




# Orchestration of Tryptophan-Kynurenine Pathway, Acute Decompensation, and Acute-on-Chronic Liver Failure in Cirrhosis

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Systemic inflammation (SI) is involved in the pathogenesis of acute decompensation (AD) and acute-on-chronic liver failure (ACLF) in cirrhosis. In other diseases, SI activates tryptophan (Trp) degradation through the kynurenine pathway (KP), giving rise to metabolites that contribute to multiorgan/system damage and immunosuppression. In the current study, we aimed to characterize the KP in patients with cirrhosis, in whom this pathway is poorly known. The serum levels of Trp, key KP metabolites (kynurenine and kynurenic and quinolinic acids), and cytokines (SI markers) were measured at enrollment in 40 healthy subjects, 39 patients with compensated cirrhosis, 342 with AD (no ACLF) and 180 with ACLF, and repeated in 258 patients during the 28-day follow-up. Urine KP metabolites were measured in 50 patients with ACLF. Serum KP activity was normal in compensated cirrhosis, increased in AD and further increased in ACLF, in parallel with SI; it was remarkably higher in ACLF with kidney failure than in ACLF without kidney failure in the absence of differences in urine KP activity and fractional excretion of KP metabolites. The short-term course of AD and ACLF (worsening, improvement, stable) correlated closely with follow-up changes in serum KP activity. Among patients with AD at enrollment, those with the highest baseline KP activity developed ACLF during follow-up. Among patients who had ACLF at enrollment, those with immune suppression and the highest KP activity, both at baseline, developed nosocomial infections during follow-up. Finally, higher baseline KP activity independently predicted mortality in patients with AD and ACLF. **Conclusion:** Features of KP activation appear in patients with AD, culminate in patients with ACLF, and may be involved in the pathogenesis of ACLF, clinical course, and mortality. (HEPATOLOGY 2019;69:1686-1701).

**T**he association of liver failure with extrahepatic organs/systems dysfunction is a characteristic feature of cirrhosis that affects morbidity and mortality. In fact, acute-on-chronic liver failure (ACLF), which is characterized by systemic inflammation (SI) and single or multiple ( $\geq 2$ )

*Abbreviations:* ACLF, acute-on-chronic liver failure; ACLF-KF, acute-on-chronic liver failure with kidney failure; AD, acute decompensation; a.u., arbitrary units; BBB, blood-brain barrier; BD, brain dysfunction; BF, brain failure; CI, confidence interval; HNA2, human nonmercaptalbumin 2; IDO, indoleamine 2,3-dioxygenase; IL, interleukin; IQR, interquartile range; KA, kynurenic acid; KD, kidney dysfunction; KF, kidney failure; KF-free ACLF, acute-on-chronic liver failure without kidney failure; KA, kynurenic acid; KP, kynurenine pathway; KYN, kynurenine; LC-MS, liquid chromatography-mass spectrometry; MAP, mean arterial pressure; NMDA, N-methyl-D-aspartic acid; PCC, plasma copeptin concentration; PRC, plasma renin concentration; QA, quinolinic acid; SI, systemic inflammation; sMR/CD206, soluble macrophage mannose receptor 1; TDO, Tryptophan 2,3-dioxygenase; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; Trp, tryptophan; Trp-KYN derivatives, tryptophan-kynurenine derivatives; 3-HK, 3-hydroxykynurenine.

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organ failures, is the main cause of death in cirrhosis.<sup>(1)</sup> The traditional paradigm of extrahepatic organ failure in cirrhosis relies on two principles: (1) They are functional disorders and (2) the pathogenesis is specific for each organ failure. However, this paradigm is changing and research has proven that SI and oxidative stress, which are well-known mechanisms of multiorgan failure in other clinical conditions, may be the common link between the diseased liver and extrahepatic organ failure in cirrhosis.<sup>(2)</sup> Patients with decompensated cirrhosis exhibit chronic SI, possibly in relation to sustained intestinal bacterial translocation. In contrast, ACLF develops in the setting of further increase of SI promoted by precipitating factors (e.g., bacterial infections). Moreover, significant parenchymal renal and cerebral lesions and inflammatory changes have been described in kidney biopsies, brain autopsies, and imaging studies in patients with cirrhosis and experimental animals with renal

failure or encephalopathy.<sup>(3-5)</sup> Extrahepatic organ failure(s) in cirrhosis could therefore be the consequence of tissue immunopathology.<sup>(6)</sup>

SI also affects metabolism and induces changes in a myriad of biologically active small molecules. Among them, the degradation of tryptophan (Trp) through the kynurenine pathway (KP) to Trp-kynurenine derivatives (Trp-KYN derivatives) is of special interest in cirrhosis (Fig. 1).<sup>(7-9)</sup> The KP is responsible for 95% of overall Trp degradation. The first and rate-limited step of KP is catalyzed by two enzymes: Tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO).<sup>(8,9)</sup> The expression of *TDO2* (the gene-encoding TDO) is biased in the liver. In contrast, *IDO1* and *IDO2* (the genes encoding IDO) are predominantly extrahepatic and expressed primarily in peripheral blood immune cells, dendritic cells, endothelial cells, macrophages and microglia, astrocytes, and epithelial cells (e.g., renal tubular cells).<sup>(8,9)</sup> Under

*An appendix with the alphabetical list of CANONIC Study Investigators is provided.*

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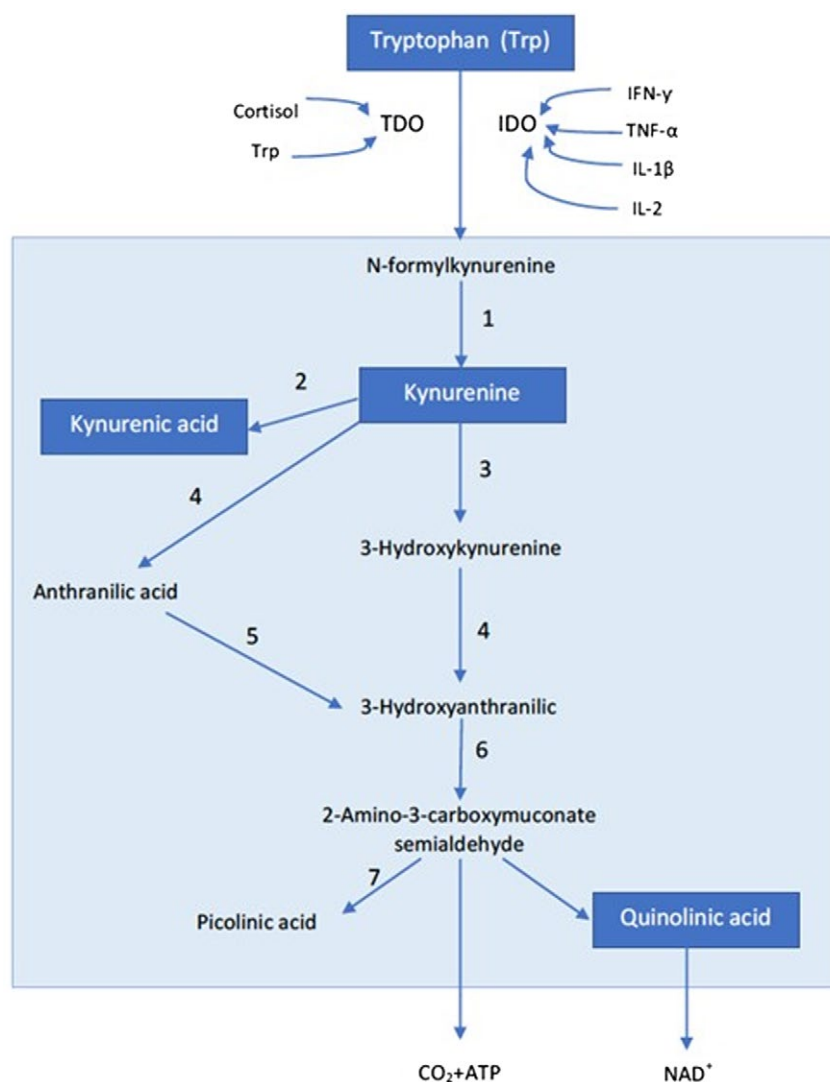
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**FIG. 1.** Trp-KP metabolic pathway including potential stimuli of TDO and IDO. Numbers indicate the enzymes participating in each biosynthetic step: 1 = formamidase; 2 = kynurenine aminotransferase; 3 = kynurenine 3-monooxygenase; 4 = kynureninase; 5 = anthranilate 3-monooxygenase; 6 = 3-hydroxyanthranilic acid dioxygenase; and 7 = 2-hydroxymuconate-6-semialdehyde dehydrogenase. The molecules measured in the study are included in the blue boxes.

