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Determination of Effective Albumin in Patients With Decompensated Cirrhosis: Clinical and Prognostic Implications

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BACKGROUND AND AIMS: Circulating albumin in cirrhosis can be dysfunctional because of accumulating structural damages, leading to the concept of effective albumin concentration (eAlb), referring to the albumin portion presenting structural and functional integrity. We aimed to estimate eAlb in patients with decompensated cirrhosis and analyze its relationships with albumin function and clinical outcomes as compared to total albumin concentration (tAlb).

APPROACH AND RESULTS: We evaluated 319 patients with cirrhosis hospitalized for acute decompensation (AD) with and without acute-on-chronic liver failure (ACLF) and 18 age- and sex-comparable outpatients with compensated cirrhosis. tAlb was quantified by standard assay, whereas eAlb was estimated combining liquid chromatography/electrospray ionization/mass spectrometry and standard methods. Albumin binding and detoxification efficiency were evaluated by electron paramagnetic resonance analysis. Circulating albumin in patients with decompensated cirrhosis displayed multiple structural abnormalities, with reversible oxidation and glycation being the most frequent. As a result, eAlb progressively declined with the worsening of cirrhosis and was superior to tAlb in stratifying patients between compensated cirrhosis, AD, and ACLF, as well as patients with and without complications. Moreover, eAlb, but not tAlb, was closely associated with binding capacities in ACLF. Finally, eAlb at admission predicted the occurrence of ACLF within 30 days and mortality at 90 days better than tAlb.

CONCLUSIONS: This large, observational study provides the evidence in patients with decompensated cirrhosis that eAlb can be quantified and differentiated from tAlb routinely measured in clinical practice. As compared to tAlb, eAlb is more closely associated with disease severity and albumin dysfunction and carries a greater prognostic power. These results prompt future research assessing eAlb as a biomarker for predicting prognosis and treatment response. (HEPATOLOGY 2021;74:2058-2073).

Human albumin is the most abundant protein in the vascular compartment, representing more than half of total plasma protein content.[1] It accounts for ~70%-80% of total plasma oncotic pressure, thus resulting as the main modulator of fluid distribution among the body compartments. Albumin also exerts many other biological properties unrelated to its oncotic power: It binds, transports, and detoxifies many endo- and exogenous molecules, is the major circulating antioxidant, modulates

Abbreviations: ACLF, acute-on-chronic liver failure; AD, acute decompensation; BE, binding efficiency; CLIFc-AD, Chronic Liver Failure Consortium-Acute Decompensation; CRP, C-reactive protein; CRR, competing risk regression; +Cys, cysteinylated; Cys-34, cysteine 34; DTE, detoxification efficiency; eAlb, effective albumin; EPR, electron paramagnetic resonance; HSA, human serum albumin; KBA, binding coefficient A; KBB, binding coefficient B; KBC, binding coefficient C; LC-ESI-MS, liquid chromatography/electrospray ionization/mass spectrometry; MELD, Model for End-Stage Liver Disease; MELD-Na, MELD-sodium; nAlb, native albumin; PC1, principal component 1; ROC, receiver operating characteristics; sHR, subdistribution HR; tAlb, total albumin.

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immune and inflammatory responses, and contributes to endothelial stabilization and vascular integrity.[2,3]

Whereas albumin oncotic power derives from highly conserved features across persons, such as its high molecular weight and negative net charge, the nononcotic properties are strictly related to its peculiar molecular structure, which presents a high degree of microheterogeneity and is affected by physio- and pathological processes.[4]

Hypoalbuminemia has long been considered a cardinal feature of decompensated cirrhosis resulting from several events, such as reduced synthesis by hepatocytes, shorter total half-life attributable to increased catabolism, and dilution attributable to total plasma volume expansion.[5] Low serum albumin concentration, measured by routine laboratory methods, has been consistently shown to be a good prognostic indicator of both mortality and morbidities in decompensated cirrhosis.[6]

In decompensated cirrhosis, besides quantitative changes, circulating albumin undergoes an extensive damage of its molecular structure because of underlying systemic inflammation and oxidative stress.[7] Reversible and irreversible oxidation of the cysteine-34 (Cys-34) residue, the main antioxidant site of the molecule, as well as nonoxidative alterations, including truncation of the C- and N- terminal portion of the molecule or glycation, are increased in advanced cirrhosis and correlate with disease severity and patient outcomes.[8,9] Because of the accumulation of these molecular alterations, the proportion of the molecule with a fully preserved structure, the "native" albumin isoform, declines in parallel with the progression of cirrhosis.[8,9] Consequently, albumin functions (i.e., binding and detoxification capacity, antioxidant function, and ability to chelate metal ions) decline in parallel with increasing severity of disease.[10,11]

The assumption that albumin global function is not only related to its circulating amount, but also to the preservation of its structural integrity led to the concept that serum "effective albumin concentration" (eAlb) in patients with advanced cirrhosis can be substantially lower than total albumin concentration (tAlb) routinely measured in clinical practice.

Based on these considerations, the aim of the present study was to translate the concept of eAlb into practice by quantitating eAlb and estimating its levels in patients with cirrhosis admitted to the hospital for acute decompensation (AD) with or without acute-on-chronic liver failure (ACLF). Moreover, we also compared the relationship between eAlb or tAlb with parameters of albumin function and clinical outcome. Finally, we assessed how the infusion of human albumin to stable patients with DC affects eAlb level and its functions.

Patients and Methods

PATIENTS AND STUDY DESIGN

The study population included patients enrolled in a prospective, observational study from January 2014

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to March 2016 at the S. Orsola-Malpighi University Hospital (Bologna, Italy).[12]

Inclusion criteria were: (1) diagnosis of cirrhosis made by clinical, laboratory, and instrumental data; (2) admission to the hospital for AD with or without ACLF; and (3) availability of a blood sample withdrawn within 24 hours from admission. AD was defined as the acute development of large ascites, HE, gastrointestinal hemorrhage, bacterial infection, or any combination of these.[13] ACLF was diagnosed according to the criteria proposed by the European Association for the Study of the Liver-Chronic Liver Failure Consortium.[13]

Exclusion criteria were age <18 years, human albumin administration for any reason in the 15 days preceding study inclusion, HCC outside the Milan criteria,[14] acute alcohol-associated hepatitis, oncohematological diseases and other extrahepatic malignancies, heart failure (New York Heart Association class >2), respiratory failure (Global Initiative for Chronic Obstructive Lung Disease III and IV), renal failure attributable to organic kidney disease, and other chronic inflammatory diseases requiring long-term specific treatment.

A group of healthy subjects and one of the patients with compensated cirrhosis under stable conditions and regularly followed at our outpatient clinic were also analyzed as reference populations.

To evaluate the impact of commercial albumin administration on eAlb, we also enrolled a group of outpatients with cirrhosis and ascites, followed at our outpatient clinic, presenting the indication to albumin administration.[15]

The study protocol was approved by the local institutional review board. Written informed consent was obtained from patients or from legal surrogates before enrollment, according to the 1975 Declaration of Helsinki.

