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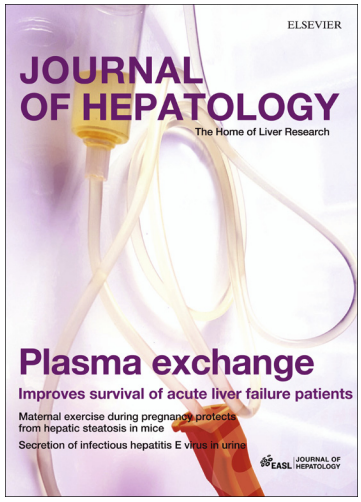

Neutrophil gelatinase-associated lipocalin is a biomarker of acute-on-chronic liver failure and prognosis in cirrhosis

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NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN IS A BIOMARKER OF ACUTE-ON-CHRONIC LIVER FAILURE AND PROGNOSIS IN CIRRHOSIS

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Keywords: Lipocalin-2; NGAL; Acute-on-Chronic liver failure; biomarker.

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LAY SUMMARY:

URINE NGAL IS A BIOMARKER OF ACUTE-ON-CHRONIC LIVER FAILURE (ACLF). NGAL is a protein that may be expressed in several tissues in response to injury. The protein is filtered by the kidneys due to its small size and can be measured in the urine. Ariza and Graupera and colleagues found in a series of 716 patients with cirrhosis that urine NGAL was markedly increased in patients with ACLF and correlated with prognosis. Moreover, NGAL gene was markedly overexpressed in the liver tissue in ACLF.

ABSTRACT

Background: Acute-on-Chronic Liver Failure (ACLF) is a syndrome that occurs in cirrhosis characterized by organ failure(s) and high mortality rate. There are no biomarkers of ACLF. *LCN2* gene and its product, neutrophil gelatinase-associated lipocalin(NGAL), are upregulated in experimental models of liver injury and cultured hepatocytes as a result of injury by toxins or proinflammatory cytokines, particularly Interleukin-6. **Aim**: To investigate whether NGAL could be a biomarker of ACLF and whether *LCN2* gene may be upregulated in the liver in ACLF. **Methods**: We analyzed urine and plasma NGAL levels in 716 patients hospitalized for complications of cirrhosis, 148 with ACLF. *LCN2* expression was assessed in liver biopsies from 29 additional patients with decompensated cirrhosis with and without ACLF. **Results**: Urine NGAL was markedly increased in ACLF vs no ACLF patients (108(35-400) vs 29(12-73) µg/g creatinine; $p<0.001$) and was an independent predictive factor of ACLF; the independent association persisted after adjustment for kidney function or exclusion of variables present in ACLF definition. Urine NGAL was also an independent predictive factor of 28-day transplant-free mortality together with MELD score and leukocyte count (AUCROC 0.88(0.83-0.92)). Urine NGAL improved significantly the accuracy of MELD in predicting prognosis. *LCN2* gene was markedly up-regulated in the liver of patients with ACLF. Gene expression correlated directly with serum bilirubin and INR ($r=0.79;p<0.001$ and $r=0.67;p<0.001$), MELD ($r=0.68;p<0.001$) and Interleukin-6 ($r=0.65;p<0.001$). **Conclusions**: NGAL is a biomarker of ACLF and prognosis and correlates with liver failure and systemic inflammation. There is remarkable overexpression of *LCN2* gene in the liver in ACLF syndrome.

INTRODUCTION

Neutrophil gelatinase-associated lipocalin (NGAL) is a 25kD protein, the product of the lipocalin-2 gene (*LCN2*) that is expressed in a number of tissues and cell types, particularly neutrophils [1]. In physiological conditions, NGAL is synthetized during the maturation process of neutrophils and then stored in cytosolic granules of adult cells. Several lines of evidence indicate that NGAL present in neutrophils plays a protective role against bacterial infections by binding to the siderophores of bacteria [2-5]. *LCN2* expression has been found at low levels in some human tissues, such as lungs, trachea, breast, colon, skin, liver, kidneys, and small intestine under physiological circumstances [1]. The physiological effects of NGAL in these tissues are incompletely understood, but it has been suggested that NGAL probably plays a role in protecting cells against different types of injury by modulating oxidative stress or through other not yet well defined mechanisms [5].

Most of the information related to the potential clinical usefulness of NGAL derives from studies investigating its possible role as biomarker of kidney injury. NGAL expression is markedly upregulated in the kidneys after injury, particularly ischemia-reperfusion or administration of contrast agents or nephrotoxic drugs [6-8]. In these conditions, NGAL is almost exclusively produced in tubular cells of the thick ascending limb or collecting duct. NGAL is found in the urine after few hours of cell injury and its concentration peaks at 6 hours and is then maintained in the urine during an extensive period of time. In this context, NGAL seems to have beneficial effects on kidney cells by reducing apoptosis and increasing proliferation of renal epithelial cells [9]. A large number of studies have shown that urinary levels of NGAL are markedly

increased in early phases of acute kidney injury (AKI) due to ischemic or nephrotoxic origin and are maintained for several days. By contrast, NGAL levels are not increased in prerenal AKI in the absence of injury of tubular cells [10]. Moreover, urine NGAL (uNGAL) levels have been shown to predict hard clinical endpoints in patients with AKI, including progression of kidney failure, need for renal replacement therapy, and mortality [10-12].