physiological conditions, most KP metabolites are constitutively synthesized within the liver by TDO; only a marginal proportion is synthesized outside the liver. However, in the setting of SI there is intense overexpression of IDO and extrahepatic production of Trp-KYN derivatives including kynurenine (KYN, which is an endothelial-derived relaxing factor and neuroactive molecule), quinolinic acid (QA, which is a neuronal *N*-methyl-D-aspartic acid [NMDA] receptor neurotoxic agonist), and kynurenic acid (KA, which is a NMDA antagonist). KYN and KA exert immunomodulatory actions by binding to aryl hydrocarbon

receptor and GPR35 in immune cells.<sup>(9,10)</sup> In addition, the KP can produce picolinic acid (a second NMDA receptor antagonist), as well as the immunosuppressive metabolites 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic acid (3-HAA).<sup>(7-9)</sup> Finally, during KP activation, there is increased generation of highly reactive oxygen and nitrogen species, which contribute to immunopathology. IDO expression can be increased by interferon  $\gamma$ , tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and other cytokines.

Circulating Trp can be transported across the cell membrane and the blood-brain barrier (BBB) only in

its free form, which represents approximately 5% of the total Trp concentration in plasma.<sup>(11)</sup> The remaining molecules circulate bound to plasma albumin. In patients with decompensated cirrhosis, however, free Trp in plasma is increased due to hypoalbuminemia, structural changes in the albumin molecule, and competition with endogenous and exogenous substances for the binding to albumin Sudlow site II,<sup>(12)</sup> including kynurenines. Once within the cells, TDO and IDO interact with Trp and initiate the KP. Although the complete set of enzymes of the KP is expressed only in the liver, kynurenines can cross the cellular membrane through L-type amino acid transporter, which is also used by Trp, and act in extrahepatic cells as intermediate substrates for the synthesis of downstream metabolites.<sup>(13)</sup> Metabolites of the KP, therefore, may act as autocrine, paracrine, and even as endocrine mediators.

Kynurenines are involved in the pathogenesis of diseases associated with acute and chronic SI, including severe sepsis, acute pancreatitis, metabolic syndrome, acute confusional states, depression, and chronic neurodegenerative diseases. Because the level of KP activation is poorly known in cirrhosis, this observational, prospective study was aimed to investigate the potential role of KP activity in acute decompensation (AD) and ACLF in cirrhosis.

## Experimental Procedures

### STUDY POPULATION

The study was performed in patients included in the CANONIC study, an European multicenter, prospective, observational investigation in 1343 consecutive patients with cirrhosis hospitalized for the treatment of AD. In this study, clinical data and bio samples were obtained not only at enrollment (which generally coincided with hospital admission), but also sequentially during a follow-up period of 28 days.<sup>(1)</sup>

This study included 342 randomly selected patients with AD and 180 with ACLF at enrollment (95 ACLF-1, 65 ACLF-2, and 19 ACLF-3). In all patients ( $n = 522$ ), the serum KP (which is mentioned as KP throughout the study) was assessed at enrollment and in 258 measurements that were repeated at the last hospital visit within the 28-day follow-up period. The urine KP was assessed in 50 patients with ACLF. A detailed description of data

and sample collection policy in the CANONIC study is given in the Supporting Information. A diagram of the patients included in the current study is shown in Supporting Fig. S1. All patients included have participated in other investigations; therefore, many data have been reported previously.<sup>(14-17)</sup>

Thirty-nine additional nonhospitalized patients with compensated cirrhosis (no previous history of AD) and 40 healthy blood donors (age: 45-65 years) were studied as controls. All patients with compensated cirrhosis had clinically significant portal hypertension, as indicated by presence of esophageal varices and/or high FibroScan liver stiffness.

Details regarding the institutional research boards that approved the CANONIC study and the current study are found in the Supporting Information.

### MEASUREMENT OF Trp AND Trp-KYN DERIVATIVES BY LIQUID CHROMATOGRAPHY COUPLED WITH MASS SPECTROMETRY

Trp and Trp-KYN derivatives in serum were first assessed in the frame of an untargeted liquid chromatography–mass spectrometry (LC-MS)-based metabolomics approach. Metabolites were extracted from serum samples following methanol-assisted protein precipitation. Therefore, detected metabolites were in their free state, not bound to albumin and/or other proteins. Extracts were analyzed by LC-MS using an Ultimate 3000 chromatographic system (Thermo Fisher Scientific, Courtaboeuf, France) coupled with an Exactive mass spectrometer (Thermo Fisher Scientific) fitted with an electrospray source and operating in the positive and negative ion modes for metabolite separations on C18 and ZIC-pHILIC columns, respectively.<sup>(18)</sup> The metabolite concentrations from the different experimental batches were standardized using the LOESS algorithm.<sup>(19)</sup> Absolute targeted quantification was performed in 234 randomly selected subset of samples from the whole group (16 healthy subjects, 20 patients with compensated cirrhosis, 100 with AD, and 98 with ACLF). Serum extracts were injected into a LC-MS/MS system consisting of a Waters ACQUITY UPLC system (Milford, MA) equipped with an UPLC charged surface hybrid column and coupled with a Waters



XEVO TQ-XS mass spectrometer operating in the positive ion electrospray multiple reaction monitoring mode. Quantification was performed using standard calibration curves and internal labeled standards ( $d_5$ -L-Trp,  $^{13}C_6$ -L-KYN,  $d_5$ -KA, and  $^{13}C_3,^{15}N_1$ -QA). To explore the renal handling of Trp and KYN derivatives in patients with ACLF, absolute serum and urine concentrations were measured by LC-MS/MS in 50 randomly selected samples from patients with ACLF (25 with kidney failure and 25 without kidney failure). Plasma and urine creatinine levels were also measured by a standard method based on Jaffé's reaction, and the fractional renal excretion of each individual metabolite was calculated based on the equation ( $[\text{urinary Trp-KYN derivative concentration}/\text{serum Trp-KYN derivative concentration}]/[\text{urinary creatinine concentration}/\text{serum creatinine concentration}] \times 100$ ). More detailed information is given in the Supporting Information.

## OTHER MEASUREMENTS

### Assessment of SI and Systemic Oxidative Stress

SI was assessed by the plasma levels of 17 cytokines and the circulating markers of macrophage activation soluble CD163 (sCD163) and mannose receptor (sMR/sCD206). Oxidative stress was assessed through the analysis of the redox state of plasma albumin (human nonmercaptalbumin 2 [HNA2]). These results have been previously reported.<sup>(15,16)</sup>

### Biomarkers of Systemic Circulatory Function

Plasma copeptin concentration (PCC) and plasma renin concentration (PRC), which estimate antidiuretic hormone release and the activity of the renin-angiotensin system, respectively, were selected as biomarkers of circulatory function.