For each patient enrolled in the study, clinical and laboratory data, including hematological, coagulation, liver and renal parameters, and serum C-reactive protein (CRP), were collected at admission. Moreover, Child-Pugh, Model for End-Stage Liver Disease (MELD), MELD-sodium (MELD-Na), and Chronic Liver Failure Consortium-Acute Decompensation (CLIFc-AD) scores were calculated to assess disease severity and prognosis. Finally, all episodes of ACLF occurring during hospitalization and 90-day survival were recorded.

At the time of inclusion in the study, a peripheral blood sample was collected from the brachial vein into pyrogen-free tubes (Vacutainer EDTA tubes and Vacutainer Lithium Heparin tubes; Becton Dickinson Italia, Milan, Italy).

To evaluate the impact of albumin administration on eAlb, a blood sample from the brachial vein was collected before the infusion of 60 g of albumin (20% solution). Blood sampling was repeated 15 minutes after infusion, from the contralateral brachial vein, and a week later. Samples of the infused albumin solution were also stored at −80°C to evaluate commercial albumin microheterogeneity.

Blood samples were centrifuged at 3,000*g* for 10 minutes; plasma was aliquoted into cryotubes (Corning Inc., Corning BV, Amsterdam, The Netherlands) and stored at −80°C until analysis.

ALBUMIN STRUCTURAL PROFILING

Albumin structural profile was assessed, slightly adapting the published methods.[16] Plasma samples were diluted 1:100 with water and filtered with a 0.22-μm filter (Merck KGaA, Darmstadt, Germany), whereas commercial albumin samples were diluted 1:2000 with water.

Liquid chromatography/mass spectrometry (LC-MS) analyses were performed by using an Agilent 1200 Series (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) interfaced with a quadrupole time-of-flight (QTOF) hybrid mass analyzer (Q-ToF Micro; Micromass, Manchester, UK), equipped with an electrospray ionization (ESI) ion source. Chromatographic separation of human serum albumin (HSA) from other serum proteins was performed on a reverse-phase C4 column (150 × 2 mm I.D.; 5 μm; 300 Å; Phenomenex Jupiter; Phenomenex Inc., Torrance, CA) thermostated at 40°C, using an elution gradient from A [water: acetonitrile: formic acid (99:1:0.1) (v/v)]/B [acetonitrile: water: formic acid (99:1:0.1) (v/v)] 70/30 (v/v), to A/B 30/70 (v/v), in 7 minutes, at a flow rate of 0.4 mL/min; the system was equipped with an autosampler, and the injection volume was 3 μL. The column was equilibrated with the mobile phase composition of the starting conditions for 10 minutes before the next injection.

The ESI-QTOF source temperature was set at 120°C, capillary voltage at 3.0 kV, and cone voltage at

35 V. Scan time was set at 1.0 second and the interscan time at 0.1 second. Mass chromatograms were recorded as total ion current, within 1,000 and 1,700 *m/z*. To characterize HSA isoforms by molecular weight determination, the HSA baseline-subtracted spectrum (*m/z*: 1,000-1,300) was deconvoluted into a true mass scale using the maximum entropy (MaxEnt1)-based software supplied with MassLynx 4.1 software (Waters Corporation, Milford, MA). Output parameters were: mass range 61,500-71,500 Da and resolution 2 Da/channel. Isoform relative abundances were calculated by dividing the isoform intensity obtained from the deconvoluted spectrum by the sum of the intensities of all isoforms and multiplying it by 100. Data were analyzed by Microsoft Excel software (Microsoft Corporation, Redmond, WA).

eAlb CONCENTRATION

eAlb concentration was estimated from the relative amount of native albumin (nAlb) quantified by LC-MS analysis and tAlb concentration determined by bromocresol green method, using a standard commercial kit, according to the following formula:

$$\text{eAlb(g/dL)} = \frac{\text{tAlb(g/dL)} \times \text{nAlb(\%)}}{100}$$

ALBUMIN BINDING FUNCTION

All samples were blinded for the clinical data and sent to the MedInnovation GmbH company (Berlin, Germany) for assessing albumin binding function by the electron paramagnetic resonance (EPR) spin-probe technique, using a commercially available EPR spectrometer (EPR-Analyzer; MedInnovation GmbH, Berlin, Germany), as described elsewhere[17] and briefly below.

Characterization of spin-probe binding affinity to albumin is possible by incubation with varying ethanol concentrations and changes in the relationship of spin probe to albumin concentration at different hydrophobic conditions. Commercial 16-doxyl stearic acid (TCI Deutschland GmbH, Eschborn, Germany) was applied as a spin probe based on its extremely high binding affinity for albumin, generally leading to >99.9% binding of this spin probe to albumin. Modification in binding affinity was induced by extrapure ethanol (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Time from thawing to analysis of the frozen serum samples was within 30 minutes. Each 50 μL of serum sample was analyzed at separate aliquot concentrations. Each aliquot received a defined concentration of ethanol and binding probe for further analysis on a microtiter plate covered by Parafilm. Followed by incubation on a microtiter shaker for 10 minutes at 37°C, aliquots were brought into capillary glass tubes for analysis within the EPR-Analyzer (temperature, 37°C). The EPR-Analyzer used a microwave power of 15 mW at a frequency of 9.4 GHz. The magnetic field strength, with a scan range of 12 mT, was 0.34 T embedding a modulation amplitude of 0.07 mT. Analysis of EPR spectra followed a complex mathematical computer simulation of its components related to a Hamilton spin function with axial anisotropy, also described in detail elsewhere.[18] The EPR spectra of the experimental probe delivered a vast number of data points, which were approximated to an ideal spectral curve during simulation. The point estimates included g-factors, hyperfine structure constants, and line widths that characterized the shape and intensity of each spectral component. These variables were then used to approximate biophysical characteristics of the 16-doxyl stearic acid spin label, including the "angle of the spin-labeled molecule axis' precession," "polarity of the environment surrounding the spin label," and the "rotation speed of the spin label."

The EPR spectra-based parameters (BE [binding efficiency]; DTE [detoxification efficiency]; KBA [binding coefficient A]; KBB [binding coefficient B]; and KBC [binding coefficient C]) provide information regarding the extent of maintained binding, transport, and detoxification of fatty acids, drugs, bilirubin, metabolites, biomarkers, and so forth. Namely, BE reflects the strength and amount of bound fatty acids under the certain conditions of ethanol concentrations, whereas DTE reflects the molecular flexibility of the patient's albumin, thus the ability of changing the conformation depending on ethanol concentration. Both are normalized to a healthy population. Binding coefficients determine the ability of binding fatty acids under the specific ethanol concentrations, with KBA at the lowest, KBB middle, and KBC at the highest ethanol concentration. With these five parameters, binding properties of albumin for every single ethanolic condition as well as their correlation are assessable.

MARKERS OF SYSTEMIC INFLAMMATION

Systemic inflammation was evaluated in outpatients and hospitalized patients by measuring IL-6 and TNF-α concentration in heparin plasma samples. Measurements were performed by a multianalyte Simple Plex cartridge kit (R&D Systems, Minneapolis, MN) run on an automated immunoassay system (ELLA; ProteinSimple, San Jose, CA), according to the manufacturer's instructions.

STATISTICAL ANALYSIS

Continuous parameters were tested for normality by the Kolmogorov-Smirnov test. Normally distributed data were summarized by the mean and SD whereas non-normally distributed parameters were summarized by the median and interquartile range. Comparisons between groups were performed by the unpaired Student *t* test or Mann-Whitney U test, as appropriate. When more than two groups were analyzed, comparisons were made by the ANOVA or Kruskal-Wallis test if the assumption of normality was violated. Significance level for *post hoc* comparison was corrected according to the Bonferroni method.