Similarly to the kidney, *Lcn2* gene is also upregulated in the liver as a consequence of liver injury, yet the available information is limited. Recent experimental studies have shown that *Lcn2* gene is upregulated in hepatocytes in conditions of liver injury, either produced by toxins, proinflammatory cytokines, and bacterial infections or after partial hepatectomy [13-16]. Hepatocytes appear to be the main cell type responsible for increased *Lcn2* production after experimental liver injury [16]. Little information exists on *LCN2* gene expression in the liver in human diseases [17] and to our knowledge there are no studies specifically assessing the potential role of NGAL as biomarker of liver diseases.

On this background, the current study assessed NGAL levels in urine and serum in human chronic liver diseases, with special emphasis on Acute-on-Chronic Liver Failure (ACLF). ACLF is a clinical syndrome recently described that occurs during the course of cirrhosis and is characterized by an acute decompensation of the disease associated with development of failure in different organs/systems and high short-term mortality [18-19]. The pathogenesis of ACLF is not yet known but several lines of evidence point towards the existence of either direct injury to liver cells and/or indirectly through a systemic inflammatory reaction [18-19]. Therefore, the working

hypothesis of the current study was that NGAL levels, either in urine or plasma, are increased in ACLF and NGAL could be a biomarker of ACLF syndrome. We also hypothesized that the liver could be a source of NGAL as a consequence of liver injury and systemic inflammation associated with ACLF.

In the current study, we first analyzed the potential usefulness of urine and plasma NGAL as biomarker of ACLF and prognosis in a series of 716 patients with and without ACLF hospitalized for an acute decompensation of cirrhosis. Subsequently, we assessed the expression of the *LCN2* gene in liver biopsies from a second group of patients encompassing the whole spectrum of chronic liver diseases, from precirrhotic conditions to ACLF. Our results show that NGAL is a biomarker of ACLF and prognosis and that there is remarkable upregulation of *LCN2* gene in the liver in ACLF.

METHODS

Study populations

The current study has two different parts:

In the first part we analyzed urine and plasma NGAL levels and its relationship with ACLF and prognosis. In the second part we assessed *LCN2* gene expression in liver biopsies of patients with different chronic liver diseases, with and without ACLF.

The first part of the study was performed in a population of patients included in the CANONIC study, as reported elsewhere [18]. Briefly, the CANONIC study was a multicenter investigation aimed at evaluating the frequency, characteristics, and outcome of ACLF in patients admitted to hospital for an acute decompensation of cirrhosis in 29 liver units from 8 European countries.

ACLF was defined according to the criteria of the CANONIC study [18], which are based on presence of organ failure(s) as defined according to CLIF-SOFA score (see Supplementary Table 1). Briefly, patients with ACLF were those with either: 1) single kidney failure; 2) single liver, coagulation, circulatory or respiratory failure associated with 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 serum creatinine ranging from ≥ 1.5 and < 2 mg/dL; or 4) two or more organ failures. Out of the 1343 patients enrolled in the CANONIC study, 716 (53.3%) had urine samples available at the time of inclusion and constitute the population of this part of the study. In the remaining patients, samples could not be collected because of logistical reasons. Patients with urinary tract infection at the time of urine collection were excluded because the urine levels of NGAL may be increased due to high leukocyte concentration in urine [20]. Out of the 716 patients, 684 (95.5%) had also a plasma sample collected at the time of inclusion.

The second part of the study was performed in an additional group of 46 patients with chronic liver diseases of different etiologies that underwent a diagnostic liver biopsy at the Liver Unit of the Hospital Clínic of Barcelona: 11 patients with chronic liver diseases without cirrhosis, 6 patients with compensated cirrhosis, 19 patients with acute decompensation of cirrhosis, and 10 patients with ACLF. Liver biopsies were performed using a percutaneous approach in patients without cirrhosis. In patients with cirrhosis a transvenous approach was used, as described elsewhere [21]. Before liver biopsy, urine and plasma samples were taken for measurement of urine and plasma NGAL and Interleukin 6 (IL-6) levels. In addition, we analyzed liver biopsies from 6 healthy

donors at the time of living donor obtained liver transplantation.

Both parts of the study were approved by the respective Institutional Review Boards of the different participating centers and patients gave written informed consent to participate.

Measurement of NGAL, KIM-1 and IL-6

Urine and blood samples were centrifuged at 1000 rpm and 2000 rpm, respectively, for 10 min and the supernatant stored at -80 ºC until analysis. All samples were processed and frozen within a maximum of 4 hours after collection. NGAL, Kidney injury molecule-1 (KIM-1) and IL-6 were measured using respective ELISAs kits. See Supplemental Material for detailed description.

RNA extraction and gene expression analysis

Total RNA was extracted from total liver tissue using trizol following manufacturer's instructions (Life Technologies, Carlsbad, CA). *LCN2* expression was evaluated by quantitative real-time PCR (qPCR) as described in the supplemental material.

Statistical analysis

Data for NGAL, KIM-1 and IL-6 were directly transcribed to a data sheet. The rest of the data were collected using an electronic case report form. Both parts were merged using the statistical analysis software. Quantitative variables are reported as mean and SD or median and interquartile range, according to their

nature. Categorical variables are reported as count and percentage in each category and total. Correlations of the non-parametric parameters with other variables are evaluated by means of the Spearman coefficient. Comparisons among groups of the non-parametric parameters are assessed with the Mann-Whitney *U* or Kruskal-Wallis tests depending on the number of strata. In post-hoc analyses, Dunn's multiple comparison test was applied in order to keep the familywise alpha value.