### Assessment of *IDO1* and *IDO2* Gene Expression in Peripheral Blood Cells

*IDO1* and *IDO2* messenger RNA (mRNA) expression was determined by real-time PCR in RNA from peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteers ( $n = 4$ ) and patients with AD cirrhosis ( $n = 8$ ) (see Supporting Information).

## DEFINITIONS

The following definitions concerning clinical features are detailed in the Supporting Information: compensated and decompensated cirrhosis, AD, ACLF, organ failures including liver failure, kidney failure (KF), brain failure (BF), coagulation failure, circulatory failure and respiratory failure, kidney dysfunction (KD), brain dysfunction (BD), ACLF grades, and clinical courses of AD and ACLF.

## STATISTICAL ANALYSIS

Results are presented as frequency and percentage for categorical variables, mean, and SD for normally distributed continuous variables, and median and interquartile range for not normally distributed continuous variables. More details are found in the Supporting Information.

## Results

### “BEDSIDE” DATA AND “BENCH” RESULTS INVESTIGATING SYSTEMIC CIRCULATORY FUNCTION AND SI AT ENROLLMENT

Age and gender were similar in the three groups of patients (compensated cirrhosis, AD, and ACLF) (Supporting Table S1). The prevalence of alcoholic cirrhosis increased and the prevalence of cirrhosis associated with hepatitis C virus (HCV) decreased progressively across the three groups of patients with cirrhosis. There were significant differences among patients with compensated cirrhosis, AD, and ACLF in standard liver and renal function tests, platelets, C-reactive protein, and white blood count. The most frequent complication associated with cirrhosis at enrollment in patients with AD and ACLF was ascites, followed by encephalopathy, bacterial infections, and gastrointestinal bleeding.

PCC, PRC, and mean arterial pressure (MAP) were normal in patients with compensated cirrhosis, markedly increased (PCC and PRC) or decreased (MAP) in patients with AD, and significantly more deteriorated in patients with ACLF (Supporting Table S2). PCC, which was more sensitive than PRC

in differentiating AD from ACLF, was used for association analysis. The plasma levels of cytokines were normal or only moderately increased in patients with compensated cirrhosis, markedly elevated in patients with AD, and significantly higher in patients with ACLF than in patients with AD (Supporting Table S2). Plasma sCD163 and sMR/sCD206 levels were increased in patients with AD and significantly higher in patients with ACLF than in patients with AD. Finally, plasma levels of HNA2 were increased to a similar degree in patients with compensated and decompensated cirrhosis, but significantly higher in patients with ACLF.

## KP IS ACTIVATED IN PATIENTS WITH AD AND ACLF

During the assessment of the whole serum metabolome using an untargeted and semi-quantitative HRMS-based approach, we detected four major components of the KP (Trp, KYN, KA, and QA) at sufficiently high levels to permit accurate and reliable measurements. KYN, KA, and QA are among the most relevant kynurenines and are produced at different steps of the KP (Fig. 1). Accordingly, measuring the serum levels of these metabolites provides comprehensive information on KP activity. In addition, the KYN to Trp ratio (KYN/Trp) is used widely to estimate the TDO and IDO activity.<sup>(20)</sup> Table 1A lists the serum levels of these metabolites, as measured by LC-MS and expressed in relative units corresponding to chromatographic peak areas, in healthy controls and patients with compensated cirrhosis, AD, and ACLF. In patients with compensated cirrhosis, the KP activity was not significantly different from healthy controls. In contrast, there were major changes in patients with AD and ACLF. KYN/Trp (Table 1A) and serum levels of KYN, KA, and QA (Table 1A, Fig. 2A) were higher, whereas Trp levels were lower (Table 1A, Fig. 2B), in patients with AD and ACLF relative to patients with compensated cirrhosis and healthy controls, which is consistent with increased Trp degradation and enhanced production of kynurenines due to overactivity of TDO or IDO. Indeed, mRNA expression for both *IDO1* and *IDO2* was significantly up-regulated in PBMC from patients with AD with respect to healthy donors (*IDO1*:  $6.2 \pm 2.7$  versus  $0.40 \pm 0.1$  arbitrary units [a.u.],  $P < 0.05$ ; *IDO2*:  $1.9 \pm 0.5$  versus  $0.3 \pm 0.2$  a.u.,  $P < 0.03$ ). Interestingly, patients

with ACLF had the lowest levels of Trp, and the highest KYN/Trp and serum levels of KYN, KA, and QA. KA and QA were the most sensitive markers of KP activation in our patients, and therefore were used thereafter for most association analyses.

Table 1B indicates that these differences in the serum levels of Trp and selected Trp-KYN derivatives between groups were similarly observed in the subset of 218 patients in whom absolute concentrations were measured using synthetic standards during the LC-MS analysis. All values detected in the serum were within the ng/mL to  $\mu\text{g/mL}$  range. The correlation coefficients between both types of measurements were Trp:  $r = 0.91$ ; KYN:  $r = 0.77$ ; KA:  $r = 0.87$ ; QA:  $r = 0.93$ ;  $P < 0.0001$  for all.

## KP ACTIVATION CORRELATES WITH SI INTENSITY

There were significant but weak positive correlations between inflammatory mediators and serum levels of KA and QA in the whole series of patients (Supporting Table S3), suggesting an association among SI, IDO, and KP overactivity. The strongest association was observed between QA and TNF $\alpha$  (Fig. 2C).

## KP ACTIVITY AND OVERALL SEVERITY OF CIRRHOSIS HAVE SIMILAR ORCHESTRATION

KP overactivity is associated with impairment of systemic circulatory function. Although KP activity rose progressively across patient groups of increasing severity (Table 1A), there were parallel progressive decline in MAP and increase in PCC and PRC (Supporting Table S2). Moreover, KA and QA levels had significant direct correlations with PCC ( $r = 0.39$ ;  $P < 0.001$  and  $r = 0.48$ ;  $P < 0.001$ , respectively). Together these findings show a positive correlation between the intensity of impairment in circulatory function and the KP activity.

## Hierarchization of the KP Activity According to the Existence of Kidney or Brain Failure or Dysfunction

Among patients with ACLF at enrollment, those who had kidney failure (ACLF-KF) exhibited

