Bacterial and fungal infections in acute-on-chronic liver failure: prevalence, characteristics and impact on prognosis

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List of Abbreviations: ACLF, acute-on-chronic liver failure; AD, acute decompensation; PAMPs, pathogen associated molecular patterns; DAMPs, damaged-associated molecular patterns; CANONIC, chronic liver failure (CLIF) Acute-on-Chronic Failure in Cirrhosis; SBP, spontaneous bacterial peritonitis; UTI, urinary tract infection; SSTI, skin and soft tissue infections; IA, invasive aspergillosis; MDROs, multidrug-resistant drug organisms; CDI, *Clostridium difficile* infection; HNA, human nonmercaptalbumin; ICU, intensive care unit; CLIF-C ACLFs, CLIF Consortium ACLF score; WBC: white blood cell; CRP: C reactive protein; MERKT: MER receptor tyrosine kinase

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Conflicts of interest

Rajiv Jalan received research funding from Vital Therapies, has served on Scientific Advisory Board for Conatus Pharma, and received lecture fees from Gambro and has on-going research collaboration with Gambro, Grifols and is the Principal Investigator of an Industry sponsored study (Sequana Medical). He is also inventor fo a drug, L-ornithine phenyl acetate which UCL has licensed to Ocera Therapeutics. Pere Ginès has received speaker honorarium and research funding from Grifols, served on the scientific advisory board for Ferring and Sequena and received research funding from Sequena. Vicente Arroyo and Javier Fernandez have received grant and research support from Grifols. All other authors declare that they have no conflict of interest.

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Abstract

Bacterial infection is a frequent trigger of ACLF, syndrome that could also increase the risk of infection. This investigation evaluated: prevalence and characteristics of bacterial and fungal infections causing and complicating ACLF; predictors of follow-up bacterial infections; and impact of bacterial infections on survival.

Patients: 407 patients with ACLF and 235 patients with acute decompensation (AD).

Results: 152 patients (37%) presented bacterial infections at ACLF diagnosis; 46% (n=117) of the remaining 255 patients with ACLF developed bacterial infections during follow-up (4 weeks). The corresponding figures in patients with AD were 25% and 18% ($p<0.001$). Severe infections (SBP, pneumonia, severe sepsis/shock, nosocomial infections and infections caused by multi-resistant organisms) were more prevalent in patients with ACLF. Patients with ACLF and bacterial infections (either at diagnosis or during follow-up) showed higher grade of systemic inflammation at diagnosis of the syndrome, worse clinical course (ACLF 2-3 at final assessment: 47% vs. 26%; $p<0.001$) and lower 90-day probability of survival (49% vs. 72.5%; $p<0.001$) than patients with ACLF without infection. Bacterial infections were independently associated with mortality in patients with ACLF-1 and 2. Fungal infections developed in 9 patients with ACLF (2%) and in none with AD, occurred mainly after ACLF diagnosis (78%) and had high 90-day mortality (71%).

Conclusion: Bacterial infections are extremely frequent in ACLF. They are severe and associated with intense systemic inflammation, poor clinical course and high mortality. Patients with ACLF are highly predisposed to develop bacterial infections within a short follow-up period and could benefit from prophylactic strategies.

What is already known about this subject?

Bacterial infections are a frequent precipitating event of ACLF. Type and severity of infections have been partially described. Other characteristics of bacterial infections, risk of bacterial and fungal infections after ACLF diagnosis, microbiology and relationship with clinical course are unknown.

What are the new findings?

- Patients with ACLF are highly predisposed to develop bacterial infections within a short follow-up period.
- Severe infections (SBP, pneumonia, severe sepsis/shock, nosocomial infections and infections caused by multi-resistant organisms) are more prevalent in patients with ACLF.
- Bacteria infections, either at diagnosis or during follow-up, are key prognostic determinants in patients with ACLF. They are associated with more severe systemic inflammation, poorer clinical course and higher mortality.
- Bacterial infections are independent predictors of 90-day mortality in patients with ACLF 1 and 2.
- Inappropriate empirical antibiotic strategies increase 90-day mortality in ACLF triggered or complicated by infection.

Impact on clinical practice

- Infection control practices are essential in the management of patients with ACLF.

- Patients with ACLF may benefit from prophylactic strategies aimed to decrease their prohibitive risk of infection.

INTRODUCTION

Acute-on-chronic fiver failure (ACLF) in cirrhosis is a syndrome characterized by acute decompensation (AD), organ failure(s) and high short-term mortality.[1] Bacterial infection is the most frequent trigger of ACLF in Western countries.[1-3]

Patients with decompensated cirrhosis present chronic systemic inflammation due to intestinal dysbiosis, loss of integrity of the intestinal mucosal barrier and sustained translocation of pathogen associated molecular patterns (PAMPs).[4-7] In patients with bacterial infections, ACLF is due to massive release of PAMPs by the infecting bacteria. PAMPs activate innate immune system leading to the release of inflammatory cytokines, vasodilatory mediators and reactive oxygen species.[4,7-9] Other precipitating events (i.e. acute alcoholic hepatitis; HBV flare) cause systemic inflammation by the release of damaged-associated molecular patterns (DAMPs) by the liver.[10] Multi-organ dysfunction/failure in ACLF develops as consequence of acute impairment in systemic circulatory function and organ hypo-perfusion and also to direct deleterious effects of inflammatory mediators in organ homeostasis, a feature known as immune-pathology.[3,4,7, 11]

It has been suggested that in addition to being a trigger of ACLF, bacterial infections may also be a specific complication of the syndrome. The hypothesis is that, as it occurs in sepsis,[1] the exaggerated systemic inflammatory response associated with ACLF may be followed by a state of immune-paralysis that predisposes to early development of secondary infections and contributes to increase mortality.[12-16] This hypothesis is supported by a single study showing a higher prevalence of bacterial infections during hospitalization in patients with ACLF (defined according to outdated criteria) in comparison to AD.[17] Other 2 studies suggest that nosocomial infections are independent predictors of ACLF.[18,19] Type and severity of infections were

partially described in these studies with no mention on other characteristics of bacterial infections, microbiology and relationship with clinical course.

The current study was performed to assess the prevalence of bacterial infections triggering and complicating ACLF, the characteristics of these infections and their impact on the clinical course and prognosis using information from the Canonic database.[1] Data on fungal infection and colonization were also analyzed.

PATIENTS AND METHODS

Study population and aims of the study

In the current investigation, only patients with complete 4-week follow-up data after diagnosis of AD or ACLF were included. We excluded 701 patients, 636 with AD without scheduled visits after diagnosis as per protocol and 65 with insufficient data at diagnosis (figure 1). Therefore, 642 patients were finally included, 407 with ACLF (292 diagnosed at enrolment and 115 during hospitalization) and 235 with AD without ACLF. Follow-up visits were performed at days 1, 2, 7, 14, 21 and 28 after diagnosis of ACLF or AD. Patients with AD developing ACLF during hospitalization completed the 28-day follow-up period after ACLF diagnosis. Data on the development of bacterial or fungal infections, including type and site of acquisition, clinical characteristics and microbiology, were recorded at diagnosis and at each visit.

Definitions related to infection

Diagnostic criteria of bacterial infections were the following. Spontaneous bacterial peritonitis (SBP): polymorphonuclear (PMN) cell count in ascitic fluid ≥250/mm^3. Urinary tract infection (UTI): abnormal urinary sediment (>10 leukocytes/field) and

positive urinary culture or uncountable leukocytes per field if negative cultures. Spontaneous bacteremia: positive blood cultures and no cause of bacteremia. Secondary bacteremia: a) catheter-related infection (positive blood and catheter cultures), b) bacteremia occurring within 24h after an invasive procedure. Pneumonia: clinical signs of infection and new infiltrates on chest x-ray. Bronchitis: clinical features of infection, no radiographic infiltrates and positive sputum culture. Skin and soft tissue infections (SSTI): clinical signs of infection associated with swelling, erythema, heat and tenderness in the skin. Cholangitis: cholestasis, right upper quadrant pain and/or jaundice and radiological data of biliary obstruction. Spontaneous bacterial empyema: PMN count in pleural fluid ≥250/mm^3. Secondary peritonitis: PMN count in ascitic fluid ≥250/mm^3 and evidence (abdominal CT/ surgery) of an intraabdominal source of infection. *Clostridium difficile* infection (CDI): positive stool toxin in a patient with diarrhea. Unproved bacterial infection: presence of fever and leukocytosis requiring antibiotic therapy without any identifiable source.[20]

Fungal infections were defined as follows. Invasive candidiasis: isolation of *Candida spp* in 1 or more blood cultures (candidemia) or from normally sterile body fluids. *Candida* colonization: isolation of *Candida spp* in non-sterile fluid in the absence of infection. Probable invasive aspergillosis (IA): detection of *Aspergillus* by direct examination and/or culture of respiratory samples in the presence of radiological imaging compatible with lung infection.[21]

Criteria used to define the site of acquisition of infection, infection resolution and appropriateness of empirical antibiotic strategies are described in the supplementary material section.

