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Serum Villin-1—A Novel Marker of Gut Barrier Damage in Acutely Decompensated Cirrhosis: A Cohort Study and Validation

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ABSTRACT

Background: Gut barrier dysfunction contributes to acute decompensation (AD) of cirrhosis progression but, it is not acknowledged in severity or prognostic scores due to a lack of appropriate assessment tools.

Aim: We investigated serum villin-1 (VIL1), an epithelial brush-border associated actin-binding protein, as a non-invasive marker for gut injury and prognosis in AD cirrhosis.

Methods: Serum VIL1 was measured in 338 cirrhosis patients (discovery $n = 130$, validation $n = 208$, including acute-on-chronic liver failure [ACLF]) from MICROB-PREDICT cohorts and 50 healthy controls (HC). Duodenal biopsies ($n = 49$ patients, $n = 11$ HC) were assessed for tissue VIL1 via immunohistochemistry. Serum cytokine profiling linked serum VIL1 levels to systemic inflammation.

Abbreviations: ACLF, acute-on-chronic liver failure; AD, acute decompensation; AKI, acute kidney injury; AUROC, area under the ROC curve; BT, bacterial translocation; CI, confidence interval; CLIF-C, chronic liver failure consortium; HC, healthy controls; HR, hazard ratio; *H*-score, histology score; IHC, immunohistochemistry; ROC, receiver operating characteristics; SDC, stable decompensated cirrhosis; SIRS, systemic inflammatory response syndrome; UDC, unstable decompensated cirrhosis; VIL1, villin-1.

David Tornai and Boglarka Balogh contributed equally to this work.

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Results: Compared to HC, both serum and tissue VIL1 levels were lower in stable AD patients. However, serum VIL1 progressively increased with AD severity, peaking in ACLF. Serum VIL1 was associated with 90-day mortality (AUROC: 0.721, $p < 0.001$; similar to MELD: 0.722, $p < 0.001$). A cut-off > 12.79 ng/mL enhanced the prognostic accuracy provided by severe disease stage, defined as CLIF-C AD score ≥ 50 or ACLF (low VIL1: 17.5% vs. high VIL1: 53.6% mortality). This threshold was associated with increased systemic inflammation, suggesting enhanced bacterial translocation. VIL1 was an independent predictor of 90-day mortality (HR: 2.52, CI: 1.648–3.848, $p < 0.001$) in Cox regression. All findings were confirmed in the validation cohort.
Conclusion: Serum VIL1 is a non-invasive indicator of gut barrier damage and short-term mortality in AD cirrhosis. Incorporating VIL1 assessment into risk stratification methods improves prognostic accuracy by capturing an essential, yet previously overlooked component of disease progression.

1 | Introduction

Enterocyte integrity, adequate mucus layer thickness, and intact mucosal immunity are essential aspects of a healthy gut barrier [1]. In patients with cirrhosis, several chronic morphological and functional alterations of these features have been demonstrated. Both portal hypertension and inflammatory processes are suggested to serve as primary pathophysiological drivers in these changes [2, 3]. Gut barrier dysfunction accompanied by persistently increased bacterial translocation (BT) has been consistently reported to play an essential role in the pathogenesis and progression of chronic liver diseases including the development of cirrhosis and its late-stage complications [4–9]. These sequelae encompass immune dysfunction [10, 11] with susceptibility to infections [12–15], development of multi-organ dysfunction [16–18], and mortality [19–22]. However, the potential deleterious effects of acute intestinal tract injury itself remain mostly unexplored in cirrhosis. It seems reasonable that during acute decompensation (AD), especially in cases progressing to acute-on-chronic liver failure (ACLF) with multi-organ dysfunction, acute intestinal injury (e.g., loss of enterocyte integrity) may occur superimposed on pre-existing intestinal damage. This 'acute-on-chronic intestinal injury' might exacerbate the inflammatory response by further increasing BT. On the other hand, it is well known that in critically ill patients, systemic inflammatory response syndrome (SIRS) can cause gut barrier injury leading to acute enhancement in BT due to the deleterious effect of the inflammatory cytokines on enterocytes [1, 23]. This may lead to the development of a vicious cycle. Hence, gut injury might emerge as both a precipitant and a consequence/complication of AD or ACLF.

Regardless of this pivotal role of the gut-liver axis in liver pathologies, acute intestinal injury is not assessed by any of the currently implemented severity scores of cirrhosis. This may be due to the lack of reliable non-invasive tools to assess this aspect. Therefore, finding appropriate markers is of paramount importance.

Villin-1 (VIL1) is a 92.5 kDa actin-bundling, severing, and capping protein [24] with a role in epithelial cell turnover [25, 26]. VIL1 expression increases in the intestinal tract as cells differentiate and migrate from the crypts to the tips of the villi [27]. VIL1 is present in the brush border of mucosal epithelial cells; thus, it is expressed along the entire length of the gut, in the proximal tubules of the kidneys and in the biliary canaliculi [24, 28]. Considering the extensive surface area of the gut compared to other organs, we hypothesised that the majority of circulating VIL-1 originates from the intestinal tract.