Outcomes considered were the presence, the development of ACLF, and 28-day transplant-free mortality. Factors associated with these outcomes were identified in a bivariate analysis. The association of quantitative variables was tested with Student t test, or Mann-Whitney *U* test, if non-parametric. To test the association of the categorical variables, the Chi-Square test was performed after checking the underlying assumptions. Those factors showing a clinically and statistically significant association in bivariate analysis were selected for the initial multivariate analysis. A logistic regression model was fitted to select the best subset of predictors of each outcome having assessed fitting characteristics. The final models were fitted using a stepwise forward method based on the improvement in model likelihood ratios. Significance levels to enter and drop model variables were adopted as 5% and 10% respectively. Non-normal distributed variables were transformed transformed using the natural logarithm. The accuracy gain in predicting 28-day transplant-free mortality of the NGAL on top of Model of end-stage liver disease (MELD) score was assessed estimating and comparing the AUCROC (95% CI) for the model with only MELD score and for the model with both variables. The significance level for all tests was set at 5%.

RESULTS

Neutrophil gelatinase-associated lipocalin as biomarker of Acute-on-Chronic Liver Failure and prognosis.

Out of the 716 patients with cirrhosis included, 148 (20.7%) had ACLF and 568 (79.3%) had acute decompensation without ACLF. The demographic, clinical, and laboratory characteristics of patients included are shown in Supplementary Table 2. The outcome of the 716 patients after 28 days was as follows: 57 patients had died, 26 had been transplanted, and 633 were alive.

NGAL levels and their relationship with complications of cirrhosis and ACLF

First, we assessed urine and plasma NGAL levels in patients with cirrhosis categorized according to presence of decompensated cirrhosis or ACLF. Patients with ACLF had markedly higher levels of both uNGAL and plasma NGAL (pNGAL) compared to those of patients with acute decompensation of cirrhosis without ACLF (uNGAL: 108 [35-400] vs 29 [12-73] µg/g of creatinine; pNGAL: 232 [147-422] vs 131 [99-187] ng/mL, respectively; $p<0.001$ for both). Interestingly, patients in whom ACLF improved during hospitalization had lower baseline uNGAL and pNGAL levels compared to those of patients with steady or worsening ACLF (uNGAL: 66 [30-143] vs 204 [57-881] µg/g of creatinine; pNGAL: 207 [139-305] vs 310 [155-557] ng/mL; $p=0.0007$ and 0.019, respectively). In the whole series of patients there was a moderately significant direct correlation between uNGAL and pNGAL levels ($r=0.42$; $p<0.001$). Overall, uNGAL and pNGAL had a moderately significant direct correlation with liver function tests, MELD score, and markers of systemic inflammation, including blood leukocytes and C-reactive protein (data not shown). There was a slight

but significant correlation between uNGAL and pNGAL and age, so that levels were higher in older patients. Moreover, uNGAL but not pNGAL levels were significantly higher in females than in males. Finally, there was no relationship between NGAL levels and etiology of cirrhosis.

NGAL levels were related with the type of decompensation of cirrhosis. Patients with hepatic encephalopathy had higher levels compared to those of patients without hepatic encephalopathy (uNGAL: 53 [21-170] vs 32 [13-87] µg/g of creatinine; pNGAL: 157 [114-235] vs 134 [99-201] ng/mL; $p<0.001$ for both). Likewise, patients with bacterial infections had higher levels than those without bacterial infections (uNGAL: 44 [20-157] vs 34 [13-96] µg/g of creatinine; pNGAL: 160 [117-305] vs 136 [100-204] ng/mL; $p=0.008$ and $p<0.001$, respectively). However, a detailed analysis indicated that increased levels of NGAL occurred in patients with bacterial infections associated with ACLF. In fact, patients with ACLF had increased uNGAL and pNGAL levels regardless of the presence or absence of bacterial infections. Moreover, patients with ACLF without bacterial infections had significantly higher uNGAL and pNGAL levels than those of patients with bacterial infections but without ACLF (Table 1). Similar findings were observed with respect to uNGAL and pNGAL levels and hepatic encephalopathy (Table 1). In sharp contrast with hepatic encephalopathy and bacterial infections, patients with gastrointestinal bleeding had lower uNGAL and pNGAL levels compared to those of patients without gastrointestinal bleeding (data not shown).

To further investigate the relationship between NGAL and ACLF we performed univariate and multivariate analysis of variables associated with the presence of ACLF at inclusion in the study. In addition to uNGAL and pNGAL, a number of

variables from clinical findings, liver and kidney function tests, systemic inflammatory parameters, and severity scores were associated with the presence of ACLF at inclusion (Table 2). In multivariate analysis, only uNGAL, pNGAL, MELD score, and hepatic encephalopathy had independent predictive value of presence of ACLF (Table 3, Model 1a).

Since kidney failure is a component of the ACLF syndrome and NGAL may be upregulated in the kidney in certain types of AKI, particularly acute tubular necrosis [9,12,19], we analyzed the relationship between NGAL and kidney function. There was a moderate statistically significant direct correlation between pNGAL and serum creatinine ($r=0.58$; $p<0.001$). By contrast, the correlation between uNGAL and serum creatinine was much weaker ($r=0.26$; $p<0.001$) (Supplementary Fig. 1). Moreover, uNGAL but not pNGAL was independently associated with ACLF even after adjusting for serum creatinine levels (Table 3, Model 1b), which suggests that the association between uNGAL and ALCF was not exclusively related to kidney function. Because some of the variables independently associated with ACLF were also included in the definition of the ACLF syndrome (bilirubin, INR, hepatic encephalopathy) we also performed a multivariate model excluding all individual variables related to ACLF syndrome as well as those related to MELD score. Again, uNGAL was independently associated with the presence of ACLF syndrome (Table 3, Model 1c).