**TABLE 1. Metabolites of the Kynurenine Pathway at Enrollment, Across Different Groups of Individuals**

Groups of individuals	Tryptophan (Trp)	Kynurenine (KYN)	KYN/Trp ratio	Kynurenic acid	Quinolinic acid
<i>Median values for metabolites of the KYN pathway (IQR) — arbitrary units corresponding to peak area/5×10<sup>4</sup></i>					
A. All study individuals					
Healthy subjects (n = 40)	469 (390-590)	6.4 (3.6-8.0)	0.012 (0.008-0.016)	0.64 (0.50-0.89)	11.1 (7.7-28.9)
Compensated cirrhosis (n = 39)	512 (429-554)	7.7 (6.2-25.5)	0.017 (0.012-0.050)	0.82 (0.46-1.00)	11.8 (7.7-22.9)
AD (n = 337)	372 (255-527)	9.8 (5.8-21.6)	0.026 (0.014-0.061)	0.86 (0.49-1.44)	24.5 (16.0-47.2)
ACLF (n = 180)	353 (240-489)	15.2 (7.5-28.3)	0.038 (0.022-0.092)	2.26 (1.06-6.66)	96.2 (30.2-184.4)
P value	<0.001	<0.001	<0.001	<0.001	<0.001
<i>Median values for metabolites of the KP (IQR) (μg/mL)</i>					
B. Subsets of study individuals					
Healthy subjects (n = 16)	10.45 (9.37-13.37)	0.32 (0.26-0.37)	0.026 (0.024-0.034)	0.0048 (0.0042-0.0059)	0.046 (0.042-0.052)
Compensated cirrhosis (n = 20)	12.82 (11.47-14.20)	0.51 (0.46-0.66)	0.047 (0.033-0.054)	0.006 (0.05-0.0081)	0.083 (0.056-0.12)
AD (n = 100)	9.48 (6.37-13.83)	0.49 (0.35-0.61)	0.049 (0.039-0.068)	0.007 (0.004-0.01)	0.15 (0.08-0.24)
ACLF (n = 98)	8.96 (6.48-11.81)	0.75 (0.53-0.98)	0.081 (0.061-0.113)	0.018 (0.009-0.039)	0.42 (0.17-0.79)
P value	0.0016	<0.001	<0.001	<0.001	<0.001
<i>Median values for metabolites of the KYN pathway (IQR) — arbitrary units corresponding to peak area/5×10<sup>4</sup></i>					
C. Patients with either AD, ACLF-KF, or KF-free ACLF					
AD (n = 337)	372 (255-527)	9.8 (5.8-21.6)	0.026 (0.014-0.061)	0.86 (0.49-1.44)	24.5 (16.0-47.2)
KF-free ACLF (n = 76)	381 (250-562)	9.9 (5.6-19.1)	0.025 (0.013-0.049)	1.14 (0.50-2.29)	39.9 (22.2-105.1)
ACLF-KF (n = 103)	332 (224-435)	19.5 (11.3-35.1)	0.057 (0.030-0.119)	4.82 (1.99-11.92)	131.2 (70.5-255.3)
P value	0.050	<0.001	<0.001	<0.001	<0.001
D. Patients with AD according to the presence or absence of brain dysfunction (BD) or kidney dysfunction (KD)					
No BD/No KD (n = 209)	376 (258-541)	8.2 (5.2-16.3)	0.021 (0.013-0.046)	0.68 (0.43-1.07)	21.1 (13.4-40.3)
BD (n = 60)	411 (293-549)	13.9 (7.4-31.8)	0.032 (0.015-0.086)	0.99 (0.63-1.54)	26.8 (18.3-43.1)
KD (n = 38)	337 (198-437)	16.6 (9.7-28.9)	0.050 (0.025-0.091)	1.89 (1.27-3.06)	55.8 (31.6-99.5)
BD and KD (n = 21)	316 (298-436)	21.7 (6.9-36.8)	0.050 (0.023-0.109)	1.51 (0.73-2.30)	45.6 (20.1-77.0)
P value	0.291	<0.001	<0.001	<0.001	<0.001
E. Patients stratified according to their clinical course by 28 days					
AD throughout the study (n = 281)	376 (253-541)	9.6 (5.6-21.1)	0.025 (0.014-0.059)	0.79 (0.46-1.28)	23.4 (15.6-44.6)
Development of KF-free ACLF (n = 23)	383 (312-507)	10.7 (5.8-16.9)	0.022 (0.012-0.062)	1.11 (0.60-1.67)	31.3 (16.4-48.2)
Development of ACLF-KF (n = 33)	332 (258-443)	12.1 (7.1-29.4)	0.042 (0.021-0.091)	1.48 (0.88-2.25)	45.6 (20.6-97.2)
P value	0.452	0.154	0.045	0.001	<0.001

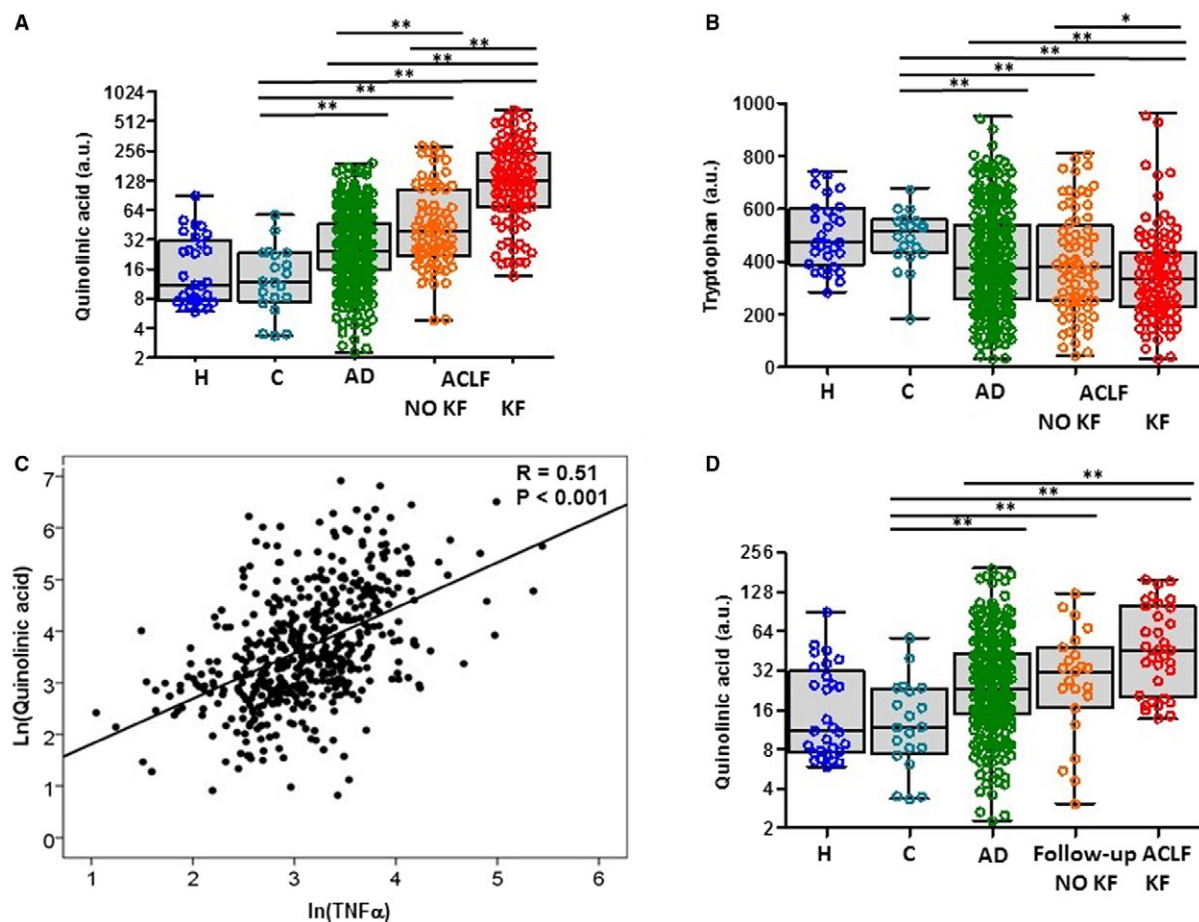
Note: P values are from the overall comparisons.

AD denotes acute decompensation; KF, kidney failure; ACLF-KF, ACLF associated with KF; KF-free ACLF, ACLF not associated with KF.

lower baseline serum levels of Trp and higher KYN, KA, and QA than those who had KF-free ACLF (Table 1C, Fig. 2A,B). These data indicate that KP overactivity is more marked in patients with KF than in those who had an ACLF form defined by the presence of extrarenal organ failures. Of note, the serum levels of KA and QA were higher in patients with KF-free ACLF than in those with AD, indicating that KF-free ACLF is also associated with KP overactivity, although to a lesser degree than in the case of ACLF-KF. Interestingly, among patients with AD, there was higher KP activity in patients with KD relative to patients free of both kidney and brain

dysfunctions (Table 1D), indicating that KP overactivity is also higher in patients with AD and KD than in those without KD.

