Research Article

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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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Background & Aims: 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. The *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. The aim of this study was 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 (AUROC 0.88(0.83–0.92)). Urine NGAL improved significantly the accuracy of MELD in predicting prognosis. The *LCN2* gene was markedly upregulated 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.

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, 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, gene coding NGAL was markedly overexpressed in the liver tissue in ACLF.

Keywords: Lipocalin-2; NGAL; Acute-on-chronic liver failure; Biomarker.
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Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; *LCN2*, lipocalin-2; AKI, acute kidney injury; uNGAL, urine NGAL; ACLF, acute-on-chronic liver failure; IL-6, interleukin 6; KIM-1, kidney injury molecule-1; qPCR, quantitative real-time PCR; MELD, model of end-stage liver disease; pNGAL, plasma NGAL; TLRs, toll-like receptors; GFR, glomerular filtration rate.

Introduction

Neutrophil gelatinase-associated lipocalin (NGAL) is a 25 kD 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 not completely 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; its concentration peaks at 6 h and is then maintained in the urine for 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.

Using this background information, 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 pre-cirrhotic 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.

Materials and 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 (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 $\geqslant$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 $\geqslant$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 °C until analysis. All samples were processed and frozen within a maximum of 4 h after collection. NGAL, Kidney injury molecule-1 (KIM-1) and IL-6 were measured using respective ELISAs kits (Supplementary materials and methods).