We then analyzed whether NGAL levels at inclusion in the study in patients with acute decompensation of cirrhosis but without ACLF correlated with the development of ACLF during hospitalization. A number of baseline variables were significantly related to development of ACLF (Supplementary Table 3).

Interestingly, patients with acute decompensation of cirrhosis without ACLF at inclusion who developed ACLF during hospitalization (n=60) had significantly higher levels of uNGAL and pNGAL at inclusion compared to those of patients with acute decompensation who did not develop ACLF. In multivariate analysis, uNGAL was an independent predictive factor of development of ACLF together with presence of ascites and MELD score (Table 3, Model 2a). uNGAL was an independent predictive factor of ACLF development even after adjustment for kidney function (Table 3, Model 2b).

Relationship between NGAL and prognosis

We then sought to determine the relationship between uNGAL and pNGAL and mortality. In univariate analysis, 28-day transplant-free mortality was associated with a number of clinical variables, liver and kidney function tests, inflammatory parameters, and severity scores (Supplementary Table 4). Interestingly, both uNGAL and pNGAL levels showed a strong association with prognosis. In multivariate analysis, independent predictive factors of 28-day transplant-free mortality were MELD score, uNGAL, and leukocyte count (Table 4, Model 1a). uNGAL was still an independent prognostic factor after adjustment for variables that could influence NGAL levels, including kidney function, leukocyte count, and presence of infection (Table 4, Model 1b). The 28-day transplant-free mortality rates according to uNGAL levels in all patients as well as those with and without ACLF are shown in Supplementary Table 5. Interestingly, uNGAL improved significantly the predictive accuracy of MELD score in the assessment of 28-day transplant-free mortality (Figure 1). To further explore the effects of uNGAL on mortality, we assessed the relationship between the probability of

death and uNGAL according to a wide range of MELD score values. Figure 2 shows a plot of the relationship between 28-day transplant-free mortality and MELD score in patients categorized in 4 quartiles of uNGAL levels. For a given value of MELD score, the probability of death increased remarkably according to uNGAL levels, particularly for MELD score values above 20. For example, for a MELD score of 30, the probability of death at 28 days was only 5% in patients with uNGAL below 15 µg/g of creatinine compared to 41% in those with uNGAL above 105 µg/g of creatinine. Therefore, uNGAL improves the ability of MELD score in predicting prognosis, particularly at high MELD score values.

Considering the strong association between NGAL and ACLF, we then assessed predictive factors of mortality only in the population of patients with ACLF at inclusion. In univariate analysis, a number of clinical and laboratory variables as well as uNGAL and pNGAL levels were associated with 28-day transplant-free mortality (data not shown). The multivariate model of 28-day transplant-free mortality in patients with ACLF at inclusion included only uNGAL and MELD score as independent predictive variables (Table 4, Model 2a). uNGAL was independently associated with mortality in patients with ACLF after adjustment for variables that could influence NGAL levels, such as kidney function and leukocyte count (Table 4, Model 2b). Finally, we performed another multivariate analysis in which instead of MELD score we included the CLIF-C ACLF score, a recently developed score for assessing prognosis in patients with ACLF [22]. This new multivariate model included only uNGAL and CLIF-C ACLF score as independent predictive factors of mortality (Table 4, Model 2c).

Lipocalin-2 gene expression in liver tissue in patients with and without ACLF.

Considering the increased urine and plasma NGAL levels in the setting of ACLF, we next sought to determine whether *LCN2* gene was upregulated in the liver in ACLF syndrome in an independent group of patients (see methods). The demographic, clinical, and laboratory characteristics of patients are shown in Supplementary Table 6. Figure 3 shows hepatic *LCN2* gene expression in the 5 groups of subjects studied. Remarkably, patients with ACLF had a striking increase in hepatic *LCN2* gene expression compared to all other groups. By contrast, patients with acute decompensation of cirrhosis without ACLF showed slightly increased, yet statistically significant, hepatic *LCN2* gene expression compared to that of healthy subjects and patients with chronic liver diseases without cirrhosis. However, the difference between *LCN2* gene expression in patients with acute decompensation without ACLF and patients with compensated cirrhosis did not reach statistical significance.

Considering all patients with cirrhosis, hepatic *LCN2* gene expression correlated directly with serum bilirubin levels, INR and MELD score, and indirectly with serum albumin concentration (Figure 4). Patients with bacterial infections had higher hepatic *LCN2* gene expression than patients without bacterial infections (Figure 4). However, this difference was due to the associated presence of ACLF. In fact, among infected patients the highest levels of hepatic *LCN2* gene expression occurred in patients with ACLF, while patients with infection without ACLF had gene expression values similar to those of patients without infection. The severity of bacterial infections, as assessed by clinical and biochemical parameters, was similar in patients with and without ACLF. The plasma and

urine levels of NGAL were significantly increased in patients with ACLF compared to the other groups and correlated directly with hepatic *LCN2* gene expression (data not shown). Finally, because IL-6 has been described as a major inducer of the *LCN2* gene in the liver [16], we evaluated IL-6 plasma levels and its relationship with *LCN2* gene expression. Patients with decompensated cirrhosis with and without ACLF had increased IL-6 levels that correlated directly with hepatic *LCN2* gene expression (Supplementary Fig.2).