Previous studies have demonstrated downregulation of VIL1 in intestinal tissue of patients with Crohn's disease, attributed to chronic injury and cellular stress [29, 30], however, circulating VIL1 levels were not evaluated in these investigations. Other studies on rats found that after ischemic-reperfusion (i.e., acute) injury, VIL1 redistributes from the brush border towards the basolateral membrane, which was proposed to facilitate VIL1 leakage into the circulation as elevated serum VIL1 levels were also detected [31, 32]. However, the impact of—AD cirrhosis associated—acute-on-chronic type injury on VIL1 levels has not been investigated.

In the present study we investigated (1) VIL1 levels in serum and intestinal mucosa according to the severity of AD cirrhosis; (2) the associations between serum VIL1 levels, kidney and liver function, and inflammatory markers; (3) the associations between serum VIL1 levels and 90-day mortality and (4) whether serum VIL1 levels could improve risk stratification provided by current scoring systems.

Our study distinguishes itself from previous research on gut permeability markers by adopting a practice-oriented approach that specifically focuses on outcomes associated with acute gut mucosal barrier injury in AD cirrhosis. Based on our results, we propose serum VIL1 as a serologic indicator of this injury, demonstrating that its incorporation enhances the prognostic accuracy of existing risk stratification methods, thus addressing a critical gap. Building on these findings, we introduce the concept of 'gut mucosal barrier failure' as a potential seventh organ failure to be added to the CLIF-SOFA score.

2 | Patients and Methods

2.1 | Patient Populations

This study included two cohorts (discovery and validation) of patients hospitalised for AD with or without ACLF, recruited consecutively within the framework of the MICROB-PREDICT project between March 2017 and July 2018. The study protocol has been published previously [33]. Clinical information was collected at baseline and scheduled follow-up visits on Weeks 1, 4, 8 and 12. In addition, unscheduled visits were performed during the study at the time of hospital readmission

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due to a new AD episode or ACLF development. The latter was followed up with another visit 1 week after the development of ACLF. AD patients were categorised according to high (≥ 50) or low (< 50) chronic liver failure consortium (CLIF-C) AD-score [34] at inclusion and according to the 3-month outcome at the end of the follow-up period, as described by the PREDICT study (i.e., stable AD [SDC], unstable AD [UDC], pre-ACLF) [33]. Patients with ACLF were identified according to EASL-CLIF-C criteria [16].

The discovery cohort consisted of 130 patients (AD: $n = 108$; ACLF: $n = 22$) who were recruited exclusively from the Division of Gastroenterology, Department of Internal Medicine of the University of Debrecen. In this cohort, no liver transplantations occurred, and all patients were followed through. The validation cohort consisted of 208 patients from 25 European hospitals, for whom serum samples were available (AD: $n = 201$; ACLF: $n = 7$). Patients in the validation cohort who underwent liver transplantation ($n = 15$) or were lost to follow-up after discharge ($n = 9$) were excluded from survival analyses. Fifty healthy controls (HC) were recruited at the University of Debrecen.

2.2 | Ethics Approval and Consent to Participate

All research was conducted in accordance with the Declarations of Helsinki and Istanbul. Ethical approval for the study, including analysis of biopsy samples collected for diagnostic purposes, was obtained from the Regional and Institutional Research Ethics Committee of the University of Debrecen and the National Scientific and Research Ethics Committee (58361-2/2016/EKU, 35281-2/2017/EKU and 41192-5/2018/EÜIG) and within the framework of the PREDICT study. Each patient or legal guardian was informed of the nature of this study and signed an informed consent form concerning participation in the study and the publication of the collected data.

2.3 | Serum Samples and Serological Measurements

Blood samples were collected at enrolment. In the discovery cohort, additional samples were obtained at ACLF development. Sera were isolated by centrifugation after half an hour of blood collection and aliquots were kept frozen at −80°C until testing. Validation samples were transferred according to biobanking instructions. Serum VIL1 and sCD163 levels were determined by commercially available solid-phase enzyme-linked immunoassays (Fine Test; Wuhan, Hubei, China, Cat.: EH3957; and IQProducts, Groningen, Netherlands, Cat.: IQP-383, respectively) using an ETI-MAX 3000 machine. The detection limit of the assay was 0.09 ng/mL. Presepsin levels were measured by a chemiluminescent PATHFAST presepsin analyser (Mitsubishi Chemical Medience Corporation, Tokyo, Japan). The detection limit was 20 pg/mL. Cytokine levels were determined using a multiplexed bead-based immunoassay (Merck Millipore, Darmstadt, Germany) on a Luminex 100 Bioanalyzer (Luminex Corp., Austin, TX). The assays were performed according to the manufacturers' instructions in a blinded fashion without prior knowledge of the patient's clinical information.