We performed additional analyses to explore the intrarenal handling of KP metabolites in the context of ACLF. For this, we measured the serum and urine creatinine and absolute serum and urine Trp and KYN, KA, and QA concentrations and the renal fractional excretion of these metabolites in 25 patients with ACLF-KF and 25 with KF-free ACLF (Table 2). In agreement with our first results expressed in relative units (Table 2), the results of additional experiments confirmed that the absolute serum concentrations



**FIG. 2.** (A) Serum levels of QA at enrollment in healthy subjects (H) and patients with compensated cirrhosis (C), AD, and ACLF without and with kidney failure (No KF and KF), respectively. (B) Serum levels of Trp at enrollment in healthy subjects (H) and patients with compensated cirrhosis (C), AD, and ACLF without and with kidney failure (No KF and KF, respectively). (C) Correlation between QA and TNF $\alpha$ . (D) Serum levels of QA at enrollment in healthy subjects (H), patients with compensated cirrhosis (C), patients with AD throughout the study, and patients with baseline AD who developed ACLF without and with kidney failure (No KF and KF, respectively) during follow-up. The values in (A) and (D) are represented in quadratic scale and in (C) in natural logarithmic scale. Because the serum levels of QA were similar in healthy subjects and compensated cirrhosis, statistical comparison among groups in (A), (B), and (D) were performed between the groups of patients with cirrhosis. \* $P < 0.05$ ; \*\* $P < 0.001$ . Values in the metabolites of the KP are in arbitrary units corresponding to peak area/ $5 \times 10^4$ .

of KYN, KA, and QA were increased significantly in patients with KF-ACLF as compared with those with KF-free ACLF. In contrast, the urinary concentration and the fractional urinary excretion of these Trp-KYN metabolites were similar in the two groups of patients (Table 2). There was yet marked heterogeneity in the urine-to-serum ratio of the levels of each Trp-KYN metabolite, within each group of patients. For example, although the urine-to-serum ratios for KA concentrations was +472 and +88 in patients with KF-free ACLF and those with ACLF-KF, respectively, the ratios were lower for QA (+116 and +19,

respectively) or even much lower for KYN (+3.3 and -0.66 fold, respectively). The urine concentration of Trp was markedly lower in patients with ACLF-KF than in patient with KF-free ACLF.

There were also close associations between KP overactivity and hepatic encephalopathy. Most patients (36 of 43 patients) with BF had ACLF, and therefore very high levels of KA (1.82 [0.90-4.39] a.u.) and QA (67.2 [26.5-156.6] a.u.). The serum levels of KA and QA were also higher in the 60 patients with AD and BD alone than in the 209 patients without KD and/or BD (Table 1D).



**TABLE 2. Serum and Urine Concentrations of Tryptophan, Kynurenine, Kynurenic Acid, and Quinolinic Acid in Patients with ACLF, According to the Presence or Absence of Kidney Failure**

Variable	No Kidney Failure	Kidney Failure	P-value
	(N=25)	(N=25)	
<b>Serum</b>			
Median values for creatinine levels (IQR) (mg/mL)	0.009 (0.007-0.010)	0.030 (0.023-0.036)	<0.001
Median values for metabolites of the KYN pathway (IQR) (μg/mL)			
Tryptophan	10.70 (5.95-12.41)	10.76 (7.68-13.78)	0.7562
Kynurenine	0.65 (0.41-0.85)	0.96 (0.52-1.33)	0.0117
Kynurenic acid	0.007 (0.005-0.013)	0.028 (0.017-0.036)	<0.001
Quinolinic acid	0.15 (0.10-0.27)	0.55 (0.42-0.78)	<0.001
<b>Urine</b>			
Median values for creatinine levels (IQR) (mg/mL)	0.658 (0.575-1.002)	0.540 (0.424-0.687)	0.0727
Median values for metabolites of the KYN pathway (IQR) (μg/mL)			
Tryptophan	14.19 (7.52-25.31)	5.53 (3.28-13.20)	0.0071
Kynurenine	2.24 (0.97-2.97)	0.68 (0.35-2.26)	0.0547
Kynurenic acid	3.31 (2.01-3.90)	3.27 (1.83-4.46)	0.8766
Quinolinic acid	13.33 (10.21-19.41)	10.71 (5.94-21.89)	0.4151
Median values for renal fractional excretion of metabolites (%)			
Tryptophan	1.8 (0.94-2.8)	3.3 (1.8-7.8)	0.0135
Kynurenine	3.2 (1.6-5.5)	4.5 (2.9-10.7)	0.1511
Kynurenic acid	439.6 (335.4-566.3)	519.2 (339.2-804.7)	0.2523
Quinolinic acid	104.4 (78.0-125.9)	98.9 (90.3-153.3)	0.6554

Together these findings indicate that, in patients with AD and in those with ACLF, the degree of KP activation is related closely to the presence of alterations in kidney and brain functions, and to their intensities.

### KP Overactivity Is Higher in Patients With Ongoing Bacterial Infections Than in Those Without

As expected, plasma cytokines<sup>(16)</sup> and markers of KP activity (serum levels of KA and QA) were significantly higher in patients with bacterial infections at enrollment than in patients without, both in the whole series of patients (Supporting Table S4) and among patients with ACLF (Supporting Table S5).

### Different Clinical Courses of AD and ACLF Are Associated With Distinct Profiles of KP Activity

There was a close association between changes in the serum levels of KA and QA and the 28-day follow-up clinical course of patients with AD and ACLF (Table 3). Improvement of ACLF (36 patients with ACLF-1, 30 with ACLF-2, and 3 with ACLF-3)

and worsening of AD or ACLF (25 patients with no ACLF, 10 with ACLF-1, and 11 with ACLF-2) were associated with parallel changes in the serum levels of KA and QA. Patients with AD steady course and those with ACLF steady course (22 patients had ACLF-1, 15 had ACLF-2, and 11 had ACLF-3) did not present significant changes in the serum levels of KA and QA during the 28-day follow-up.

### ANTECEDENCE OF INCREASED KP ACTIVITY RELATIVE TO THE DEVELOPMENT OF ACLF

Among the 337 patients with AD at enrollment, those who developed ACLF-KF during the 28-day follow-up period had higher baseline KA and QA concentrations than those who developed KF-free ACLF (Table 1E, Fig. 2D). The baseline concentrations of these kynurenines were also higher in patients developing KF-free ACLF relative to patients without ACLF throughout the study (Table 1, Fig. 2D), although differences were not statistically significant. Our findings therefore show an antecedence of an increase in KP activity relative to the development of ACLF-KF.