DISCUSSION

The main findings of the current study are that NGAL, the product of the *LCN2* gene, either in the urine or plasma, is a biomarker of ACLF and prognosis in patients with acute decompensation of cirrhosis. Furthermore our study shows that ACLF is characterized by a remarkable upregulation of the *LCN2* gene in the liver, a feature not present in patients with acute decompensation of cirrhosis without ACLF. Therefore, NGAL should be considered the first biomarker of the ACLF syndrome [18].

Experimental studies have demonstrated that *Lcn2* gene is overexpressed in the liver tissue of animals with experimental models of liver injury and in cultured hepatocytes as a consequence of damage induced by toxins or proinflammatory cytokines in a manner similar to what occurs in tubular cells of the kidneys [6-8, 13-16]. Our study extends these observations to human liver diseases by assessing *LCN2* expression in the whole spectrum of liver diseases, from precirrhotic conditions to ACLF. Our results show that hepatic *LCN2* gene expression was markedly up-regulated in the liver of patients with ACLF compared to that of patients without ACLF and that gene expression

correlated with liver function parameters, including serum bilirubin, albumin levels, INR, and MELD score, the most commonly used severity index of cirrhosis in clinical practice [23]. Moreover, the findings of the current study clearly show that uNGAL levels are independently associated with the presence of ACLF. Furthermore, in those patients without ACLF at admission, uNGAL was an independent predictive factor of development of ACLF during hospitalization. Finally, uNGAL was also an independent prognostic factor of 28-day transplant-free mortality.

A number of investigations have shown that NGAL expression is up-regulated in inflammatory conditions [1]. In fact, NGAL expression can be induced by several cytokines and growth factors [1]. In hepatocytes, several cytokines, particularly IL-6, have been shown to up-regulate *Lcn-2* gene expression [13,14,16]. Moreover, toll-like receptors (TLRs) also play a role in the regulation of *Lcn2* expression [4,24]. In this regard, decompensated cirrhosis is characterized by an inflammatory state, with frequently increased levels of proinflammatory cytokines and circulating bacterial DNA, probably related to bacterial translocation from the intestinal lumen to mesenteric lymph nodes [25-29]. This inflammatory state is likely further increased in patients with ACLF [18,19] [Ginès P, unpublished observations]. Therefore, it could be speculated that up-regulation of *LCN2* gene expression found in patients with ACLF is related, at least in part, to the proinflammatory state characteristic of ACLF. The direct relationship between IL-6 plasma levels and hepatic *LCN2* gene expression found in the current study supports this hypothesis.

The results of the current study also confirm the hypothesis that *LCN2* gene expression is up-regulated in the liver of patients with ACLF, raising the possibility that an hepatic production of NGAL could contribute to the increased pNGAL and uNGAL levels found in ACLF syndrome. Considering that ACLF is characterized by failure of different organs/systems, the increased NGAL levels could theoretically derive from the kidney, leukocytes, liver, and/or other organs involved in ACLF [1]. The results of the current investigation do not allow answering the question as to what extent the liver contributes to the increased NGAL levels. However, the findings that uNGAL was independently associated with ACLF as well as prognosis in multivariate analysis after adjustment for potential confounders, suggest that the liver likely plays a role in the increased NGAL levels. The definitive answer to the question of the possible contribution of different organs to increased NGAL levels in ACLF would require assessment of gene expression in different organs, which is not feasible in human disease. In any case, regardless of the potential source(s), the results of the current study indicate that NGAL is a good biomarker of ACLF and prognosis in human cirrhosis.

The potential relationship between bacterial infections and increased *LCN2* gene expression in ACLF should be discussed, because ACLF is frequently associated with bacterial infections. In animal models of sepsis without liver disease there is marked up-regulation of the *Lcn2* gene and patients with bacterial infections without liver diseases have increased urine and plasma NGAL levels that correlate with disease severity and mortality [4,30,31]. Moreover, *Lcn2* knockout mice have increased susceptibility to bacterial

infections and an enhanced bacterial translocation from the intestinal lumen to mesenteric lymph nodes [16,32,33]. In this regard, the possible relationship between bacterial infections and increased *LCN2* gene expression and NGAL levels in cirrhosis deserves discussion, particularly because of the increased susceptibility to infections characteristic of cirrhosis [34,35]. In the current study, urine and plasma NGAL levels correlated with the presence of bacterial infections. However, a closer look at this relationship showed that patients with bacterial infections associated with ACLF, but not those without ACLF, had increased levels of NGAL. Moreover, patients with ACLF without bacterial infections had NGAL levels greater than those of patients with bacterial infections but without ACLF. Therefore, increased NGAL levels appear to be more closely related to ACLF than to bacterial infections. Importantly, in multivariate analysis, uNGAL was independently associated with presence of ACLF after adjustment for bacterial infections. Moreover, data in patients in whom *LCN2* gene expression was assessed in liver tissue, showed that ACLF and not bacterial infections was the main determinant of increased gene expression.

We believe that the findings of our study are of interest from a clinical perspective. NGAL levels could be useful to monitor the risk and progression of ACLF in clinical practice. Moreover, they could also be useful for risk stratification of patients with decompensated cirrhosis admitted to hospital. Finally, NGAL levels may add information to the prognostic value of the commonly used scores to assess prognosis in patients with cirrhosis: MELD and CLIF-C ACLF scores. In this regard, it is important to point out that uNGAL

improved the accuracy of MELD score in predicting 28-day mortality. Because organ allocation for liver transplantation in many countries is performed according to MELD score [23], the use of uNGAL together with MELD score may help improve clinical decisions about organ allocation and transplantation.