2.4 | Biopsy Samples and VIL1 Immunohistochemistry

In a subset of patients ($n = 49$) and HCs ($n = 11$), after routine pathological evaluation (including quantification of immune cell infiltration), residual duodenal biopsy specimens obtained at diagnostic endoscopic procedures at enrolment were available and were subjected to immunohistochemical staining (IHC) for VIL1. Anti-human VIL1 primary antibody (Proteintech, UK, Cat.: 66096-1-Ig, RRID: AB_2881495) and a mouse-specific secondary antibody (R&D Systems, MN, USA, Cat.: VC001, RRID: AB_2927375) were used. Visualisation was performed with DAB Eqv (Vector Labs, MA, USA, Cat.: SK-4103). Results were evaluated by an experienced gastrointestinal pathologist (AB) using the semi-quantitative histology (*H*-)score method [35] in a blinded fashion. Biopsies were obtained based on endoscopic results to support diagnosis, always considering the safety of sampling on a case-by-case basis.

2.5 | Statistical Analysis

Variables were tested for normality using the Shapiro–Wilk's W test. Categorical variables were summarised as frequencies and percentages and compared with the χ^2 test. Continuous variables were summarised as medians and interquartile range (IQR, 25th–75th percentiles) and were compared with the Mann–Whitney U test or Kruskal–Wallis H-test with Dunn's multiple comparison post hoc analysis. The Spearman's nonparametric rank correlation test was used to determine correlations. Cases with missing data were excluded. The ability of VIL1 to discriminate between survivors and non-survivors was assessed by receiver operating characteristics (ROC) curve analysis plotting sensitivity% vs. 100-specificity%. The area under the curve (AUROC) and the corresponding 95% confidence intervals (CI) were calculated. The Youden index, indicating the maximum value of sensitivity + specificity, was chosen to estimate the best discriminative threshold. In the validation cohort, both cut-off values—derived from the discovery and the validation cohorts—were applied in subsequent analyses. Kaplan–Meier survival curves were plotted to estimate the cumulative probability of 90-day survival in different groups. Differences in observed survival rates were assessed using the log-rank test. The association between logarithmically transformed VIL1 levels and mortality during the follow-up was evaluated by univariable Cox-regression analysis to avoid optimised cut-off value related bias. Multivariable analysis with forced entry method was performed to adjust for the severity of AD (CLIF-C AD score of < 50, ≥ 50, and ACLF) and number of precipitating events. Subsequently, other variables were also tested with forward or backward stepwise procedures. Associations are given as hazard ratio (HR) with 95% confidence intervals (CI). For statistical analysis and graphical presentation, the SPSS v.29.0 (SPSS, Chicago, IL), and GraphPad Prism 10.2.1 (San Diego, CA) programs were used. A two-sided probability (p) value of < 0.05 was considered to be statistically significant.

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3 | Results

3.1 | Baseline Parameters of Patients

Clinical and laboratory characteristics of patients at admission as well as outcome data are summarised in Table 1 for all patients and in Table S1 for a subpopulation with duodenal biopsy samples of the discovery cohort. Differences were observed between the discovery and validation cohorts in the proportion of ACLF patients (16.9% vs. 3.4%) etiological causes, number of identified precipitating events and the extent of inflammation. Tables S2 and S3 provide these details broken down by severity groups

TABLE 1 | Clinical and laboratory characteristics of patients with acutely decompensated cirrhosis at admission and short-term mortality rates.

	Discovery cohort ($n=130$)	Validation cohort ($n=208$)	p
Age	60 (53–68)	59 (52–68)	0.931
Sex (m/f)	86/44 (66.2/33.8)	137/71 (65.9/34.1)	>0.999
Aetiology (alcohol/viral/both/other)	109/5/5/11 (83.9/3.8/3.8/8.5)	109/24/17/58 (52.4/11.5/8.2/27.9)	**<0.001**
SDC/UDC/pre-ACLF/ACLF	68/18/22/22 (52.3/13.8/16.9/16.9)	114/55/32/7 (54.8/26.4/15.4/3.4)	**<0.001**
CLIF-C AD score <50/≥50	39/69 (30.5/53.9)	75/127 (36.1/61.1)	0.902
CLIF-C AD score	51.5 (47.3–59)	52 (47–56.5)	0.464
CLIF-C ACLF score	48 (43–51)	46.5 (43.8–47.8)	0.431
MELD score	16 (12–21)	16 (12–20)	0.416
Child-Pugh A/B/C	17/57/56 (13.1/43.8/43.1)	18/101/73 (8.7/48.6/35.1)	0.261
White blood cells	7.78 (5.30–10.18)	6.2 (4.113–8.4)	**<0.001**
Platelets	126 (85–187)	98 (64–141)	**<0.001**
INR	1.35 (1.13–1.65)	1.4 (1.24–1.69)	**0.032**
Albumin (g/L)	30 (25–35)	28 (25–32)	0.121
AST (U/L)	74 (36–148)	53 (33–81)	**0.002**
ALT (U/L)	33 (120–56)	29 (19–43.3)	0.092
ALP (U/L)	154.5 (101–224)	127.5 (95–189.3)	**0.013**
γGT (U/L)	214 (75–461)	77 (40.4–171.6)	**<0.001**
Bilirubin (μmol/L)	60.8 (18.7–141.8)	39.6 (23.1–82.7)	0.106
Creatinine (μmol/L)	79 (52–110)	80 (65–108)	0.319
CRP (mg/L)	27.1 (9.96–52.35)	17.30 (7.11–39.6)	**0.024**
Ascites	93 (71.5%)	118 (56.7%)	**0.005**
Hepatic encephalopathy	32 (24.6%)	59 (28.4%)	0.529
GI bleeding	30 (23.1%)	20 (9.6%)	**<0.001**
Precipitating events			
Any	76 (58.5)	75 (36.1)	**<0.001**
Single	63 (82.9)	67 (89.3)	0.347
Multiple	13 (17.1)	8 (10.7)	
Severe AH	36 (27.7)	28 (13.5)	**0.002**
Bacterial infection	40 (30.8)	50 (24.0)	0.206
GIB with shock	7 (5.4)	5 (2.4)	0.225
Mortality Day-28	14 (10.8%)	19 (9.1%)	0.999
Mortality Day-90	27 (20.8%)	39 (18.8%)	0.887