Bacterial infections were considered as potential triggers of ACLF when they were detected prior or at the time of diagnosis of the syndrome (day 0). Infections were qualified as complications of ACLF when they were detected between day 1 and day 28 after the diagnosis of the syndrome. These criteria were based in the foreseeable sequence of events of ACLF triggered by bacterial infections. First, infections causing ACLF precede the onset of the syndrome and ACLF development frequently precedes hospital admission. Second, in the Canonic study there was an additional 1-day delay between hospital admission, study enrolment and ACLF diagnosis in all patients as per protocol design and a delay of two or more additional days in 40% of patients for other reasons[1]. Finally, the Canonic protocol included a complete diagnostic work-up of bacterial infections at study enrolment. The same criteria were used to qualify bacterial infections in patients with AD without ACLF.

Supplementary table 1 shows the bacteria and fungi isolated in patients with and without ACLF. Criteria used to define multidrug-resistant organism (MDROs) have been previously described. [20]

Definitions related to ACLF

Diagnostic criteria of organ failure was based on the CLIF-C organ failure score.[1,2] ACLF grade 1 (ACLF-1) defines the presence of renal failure alone or of any other single organ failure if associated to renal dysfunction and/or cerebral dysfunction. ACLF grade 2 and grade 3 (ACLF-2 and 3) define the presence of 2 and 3 to 6 organ failures, respectively.[1,2] The clinical course of ACLF was defined as good-relatively good when the ACLF grade at final assessment was 0 or 1 and severe when it was 2 or 3.[22]

Assessment of systemic inflammation and of oxidative stress at diagnosis of ACLF and AD

Systemic inflammation was assessed by measuring the plasma levels of five inflammatory cytokines involved in innate immune responses[8] and systemic oxidative stress by the determination of the redox state of human serum albumin.

Cytokines were measured using a multiplexed bead-based immunoassay on a Luminex 100 Bioanalyzer. Non-oxidized (human mercaptalbumin, HMA), and reversible and irreversible oxidized (normercaptalbumins HNA1 and HNA2) albumin forms in plasma were separated by high performance liquid chromatography and detected by fluorescence. Normal values in healthy subjects have been previously described.[8]

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 for not normally distributed continuous variables. 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 identify predictors of infection in ACLF 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 dropping variables. The proportional-hazards model for Competing-Risks proposed by Fine and Gray[23] was used to identify independent predictors of

mortality. This model was chosen in order to account for liver transplantation as an event ‘competing’ with mortality. In all statistical analyses, significance was set at $p<0.05$. Analyses were done with SPSS (version 23.0; SPSS, Inc. Chicago, IL) and SAS (version 9.4; SAS Institute Inc.; Cary, NC) statistical packages.

RESULTS

Overall bacterial infections

Figure 1 shows the flow chart of patients included (n=642) and excluded (n=701) from the study. A total of 360 patients (56%) presented bacterial infections during the study. In 211 patients (152 patients with ACLF and 59 with AD) infection was present at diagnosis. In the remaining 149 patients, infection was diagnosed during follow-up. Thirty-one patients with bacterial infections at diagnosis developed new bacterial infections during follow-up. Twenty-two patients with ACLF complicated by infection developed reinfection (reinfections are not included in the analysis of the results).

Bacterial infections triggering ACLF

Prevalence and characteristics

Two hundred and eleven patients (33%) presented bacterial infections at diagnosis of ACLF or AD. Prevalence was significantly higher in patients with ACLF (overall infections: 37% vs. 25%; proved infections: 33.5% vs. 19%; p< 0.001 each). All types of infection except for SSTI, CDI and unproved infections were more frequent in patients with ACLF. Differences were significant for pneumonia (7.7% vs. 3%, p=0.015) and secondary peritonitis (2.6% vs. 0%, p=0.009) (Table 1). The prevalence of infections at ACLF diagnosis was significantly higher (p=0.016) in patients with ACLF-3 (52%; Supplementary table 2).

Progression to severe sepsis/septic shock was more frequently observed in infections present at diagnosis of ACLF than in those associated with AD (49% vs. 2%; p<0.001). Prevalence of nosocomial infections (53% vs. 22%; p<0.001) and of infections caused by MDROs (16% vs. 3%: p=0.01) was also significantly higher in

ACLF (Table 1). Significant differences were also observed when the analysis was restricted to patients with ACLF diagnosed at enrolment (data not shown).

Impact of infection on the severity of ACLF, clinical course and mortality

The grade of systemic inflammation (WBC count, serum CRP levels and plasma concentration of inflammatory cytokines) was more intense in patients with infections at ACLF diagnosis than in those without (Table 2). Severity of the syndrome was also higher in patients with ACLF precipitated by bacterial infections, as indicated by a higher prevalence of encephalopathy, circulatory, respiratory and cerebral failure at diagnosis of the syndrome, a higher baseline CLIF-C ACLF score, and higher requirements of organ support during hospitalization (Table 2). Similar results were observed when patients with unproved infections were considered as non-infected (Supplementary Table 3).

The clinical course of ACLF, as estimated by the final ACLF grade, was also significantly worse in patients with ACLF caused by bacterial infections. Twenty-eight day and 90-day mortality rates were also higher in patients with ACLF triggered by bacterial infection (overall or proved episodes), differences being statistically significant only at 90 days (Table 2, Supplementary Table 3).

In order to confirm that infection-triggered ACLF portends a worse prognosis, we examined data on the 115 patients with AD who developed ACLF during follow-up. Cases triggered by infection showed higher organ support requirements, worse clinical course of ACLF and higher 28 and 90-day mortality rates than those caused by other precipitating events (Supplementary Table 4).

Infection resolution and patient mortality according to the type and characteristics of bacterial infections detected at ACLF diagnosis.

The resolution rate of bacterial infections detected at diagnosis was significantly lower in patients with ACLF than in those with AD (71.1% vs. 98.3%; p<0.001). Type of infection influenced infection resolution and mortality (Table 3). SSTI and unproved infections showed the lowest resolution rates and SSTI and SBP the highest mortality rates. The presence of severe sepsis/septic shock and the isolation of MDROs also influenced negatively infection resolution and prognosis.

Bacterial infections complicating ACLF not triggered by infection

Incidence and characteristics

Patients with ACLF not triggered by infections presented significantly higher incidence of bacterial infection during follow-up than patients with AD (46% vs. 18%, p<0.001) (Table 1). This feature was observed throughout the entire 28-day follow-up period (Figure 2A). The risk of developing bacterial infections correlated directly with the grade of ACLF (Figure 2B and supplementary table 2). Similar results were observed when patients with unproved infections were considered as non-infected (Supplementary Figures 1A and 1B).

All types of bacterial infections were more frequent in patients with ACLF than in patients with AD except for CDI (Table 1). Differences were statistically significant for pneumonia (8.6% vs. 1.7%, p<0.001), SBP (8.6% vs. 3.4%, p=0.03) and bacteremia (3.9% vs. 0.6%, p=0.03). Follow-up infections were also more severe in patients with ACLF as indicated by the higher prevalence of sepsis and severe sepsis/septic shock (41.9% vs. 6.2%, p< 0.001) and of infections caused by MDROs (18.8% vs. 3.1%, p=0.02) (Table 1).

Risk factors of follow-up bacterial infections in ACLF and impact of infection on clinical course and mortality

Patients with ACLF developing bacterial infections during follow-up were those with higher grade of systemic inflammation and higher severity of ACLF at diagnosis as indicated by higher WBC count and higher plasma levels of CRP and cytokines, higher frequency of hepatic encephalopathy, cerebral and respiratory failure and mechanical ventilation, and higher CLIF-C ACLF score. They also presented worse clinical course and higher 28-day and 90-day mortality rates (Table 2).