The current study has some limitations that should be acknowledged. First, uNGAL levels cannot be used to assess prognosis in patients with urinary tract infections because they are associated with increased urinary concentration of NGAL [20]. Second, although the predictive value of uNGAL was adjusted in multivariate analyses for kidney function, it is important to emphasize that serum creatinine is not the gold standard for glomerular filtration rate (GFR) assessment in cirrhosis. Nonetheless, it is the most useful and frequent way of assessing GFR in clinical practice. Finally, the findings of the current investigation were derived from patients recruited in liver units from large university hospitals with expertise in the management of complications of cirrhosis that were part of the EASL-CLIF Consortium [18]. Therefore, we do not know whether findings could be extrapolated completely to other centers with less expertise in the management of patients with advanced liver diseases.

In conclusion, the results of the current study demonstrate a remarkable overexpression of the *LCN2* gene in the liver of patients with ACLF compared to patients with compensated cirrhosis and those with acute decompensation of cirrhosis without ACLF. The expression of the *LCN2* gene in the liver correlated directly with liver function tests and MELD score. Moreover, our study also indicates that uNGAL is an excellent biomarker of prognosis in patients

hospitalized for acute decompensation of cirrhosis and improves the prognostic accuracy of existing prognostic markers, especially MELD score. Measurement of uNGAL may be helpful in clinical practice for decision-making in patients with cirrhosis.

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Author names in bold designate shared co-first authorship.

FIGURE LEGENDS

Figure 1. Area under the receiver operating curve of 28-day transplant-free mortality of patients included in the study categorized according to MELD score (0.81 [0.74-0.87]) and MELD plus urine NGAL [log-transformed] (0.86 [0.82-0.91]); p=0.017 for comparison between AUCROC curves.

Figure 2. Plots of relationship of MELD score and 28-day transplant-free mortality in patients categorized according to quartiles of urine NGAL.

Figure 3. Individual values of Lipocalin-2 (*LCN2*) gene expression in human livers. Hepatic lipocalin-2 gene expression in patients with chronic liver diseases without cirrhosis (n=11), compensated cirrhosis (n=6), decompensated cirrhosis (n=19), Acute-on-Chronic Liver Failure (n=10) and healthy controls (n=6).

*p<0.001 vs all other groups; **p<0.05 vs healthy controls and patients with chronic liver diseases without cirrhosis.

Figure 4. Relationship between hepatic Lipocalin-2 (*LCN2*) gene expression and liver function tests, MELD score and bacterial infections in patients with cirrhosis. Panels **A, B, C and D** show the correlation between hepatic *LCN2* gene expression in patients with cirrhosis

(compensated, decompensated and ACLF) and serum bilirubin **(A)**, INR **(B)**, MELD score **(C)**, and serum albumin **(D)**. Panel **E** shows individual values of hepatic *LCN2* gene expression in patients categorized according to the presence or absence of bacterial infections. Open circles represent patients with ACLF while closed circles represent patients without ACLF.

TABLES

Table 1. Urine and plasma NGAL levels in patients categorized according to presence or absence of ACLF with or without bacterial infection (upper table) or hepatic encephalopathy (lower table).

	No ACLF		ACLF		
	No bacterial infection (n=471)	Bacterial infection (n=95)	No bacterial infection (n=104)	Bacterial infection (n=43)	P value
Plasma NGAL (ng/mL)	129 (97-184)	144 (107-191)	200 (132-403)[¥]	306 (204-587)	**< 0.001**
Urine NGAL (µg/g creatinine)	29 (12-74)	33 (16-62)	92 (31-280)[¥]	159 (54-470)	**< 0.001**

	No ACLF		ACLF		
	No HE (n=408)	HE (n=155)	No HE (n=77)	HE (n=70)	P value
Plasma NGAL (ng/mL)	128 (96-177)	142 (107-208)	257 (136-434)[¥]	221 (164-417)	**< 0.001**
Urine NGAL (µg/g creatinine)	27 (12-61)	39 (15-111)	116 (36-318)[¥]	105 (37-436)	**< 0.001**

NGAL, Neutrophil gelatinase-associated lipocalin; ACLF, Acute-on-Chronic Liver Failure; HE, hepatic encephalopathy.

Values are median and IQ ranges.

[¥]Post-hoc comparisons (Dunn's test) statistically significant with respect to the groups of patients without ACLF.

Table 2. Comparison of baseline characteristics of patients included categorized according to absence or presence of ACLF at inclusion.