Note: Data are presented as median (interquartile range) or *n* (%). Statistically significant *p* valuse are indicated with bold numbers.
Abbreviations: γGT, gamma glutamyl transferase; ACLF, acute-on-chronic liver failure; ALP, Alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; GI, gastrointestinal; INR, international normalised ratio; SDC, stable decompensated cirrhosis; UDC, unstable decompensated cirrhosis.

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in both cohorts. No differences between the relative number of organ failures were found between ACLF populations of the two cohorts at inclusion (Table S4).

3.2 | Villin-1 Levels in Duodenal Tissue and Serum

In duodenum tissue samples, we observed a significant decrease in VIL1 protein expression according to *H*-score in all outcome-related severity groups of patients with cirrhosis compared to HC (Figure 1A). This finding corresponds to VIL1 down-regulation due to chronic injury described in the literature [29]. The most differentiated cells at the villus tip were less affected than more immature cells at the crypts. Additionally, a negative correlation was observed between VIL1 *H*-score and tissue plasma cell ($r = -0.385$, $p = 0.006$) and lymphocyte counts ($r = -0.343$, $p = 0.016$).

In line with the IHC results, decreased serum VIL1 levels were observed in the least severe patient group (SDC) in the discovery

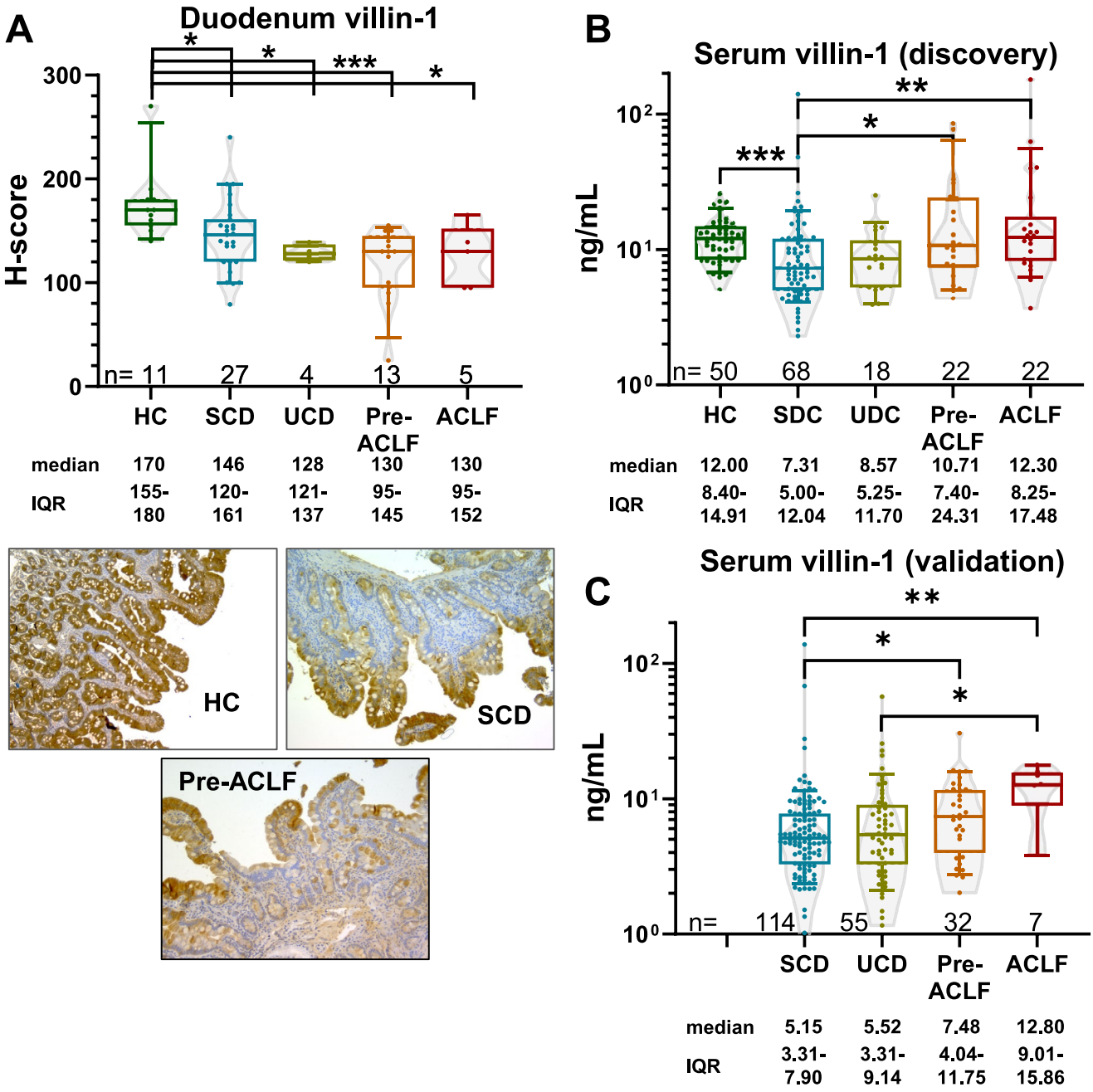

FIGURE 1 | Duodenum and serum villin-1 levels in various severity groups and healthy controls. Villin-1 level was decreased in patients with stable decompensated cirrhosis (SDC) both in duodenum tissue and serum compared to healthy controls (HC). In the tissue, similar villin-1 levels were observed across severity groups. Representative histological images are shown with 200-fold magnification (A). In serum, villin-1 levels increased parallel to severity (B, C). Patient numbers in subgroups are indicated in Table 1. Box-and-whiskers graphs represent median with interquartile range (IQR), 10th and 90th percentile. Only statistically significant differences are indicated: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. ACLF, acute-on-chronic liver failure; *H*-score, histology-score; UDC, unstable decompensated cirrhosis.