Supplementary figure 2 shows the individual plasma concentrations of cytokines measured at diagnosis of the syndrome in patients with ACLF triggered by infection, ACLF complicated by infection and ACLF without infections during the whole study period. Although concentrations were higher in infected patients a marked overlap among groups was observed.

Multiple regression analysis identified CLIF-C ACLF score (n=167; OR=1.10, 95% CI=1.01-1.08; p=0.017) and HNA2 (n=68; OR=1.15, 95% CI=1.04-1.27; p<0.005) at diagnosis as independent risk factors of follow-up bacterial infections.

The resolution rate of follow-up bacterial infections in patients with ACLF was 78.6% vs. 98.8% in AD (Table 3, p<0.001). Resolution rate and mortality rates associated with bacterial infections at follow-up were not significantly influenced by the type and severity of the infections.

Rate and characteristics of bacterial infections occurring in ACLF according to the precipitating event and the need for critical care

Rate and characteristics of bacterial infections that triggered or complicated ACLF differed between patients hospitalized in the ICU and those admitted to the regular

ward. In contrast, type of precipitating event did not influence these parameters (Supplementary Table 5 and 6). Prevalence of infection was significantly higher in patients with ACLF triggered by infection requiring ICU admission. Pneumonia was more prevalent in critical care while UTI and SSTI were more frequent in the regular ward. As expected, severity of infection was higher in the ICU.

Overall impact of bacterial infections on clinical course and survival in patients with ACLF

The clinical course (ACLF 2-3 at final assessment: 47% vs. 26%; $p<0.001$) was significantly worse and the probability of 90-d transplant-free survival significantly shorter (Figure 3A) in patients with ACLF and bacterial infection (either at diagnosis or during follow-up) than in those without (45% vs. 70%, $p<0.001$). Similar results were obtained when only patients developing proved infections were considered as infected (Supplementary Figure 3). Infections had a great impact on the prognosis of patients with the less severe forms of ACLF (Figures 3B and 3C). Infected patients with ACLF-1 and ACLF-2 showed a lower 90-d probability of survival than those without infection. In contrast, patients with ACLF-3 with and without infections did not show differences in prognosis. Patients with AD with and without bacterial infections (overall, Figure 3A, and proved, Supplementary Figure 3) also showed a similar prognosis, since patients with AD developing ACLF during hospitalization were included in the ACLF group.

Appropriateness of empirical antibiotic strategies also had an impact on clinical course and survival of patients with ACLF. Appropriate empirical antibiotic therapy was administered in 74% and 72% of bacterial infections triggering and complicating ACLF, respectively. Adequacy of initial antibiotic strategies was associated with

lower critical care requirements, better evolution of the syndrome in infection-triggered ACLF and lower 28 and 90-d mortality (Table 4).

Predictors of mortality

Supplementary Table 7 shows factors associated with 90-day transplant-free mortality in the univariate and multivariate analysis in the whole series of patients with ACLF. Age (HR: 1.03), hepatic encephalopathy (HR: 1.98), serum bilirubin (HR: 1.03), INR (HR: 1.38) and serum creatinine (HR: 1.27) at diagnosis of the syndrome were identified as independent predictors of death. When the analysis was restricted to patients with ACLF-1 and 2 (Table 5, first model), serum bilirubin (HR: 1.03; 95% CI: 1.01-1.05; p<0.001), age (HR: 1.03; 95% CI: 1.00-1.05; p=0.02), bacterial infection at diagnosis or during follow-up (HR: 1.79; 95% CI: 1.08-2.96; p=0.02) and serum creatinine (HR: 1.14; 95% CI: 1.01-1.29; p=0.04) were identified as independent predictors. When appropriateness of initial antibiotic therapy was introduced in the model (Table 5, second model), this factor but not bacterial infection remained as independent predictor of survival in patients with ACLF-1 and 2 (HR: 0.40; 95% CI: 0.26-0.63; p<0.001). WBC count and mechanical ventilation were not entered in the regression models because of their potential collinearity with infection.

Fungal infection and colonization

Fungal isolation was infrequent and mainly observed in patients with ACLF (3.9% vs. 0.4%, p=0.005). Of the 16 patients with ACLF and fungal isolation, seven corresponded to invasive candidiasis (5 candidemias and 2 secondary peritonitis), one to probable IA and 8 to colonization by candida. The single isolation in patients

with AD consisted of a urinary colonization by *Candida*. Six out of the eight invasive fungal infections were diagnosed during follow-up in patients with ACLF. In the remaining two patients (a secondary peritonitis and an IA) diagnosis was performed at ACLF diagnosis. Only nineteen patients (6 of them with candida colonization) received antifungal prophylaxis. Mortality rates associated with invasive fungal infection and colonization were 57% and 44% at 28-day and 71% and 67% at 90-day, respectively.

DISCUSSION

The results of our study indicate that bacterial infection is a major problem and a key prognostic determinant in ACLF. The overall prevalence of infections in patients with ACLF was extremely high (66.1%). Two-thirds of ACLF patients presented infections at diagnosis or within follow-up. In contrast, the overall prevalence of infection in patients with AD was of 38.7%. The severity of bacterial infections, as indicated by the frequency of SBP, pneumonia, severe sepsis, nosocomial infections and infections caused by MDROs, was also significantly higher in patients with ACLF. Not surprisingly, the clinical course of ACLF, as estimated by the percentage of patients with ACLF grade 2 or 3 at final assessment, was significantly worse in patients with bacterial infections than in those without (45% vs. 25%).

The prevalence of bacterial infections at ACLF diagnosis in our series was 37.3%. These infections are important because they promote a burst of systemic inflammation that precipitates the development of the syndrome.[1,3,7] In the current study we compared for the first time the severity of ACLF triggered by bacterial infections and by other precipitating events. Our results clearly show a greater severity of systemic inflammation and of ACLF in patients with infections. The clinical course of ACLF was also significantly worse in these patients.

One of the most outstanding findings of our study was the extremely high incidence of follow-up bacterial infections (46%) observed in the 255 patients without infections at ACLF diagnosis. This represents that approximately one every two non-infected patients with ACLF will develop bacterial infections within 4 weeks after diagnosis. This figure contrasts sharply with the 18% incidence of follow-up infections in non-

infected patients with AD. Bacterial infections are, therefore, not only a frequent trigger of ACLF but also an extremely common complication of the syndrome.

The mechanism of this high risk of follow-up bacterial infections in patients with ACLF is likely multifactorial. Severity of systemic oxidative stress (HNA2 levels) and of ACLF (CLIF-C ACLF score) at diagnosis were significantly associated with the development of follow-up bacterial infections in the current study. Systemic inflammation may increase bacterial translocation either directly [24] or indirectly (by increasing circulatory dysfunction and homeostatic stimulation of sympathetic nervous system). The secondary release of norepinephrine at the intestinal mucosa impairs the local immune system function and induces qualitative and quantitative changes of the intestinal microbiota towards a phenotype associated with bacterial translocation.[25] The reduction of the amount of bile acid secretion secondary to liver failure is another factor favoring intestinal bacterial overgrowth.[26] Finally, the frequent instrumentation of patients with cerebral, respiratory or renal failure with intravenous, intra-arterial and urinary catheters and the frequent use of artificial organ support devices are other major factors increasing the rate of follow-up bacterial infections in these patients.[27,28] In fact, the more prevalent infections complicating ACLF were spontaneous bacteremia and spontaneous bacterial peritonitis, which are caused by bacterial translocation, and pneumonia and secondary bacteremia, which are commonly observed in patients undergoing invasive therapeutic procedures.