Variable	No ACLF (n=568)	ACLF (n=148)	P value
Age (years)	58 ± 12	57 ± 11	0.430
Male sex (%)	65	66	0.776
Alcoholic cirrosis (%)	46	59	**0.007**
Previous decompensation (%)	73	83	**0.012**
Alcohol consumption within previous 3 months (%)	12	17	0.082
Ascites (%)	59	66	0.107
Hepatic encephalopathy (%)	28	48	**< 0.001**
Gastrointestinal bleeding (%)	19	12	**0.035**
Bacterial infection (%)	17	29	**0.001**
Serum bilirubin (mg/dL)	4.5 ± 5.3	10.4 ± 10.9	**< 0.001**
INR	1.5 ± 0.4	2.0 ± 0.9	**< 0.001**
Serum albumin (g/dL)	2.9 ± 0.6	2.9 ± 0.7	0.626
Serum creatinine (mg/dL)	1.0 ± 0.4	2.3 ± 1.5	**< 0.001**
Serum sodium (mmol/L)	136 ± 6	134 ± 7	**0.039**
Urine KIM-1 (µg/g creatinine)	3.9 (2.2-6.8)	5.8 (3.0-11.6)	**< 0.001**
Leukocyte count ($x10^9$/L)	6.7 ± 4.2	9.1 ± 5.6	**< 0.001**
Platelet count ($x10^9$/L)	107 ± 72	91 ± 60	**0.005**
C-reactive protein (mg/L)	27 ± 33	33 ± 30	**0.002**
MELD score	16 ± 5	27 ± 7	**< 0.001**
Child-Pugh score	9 ± 2	11 ± 2	**< 0.001**
Urine NGAL (µg/g creatinine)	29 (12-73)	108 (35-400)	**< 0.001**
Plasma NGAL (ng/mL)	131 (99-187)	232 (147-422)	**< 0.001**

ACLF, Acute-on-Chronic Liver Failure; INR, international normalized ratio; KIM-1, kidney injury molecule 1; MELD, Model for End-Stage Liver Disease; NGAL, Neutrophil gelatinase-associated lipocalin.

Values for urine KIM-1, urine NGAL and plasma NGAL are median and IQ ranges. The values of the remaining variables are mean ± SD or percentage.

Table 3. Multivariate analysis of ACLF at inclusion (models 1) and development of ACLF during hospitalization (models 2).

Patients with ACLF at inclusion in the study.

Model 1a.

Variable	Odds Ratio	95 % CI
MELD score	1.30	1.24-1.37
Plasma NGAL (ng/mL)*	2.31	1.46-3.68
Urine NGAL (µg/g creat)*	1.39	1.13-1.71
Presence of Hepatic encephalopathy	2.34	1.36-4.03

*log- transformed AUCROC (95% CI): 0.92 (0.89-0.94)

Model 1b.

Variable	Odds Ratio	95 % CI
Urine NGAL (µg/g creat)*	1.34	1.11-1.62
Presence of Hepatic encephalopathy	2.21	1.27-3.85
Serum bilirubin (mg/dL)*	1.82	1.30-2.55
Serum creatinine (mg/dL)*	39.02	18.96-80.29
INR*	17.05	5.74-50.68

*log- transformed AUCROC (95% CI): 0.92 (0.89-0.95)

Model 1c.

Variable	Odds Ratio	95 % CI
Urine NGAL (µg/g creat)*	1.78	1.54-2.06
Alcoholic cirrosis	2.37	1.51-3.73
Platelet count ($x10^9$/L)*	0.37	0.25-0.54
Leukocyte count ($x10^9$/L)*	2.33	1.57-3.45

*log- transformed AUCROC (95% CI): 0.79 (0.75-0.83)

Table 3 (cont.)

Patients with ACLF during hospitalization who did not have ACLF at inclusion.

Model 2a.

Variable	Odds Ratio	95 % CI
MELD score	1.13	1.07-1.19
Urine NGAL (µg/g creat)*	1.36	1.10-1.69
Presence of Ascites	2.13	1.10-4.15

*log- transformed AUCROC (95% CI): 0.74 (0.66-0.81)

Model 2b.

Variable	Odds Ratio	95 % CI
Urine NGAL (µg/g creat)*	1.31	1.05-1.63
Serum creatinine (mg/dL)*	2.89	1.28-6.51
INR*	15.40	4.82-49.19
Leukocyte count ($x10^9$/L)*	1.79	1.07-2.98

*log- transformed AUCROC (95% CI): 0.78 (0.72-0.84)

ACLF, Acute-on-Chronic Liver Failure; MELD, Model for End-Stage Liver Disease; NGAL, Neutrophil gelatinase-associated lipocalin; INR, international normalized ratio.

Table 4. Multivariate analysis of mortality in all patients (models 1) and in patients with ACLF at inclusion (models 2).

28-day transplant-free mortality in all patients.

Model 1a.

Variable	Odds Ratio	95 % CI
MELD score	1.12	1.07-1.17
Urine NGAL (µg/g creat)*	1.77	1.42-2.21
Leukocyte count ($x10^9$/L)*	2.16	1.26-3.72

*log- transformed AUCROC (95% CI): 0.88 (0.83-0.92)

Model 1b.

Variable	Odds Ratio	95 % CI
Urine NGAL (µg/g creat)*	2.05	1.63-2.57
Leukocyte count ($x10^9$/L)*	2.35	1.34-4.13
Serum bilirubin (mg/dL)*	1.57	1.07-2.29
INR*	7.99	2.30-27.80

*log- transformed AUCROC (95% CI): 0.88 (0.84-0.93)

Table 4 (cont.)

28-day transplant-free mortality in patients with ACLF at inclusion.

Model 2a.

Variable	Odds Ratio	95 % CI
Urine NGAL (µg/g creat)*	1.84	1.35-2.50
MELD score	1.14	1.05-1.23

*log- transformed AUCROC (95% CI): 0.83 (0.76-0.91)

Model 2b.