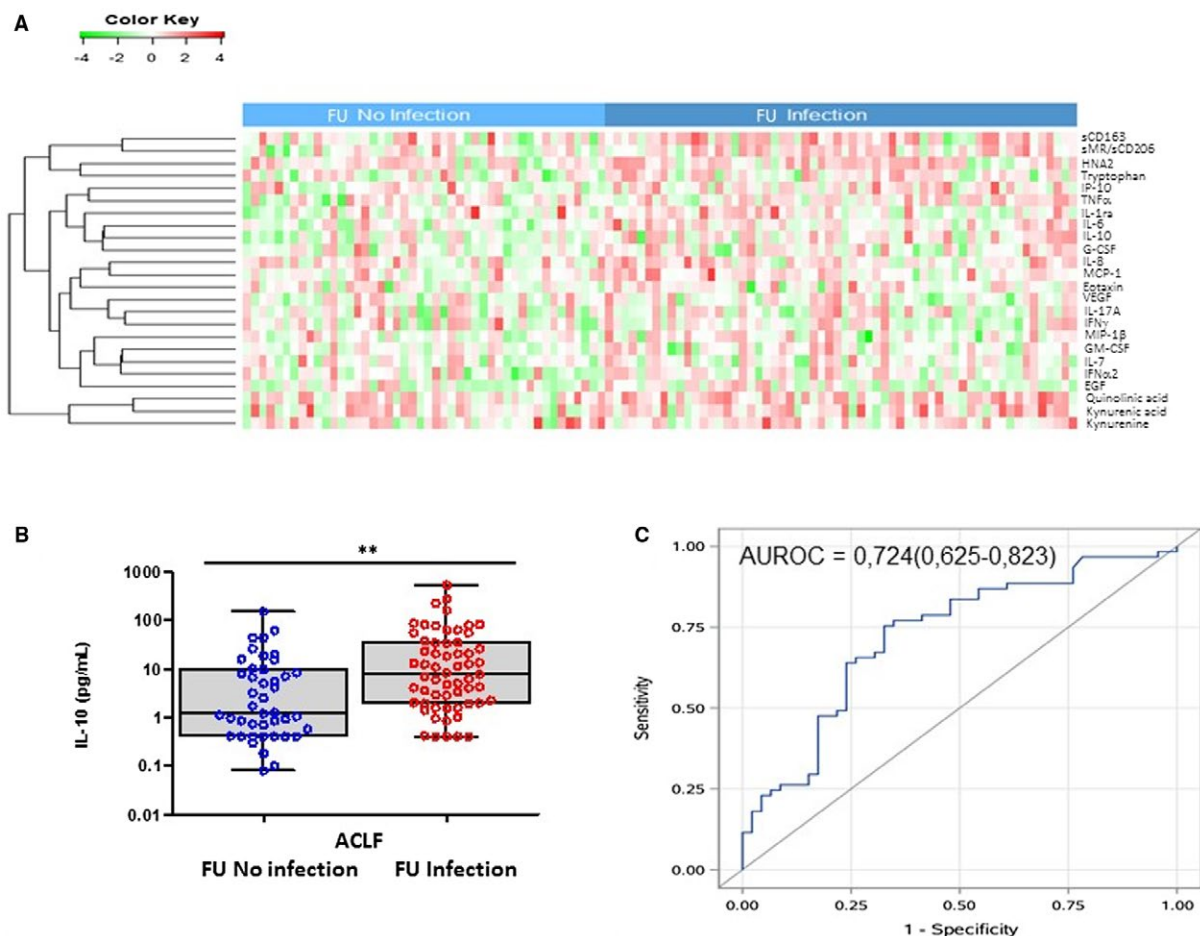
**TABLE 3. Metabolites of the KP at Enrollment and Last Follow-Up Assessment Among Groups of Patients Who Differed in Their Clinical Course (Improvement, Worsening, Steady State)**

Patient Groups	n	Enrollment	Last Assessment	P Value
<i>Median values for Trp levels (IQR) (a.u. correspond to peak area/5 × 10<sup>4</sup>)</i>				
Improvement of ACLF	69	395 (251-507)	397 (291-509)	0.560
ACLF steady course	48	355 (235-473)	354 (216-547)	0.952
Worsening of AD or ACLF	46	330 (228-492)	369 (244-569)	0.307
AD steady course	95	379 (272-634)	430 (291-645)	0.258
<i>Median values for KYN levels (IQR) (a.u. correspond to peak area/5 × 10<sup>4</sup>)</i>				
Improvement of ACLF	69	12.5 (6.1-29.1)	10.8 (6.5-23.5)	0.412
ACLF steady course	48	17.7 (9.8-32.8)	14.0 (8.3-22.9)	0.058
Worsening of AD or ACLF	46	13.5 (7.3-27.6)	13.9 (8.1-18.0)	0.203
AD steady course	95	9.8 (5.2-17.4)	8.7 (5.3-15.3)	0.543
<i>Median values for the KYN to Trp ratio (IQR)</i>				
Improvement of ACLF	69	0.031 (0.020-0.064)	0.029 (0.017-0.067)	0.372
ACLF steady course	48	0.048 (0.024-0.117)	0.045 (0.015-0.091)	0.214
Worsening of AD or ACLF	46	0.043 (0.020-0.093)	0.032 (0.024-0.066)	0.081
AD steady course	95	0.021 (0.012-0.054)	0.019 (0.012-0.037)	0.096
<i>Median values for KA levels (IQR) (a.u. correspond to peak area/5 × 10<sup>4</sup>)</i>				
Improvement of ACLF	69	1.60 (0.93-2.84)	0.84 (0.48-1.47)	<0.001
ACLF steady course	48	5.37 (1.90-10.39)	3.62 (1.05-18.28)	0.310
Worsening of AD or ACLF	46	1.63 (0.88-2.94)	2.36 (1.15-4.68)	0.009
AD steady course	95	0.77 (0.49-1.13)	0.64 (0.45-0.97)	0.509
<i>Median values for QA levels (IQR) (a.u. correspond to peak area/5 × 10<sup>4</sup>)</i>				
Improvement of ACLF	69	60.5 (29.9-120.2)	43.7 (22.6-79.9)	<0.001
ACLF steady course	48	127.3 (50.7-272.5)	104.8 (48.4-246.8)	0.896
Worsening of AD or ACLF	46	53.9 (25.0-117.7)	89.0 (49.9-159.1)	0.023
AD steady course	95	22.7 (16.4-45.2)	27.5 (15.8-43.1)	0.126

## ANTECEDENCE OF INCREASED KP ACTIVITY RELATIVE TO THE DEVELOPMENT OF ACLF-RELATED INFECTIOUS COMPLICATIONS

As expected,<sup>(17)</sup> a high proportion (56%) of the 108 patients who had ACLF but no infection at enrollment subsequently developed bacterial infection during the 28-day follow-up. Of note, none of the baseline characteristics obtained at bedside with the exception of serum albumin could predict the development of bacterial infection (Supporting Table S6). In contrast, at baseline, several “bench” values assessing SI intensity were higher in patients who developed infection during follow-up than in those who did not develop this complication (Fig. 3A, Table 4). Moreover, significantly higher baseline levels of signals for immune suppression (i.e., interleukin [IL]-10 [Fig. 3B] and sMR/sCD206) were detected. Among the variety of SI markers evaluated at baseline, only IL-10 and

sMR/sCD206 were independently associated with follow-up bacterial infections in patients with ACLF who were “uninfected” at enrollment (odds ratio [95% confidence interval (CI)]: Log (IL-10), 1.35 (1.04-1.73),  $P = 0.022$ ; Log (sMR/sCD206), 2.80 (1.13-6.90),  $P = 0.026$ ). The area under receiver operating characteristic curves for predicting the development of infection was 0.695 (95% CI, 0.595-0.796) for IL-10 and 0.679 (95% CI, 0.575-0.784) for sMR/sCD206. In addition, the combination of these two markers of immunosuppression ( $-0.083 + 0.296 \ln[\text{IL-10}] + 1.028 \ln[\text{sMR/sCD206}]$ ) significantly predicted the risk of bacterial infections (Fig. 3C). Together these findings support the existence of immune suppression in patients with ACLF, providing a rational explanation for the high risk of developing bacterial infection in these patients.<sup>(17)</sup> Because KP activation can cause immune suppression through different mechanisms,<sup>(21)</sup> we compared, among patients with ACLF and no infection at enrollment, the baseline levels of KP metabolites of patients who developed infection during follow-up with those



**FIG. 3.** (A) Differential baseline patterns of inflammatory mediators/markers and metabolites of the KP in ACLF patients who did or did not develop follow-up bacterial infections. The pattern identifies higher markers of SI, immunosuppression (sMR/sCD206 and IL-10), and KP activity in patients who developed infections during FU. (B) Individual baseline values of IL-10 in ACLF patients who did or did not develop follow-up bacterial infections (values represented in quadratic scale). \*\* $P < 0.001$ . (C) Accuracy of combining the baseline plasma levels of IL-10 and sMR/sCD206 in predicting 28-day follow-up bacterial infections in “uninfected” patients with ACLF. Abbreviations: AUROC, area under the receiver operating characteristic curve; FU, follow-up.

patients who did not develop this complication. Thus, serum QA levels were higher in patients who developed infections during follow-up (Table 4), indicating that among patients with ACLF, an increase in KP activity precedes the onset of nosocomial bacterial infection.