There are many similarities between ACLF and severe sepsis. Both conditions develop in the setting of intense systemic inflammation and oxidative stress. In patients with sepsis, systemic inflammation is initiated by an acute release of PAMPS by bacteria and secondary activation of the innate immune system cells.[29-32] Approximately 40% of patients with ACLF share this pathophysiological

mechanism.[1,8,33,34] The second similarity is that patients with ACLF and with severe sepsis develop organ failure(s) and that this correlates closely with prognosis.[1,19,32,35] Finally, our study suggests that the third feature shared by patients with ACLF and with severe sepsis is that they both are highly predisposed to develop bacterial infections shortly after diagnosis. There are many evidences supporting a two-phase immune response in patients with severe sepsis.[36-39] Following a short initial period (few days after diagnosis) of severe systemic inflammation patients develop a second period of immune-suppression due to impairment of immune cell function and apoptotic depletion of immune cells.[39] During this period, aggravation of the primary infection or development of new secondary infections is common.[40]

The 117 non-infected patients with ACLF at diagnosis of the syndrome represent a unique population to assess if this sequence of events also occurs in ACLF, since in this group of patients the temporal relationship between systemic inflammation, ACLF development and follow-up bacterial infections is not interfered by antibiotic therapy. Our results support a two-phase clinical course in non-infected patients with ACLF. The first-phase, probably very short, is characterized by acute development of severe systemic inflammation and organ/system failure(s). ACLF is diagnosed at the end of this phase. The second-phase, of longer duration, is characterized by a remarkable high incidence of bacterial infections that mainly develop within the first week after the diagnosis of ACLF. Whether immune-suppression is involved in the pathogenesis of this second phase is currently unknown, but impaired pathogen killing activity and reactive oxygen species release by macrophages and neutrophils has been reported in these patients.[41,42] Recent studies have also shown that patients with ACLF have increased numbers of immunoregulatory monocytes and macrophages that express MERTK and elevated plasma levels of prostaglandin E2,

alterations that suppress the innate immune response to microbes and could increase the risk of infection. [43,44]

The high incidence of bacterial infection after ACLF diagnosis justifies the implementation of infection control practices such as bundles on prevention of ventilator-associated pneumonia and catheter-related bacteremia and hand hygiene.[45] Selective intestinal decontamination with non-absorbable antibiotics could also prevent nosocomial infections in ACLF patients but could also promote the development of MDROs.[46,47] Treatments aimed at restoring the patients' immune function could also be beneficial in these patients.[48,49] Our study also demonstrates that adequacy of empirical antibiotic strategies is also a key factor in the management of infected patients with ACLF. Inappropriate first line therapies were associated with increased mortality. Therefore, broad antibiotic schemes covering all potential pathogens should be applied at high doses within the first 48-72h after the diagnosis of infection to improve clinical efficacy and minimize the selection of resistant strains.[45]

We observed significantly higher mortality rate and shorter probability of survival in patients with ACLF triggered or complicated by bacterial infections than in patients with ACLF without bacterial infections throughout the entire period of observation, suggesting that bacterial infections has a major impact on the prognosis of patients with ACLF. This is also supported by the observation that infection was an independent predictor of mortality in patients with ACLF grade 1 and 2. The overall prevalence of bacterial infections in patients ACLF-3 was so high (91%) that they did not impact prognosis.

The prevalence of fungal infections in our patients with ACLF was low (2%) and mainly occurred during the follow-up period after ACLF diagnosis. This figure is in

line with recent studies showing a low incidence of invasive fungal infections in patients with cirrhosis admitted to ICU (1%).[50] However, fungal infections could have been under-estimated in our study since specific cultures were not performed. The relatively low rate of patients with ACLF-3 included in the Canonic series (20%) could also explain this finding.

In summary, bacterial infections are a significant problem and a major prognostic determinant in patients with ACLF. Infections are detected at ACLF diagnosis in one-third of the patients. Among the remaining patients with ACLF, approximately half develop bacterial infections within a follow-up period 4-week. The severity of systemic inflammation and of ACLF is significantly higher, the clinical course significantly worse and mortality significantly higher in patients with ACLF and bacterial infections than in those without. Adequate empirical antibiotic strategies, infection control practices and prophylactic measures are essential in the management of patients with ACLF.

FIGURE LEGENDS

Figure 1

Flow chart of the patients included and excluded from the study. In total, 642 patients were included. Three hundred sixty patients developed infections throughout the study: 152 with ACLF and 59 with AD presented an infection at diagnosis, 149 patients without infections at diagnosis developed infections during follow-up (117 with ACLF and 32 with AD). Finally, 53 patients with bacterial infections at diagnosis or during follow-up developed new bacterial infections.

Figure 2A

Probability of developing bacterial infections during follow-up in patients with ACLF (red line) and AD (green line) without infections at diagnosis. Probability was significantly higher in patients with ACLF, especially in the first week after diagnosis.

Figure 2B

Incidence of bacterial infections within follow-up in patients with AD and with ACLF-1, ACLF-2 and ACLF-3 without bacterial infections at diagnosis. Incidence correlated with the grade of ACLF, being extremely high in patients with ACLF-3.

Figure 3A

Probability of 90-day transplant-free survival in patients with AD and ACLF with and without bacterial infections. Survival was significantly shorter ($p<0.001$) in patients with ACLF and bacterial infections [either at diagnosis (ACLF-BiD) or during follow-up (ACLF-BiFu); continuous red and orange lines, respectively] than in patients with

ACLF without bacterial infections (discontinuous red line; ACLF-NoBi) and in patients with AD with (continuous green line; AD-Bi) and without bacterial infections (discontinuous green line; AD-NoBi).

Figure 3B

Probability of 90-d transplant-free survival in patients with ACLF-1 (green), ACLF-2 (blue) and ACLF-3 (red) with (continuous lines) and without (discontinued lines) bacterial infections (either at diagnosis or during follow-up). Patients with ACLF 1 and ACLF-2 without bacterial infections showed a higher probability of survival than those with infection (p=0.004 and p=0.024, respectively).

Figure 3C

90-d mortality rate of patients with ACLF 1-2 and with ACLF-3 with (red) and without (blue) bacterial infections (either at admission or during follow-up). Difference was statistically significant in patients with ACLF 1-2 ($p<0.001$) but not in patients with ACLF-3.

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Table 1. Prevalence and characteristics of bacterial infections present at diagnosis or developed during follow-up and associated mortality in patients with AD and ACLF

	At diagnosis*		During follow-up**	
	AD (n=235)	**ACLF (n=407)**	**AD (n=176)**	**ACLF (n=255)**
Prevalence and types of infection				
Prevalence of infections (n/%)	59(25.1)	152(37.3)***	32(18.2)	117(45.9)***
Types of infection (n/%)				
Spontaneous bacterial peritonitis	16(6.8)	41(9.8)	6(3.4)	22(8.6)*
Urinary infections	10(4.3)	25(6.0)	12(6.8)	27(10.6)
Pneumonia	7(3.0)	32(7.7)*	3(1.7)	22(8.6)***
Unproved infections	14(6.0)	16(3.8)	7(4.0)	18(7.0)
SSTI	7(3.0)	12(2.9)	1(0.6)	7(2.7)
Spontaneous/secondary bacteraemia	1(0.4)	9(2.2)	1(0.6)	10(3.9)*
Secondary bacterial peritonitis	-	11(2.6)**	-	2(0.8)
Other[1]	3(1.3)	6(1.4)	1(0.6)	8(3.1)
Clostridium difficile infection	1(0.4)	-	1(0.6)	1(0.4)
Characteristics of bacterial infections				
Site of acquisition (n/%)				
Community-acquired	32(54.2)	38(25.0)***	-	-
Healthcare-associated	14(23.7)	34(22.4)	-	-
Nosocomial	13(22.0)	80(52.6)	32(100.0)	117(100.0)
Infections caused by MDROs (n/%)				
No	57(96.6)	128(84.2)*	31(96.9)	95(81.2)*
Yes	2(3.4)	24(15.8)	1(3.1)	22(18.8)
Sepsis (n/%)				
No sepsis	46(78.0)	78(51.3)***	30(93.8)	68(58.1)***
Sepsis	12(20.3)	-	1(3.1)	26(22.2)
Severe sepsis or shock	1(1.7)	74(48.7)	1(3.1)	23(19.7)
Mortality (n/%)				
28-day mortality	1(1.7)	54(35.5)***	2(6.3)	45(38.5)***
90-day mortality	6(10.2)	77(50.7)***	3(9.4)	60(51.3)***