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cohort compared to HCs. However, in accordance with the increasing VIL1 leakage due to more severe acute mucosal injury in more severe AD patients (UDC, pre-ACLF and ACLF), a gradual and significant increase in serum VIL1 levels was detected (Figure 1B). We made the same observation in sensitivity analysis when we only included patients with duodenal biopsy samples (Figure S1). In the validation cohort, the absolute serum VIL1 values were lower compared to the discovery cohort except for the ACLF group; however, the significant gradual increase among the AD groups was confirmed (Figure 1C).

3.3 | Increased Serum Villin-1 Levels Are Associated With ACLF

Serum VIL1 levels were highest in ACLF patients (Figure 1B,C) but were also significantly elevated in pre-ACLF patients compared to those who did not develop ACLF within 3 months of inclusion (SDC and UDC groups; Figure 2A,B), indicating that VIL1 levels start to increase in a portion of patients prior to ACLF development. Longitudinal investigation revealed that the increase in serum VIL1 levels observed at ACLF development was not statistically significant compared to pre-ACLF admission concentrations. However, in ACLF patients we observed a significant gradual increase in serum VIL1 levels in parallel with ACLF grade (Figure 2C). All of these findings suggest an association between acutely increased serum VIL1 levels and the extent of AD-related systemic injury.

3.4 | Serum Villin-1 Levels Correlate With CLIF-C Kidney but Not Liver Failure Score

VIL1 levels were found to be mildly correlated with white blood cell ($r=0.279$, $p=0.001$) and neutrophil counts ($r=0.261$, $p=0.003$). They were also positively correlated with creatinine