The association between total and effective albumin and clinical scores, including MELD, MELD-Na, Child-Pugh, and CLIFc-AD scores, was evaluated by the Spearman Rho correlation analysis. Principal component analysis (PCA) was applied to summarize albumin functional parameters measured by EPR and explore their relationship with tAlb, eAlb, and disease severity scores. Multivariable linear regression analysis was used to test the association between functional parameters summarized by the principal component (PC) and tAlb, eAlb, and severity of cirrhosis in patients with AD or ACLF at hospital admission. Multicollinearity was evaluated by calculating the variance inflation factor for each variable.

Multivariable competing risk regression (CRR) analysis, with backward elimination ($P > 0.05$) according to the Fine and Gray method, was used to identify predictors of the development of ACLF within 30 days from admission and 90-day mortality. Baseline tAlb, eAlb, clinical features, and routinely measured biochemical parameters significantly associated ($P < 0.05$) with the specific outcome at univariate analysis were included in the initial model. The disease severity score to be included in each analysis was selected based on the highest AUC at receiver operating characteristics (ROC) curve analysis. Parameters included in the calculation of the selected score were excluded from the model to avoid multicollinearity.

For Kaplan-Meier survival analysis eAlb was categorized according to the best cutoff for the development of ACLF within 30 days from admission and 90-day mortality as determined by the ROC curve analysis. Comparisons between curves were performed by the log-rank test. Changes of tAlb, nAlb, eAlb, and albumin functional parameters following albumin administration were analyzed by the Friedman test. The Bonferroni adjustment was used for *post hoc* comparisons. All tests were two sided, and *P* values <0.05 were considered as statistically significant. Analyses were performed using the Statistical Package for Social Sciences (SPSS version 25; IBM Corp., Armonk, NY) and R software (version 3.5.1; www.r-project.org).

Results

STUDY POPULATIONS

Three hundred nineteen patients with cirrhosis hospitalized because of AD were included in the present analysis. Of these, 78 (24%) presented ACLF at admission. Eighteen outpatients with compensated cirrhosis were also enrolled (Supporting Fig. S1, Supplementary Appendix). Age, sex, etiology of cirrhosis, biochemical parameters, markers of systemic inflammation, prognostic scores, and clinical features of outpatients and hospitalized patients are reported in Table 1.

A separate group of 8 outpatients with cirrhosis and ascites (4 males, 4 females; age range 44-72 years) were enrolled to assess the effect of human albumin administration on eAlb. Finally, 21 healthy controls (14 males, 7 females; age range 35-73 years) were also included as a reference population.

TOTAL SERUM ALBUMIN CONCENTRATION AND RELATIVE AMOUNT OF nAlb

Values of tAlb and the relative amount of nAlb in healthy controls were 4.5 (4.3-4.7) g/dL and 49.0 ± 3.5%, respectively.

TABLE 1. Anthropometric and Clinical Data of Outpatients and Patients With AD or ACLF at Hospital Admission

	Outpatients (n = 18)	AD (n = 241)	ACLF (n = 78)	*P* Value
Anthropometric data				
Age (years)	59 (53-77)	63 (51-75)	62 (56-74)	0.800
Male sex	15 (83)	144 (60)	51 (65)	0.112
Etiology of cirrhosis				
Viral	8 (44)	118 (49)	28 (36)	0.131
Alcohol	5 (28)	41 (17)	17 (22)	0.383
NASH	0 (0)	17 (7)	7 (9)	0.409
Other or mixed	5 (28)	65 (27)	26 (33)	0.556
Hematology, biochemistry, and prognostic scores				
Hemoglobin (g/dL)	13.9 (12.1-15.7)	10.8 (9.5-12.2)	10.1 (9.1-11.1)	<0.001
Leukocyte (10^9/L)	5.0 (3.8-6.7)	5.5 (3.6-8.5)	7.5 (5.1-9.6)	0.006
CRP (mg/dL)	—	1.1 (0.4-3.6)	1.2 (0.7-2.7)	0.204
Platelets (10^9/L)	139 (101-159)	95 (59-158)	86 (55-144)	0.102
Sodium (mmol/L)	141 (138-142)	137 (134-139)	136 (132-139)	0.001
Bilirubin (mg/dL)	0.9 (0.5-1.4)	2.2 (1.1-3.7)	6.8 (2.1-14.5)	<0.001
Creatinine (mg/dL)	0.8 (0.7-0.9)	0.9 (0.7-1.2)	1.9 (1.4-2.5)	<0.001
INR	1.1 (1.1-1.3)	1.4 (1.2-1.5)	1.6 (1.3-2.2)	<0.001
IL-6 (pg/mL)	4.0 (2.1-6.3)	21.6 (10.5-51.7)	37.5 (23.0-65.1)	<0.001
TNF-α (pg/mL)	9.2 (8.2-13.1)	15.2 (11.9-19.8)	19.3 (14.5-27.5)	<0.001
MELD score	9 (7-10)	14 (10-17)	26 (22-30)	<0.001
MELD-Na score	10 (7-12)	16 (13-20)	27 (23-32)	<0.001
Child-Pugh score	5 (5-6)	8 (7-10)	11 (9-12)	<0.001
CLIFc-AD score	—	51 (45-56)	—	—
Clinical data				
Ascites	—	147 (61)	55 (71)	0.130
Encephalopathy III/IV	—	33 (14)	28 (36)	<0.001
Renal dysfunction	—	20 (8)	51 (65)	<0.001
GI bleeding	—	18 (8)	6 (8)	0.948
Bacterial infection	—	65 (27)	14 (18)	0.109
HCC	3 (17)	65 (27)	15 (19)	0.271
Diabetes	6 (33)	88 (37)	25 (32)	0.761

Data are reported as median and interquartile range or absolute number and frequencies.
Abbreviations: INR, international normalized ratio; GI, gastrointestinal.

As expected, tAlb was slightly lower in outpatients (4.2 [3.6-4.7] g/dL) than in healthy controls ($P = 0.119$), whereas it was significantly reduced in hospitalized patients as compared to both healthy controls and outpatients (3.0 [2.5-3.5] g/dL; $P < 0.001$). However, no significant difference was observed between those presenting AD or ACLF at admission (3.0 [2.5-3.5] vs. 2.9 [2.4-3.4] g/dL; $P = 1.000$; Fig. 1A).

LC-ESI-MS analysis revealed that patients presented 11 damaged isoforms of albumin, each of which was characterized by specific oxidative and/or nonoxidative structural alterations (Supporting Table S1, Supplementary Appendix). Reversible oxidation of Cys-34 residue, alone or in combination with other structural changes, was the most abundant alteration in hospitalized patients. Namely, the relative amounts of cysteinylated (HSA + Cys) and cysteinylated and glycated (HSA + Cys + Glyc) isoforms were significantly increased in AD patients as compared to outpatients and controls and in ACLF patients with respect to those with AD. Moreover, the cysteinylated and N-terminal truncated (HSA + Cys-DA) isoform was significantly increased in ACLF patients as compared to all other groups of subjects.