*All patients included

**Only patients without bacterial infections at diagnosis

SSTI Skin and soft tissue infections ; MDROs: Multidrug resistant organisms

[1] Other infections at diagnosis of ACLF: tracheobronchitis (4), spontaneous bacterial empyema (n=1), cholangitis (1),undefined (3). Other infections during follow-up: dental infection (1), undefined (8)

*p-value<0.05; **p-value<0.01; ***p-value<0.001

Table 2. Clinical and laboratory data at ACLF diagnosis, clinical course, and mortality in patients with and without bacterial infection at diagnosis or during follow-up#

	Bacterial infection at ACLF diagnosis (n=152)	No bacterial infection at ACLF diagnosis (n=255)	Bacterial infection during follow-up## (n=117)	No bacterial infection during follow-up## (n=138)
Cause of admission				
GI bleeding	7(8.3)	26(19.7)*	14(21.2)	12(18.2)
Infection	31(36.9)	25(18.9)**	16(24.2)	9(13.6)
Encephalopathy	10(11.9)	33(25.0)*	20(30.3)	13(19.7)
HRS	2(2.4)	7(5.3)	2(3.0)	5(7.6)
Ascites	19(22.6)	27(20.5)	9(13.6)	18(27.3)
Other	15(17.9)	14(10.6)	5(7.6)	9(13.6)
Clinical and laboratory data				
Age (years)	56±13	56±11	54±11	57±11*
Alcoholic cirrhosis (%)	80(53.7)	148(61.4)	63(57.8)	85(64.4)
No prior decompensation (%)	43(29.7)	53(22.3)	25(22.3)	28(22.2)
Ascites with surrogates (%)	147(96.7)	252(98.8)	116(99.2)	136(98.6)
Encephalopathy (%)	83(61.0)	111(50.2)*	62(60.8)	49(41.2)**
WBC (x10^9/L)	9.9(6.1-15.4)	6.8(4.6-11.7)***	7.5(5.0-13.2)	6.5(4.6-9.9)*
Serum CRP (mg/L)	36(21- 77)	25(11-46)***	29(16-51)	19(9-40)*
Serum bilirubin (mg/dL)	6.8(3.3-14.6)	8.5(2.6-19.4)	11.0(2.7-22)	6.6(2.5-16.5)
INR	1.9(1.5-2.7)	1.9(1.4-2.6)	2.0(1.5-2.5)	1.8(1.4-2.6)
Serum creatinine (mg/dL)	1.7(1.0-2.6)	1.9(1.0-2.6)	1.9(1.1-3.0)	1.8(0.9-2.4)
Plasma sodium (mEq/L)	134±7	135±6	135±6	134±6
Serum albumin	2.8(2.4-3.2)	2.9(2.5-3.4)	2.9(2.6-3.3)	2.9(2.5-3.5)
Renal failure (%)	72(52.2)	112(50.9)	52(51.5)	60(50.4)
Cerebral failure (%)	42(30.9)	38(17.2)**	24(23.3)	14(11.9)*
Respiratory failure (%)	25(20.5)	18(10.2)*	13(15.7)	5(5.4)*
Circulatory failure (%)	45(34.1)	39(18.0)***	22(22.2)	17(14.4)
Coagulation failure (%)	52(39.1)	65(30.5)	27(27.6)	38(33.0)
Liver failure (%)	48(35.0)	93(42.9)	46(46.0)	47(40.2)
MELD score	28±7	27±7	28±7	27±7
CLIF-C ACLF score	54±11	48±9**	50±9	46±9*
NASCELD criteria for ACLF$	22(14.5)	22(8.6)	17(14.5)	5(3.6)**
ACLF-1(%)	71(46.7)	133(52.2)	50(42.7)	83(60.1)***
ACLF-2(%)	52(34.2)	95(37.3)	45(38.5)	50(36.2)
ACLF-3(%)	29(19.1)	27(10.6)	22(18.8)	5(3.6)
Inflammatory cytokines				
TNF (pg/ml)	37(26-50)	29(17-39)**	31(18-42)	25(15-35)*
IL-6 (pg/ml)	101(34-466)	29(13-75)***	33(16-100)	26(11-43)*
IL-8 (pg/ml)	117(66-225)	75(38-165)**	87(45-165)	60(32-169)
IL-10 (pg/ml)	18(7-58)	6(2-19)***	7(3-34)	4(1-14)**
IL-1ra (pg/ml)	39(14-108)	16(8-42)**	23(9-57)	14(7-30)*
Albumin oxidation fractions&				
HMA (%)	42(30-58)	46(34-58)	42(33-58)	48(35-56)
HNA1+HNA2 (%)	56(42-68)	52(41-64)	51(41-65)	52(43-64)
HNA2 (%)	11(8-15)	11(6-15)	12(7-17)	8(5-12)*
Need for critical care (28-d)				
ICU	95(62.5)	112(43.9)***	66(56.4)	46(33.3)***
Mechanical ventilation	58(38.2)	56(22.0)***	41(35.0)	15(10.9)***
Renal replacement therapy	51(33.6)	55(21.6)**	31(26.5)	24(17.4)
NASCELD criteria for ACLF$	63(41.5)	63(24.7)***	46(39.3)	17(12.3)***
Clinical course of ACLF				
No ACLF or ACLF-1 at final assessment	74(51.0)	151(64.8)**	61(54.5)	90(74.4)**
ACLF 2-3 at final assessment	71(49.0)	82(35.2)	51(45.5)	31(25.6)
28-day transplant free mortality	54(35.5)	71(27.8)	45(38.5)	26(18.8)***
90-day transplant free mortality	77(50.7)	98(38.4)*	60(51.3)	38(27.5)***

Patients are divided in two groups: A: patients with and without bacterial infections at diagnosis of ACLF, B: patients with ACLF without bacterial infections at diagnosis who did and did not develop bacterial infections during follow-up.

Patients with ACLF and bacterial infection at diagnosis of the syndrome were excluded from this analysis

$: two or more of the following: vasopressors, renal replacement therapy, mechanical ventilation, grade 3-4 hepatic encephalopathy
& According to the redox state at cysteine 34
*P-value<0.05; **P-value<0.01; ***P-value<0.001

Table 3. Type and characteristics of bacterial infections at ACLF diagnosis or during follow-up: relationship with infection resolution and patient mortality

	Patients with bacterial infections at ACLF diagnosis				Patients with bacterial infections during follow-up			
	N	Resolution Rate	Mortality at 28 days	Mortality at 90 days	N	Resolution Rate	Mortality at 28 days	Mortality at 90 days
Prevalence and types of infection								
Prevalence of bacterial infections (n/%)	152	108(71.1)	54(35.5)	77(50.7)	117	92(78.6)	45(38.5)	60(51.3)
Type of Infections (n/%)								
Spontaneous bacterial peritonitis	41	26(63.4)**	19(46.3)	24(58.5)*	22	16(72.7)	10(45.5)	13(59.1)
Urinary infections	25	24(96.0)	5(20.0)	11(44.0)	27	23(85.2)	8(29.6)	11(40.7)
Pneumonia	32	20(62.5)	12(37.5)	18(56.3)	22	15(68.2)	12(54.6)	14(63.6)
Unproved infections	16	9(56.3)	7(43.8)	8(50.0)	18	16(88.9)	11(61.1)	12(66.7)
SSTI	12	6(50.0)	7(58.3)	9(75.0)	7	6(85.7)	0(0.0)	1(14.3)
Spontaneous/secondary bacteraemia	9	7(77.8)	2(22.2)	3(33.3)	10	8(80.0)	2(20.0)	5(50.0)
Secondary bacterial peritonitis	11	10(90.9)	2(18.2)	4(36.5)	2	0(0.0)	1(50.0)	2(100.0)
Other[1]	6	6(100.0)	0(0.0)	0(0.0)	8	7(87.5)	1(12.5)	2(25.0)
Clostridium difficile infection	0	-	-	-	1	1(100.0)	0(0.0)	0(0.0)
Characteristics of bacterial infection								
Site of acquisition (n/%)								
Community-acquired	38	27(71.1)	15(39.5)	16(42.1)	-	-		
Healthcare-associated	34	20(58.8)	14(41.2)	19(55.9)	-	-		
Nosocomial	80	61(76.3)	25(31.3)	42(52.5)	117	92(78.6)	45(38.5)	60(51.3)
Multiresistant bacterial infection (n/%)								
No	128	94(73.4)	43(33.6)	60(46.9)*	95	77(81.1)	36(37.9)	47(49.5)
Yes	24	14(58.3)	11(45.8)	17(70.8)	22	15(68.2)	9(40.9)	13(59.1)
Severity of infection (n/%)								
No sepsis	78	63(80.8)**	23(29.5)	37(47.4)	68	51(75.0)	23(33.8)	31(45.6)
Sepsis	0	0(0.0)	0(0.0)	0(0.0)	26	20(76.9)	10(38.5)	15(57.7)
Severe sepsis or shock	74	45(60.8)	31(41.9)	40(54.1)	23	15(65.2)	12(52.2)	14(60.9)