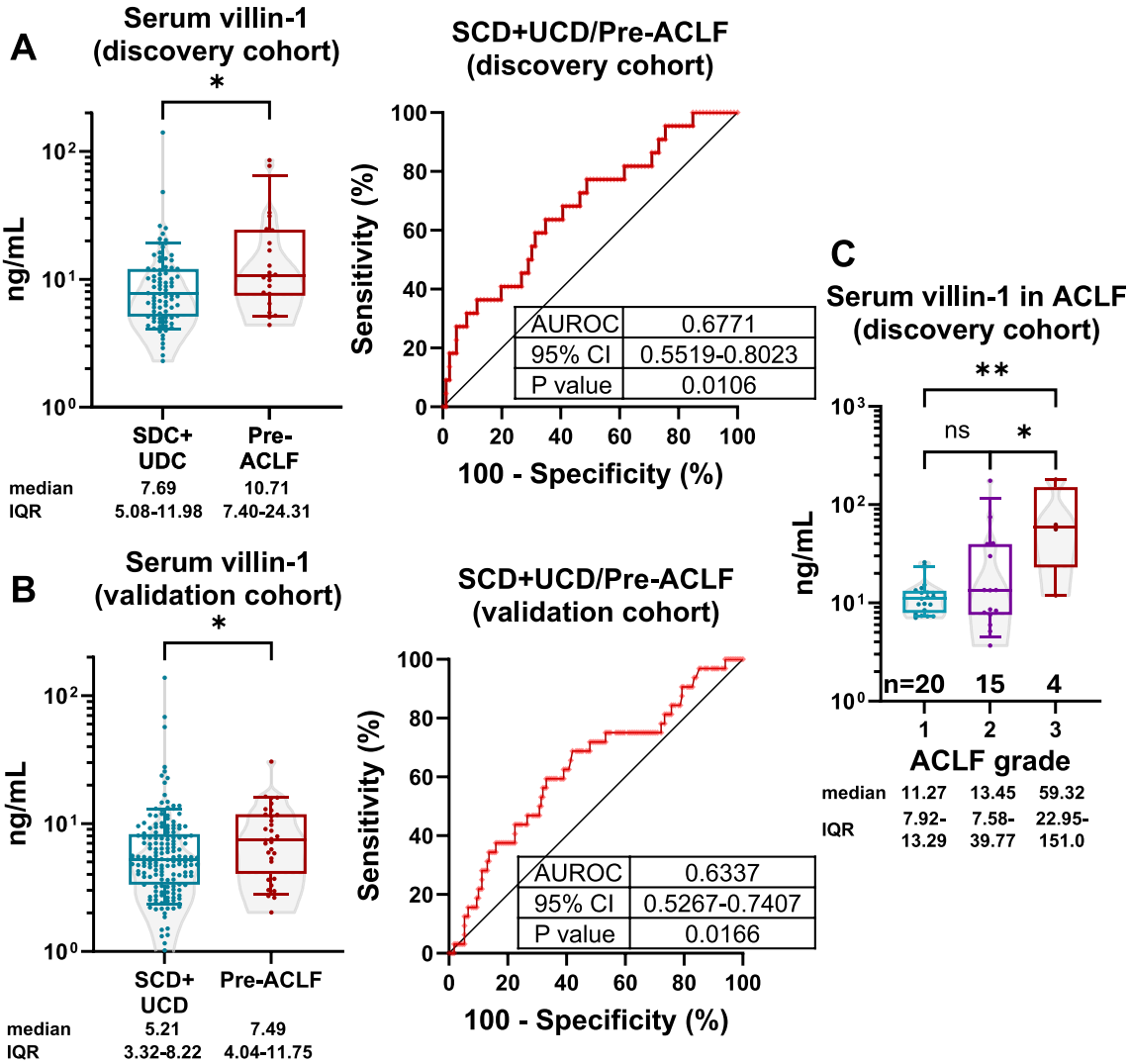

FIGURE 2 | Serum villin-1 levels are increased prior to (pre-ACLF) and decreased after ACLF. Serum villin-1 levels are significantly elevated in pre-ACLF patients compared to patients who did not develop ACLF within 3 months of inclusion, that is, patients with stable (SDC) and unstable (UDC) decompensated cirrhosis (A, B). In ACLF patients significant gradual increase in serum VIL1 levels is demonstrated in parallel with ACLF grade (C). Box-and-whiskers graphs represent median with interquartile range (IQR), 10th and 90th percentile. *Indicates <0.05, **indicates <0.01 and ns indicates non-significant p value. ACLF, acute-on-chronic liver failure, AUROC, area under the receiver operating characteristic curve; CI, confidence interval.

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($r = 0.344$, $p < 0.001$). Consequently, serum VIL1 levels showed an increasing trend by CLIF-C renal failure score reaching statistical significance between patients with renal failure scores of 1 and 3 (but not 2) (Figure S2A,B). However, VIL1 did not correlate with liver enzymes (AST, ALT, γGT, ALP) or liver function tests (albumin, INR or total bilirubin) and we observed no association between VIL1 levels and CLIF-C liver failure score (Figure S2C). Based on these results, the liver is an unlikely source of serum VIL1, but kidneys might contribute to elevated levels when severely injured. To further dissect this possibility, we divided patients into the following subgroups: (1) non-ACLF AD; (2) ACLF with only kidney failure; (3) ACLF with no kidney failure and (4) ACLF with kidney and other organ failures. This analysis revealed an increasing trend and demonstrated that 'other' organ failures in general were associated with serum VIL1 elevation comparably to renal failure, with an additive effect observed (Figure S2D). Additionally, aetiology of cirrhosis was not associated with VIL1 levels (data not shown).