### KP OVERACTIVITY IS AN INDEPENDENT PREDICTOR OF SURVIVAL IN CIRRHOSIS

Based on results of univariate analysis (Supporting Tables S7 and S8), multivariate analysis identified 11 independent baseline predictors of 90-day mortality in the whole series of patients (Table 5). Among these

predictors, there were only four variables obtained at bedside, including older age, higher bilirubin, higher international normalized ratio, and lower serum sodium and 7 “bench” variables, including higher levels of KYN, IL-8, endothelial growth factor, vascular endothelial growth factor, and sMR/sCD206, and lower levels of Trp and INF $\gamma$ . Based on results of the univariate analysis (Supporting Tables S9 and S10), we identified four independent baseline predictors of 28-day mortality in patients with ACLF: older age, higher bilirubin, higher KYN, and higher sCD163 (Table 5). Together, these findings indicate that increased KP activity is a major predictor of poor outcome in patients with AD and in those with ACLF.

**TABLE 4. Plasma Cytokines, Biomarkers of Macrophage Activity and Systemic Inflammation, and KP Metabolites in Patients With ACLF, Without Infection at Enrollment, Who Did and Did Not Develop Infection During 28-Day Follow-up**

	Bacterial Infection During Follow-up		P Value
	Absent	Present	
	n = 47	n = 61	
Median values for cytokines (IQR) (pg/mL)			
Proinflammatory cytokines			
TNF $\alpha$	25.3 (14.9-36.0)	30.3 (17.7-41.8)	0.076
IL-6	25.2 (9.4-42.9)	34.0 (13.8-103.8)	0.015
IL-8	71.2 (32.5-204.6)	89.3 (50.5-149.5)	0.315
MCP-1	396.7 (261.9-572.3)	440.6 (348.6-629.5)	0.109
IP-10	1021.0 (596.4-1571.0)	1457.0 (735.3-2265.0)	0.017
MIP-1 $\beta$	23.3 (12.9-37.0)	24.8 (17.9-54.8)	0.296
G-CSF	20.5 (11.7-40.7)	33.1 (11.1-70.5)	0.188
GM-CSF	5.1 (2.4-8.0)	6.2 (3.8-16.0)	0.081
Anti-inflammatory cytokines			
IL-10	1.7 (1.0-8.2)	7.7 (2.0-34.0)	0.001
IL-1ra	13.5 (6.5-30.6)	19.4 (8.4-49.4)	0.105
Other cytokines			
IFN $\gamma$	5.9 (1.7-24.4)	5.1 (2.2-17.1)	0.865
IFN $\alpha$ 2	10.8 (2.9-32.2)	23.4 (8.0-52.9)	0.041
Eotaxin	115.1 (77.2-179.9)	122.3 (90.4-167.7)	0.401
IL-17A	3.1 (1.3-13.6)	3.0 (1.4-15.6)	0.568
IL-7	2.4 (1.4-5.2)	2.1 (1.4-7.5)	0.795
EGF	3.2 (2.8-21.4)	6.9 (2.8-27.4)	0.205
VEGF	62.0 (26.3-196.3)	44.0 (26.3-150.7)	0.990
Median values for irreversibly oxidized albumin fraction (IQR) (%)			
HNA2	8.3 (5.0-12.4)	11.2 (7.0-16.5)	0.075
Median values for markers of macrophage activation (IQR) (mg/L)			
sCD163	9.4 (5.4-15.5)	14.2 (8.2-19.2)	0.011
sMR/sCD206	0.7 (0.6-1.1)	1.1 (0.8-1.5)	0.002
Median values for markers of systemic inflammation (IQR)			
White cell count ( $\times 10^9$ cells/L)	8.2 (5.2-12.2)	7.4 (5.6-13.4)	0.902
C-reactive protein concentration (mg/L)	19.0 (9.3-43.0)	27.0 (14.1-52.3)	0.141
Median values for metabolites of the KP (IQR) (a.u. correspond to peak area/5 $\times 10^4$ )			
Trp	345 (200-456)	414 (278-509)	0.069
KYN	19.0 (7.9-39.3)	17.9 (11.2-32.6)	0.848
KYN/Trp ratio	0.055 (0.023-0.119)	0.044 (0.024-0.090)	0.701
KA	1.71 (0.67-5.27)	2.50 (1.12-5.23)	0.080
QA	67.2 (25.3-127.0)	102.3 (41.8-206.4)	0.037

Abbreviations: EGF, epidermal growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1ra, interleukin-1 receptor antagonist protein; IP-10, 10 kDa interferon gamma-induced protein; MCP-1, monocyte chemoattractant protein 1; MIP-1 $\beta$ , macrophage inflammatory protein 1-beta; VEGF, vascular endothelial growth factor.

## Discussion

There are several investigations showing activation of KP in plasma, cerebrospinal fluid and brain tissue in primary biliary cirrhosis, HCV-associated

chronic hepatitis, cirrhosis, hepatocellular carcinoma, and hepatic encephalopathy.<sup>(22-25)</sup> Our study is a prospective observational investigation to assess Trp degradation through the KP in a large series of patients with cirrhosis at different stages of the disease and its



**TABLE 5. Independent Baseline Predictors of 90-Day Mortality in the Whole Series of Patients and 28-Day Mortality in Patients With ACLF**

Variable	Whole Series of Patients		Patients With ACLF	
	HR for Death at 90 days (95% CI)	P Value	HR for Death at 28 days (95% CI)	P Value
Ln(Trp)	0.71 (0.53-0.97)	0.029	—	
Ln(KYN)	1.36 (1.14-1.62)	<0.001	1.66 (1.29-2.15)	<0.001
Ln(IL-8)	1.26 (1.00-1.58)	0.047	—	
Ln(IFN $\gamma$ )	0.82 (0.71-0.95)	0.008	—	
Ln(EGF)	0.85 (0.72-0.99)	0.041	—	
Ln(VEGF)	1.29 (1.10-1.51)	0.001	—	
Age	1.04 (1.02-1.06)	<0.001	1.06 (1.03-1.09)	<0.001
Ln(Bilirubin)	1.32 (1.04-1.70)	0.025	1.77 (1.23-2.56)	0.002
Ln(INR)	2.90 (1.62-5.18)	<0.001	—	
Ln(sCD163)	—		2.07 (1.12-3.83)	0.020
Ln(sMR/sCD206)	2.36 (1.42-3.91)	<0.001	—	
Serum sodium	0.96 (0.93-1.00)	0.033	—	

Abbreviations: EGF, epidermal growth factor; HR, hazard ratio; INR, international normalized ratio; Ln, natural logarithm; VEGF, vascular endothelial growth factor.

relationship with the severity of the disease, clinical course, and survival. We report six important findings. First, KP activity was normal in patients with compensated cirrhosis but markedly increased in patients with AD and particularly in those with ACLF. Second, our results were consistent with activation of KP as a result of SI. Third, KP activity and overall severity of cirrhosis followed similar orchestration. Indeed, among patients with AD, KP activity was higher in patients with KD or BD than in those without these dysfunctions. Among patients with ACLF, KP activity was remarkably higher in patients with ACLF-KF than in patients with KF-free ACLF. Fourth, we found an antecedence of increased KP activity relative to the development of ACLF. Fifth, we observed an antecedence of KP overactivity relative to ACLF-related infectious complications. Finally, higher KP activity was an independent predictor of death in patients with AD (at 90 days) and in those with ACLF (at 28 days).

The first issue to be discussed is the strong association between KF and high circulating levels of Trp-KYN derivatives in patients with ACLF, which has been proposed to be related to a reduction in the renal excretion of Trp-KYN derivatives and not to the mechanism of kidney failure. Our data do not support this contention. Indeed, the urinary concentration of KYN, KA, and QA were similar in our patients with KF-free ACLF and in those with ACLF-KF despite marked differences in serum concentrations of

Trp-KYN derivatives. Moreover, the fractional excretion of KYN, KA and QA, which estimates the percentage of the filtered Trp-KYN derivatives excreted by the urine, were also similar between groups. Therefore, the difference in the levels of circulating Trp-KYN derivatives between patients with ACLF and KF and those without KF cannot be explained by differences in the renal excretion of Trp-KYN derivatives.