SSTI Skin and soft tissue infections [1] Other infections at diagnosis of ACLF: tracheobronchitis (4), spontaneous bacterial empyema (n=1), cholangitis (1), undefined (3).
Other infections during follow-up: dental infection (1), undefined (8)
*p-value<0.05; **p-value<0.01; ***p-value<0.001

Table 4. Clinical course and mortality of patients with ACLF triggered or complicated by infection receiving appropriate or inappropriate empirical antibiotic treatment#

	Bacterial infection at ACLF diagnosis (n=152)		Bacterial infection during follow-up## (n=117)	
	Inappropriate empirical antibiotic treatment (n=35)	Appropriate empirical antibiotic treatment (n=112)	Inappropriate empirical antibiotic treatment (n=24)	Appropriate empirical antibiotic treatment (n=84)
ICU admission and organ support				
ICU	26(74.3)	68(60.7)	18(75.0)	42(50.0)*
Mechanical ventilation	17(48.6)	40(35.7)	10(41.7)	28(33.3)
Renal replacement therapy	15(42.9)	35(31.3)	7(29.2)	20(23.8)
ACLF evolution				
No ACLF or ACLF-1 at final assessment	12(35.3)	59(55.1)*	12(54.6)	47(57.3)
ACLF 2-3 at final assessment	22(64.7)	48(44.9)	10(45.4)	35(42.7)
28-day transplant free mortality	19(54.3)	32(28.6)**	11(45.8)	26(31.0)
90-day transplant free mortality	26(74.3)	47(42.0)***	16(66.7)	36(42.9)*

According to microbiological results or the need for escalation of initial antibiotic treatments in culture negative infections. Data on empirical antibiotic therapy were not available in 14 patients.
Patients with ACLF and bacterial infection at diagnosis of the syndrome were excluded from this analysis
*P-value<0.05; **P-value<0.01; ***P-value<0.001

Table 5. Predictors of 90-day mortality in the univariate and multivariate analysis in patients with ACLF 1 and ACLF 2.

Model 1. Without considering appropriateness of empirical antibiotic therapy

	Univariate Analysis		Multivariate analysis#	
Predictors	**HR (CI 95%)**	**p-value**	**HR (CI 95%)**	**p-value**
Infection (at ACLF diagnosis or during follow-up)	1.65(1.05-2.60)	0.031	1.79(1.08-2.96)	0.023
Age (years)	1.01(0.99-1.03)	0.147	1.03(1.00-1.05)	0.018
Encephalopathy (%)*	1.56(1.03-2.37)	0.036	-	-
Leukocytes (x10^9/L)*	1.07(1.04-1.11)	<0.001	-	-
Bilirubin (mg/dL)*	1.02(1.01-1.04)	0.007	1.03(1.01-1.05)	<0.001
INR*	1.11(0.91-1.36)	0.299	-	-
Creatinine (mg/dL)*	1.12(0.98-1.28)	0.098	1.14(1.01-1.29)	0.041
Heart rate (b.p.m)*	1.02(1.01-1.03)	0.001	-	-
Mechanical ventilation**	2.55(1.62-4.01)	<0.001	-	-
Renal replacement therapy**	2.32(1.51-3.57)	<0.001	-	-

Model 2. Considering appropriateness of empirical antibiotic therapy

Infection (at ACLF diagnosis or during follow-up)	1.65(1.05-2.60)	0.031	1.99(0.65-6.10)	0.228
Appropriate empirical antibiotic therapy	0.41(0.27-0.62)	<0.001	0.40(0.26-0.63)	<0.001
Age (years)	1.01(0.99-1.03)	0.147	1.02(1.00-1.04)	0.037
Encephalopathy (%)*	1.56(1.03-2.37)	0.036	-	-
Leukocytes (x10^9/L)*	1.07(1.04-1.11)	<0.001	-	-
Bilirubin (mg/dL)*	1.02(1.01-1.04)	0.007	1.03(1.01-1.05)	0.009
INR*	1.11(0.91-1.36)	0.299	-	-
Creatinine (mg/dL)*	1.12(0.98-1.28)	0.098	-	-
Heart rate (b.p.m)*	1.02(1.01-1.03)	0.001	-	-
Mechanical ventilation**	2.55(1.62-4.01)	<0.001	-	-
Renal replacement therapy**	2.32(1.51-3.57)	<0.001	-	-

* At ACLF diagnosis; ** Within the 4-weeks follow-up period

#Mechanical ventilation and leukocytes were not included in the multivariate model because of potential collinearity with infection