3.5 | Increased Serum Villin-1 Levels Predict 90-Day Mortality

Serum VIL1 levels were significantly increased in non-survivor patients compared to survivors (median [IQR]: 13.94 [7.95–31.19] vs. 8.23 [5.27–12.07] ng/mL; Figure 3A). ROC analysis demonstrated an AUROC of 0.721 (Figure 3B), which was comparable with the ROC curve of Model for End-Stage Liver Disease (MELD) score (AUROC: 0.722; difference: 0.001, $p = 0.988$; Figure S3) and surpassed AUROCs of creatinine (0.656) and bilirubin (0.658) utilised to identify kidney and liver failure, respectively. Based on serum VIL1's best discriminatory cut-off value (> 12.79 ng/mL; associated sensitivity: 56%, specificity: 81.55%), we plotted Kaplan–Meier curves that showed a significantly higher incidence of mortality in the group with high VIL1 levels compared to that with low levels (44.2% vs. 12.5%, log-rank $p < 0.001$). This was also associated with shorter mean time to death in the high VIL1 group (62.5 ± 5.9 vs. 82.4 ± 2.1 days; Figure 3C). These results were confirmed in the validation cohort (Figure 3D–F). However, the best discriminative threshold, in line with generally lower VIL1 levels, was 7.04 ng/mL in this cohort. Therefore, we performed the Kaplan–Meier analysis with both cut-off values and observed a significantly increased mortality rate in patients with increased serum VIL1 levels defined by either threshold (Figure 3F, Figure S4). Interestingly, both cut-off levels were approximately at the second tertile of the corresponding patient population (discovery: 11.91; validation: 7.77 ng/mL).

Since gut barrier injury-driven mortality is expected to be associated with higher levels of inflammation, next, we investigated whether patients with elevated VIL1 levels have increased concentrations of inflammatory markers. Patients with > 12.79 ng/mL serum VIL1 levels exhibited increased levels of IL-6, TNF-α, IL-10, IL-1Ra, IL-8, MCP-1, MIP-1α, MIP-1β, sCD163, presepsin, procalcitonin and decreased levels of IL-4 (Figure 4).

We also investigated whether VIL1 can provide incremental prognostic value in addition to these systemic inflammatory indicators. Therefore, we evaluated the 90-day mortality predicting ability of these markers. Most molecules' performance was mild or moderate (data not shown) but IL-6 emerged as the best predicting marker among them with an AUROC of 0.749 (CI: 0.655–0.844), $p < 0.001$. Therefore, we stratified patients by the median IL-6 level and assessed the prognostic value of VIL1 within both the low and high inflammation subgroups. In the subgroup of patients with low systemic inflammation (IL-6 < median), we observed that the overall 90-day mortality rate was low (10.9%). Consequently, increased serum VIL1 levels (> 12.79 ng/mL) could not provide significant stratification in this subgroup. Conversely, increased serum VIL1 demonstrated strong prognostic discriminative power in the high IL-6 subgroup. Within this cohort of patients—already at high risk due to severe systemic inflammation—VIL1 effectively stratified outcomes based on its cut-off value (12.79 ng/mL). Patients with elevated IL-6 and VIL1 levels had a substantially higher 90-day mortality compared to those with high IL-6 but low VIL1 levels (56.5% vs. 14.6%, log-rank $p < 0.001$; Figure 5).

3.6 | Increased Serum Villin-1 Levels Improve the 90-Day Mortality Predicting Ability of AD Severity Groups and the Number of Precipitating Events

Next, we investigated whether the addition of high VIL1 level (> 12.79 ng/mL) could increase the 90-day mortality-predicting capability of currently used severity measures. For this, we categorised patients into three severity groups: CLIF-C AD score < 50, ≥ 50 and ACLF (Figure S5). Since in our discovery cohort, there was only one death among patients with CLIF-C AD score < 50 we didn't expect any additional risk stratification benefit by further sorting this group. Therefore, we only applied additional categorization based on VIL1 cut-off to the 2 more severe groups (Figure S6). The mortality rates in these groups were primarily separated based on VIL1 level rather than the presence of ACLF; thus, we combined the groups accordingly. This final graph (Figure 6A) revealed that patients with severe AD (either CLIF-C AD score ≥ 50 or ACLF) and high VIL1 exhibited a markedly higher incidence of mortality compared to others. This was confirmed in the validation cohort using both cut-off levels (Figure 6B, Figure S7).

Next, we tested whether increased VIL1 level can improve risk stratification provided by ACLF grades. Using > 12.79 ng/mL serum VIL1 as a threshold for defining an additional organ (gut mucosal barrier) failure, a significant portion of higher-risk patients were identified and reclassified to a higher risk (ACLF grade) category, most apparently from group 0 to 1 and from 2 to 3 (Figure S8).

Additionally, we investigated VIL1 levels according to the resolution of ACLF. We divided ACLF patients into three groups: patients who were discharged and survived over 1 year, those discharged but not stabilised and died within 1 year and those who died during the same hospitalisation. In this comparison, we observed an increasing trend and a significant difference between the 'recovered' and the 'in-hospital mortality' groups (Figure S9).