Besides the reversible Cys-34 oxidation, the glycated albumin isoforms (HSA + Glyc and HSA + 2Glyc) also significantly increased in parallel with severity of cirrhosis. These abnormalities were more evident in patients with

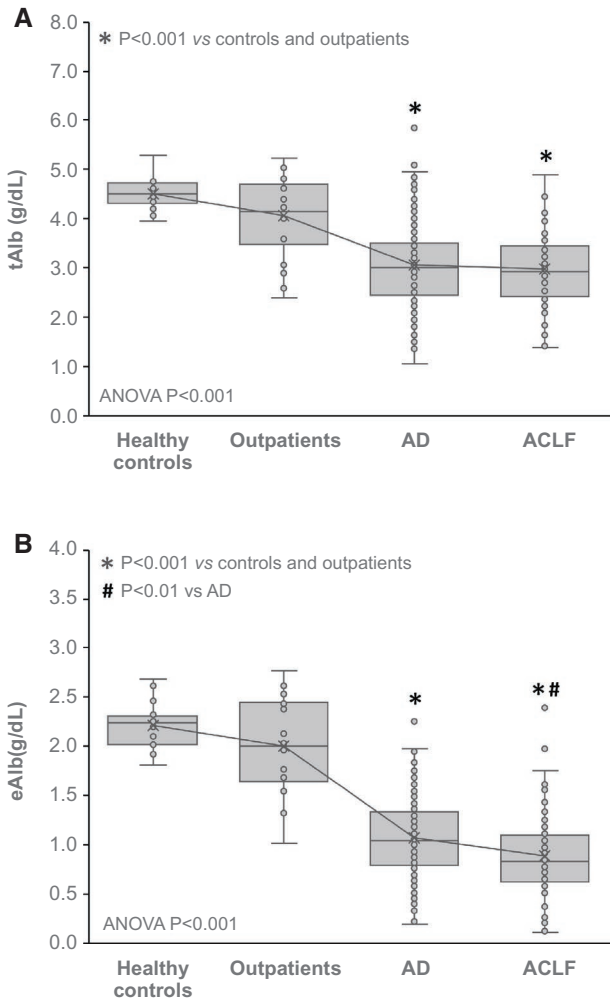

FIG. 1. tAlb concentration (A, g/dL) and eAlb concentration (B, g/dL) in healthy controls (n = 21), outpatients with compensated cirrhosis (n = 18), and hospitalized patients with AD (n = 241) or ACLF (n = 78) at admission.

diabetes mellitus (Supporting Table S2, Supplementary Appendix). However, even in nondiabetic patients, the glycated isoforms were progressively and significantly more abundant from outpatients to patients with AD or ACLF (Supporting Table S3, Supplementary Appendix). In contrast, the relative abundance of sulfinylated HSA (HSA + SO_2H) was significantly higher in outpatients with respect to hospitalized patients with or without ACLF, whereas sulfonylated HSA (HSA + SO_3H) was higher in patients than in healthy controls, without differences among patient groups. Finally, the HSA (dehydroalanine; DHA) isoform, in which the cysteine-487 residue is converted into a DHA, was significantly reduced from healthy controls to outpatients and patients with AD or ACLF (Supporting Table S1, Supplementary Appendix).

As a result of the above changes, the proportion of nAlb was significantly lower in hospitalized patients as compared to outpatients and healthy controls (33.9 ± 9.7% vs. 48.8 ± 3.8% and 49.0 ± 3.5%; ANOVA, $P < 0.001$). In contrast to what was observed for tAlb, a significant further reduction was observed in patients with ACLF as compared to those with AD admission (35.3 ± 8.8% vs. 29.6 ± 11.0%; $P < 0.001$).

eAlb CONCENTRATION

eAlb progressively declined in patients admitted with AD as compared to outpatients and healthy controls (1.0 [0.7-1.3] vs. 2.0 [1.8-2.4] and 2.2 [2.0-2.3] g/dL; ANOVA, $P < 0.001$) and, contrary to tAlb, in patients admitted with ACLF as compared to those with mere AD (0.8 [0.6-1.1] vs. 1.0 [0.7-1.3] g/dL; $P = 0.001$; Fig. 1B).

In hospitalized patients, both tAlb and eAlb correlated with severity of cirrhosis, but eAlb was more closely associated: MELD (tAlb, Rho –0.126; $P = 0.025$; eAlb, Rho –0.353; $P < 0.001$), MELD-Na (tAlb, Rho –0.129; $P = 0.021$; eAlb, Rho –0.370; $P < 0.001$), Child-Pugh (tAlb, Rho –0.134; $P = 0.016$; eAlb, Rho –0.344; $P < 0.001$), and CLIF-c AD (tAlb, Rho –0.170; $P = 0.002$; eAlb, Rho –0.318; $P < 0.001$) scores. Interestingly, tAlb, nAlB, and eAlb were inversely correlated with serum levels of proinflammatory cytokines IL-6 and TNF-α. Once more, a closer association was observed with eAlb (Supporting Table S4, Supplementary Appendix).

Finally, eAlb was significantly lower than tAlb in patients with bacterial infection, renal failure, ascites, and grade III and IV HE at hospital admission, whereas no differences were observed between patients with or without renal dysfunction (Supporting Table S5, Supplementary Appendix).

ALBUMIN BINDING AND DETOXIFICATION EFFICIENCY

Several functional parameters of albumin molecule, including BE, DTE, KBA, KBB, and KBC, were analyzed in patients with cirrhosis. All these parameters progressively and significantly decreased from outpatients to patients with AD and those with ACLF at admission (Fig. 2). The extent of association between