Variable	Odds Ratio	95 % CI
Urine NGAL (µg/g creat)*	2.31	1.63-3.27
Serum bilirubin (mg/dL)*	2.73	1.68-4.44

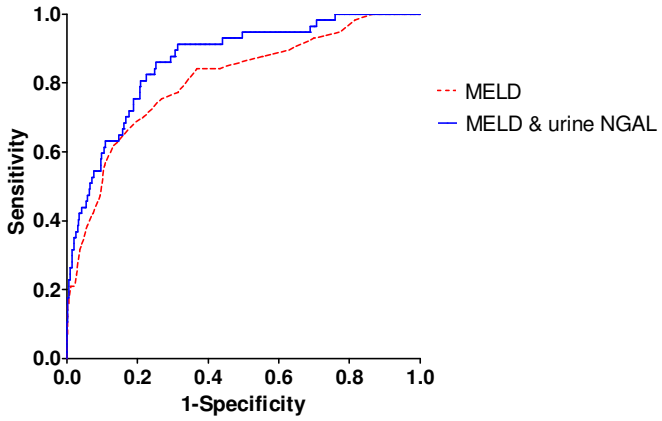
*log- transformed AUCROC (95% CI): 0.87 (0.80-0.94)

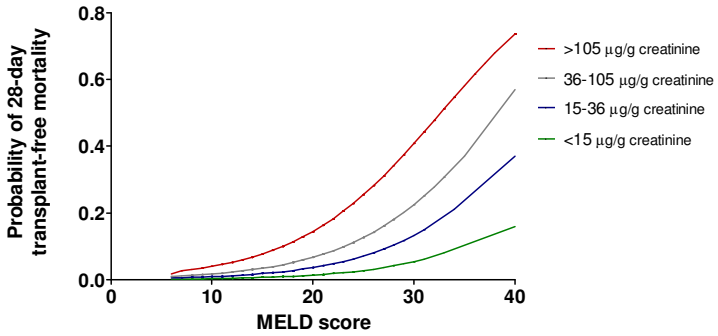
Model 2c.

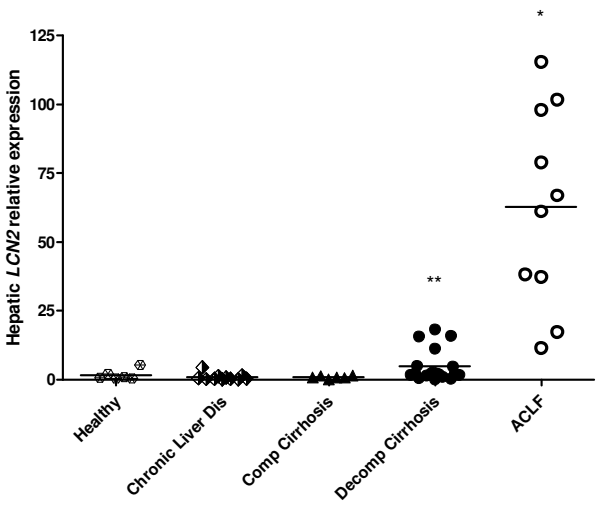
Variable	Odds Ratio	95 % CI
CLIF-C ACLF score	1.16	1.07-1.26
Urine NGAL (µg/g creat)*	1.64	1.16-2.34

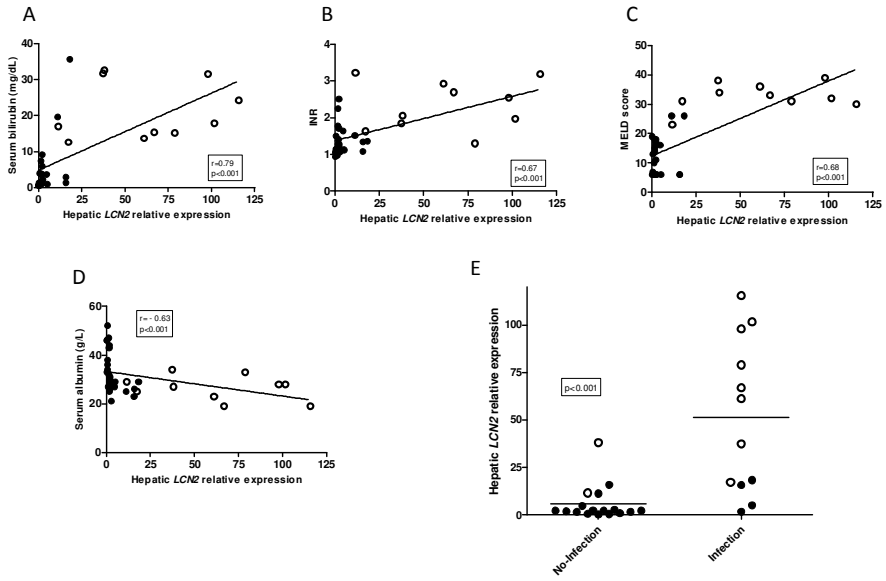
*log- transformed AUCROC (95% CI): 0.87 (0.80-0.94)

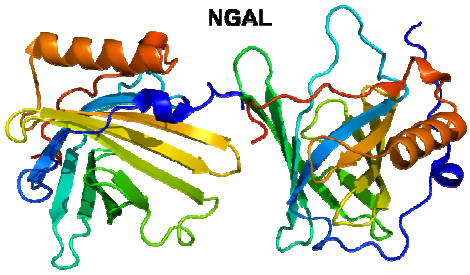
ACLF, Acute-on-Chronic Liver Failure; MELD, Model for End-Stage Liver Disease; NGAL, Neutrophil gelatinase-associated lipocalin; INR, international normalized ratio.











Relationship of MELD score and 28-day transplant-free mortality in patients categorized according to quartiles of urine NGAL.