The heterogeneity of urine Trp-KYN derivative profiles in patients with ACLF was a key observation that may help to understand the potential mechanism of the extremely higher activation of the KP associated with ACLF-KF relative to KF-free ACLF, the absence of differences between the urine levels of the Trp-KYN derivatives in both conditions, the mechanism of the specific urine profile of each Trp-KYN derivative, and the pathogenesis of the marked activation of the renal KP in patients with ACLF-KF.

Whereas the urine-to-serum ratios for KA concentrations were strikingly increased in both groups of patients with ACLF, the increases in ratios for QA concentrations, although important, were significantly less marked, and the increases in ratios for KYN concentrations were either mild or inexistent. These differences in the urine-to-serum ratios between Trp-KYN derivatives were poorly related with glomerular filtration rate (as estimated by serum creatinine) and their corresponding serum levels. Together, these

findings strongly suggest a tubular mechanism (e.g., tubular secretion) that is unrelated to renal dysfunction as the most likely explanation for high urine-to-serum ratios of some Trp-KYN metabolites, but not all.

The renal tubular cells are among the most sensitive cells for expressing the Trp-consuming enzyme IDO in response to inflammatory cues.<sup>(26)</sup> Here, we found that the urine concentration of Trp was markedly lower in patients with ACLF-KF than in those with KF-free ACLF, suggesting that Trp consumption is related to higher degree of renal inflammation in the former group of patients. Therefore, it is likely that in patients with ACLF-KF, kidney inflammation could lead to increased tubular release of Trp-KYN derivatives into the urine and perhaps also to spillover in the systemic circulation. The differences in profile of Trp-KYN derivatives in serum and urine probably reflects differences in their origin (primarily the tubular cells in the urine and from many different types of cells, including the tubular cells, in serum). The development of KF and associated tubular release of Trp-KYN derivatives would depend on the severity of renal inflammation. This feature was detected in serum, but not in urine, probably because the severity of renal failure impairs the mechanisms influencing the tubular release of endogenous metabolites, especially the tubular urine flow rate.

Kynurenines have deleterious effects on brain function and participate in the pathogenesis of a variety of acute and chronic brain disorders and diseases associated with SI.<sup>(13,27)</sup> Because the expression of IDO in the brain is low, tissue concentration of kynurenines depends largely on the transport of circulating kynurenines across the BBB. KYN and 3-HK, but not KA and QA, are readily transported across the BBB. Once in the brain, they are further degraded to produce KA and QA and other kynurenines. QA and, with less intensity, 3-HK and 3-HAA have neurotoxic effects due to their capacity to generate reactive oxygen species. QA also causes neuronal excitotoxicity through the activation of NMDA (glutamate) receptors. The ultimate consequences of these mechanisms are brain inflammation and oxidative stress, changes in neurotransmission, and neuronal dysfunction and apoptosis.

There is evidence from both clinical and experimental studies that brain inflammation and hyperammonemia are major mechanisms of hepatic

encephalopathy.<sup>(28)</sup> There are some preliminary data suggesting that activated KP may also play a contributory role. Two-fold to 10-fold elevations of cerebrospinal fluid QA concentrations have been reported in 4 neonates with hepatic coma and in 6 patients with cirrhosis who died with hepatic encephalopathy.<sup>(24,25)</sup> The cortical content of QA was also 3 times higher in the latest group of patients than in a control group of patients with cirrhosis dying from other causes. In addition, high levels of KYN in cerebrospinal fluid and plasma have recently been reported in patients with cirrhosis and hepatic encephalopathy. Our data showing a close association between high KP activity and the presence of BD and BF in patients with AD and ACLF, respectively, are in keeping with this suggestion. The neurotoxic effects of QA and other kynurenines could also explain the surprisingly extended areas of gray and white matter losses described in patients with decompensated cirrhosis.<sup>(5)</sup>

KYN has been identified as an endothelium-derived relaxing factor, the release of which is promoted by the effect of the inflammatory mediators on IDO, leading to activation of soluble guanylyl cyclase in the underlying arteriolar smooth muscle cells and vasodilation.<sup>(29)</sup> Inhibition of IDO activity protects animals with sepsis from hypotension, indicating that KP is a major contributory mechanism of systemic circulatory dysfunction in SI.<sup>(30)</sup> Our results suggest that this may also be the case in patients with cirrhosis with AD and ACLF, as the circulating levels of QA and other kynurenines correlated directly with the degree of systemic circulatory dysfunction. Angiotensin II also increases the expression of IDO, activates the KP, and promotes oxidative stress and apoptosis in endothelial cells.<sup>(30)</sup> This mechanism may be important in patients with cirrhosis with ACLF, because the renin-angiotensin system is markedly activated in these patients. Therefore, the sequential occurrence of acute and severe SI, increased endothelial IDO and KP activity and oxidative stress, KYN mediated arteriolar vasodilation, homeostatic stimulation of the renin-angiotensin system, and further increase in the KP activity and oxidative stress represents a potential vicious pathophysiological circle that could lead to progressive endothelial cell apoptosis, microcirculatory dysfunction, and organ failure.

The KYN/Trp ratio and the plasma levels of kynurenines are accurate predictors of multiorgan failure and mortality in critically ill patients hospitalized

by acute BF of different etiologies,<sup>(31)</sup> severe trauma,<sup>(32)</sup> acute pancreatitis<sup>(33)</sup> and sepsis,<sup>(34)</sup> suggesting that KP activity is a critical determinant in the evolution of diseases associated with acute SI. In fact, the blockade of the two key enzymes of the KP, IDO, and KMO reduces the rate of multiorgan failure and mortality in experimental sepsis or acute pancreatitis.<sup>(30,35,36)</sup> Our results indicate that the course of ACLF correlated closely with changes in plasma KA, QA, and Trp concentrations, and that kynurenines were independent predictors of mortality both in patients with AD and with ACLF. This supports the idea that KP activity might be an important mechanism of multiorgan failure and mortality in cirrhosis.

Fernández et al. recently showed that patients hospitalized with ACLF not triggered by infections are highly predisposed to develop nosocomial infections, suggesting immune paralysis.<sup>(17)</sup> Our data support this contention, as the higher circulating levels of IL-10 and sMR/sCD206 in patients with ACLF were the only independent predictors of nosocomial infections in these patients. The activation of IDO is a potent immunosuppressive mechanism<sup>(10,37,38)</sup> and increases IL-10 release. Because the baseline values of KP activity were also higher in patients with ACLF developing nosocomial infections than in those who did not develop infections, our data might indicate that kynurenines contribute to the immunosuppression present in patients with ACLF.

Our study has two important limitations. First, it is an observational investigation looking into associations between KP activity and patients' characteristics or clinical course, and although significant associations may be suggestive, they do not ensure cause-to-effect relationships. Second, some active KP derivatives were not detectable using our untargeted metabolomics workflow (essentially due to sensitivity limitations), and our data are discussed in terms of KP activity rather than specific Trp-KYN derivatives.

In summary, we show that KP is activated in patients with AD, especially in those with ACLF, likely as a result of SI and secondary activation of IDO enzymes. We also observed close associations between the KP activity and the presence and/or development of kidney and brain dysfunction/failure, ACLF, impairment in systemic circulatory function, nosocomial bacterial infections, and mortality. Trp-KYN derivatives, which are known to have neurotoxic, pro-oxidant, immunosuppressive, endothelial

dysfunctional and pro-apoptotic properties, could play an active role in these associations.

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