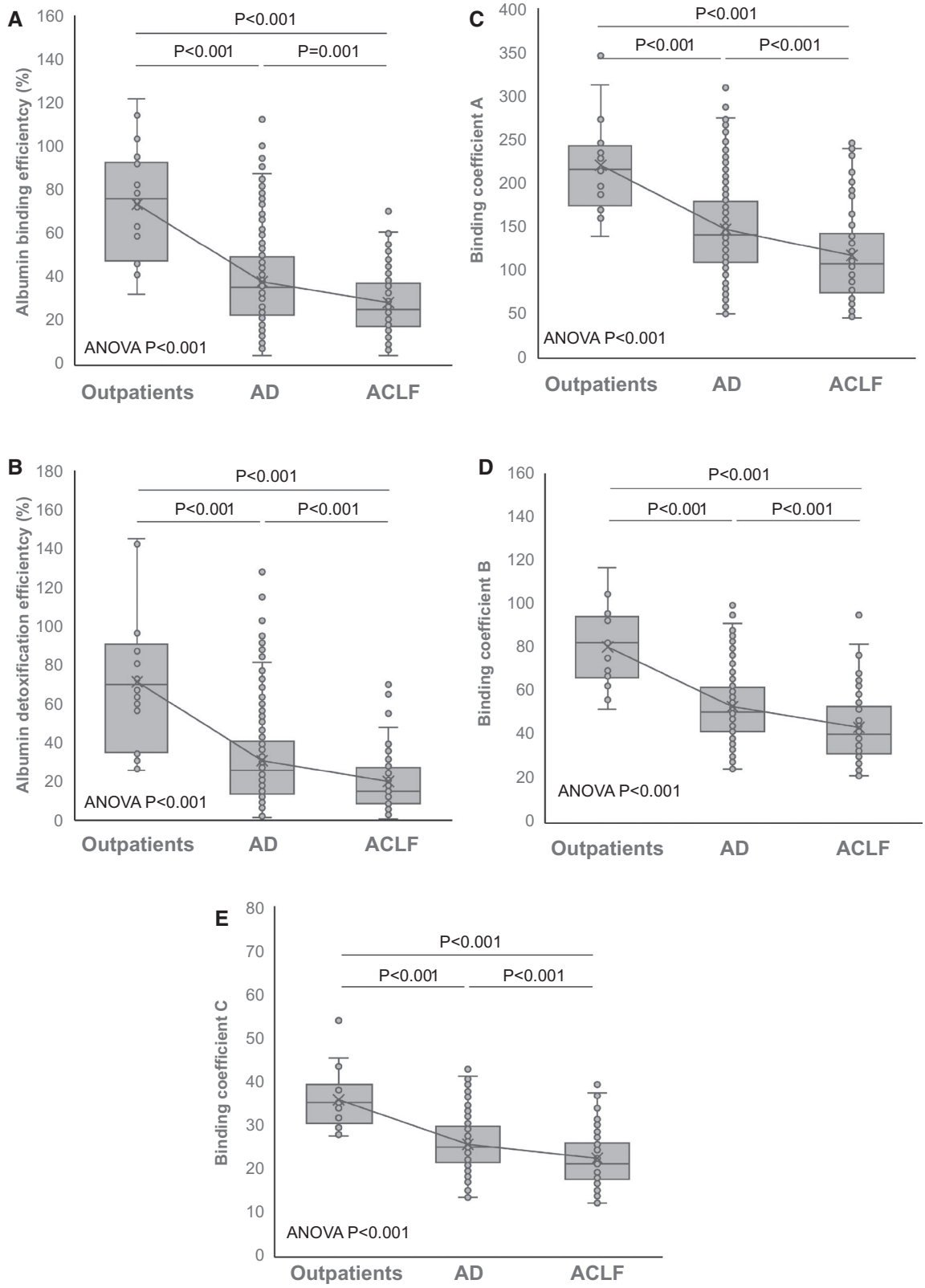

FIG. 2. Binding efficiency (A), detoxification efficiency (B), binding coefficient A (C), binding coefficient B (D), and binding coefficient C (E) of the albumin molecule in plasma samples from outpatients (n = 18) and hospitalized patients with AD (n = 241) or ACLF (n = 78) at admission.

these parameters and principal components 1 (PC1) and 2, was far closer with the former, which accounted for 89% and 86% of variability in the patient populations with AD or ACLF at admission, respectively (Supporting Fig. S2). Therefore, PC1 was chosen to express the albumin residual function and analyze its relationship with tAlb, eAlb, and severity of cirrhosis, as assessed by MELD, MELD-Na, and Child-Pugh scores. In both patients with AD or ACLF, these prognostic scores, tAlb and eAlb, were significantly correlated with PC1 (Supporting Fig. S2). A subsequent multiple linear regression analysis showed that MELD score, tAlb, and eAlb independently predicted the residual function of albumin in AD patients, whereas eAlb and MELD-Na, but not tAlb, were independent predictors of albumin function in patients with ACLF (Table 2).

EFFECTS OF HUMAN ALBUMIN ADMINISTRATION TO PATIENTS WITH CIRRHOSIS AND ASCITES

Alongside the effects of human albumin infusion to patients with stable cirrhosis and ascites, we assessed nAlb in commercial albumin vials and found it lower than in both healthy controls and outpatients (39.0 ± 4.5% vs. 49.0 ± 3.5 and 48.8 ± 3.8%; ANOVA, $P < 0.001$).

Administration of 60 g of albumin (20% solution) led to a transient increase of tAlb immediately after infusion, which faded at the 1-week control (Supporting Fig. S3A, Supplementary Appendix). Changes in nAlB were modest and not significant. However, a trend to higher levels was observed after a week (Supporting Fig. S3B, Supplementary Appendix). As a result, the significant increase in eAlb found that postinfusion was maintained also at the 1-week control (Supporting Fig. S3C, Supplementary Appendix). Despite this, a favorable effect on both BE and DTE only reached statistical significance in the post-infusion evaluation (Supporting Fig. S4, Supplementary Appendix).

TABLE 2. Multiple Linear Regression Analysis of the Association Between tAlb, eAlb, and Disease Severity Scores, With Functional Parameters of Albumin Summarized by PCA in Hospitalized Patients With AD or ACLF at Hospital Admission

	Standardized Coefficient	*P* Value
Hospitalized patients with AD (n = 241)		
tAlb (g/dL)	0.386	<0.001
eAlb (g/dL)	0.259	0.001
MELD score	–0.165	0.002
Hospitalized patients with ACLF (n = 78)		
tAlb (g/dL)	—	—
eAlb (g/dL)	0.350	0.001
MELD-Na score	–0.311	0.003

The disease severity score included in each analysis was most closely associated with the first PC.

EFFECTIVE ALBUMIN CONCENTRATION AND CLINICAL OUTCOMES

Risk of 30-Day Occurrence of ACLF in Patients Admitted With Only AD

Among hospitalized patients with AD at admission (241; 76%), 32 (13%) developed ACLF during their hospital stay (Supporting Fig. S1, Supplementary Appendix). When compared to those remaining free from ACLF, the baseline parameters of patients who later developed ACLF (Table 3) were characterized by a significantly higher leucocyte count, CRP level, IL-6, and TNF-α, a lower hemoglobin level, and a more advanced disease, as witnessed by the significantly higher disease severity scores. Moreover, patients who developed ACLF within 30 days showed significantly lower tAlb and eAlb.

We also performed a multivariable CRR analysis to identify independent predictors of ACLF within 30 days from admission, considering in-hospital death, liver transplantation, and hospital discharge as competing events. Besides the two albumin-related parameters and hemoglobin level, MELD was included in the analysis because of the higher discriminating performance against ACLF development at ROC curve analysis, as compared to the other disease severity scores (Supporting Table S6, Supplementary Appendix), whereas, among the non-specific markers of inflammation, CRP was selected because it showed a lower *P* value than leukocyte count at univariate analysis. Multivariable analysis showed that MELD score (subdistribution HR [sHR], 1.16; 95% CI, 1.08-1.24; $P < 0.001$), CRP level (HR, 1.09; 95% CI, 1.02-1.16; $P = 0.010$), and

TABLE 3. Univariate Analysis of Baseline Parameters Associated With the Development of ACLF Within 30 Days in Hospitalized Patients Without ACLF at Admission