FIGURE 1

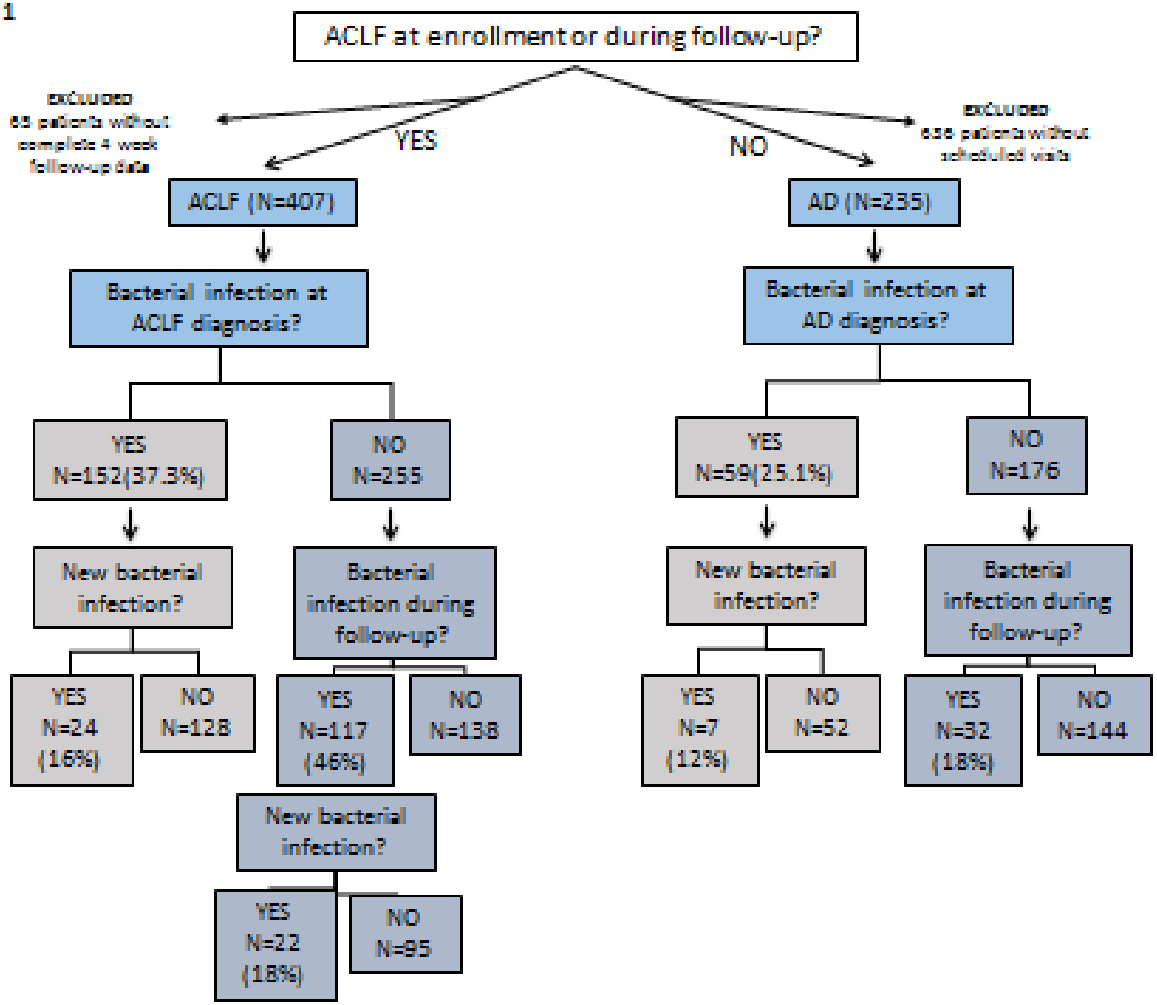

ACLF at enrollment or during follow-up?
YES
NO
EXCLUDED
65 patients without complete 4 week follow-up data
EXCLUDED
636 patients without scheduled visits
ACLF (N=407)
AD (N=235)
Bacterial infection at ACLF diagnosis?
Bacterial infection at AD diagnosis?
YES N=152(37.3%)
NO N=255
YES N=59(25.1%)
NO N=176
New bacterial infection?
Bacterial infection during follow-up?
New bacterial infection?
Bacterial infection during follow-up?
YES N=24 (16%)
NO N=128
YES N=117 (46%)
NO N=138
YES N=7 (12%)
NO N=52
YES N=32 (18%)
NO N=144
New bacterial infection?
YES N=22 (18%)
NO N=95

FIGURE 2A

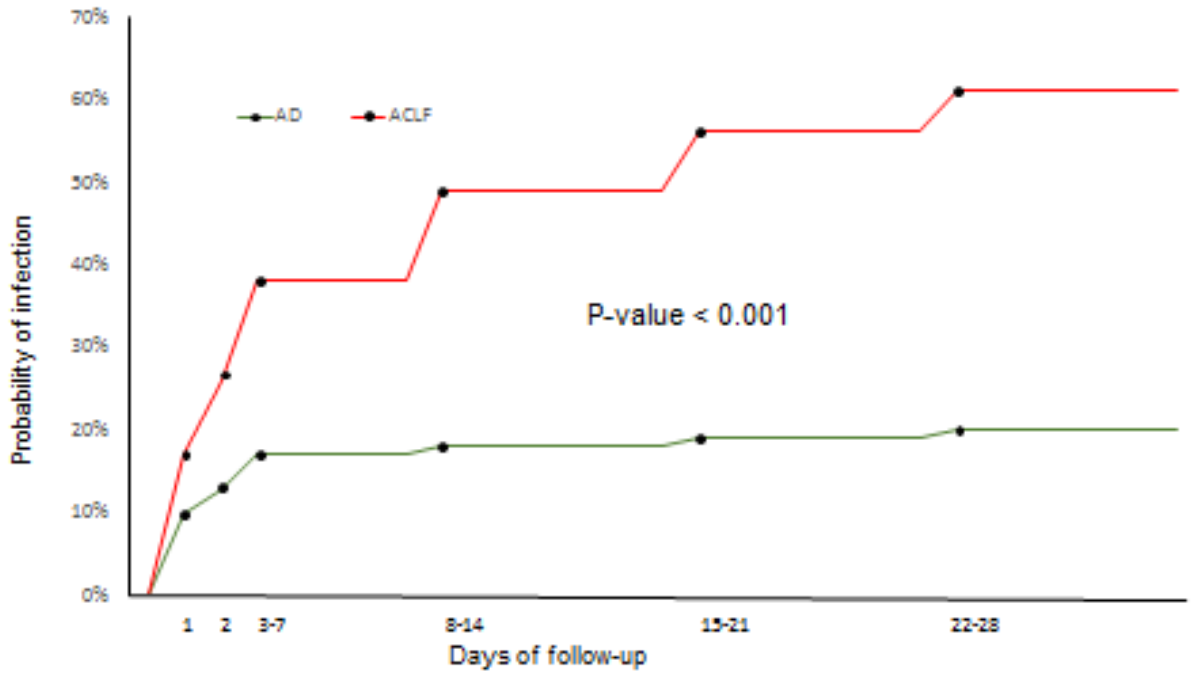

70%
60%
50%
40%
30%
20%
10%
0%
AD
ACLF
P-value < 0.001
Probability of infection
1
2
3-7
8-14
15-21
22-28
Days of follow-up

FIGURE 2B

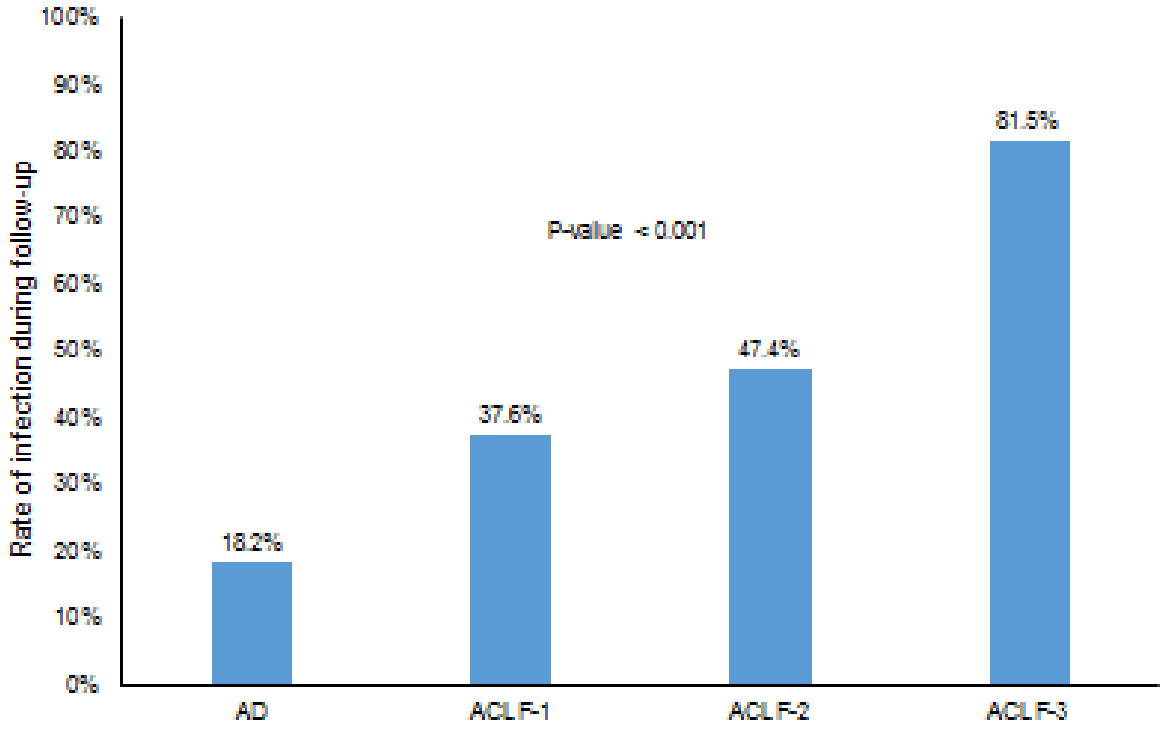

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Rate of infection during follow-up
P-value < 0.001
18.2%
37.6%
47.4%
81.5%
AD
ACLF-1
ACLF-2
ACLF-3

