0.633-0.822) |
| 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-C AD + Ln(cK18+K18) | 0.744 (0.660-0.827) | ns |

Abbreviations: CI, confidence interval; ns, not significant.
of K18 markers to markers of hepatic injury, bilirubin and ALT, and not markers of nonhepatic 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 patients with ACLF as opposed to those without. The data suggest 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

| 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-Day Mortality C-Index (95% CI) P Value 90-Day Mortality C-Index (95% CI) P Value |
| Ln(cK18) 0.571 (0.408-0.732) 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 (0.644-0.864) 0.752 (0.675-0.828) |
| CLIF-C AD + (cK18) 0.787 (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 |

Abbreviation: CI, confidence interval.

FIG. 2. Kaplan-Meier analysis defining cumulative mortality according to measurements of cK18, K18, and cK18:K18 ratio in patients with (A) AD (no ACLF) and (B) ACLF.
important in its pathogenesis. Whether there is an 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 observed in patients with ACLF, as compared to those with AD, suggests that although levels of both apoptotic and nonapoptotic modes of cell death markedly increase with clinical severity, it is nonapoptotic, 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, and the data presented here confirm and broaden this observation to ACLF. The shift in the dominant mode of cell death from apoptosis to nonapoptotic forms with increasing clinical severity also possibly

**FIG. 3.** (A) TUNEL staining of liver biopsies of patients with AH without and with ACLF (magnification, 40×). (B) TUNEL staining of liver biopsies of patients with HBV without and with ACLF (magnification, 40×). Numbering of images reflects the patient number as given in Supporting Table S2.
explains the limited effect of the pan-caspase inhibitor, Emricasan, when used in ACLF patients.\(^{(38)}\)

Current hypotheses describe ACLF as a syndrome driven by systemic inflammation.\(^{(3-5)}\) In keeping with past 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 (IL-8) and the activation of anti-inflammatory strategies to limit the immunological consequences of cell death (IL-10, IL1-RA, and sCD163). Although 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 a 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 AH\(^{(40)}\) and necrosis predominates in patients with HBV-related ACLF.\(^{(41)}\) The data presented confirm the recent observation by Bissonnette et al., that patients with AH have elevated levels of K18 and its fragments, but, however, argues for caution in using elevation of cK18 and K18 levels as diagnostic of AH without considering the clinical severity of the presentation. Patients with a clinically severe presentation of etiologies other than AH 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 around 30\%-40\% patients.\(^{(6)}\) The mechanisms underlying this are not clear, but the data from the present study are against the idea that cell death is the defining mechanism.

The data also describe distinct patterns of severity of cell death in different subpopulations of patients with AD and ACLF, suggesting that therapeutic approaches may need to be different depending upon the predisposing factors, precipitating illness, and 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 undescribed mechanism. The data also show 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 hepatocytes resistance to apoptosis given that 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, 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 subgroups outlined above, it is clear that, although 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 analyzed group were not statistically different to that of the original study (Supporting Table S2). 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 observed that liver failure is associated with a marked elevation in K18 and its fragments and such elevations are not observed 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 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 nonapoptotic cell death, but 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 AD 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 suggest that although there is a dramatic increase in levels of both apoptotic and nonapoptotic cell death with increasing clinical severity of decompensation, progression from AD to ACLF is associated with a relatively greater rise in nonapoptotic 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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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29581/suppinfo.