	Patients Remaining Free From ACLF (n = 209)	Patients Who Developed ACLF (n = 32)	*P* Value
Anthropometric data			
Age (years)	64.0 (51.0-76.0)	58.0 (50.5-68.0)	0.253
Male sex	123 (59)	21 (66)	0.467
Etiology of cirrhosis			
Viral	104 (50)	14 (44)	0.526
Alcohol	35 (17)	6 (19)	0.779
NASH	15 (7)	2 (6)	0.849
Other or mixed etiology	55 (26)	10 (31)	0.558
Hematology, biochemistry, and prognostic scores			
Hemoglobin (g/dL)	10.9 (9.6-12.2)	9.9 (9.0-11.5)	0.030
Leukocyte (10^9/L)	5.2 (3.5-8.2)	7.0 (5.3-11.8)	0.001
CRP (mg/dL)	0.9 (0.3-2.8)	3.1 (1.5-8.0)	<0.001
Platelets (10^9/L)	92.5 (59.5-154.0)	107.5 (59.0-164.0)	0.865
Sodium (mmol/L)	137.0 (134.0-139.0)	137.5 (133.0-141.0)	0.931
Bilirubin (mg/dL)	1.9 (1.1-3.4)	3.9 (2.2-10.9)	<0.001
Creatinine (mg/dL)	0.9 (0.7-1.2)	1.1 (0.9-1.4)	0.006
INR	1.3 (1.2-1.5)	1.5 (1.4-1.8)	<0.001
IL-6 (pg/mL)	19.9 (9.2-40.7)	60.7 (20.3-97.1)	<0.001
TNF-α (pg/mL)	14.7 (11.8-19.2)	18.80 (14.5-24.2)	0.014
MAP (mm Hg)	86.7 (80.0-96.0)	86.7 (77.5-91.5)	0.290
HR (bpm)	78.0 (68.0-88.0)	80.5 (73.5-92.5)	0.089
MELD score	13.0 (10.0-17.0)	19.0 (14.5-23.0)	<0.001
MELD-Na score	15.4 (12.5-19.3)	21.6 (16.4-25.2)	<0.001
Child-Pugh score	8.0 (6.0-9.0)	9.0 (8.0-11.0)	<0.001
CLIFc-AD score	49.7 (44.7-54.7)	56.9 (51.3-61.8)	<0.001
Albumin data			
Total albumin (g/dL)	3.1 (2.5-3.5)	2.7 (2.2-3.2)	0.002
Effective albumin (g/dL)	1.1 (0.8-1.4)	0.8 (0.6-1.1)	0.001
Clinical data			
Ascites	125 (60)	22 (69)	0.334
Encephalopathy III/IV	30 (14)	3 (9)	0.445
Renal dysfunction	15 (7.2)	5 (16)	0.107
GI bleeding	16 (8)	2 (6)	0.778
Bacterial infection	53 (25)	12 (38)	0.150
HCC	56 (27)	9 (28)	0.887
Diabetes	75 (36)	13 (41)	0.604

Data are reported as median and interquartile range or absolute number and frequencies.
Abbreviations: INR, international normalized ratio; MAP, mean arterial pressure; HR, heart rate; GI, gastrointestinal.

eAlb (sHR, 0.30; 95% CI, 0.10-0.88; $P = 0.028$), but not tAlb, were the independent predictors of ACLF development within 30 days from hospital admission (Fig. 3A).

Finally, we categorized eAlb according to the best cutoff for development of ACLF within 30 days from admission by ROC curve analysis, which indicated the value of 0.81 g/dL. Kaplan-Meier analysis showed that the cumulative incidence of 30-day ACLF was significantly higher in patients with eAlb at admission lower or equal to 0.81 g/dL than in patients with higher levels (49% vs. 35%; $P = 0.001$; Fig. 3B).

Risk of 90-Day Mortality

Among the 319 patients admitted to the hospital, 17 (5%) subjects underwent liver transplantation and 71 (22%) died within 90 days from admission. Patients in the nonsurvivor group were older, presented a significantly higher degree of systemic inflammation, and had worse disease severity scores when compared to those still alive 90 days after admission (Table 4). Regarding the clinical data at admission, ascites, grade III/IV encephalopathy, renal dysfunction, and bacterial infections were more frequent in nonsurvivors, whereas gastrointestinal bleeding was less frequent in the same group as compared to survivors. Finally, nonsurvivors were also characterized by significantly lower levels of tAlb and eAlb (Table 4).

We also performed a multivariable competing risk-regression analysis, considering liver transplantation as a competing event, to identify independent predictors of 90-day mortality. Among disease severity scores, the CLIFc-AD score was included in the model, because it showed the highest discriminating performance against survivors and nonsurvivors at ROC curve analysis (Supporting Table S7, Supplementary Appendix). The analysis showed that the CLIFc-AD score (sHR, 1.08; 95% CI, 1.05-1.10; $P < 0.001$), grade III/IV encephalopathy at admission (sHR, 2.28; 95% CI, 1.31-3.98; $P = 0.004$), CRP level (sHR, 1.08; 95% CI, 1.03-1.12; $P = 0.001$), total bilirubin (sHR, 1.04; 95% CI, 1.01-1.07; $P = 0.003$), and eAlb (sHR, 0.49; 95% CI, 0.26-0.90; $P = 0.021$), but not tAlb, were independent predictors of 90-day mortality (Fig. 4A).

Finally, we categorized eAlb according to the best cutoff for the risk of 90-day mortality by ROC curve analysis, which indicated the value of 0.77 g/dL. Kaplan-Meier analysis showed that cumulative incidence of mortality was almost doubled in patients with eAlb ≤0.77 g/dL than in patients with higher levels (37% vs. 18%; $P < 0.001$; Fig. 4B).

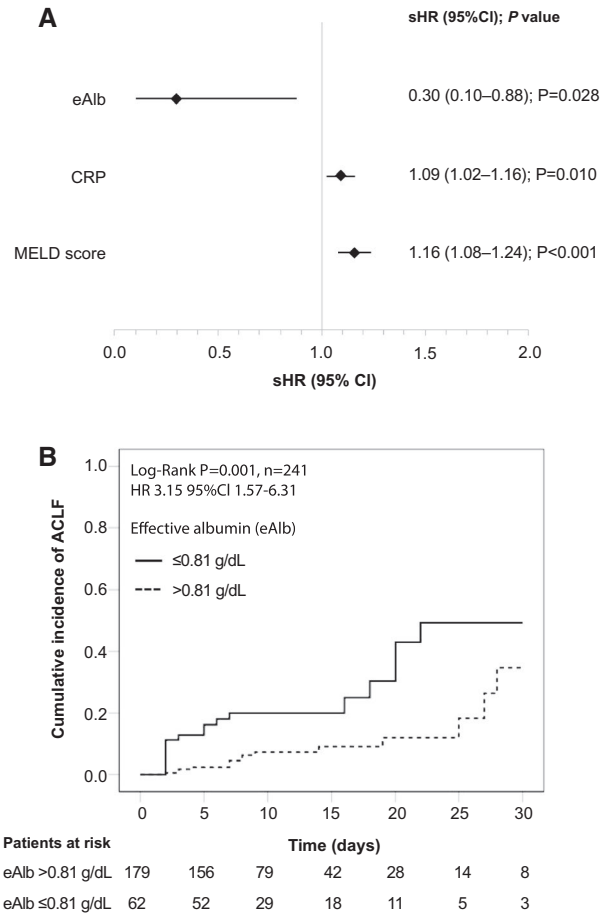

FIG. 3. (A) Multivariable competing risk-regression analysis with backward selection of factors associated with the development of ACLF within 30 days from admission in hospitalized patients with AD (n = 241). In-hospital death, liver transplant and hospital discharge were considered as competing events. Data are presented as sHR and 95% CI. Variables entered in the model were CRP, hemoglobin, MELD score, tAlb, and eAlb. (B) Probability of developing ACLF within 30 days from admission in subjects with AD (n = 241) according to the cut-off level of eAlb concentration equal to 0.81 g/dL, as determined by ROC curve analysis.