FIGURE 3A

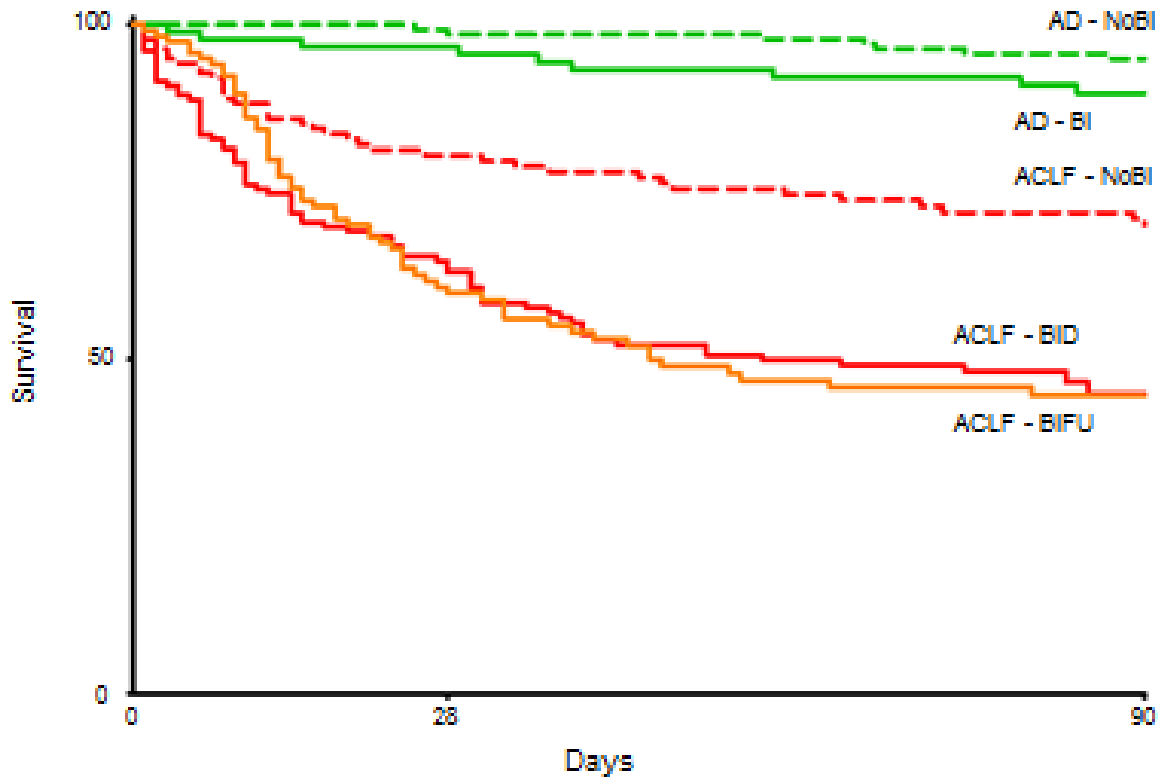

100
50
0
Survival
0
28
90
Days
AD - NoBI
AD - BI
ACLF - NoBI
ACLF - BID
ACLF - BIFU

FIGURE 38

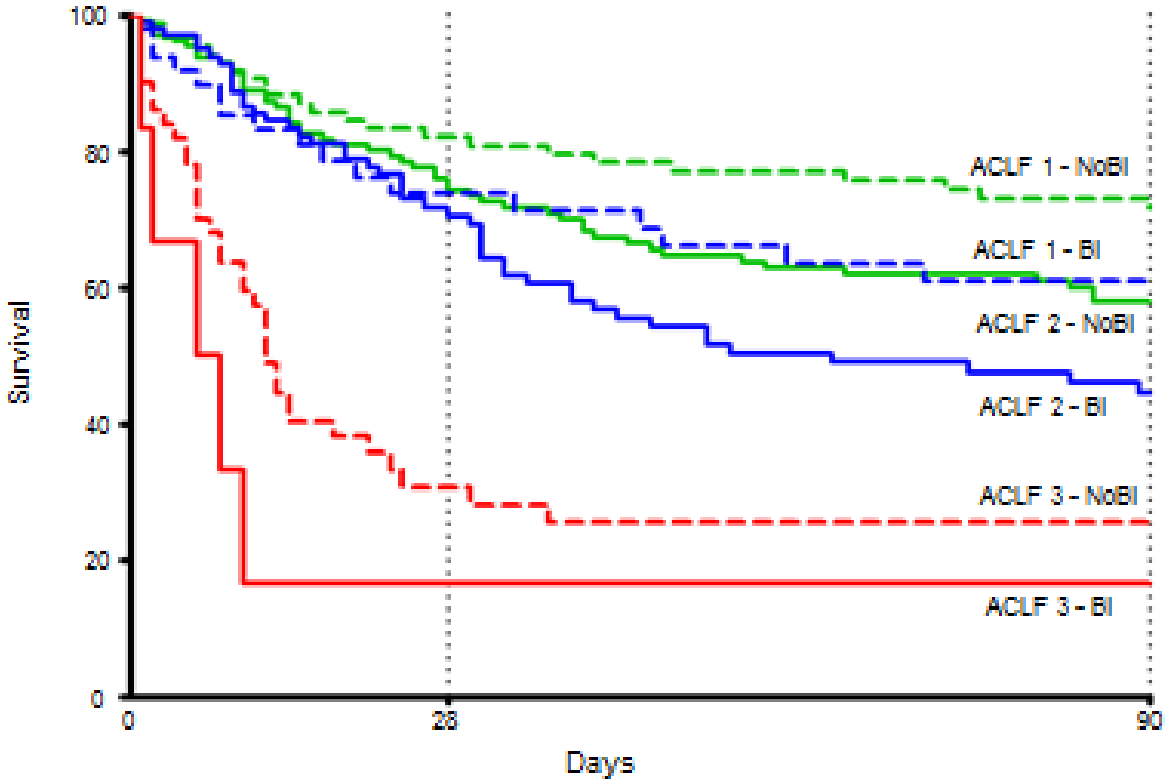
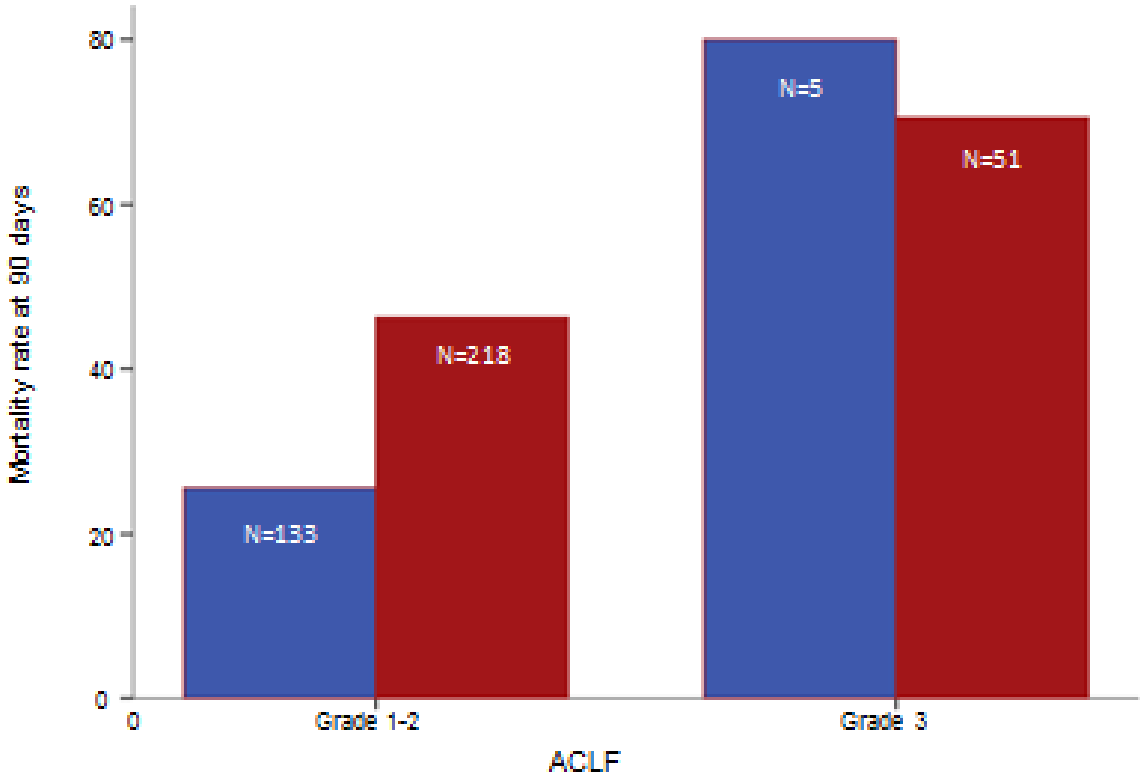

100
80
60
40
20
0
Survival
0
28
90
Days
ACLF 1 - NoBI
ACLF 1 - BI
ACLF 2 - NoBI
ACLF 2 - BI
ACLF 3 - NoBI
ACLF 3 - BI

FIGURE 3C


80
60
40
20
0
Mortality rate at 90 days
N=133
N=218
N=5
N=51
0
Grade 1-2
Grade 3
ACLF