Discussion

Over the last decade, a strong evidence emerged that circulating albumin in patients with decompensated cirrhosis can be dysfunctional because of accumulating posttranslational damages. These findings led to the concept of "effective albumin concentration",[2] which refers to albumin isoform presenting structural and functional integrity. The present observational study in a large cohort of patients admitted to the hospital for an episode of AD with or without ACLF provides the evidence that eAlb can be quantified and differentiated from tAlb routinely measured in clinical practice through standard methods.

In detail, the major findings of this study can be summarized as follows: (1) we confirmed that circulating albumin in patients with decompensated cirrhosis undergoes multiple structural abnormalities. Among these, reversible oxidation and glycation

TABLE 4. Univariate Analysis of Factors Associated With 90-Day Mortality at Hospital Admission

	Alive (n = 248)	Dead (n = 71)	*P* Value
Anthropometric data			
Age (years)	61.0 (51.0-73.0)	70.0 (61.0-79.0)	<0.001
Male sex	154 (62)	41 (58)	0.507
Etiology of cirrhosis			
Viral	110 (44)	36 (51)	0.344
Alcohol	44 (18)	14 (20)	0.703
NASH	19 (8)	5 (7)	0.862
Other or mixed etiology	75 (30)	16 (23)	0.205
Hematology, biochemistry, and prognostic scores			
Hemoglobin (g/dL)	10.7 (9.5-12.0)	10.6 (9.1-12.3)	0.883
Leukocyte (10^9/L)	5.5 (3.6-8.2)	8.3 (5.1-10.6)	<0.001
CRP (mg/dL)	1.0 (0.4-2.7)	2.2 (0.9-6.4)	<0.001
Platelets (10^9/L)	94.0 (58.5-150.0)	91.0 (60.0-152.0)	0.982
Sodium (mmol/L)	137.0 (134.0-139.0)	135.0 (132.0-139.0)	0.078
Bilirubin (mg/dL)	2.2 (1.1-4.1)	3.8 (2.0-9.4)	<0.001
Creatinine (mg/dL)	0.9 (0.7-1.4)	1.3 (0.9-2.0)	<0.001
INR	1.4 (1.2-1.6)	1.5 (1.3-1.8)	0.006
MAP (mm Hg)	86.7 (80.0-93.7)	83.3 (75.8-93.2)	0.072
HR (bpm)	78.0 (68.0-88.5)	80.0 (72.5-88.5)	0.100
MELD score	15 (11-19)	22 (16-27)	<0.001
MELD-Na score	17 (13-22)	24 (19-30)	<0.001
Child-Pugh score	8 (7-10)	10 (9-11)	<0.001
CLIFc-AD score	51 ± 9	61 ± 10	<0.001
Albumin data			
Total albumin (g/dL)	3.2 (2.8-3.5)	3.0 (2.6-3.4)	0.016
Effective albumin (g/dL)	1.1 (0.8-1.4)	1.0 (0.6-1.2)	0.001
Clinical data			
Ascites	148 (60)	54 (76)	0.012
Encephalopathy III/IV	39 (16)	22 (31)	0.004
Renal dysfunction	45 (18)	26 (37)	0.001
GI bleeding	23 (9)	1 (1)	0.027
Bacterial infection	87 (35)	38 (54)	0.005
HCC	61 (25)	19 (27)	0.665
Diabetes	89 (36)	24 (34)	0.746

Data are reported as median and interquartile range or absolute number and frequencies.
Abbreviations: INR, international normalized ratio; GI, gastrointestinal.

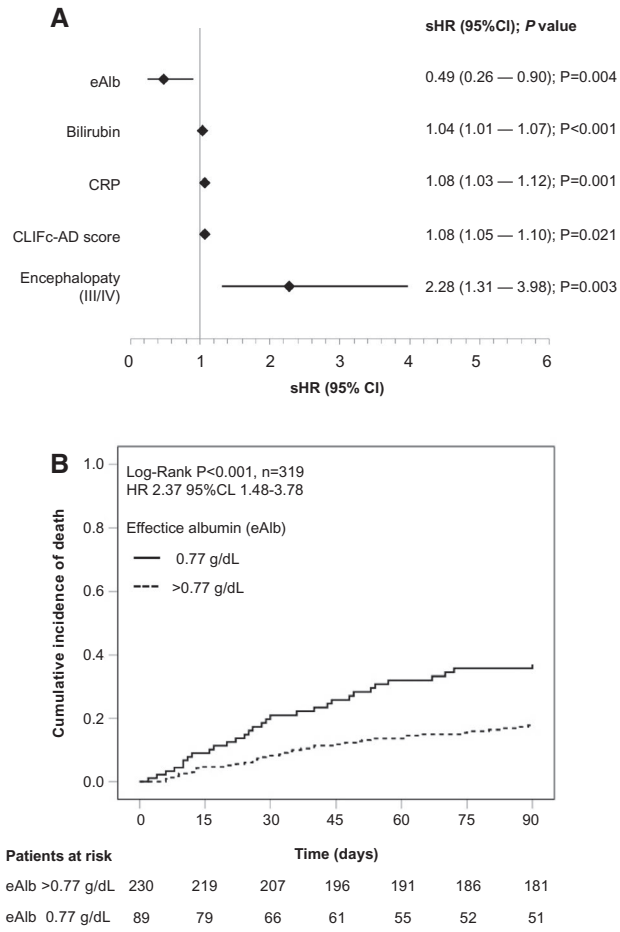

FIG. 4. (A) Multivariable CRR analysis, considering liver transplant as a competing event, with backward selection of factors associated with 90-day mortality in hospitalized patients (n = 319). Data are presented as sHR and 95% CI. Variables entered in the first step of the analysis were tAlb and eAlb concentration, CRP and bilirubin level, grade III/IV encephalopathy, bacterial infections, ascites, and gastrointestinal bleeding at admission. The disease severity score (CLIF-C AD) to be included in the analysis was selected by the ROC analysis. (B) Probability of 90-day mortality according to the cut-off level of eAlb concentration equal to 0.77 g/dL, as determined by ROC curve analysis.

prevail; 2) eAlb progressively declines in parallel with the worsening of cirrhosis, providing a better patient stratification than tAlb; 3) this is also true comparing patients with or without complications, such as bacterial infection, renal failure, ascites, and HE; 4) eAlb, but not tAlb, is closely associated with residual albumin functions, such as BE and DTE, in patients with ACLF; 5) human albumin administration to stable patients with cirrhosis and ascites lead to a short-lived improvement in tAlb. Despite that an improvement in eAlb can be traced up to a week after infusion, an amelioration in albumin binding and detoxification functions was also short-lived; and (6) eAlb better

predicts the occurrence of ACLF within 30 days and 90-day mortality than tAlb in patients with cirrhosis and AD.

The present study confirmed that circulating albumin in patients with cirrhosis undergoes multiple posttranslational abnormalities that accumulate as disease progresses.[8] The reversible oxidation of Cys-34, alone or in combination with other structural changes, was the most frequent alteration. This is relevant, given that nonmercaptalbumin-1, which includes all albumin isoforms with reversible oxidation of Cys-34 including the cysteinylated isoforms, triggers inflammatory response in peripheral blood mononuclear cells isolated from both healthy volunteers and patients with decompensated cirrhosis.[19] In contrast, sulfinylated and sulfonylated isoforms, which constitute the irreversibly oxidized nonmercaptalbumin-2, underwent less defined changes. Only the sulfonylated isoform was increased in patients, but without differences related to the severity of their conditions, and the sulfinylated isoform was even more abundant in compensated outpatients than in hospitalized patients. These results appear to be at variance with previous reports and may be attributable to methodological reasons (high-performance liquid chromatography [HPLC] vs. HPLC-ESI-MS), different ways for expressing results (abundance of grouped vs. single isoforms), and different prevalence of patients with active alcohol use disorder.[7,20-22] Another interesting finding was that albumin glycation was also very frequent and able to stratify both diabetic and nondiabetic patients according to severity of their liver disease.

The present study quantifying eAlb clearly shows that it declines in parallel with severity of underlying cirrhosis. Indeed, serum eAlb concentration better stratified patients with compensated and decompensated cirrhosis and, among the latter, AD and ACLF with respect to tAlb, which did not differ in these patient populations. Moreover, the relationships with the several prognostic scores assessed in our study were substantially closer with eAlb than tAlb. These findings suggest that eAlb is a better biomarker of the severity of cirrhosis than tAlb. Consistent with this assumption, the inverse relationship between eAlb and markers of systemic inflammation was closer with respect to tAlb, which may be relevant considering that a progressive increase in proinflammatory cytokines marks the transition from stable decompensated cirrhosis to AD and ACLF.[23] Furthermore, eAlb was more significantly reduced than tAlb in patients with complications, especially renal failure, ascites, and bacterial infections.

The present study also confirmed, in a far larger series than in previous reports,[10] that BE and DTE are substantially and progressively reduced in patients with AD and ACLF with respect to those with compensated cirrhosis. EPR spectroscopy, using 16-doxyl stearic acid as a spin probe, assesses the albumin capacity of binding fatty acids and provides indirect information on BE and DTE of many substances such as drugs, bilirubin, eicosanoids, toxic compounds of bacterial origin, including lipopolysaccharide, lipoteichoic acid, peptidoglycan, and other proinflammatory substances, and mediators of inflammation.[1,24-26] The consequences of an impairment of albumin BE and DTE are numerous and highly relevant in the pathophysiological picture of advanced cirrhosis.[5] Indeed, several immune-modulating substances can circulate as free components, thus reacting arbitrarily instead of being transported to specific binding sites, and the pharmacokinetics and pharmacodynamics of many drugs, including β-lactams and macrolides, can be even substantially modified.[26] The significant relationship between residual albumin function and both tAlb and eAlb found in the present study could be considered an expected finding, given that a reduction in serum albumin concentration cannot be without effects on the functional activities of the albumin pool. However, the finding that eAlb, but not tAlb, was significantly associated with residual albumin function in patients with ACLF is more intriguing, and suggests that when extreme albumin pool dysfunction occurs, qualitative abnormalities prevail over quantitative deficiency. Thus, another advantage of eAlb measurement in these contexts is that it likely provides a better representation of albumin dysfunction with respect to tAlb dosage. The practical implications of this result would obviously need to be addressed by specifically focused studies.

The results of our ancillary study on the effects of human albumin infusion to patients with stable cirrhosis and ascites showed that the improvement in tAlb and albumin detoxification and binding efficiency were short-lived, given that they were only observed in the postinfusion evaluation and faded a week after. However, a more prolonged effect on eAlb was noted. These results may appear disappointing, but are not

unexpected. Indeed, nAlb in the commercial albumin vials was significantly lower with respect to not only healthy controls, confirming previous reports,[27-29] but also patients with compensated cirrhosis.

The series of results related to eAlb predicting power of clinical outcomes possibly represent the most relevant findings of the present study from a clinical standpoint. An interesting result is that eAlb could help in predicting the occurrence of ACLF within 30 days from admission in patients hospitalized because of AD. Predicting short-term occurrence of ACLF in these patients is still a not fully solved issue, even though we recently reported that different combinations of MELD, leukocyte count, and blood hemoglobin thresholds could help in stratifying the risk of nosocomial ACLF.[30] What we found in this study at univariate analysis, besides confirming our previous data obtained in a different patient population and within a different time frame,[30] was that both tAlb and eAlb differed in patients with uncomplicated AD or AD complicated by the occurrence ACLF at a later time, being significantly lower in the latter. Notably, eAlb, but not tAlb, was an independent predictor of 30-day ACLF at a multivariable CRR analysis considering in-hospital death, liver transplantation, and hospital discharge as competing events. Furthermore, the best cut-off value of eAlb measured at admission in patients with AD identified two populations with a significantly different cumulative incidence of ACLF: Those whose eAlb was lower than 0.81 g/dL had a >3 times higher risk of developing this complication within 30 days.

Baseline tAlb and eAlb were also lower in patients who died within 90 days. Similar to what was observed for the occurrence of 30-day ACLF, the multivariable CRR analysis considering liver transplantation as a competing event revealed that eAlb, but not tAlb, was among the independent predictors of 90-day mortality. Furthermore, the best cut-off value of eAlb measured at admission identified two populations of patients with AD whose risk of 90-day mortality was significantly different. Indeed, in patients whose eAlb was lower than 0.77 g/dL, the risk of 90-day mortality was more than doubled. These results indicate that eAlb, in patients hospitalized because of AD, is an independent predictor of future adverse events, such as the short-term development of ACLF or medium-term mortality, far better than tAlb. Thus, evaluating the impact of the inclusion of eAlb in prognostic scores would warrant future investigation.

There are some limitations of the present study that merit to be recognized, the most relevant likely being the absence of a confirmation of the main findings in a validation cohort. The generalizability of the results may also be limited by the prevalent viral etiology of cirrhosis and the relatively low prevalence of active alcohol use disorder in patients enrolled in the study, given that albumin molecule abnormalities and functions are more frequent in this population.[22,31] Moreover, it must be recognized that the high-throughput method used in this study to determine the relative amount of the albumin isoforms in plasma samples is not yet implemented at the routine level. However, it provides results in a relatively short time at a reduced cost, so that it could be used as a second-level evaluation. Finally, the number of patients with AD who developed ACLF within 30 days from the index admission to the hospital was relatively low, and the predictive power of eAlb would need to be reassessed in a larger population.

In conclusion, the present study quantitated eAlb, translating the concept of effective albumin concentration into practice. It also showed that measuring eAlb provides several advantages over the routine assessment of tAlb. Indeed, contrary to tAlb, eAlb enables one to distinguish patients with AD from those with ACLF and is among the independent predictors of the 30-day occurrence of ACLF and 90-day mortality. Furthermore, eAlb is more closely associated with the extent of albumin dysfunction. Altogether, these results further strengthen the potential usefulness of assessing eAlb in clinical practice as a biomarker for prognostic assessments as well as a means to guide treatments to a more personalized approach. This would not only pertain to albumin administration for patients with decompensated cirrhosis, but also drugs whose pharmacokinetics and pharmacodynamics can be expected to be modified by a dysfunction of albumin binding/detoxification capacity, such as antibiotics. In this respect, our results seem to provide a solid background for future research in these fields.

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Supporting Information

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