Since acute gut mucosal barrier injury/failure with increased intestinal permeability can be not only a complication of AD but also a triggering factor, in the next analysis, we considered high serum VIL1 level as the precipitating event and added it to the

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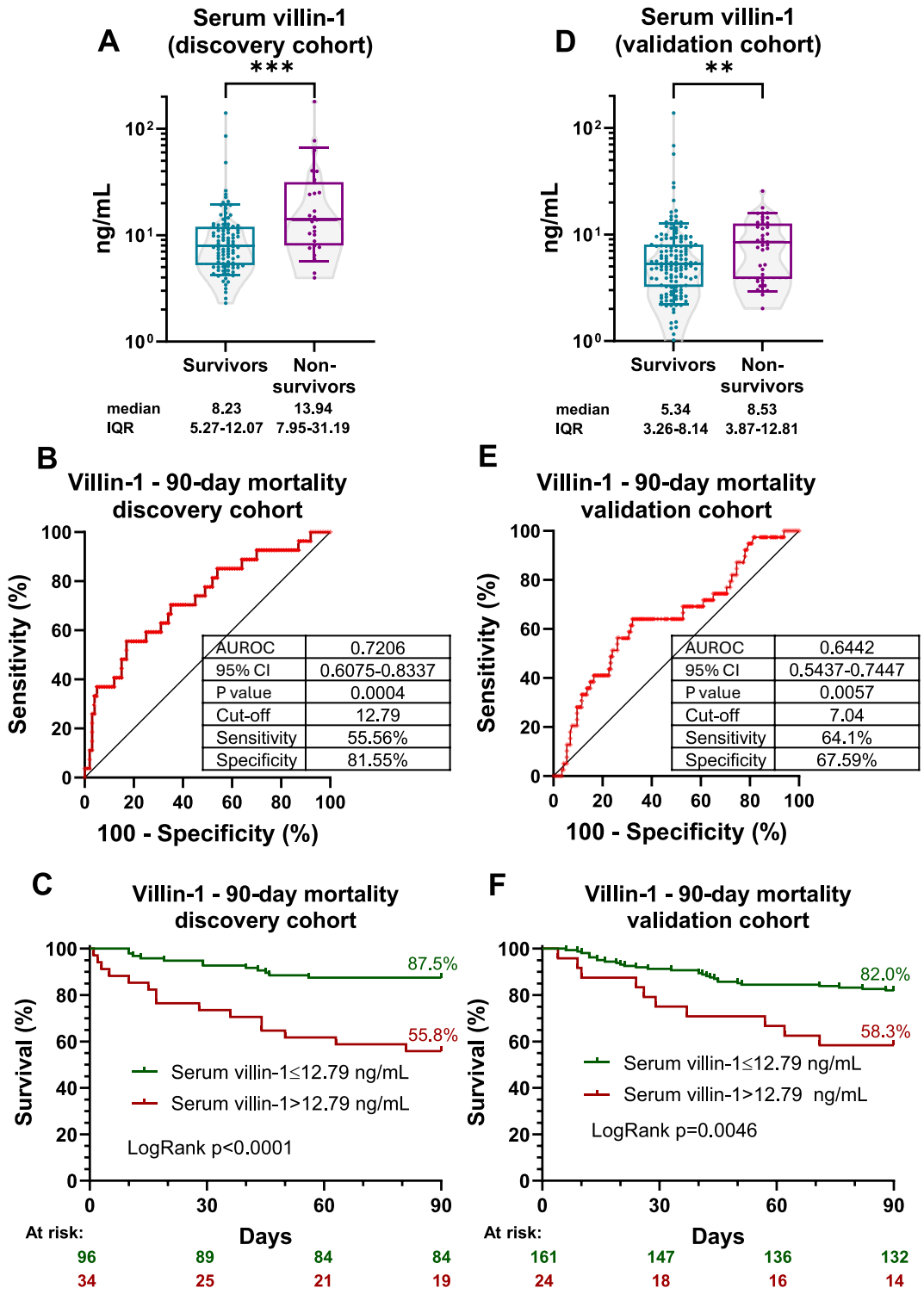

FIGURE 3 | Increased serum villin-1 levels are associated with increased 90-day mortality rate. Serum villin-1 levels are significantly increased in non-survivor patients compared to survivors (A, D). In receiver operating characteristic (ROC) analysis, increased villin-1 levels could discriminate between survivors and non-survivors. Best discriminatory cut-off levels are defined (B, E). Based on this cut-off, increased villin-1 levels are associated with increased incidence of 90-day mortality in Kaplan–Meier analysis. Numbers of patients at risk are displayed at every 30 days (C, F). Box-and-whiskers graphs represent median with interquartile range (IQR), 10th and 90th percentile. **Indicates < 0.01 and ***indicates < 0.001 for *p* value. ACLF, acute-on-chronic liver failure; AUROC, area under the receiver operating characteristic curve; CI, confidence interval.

number of the previously identified ones. Kaplan–Meier curves showed significantly improved discrimination between groups in the discovery cohort (Figure 6C), however, in the validation cohort, the number of precipitating events was not predictive for mortality (Figure S10); therefore, we could not carry out this analysis in this cohort.

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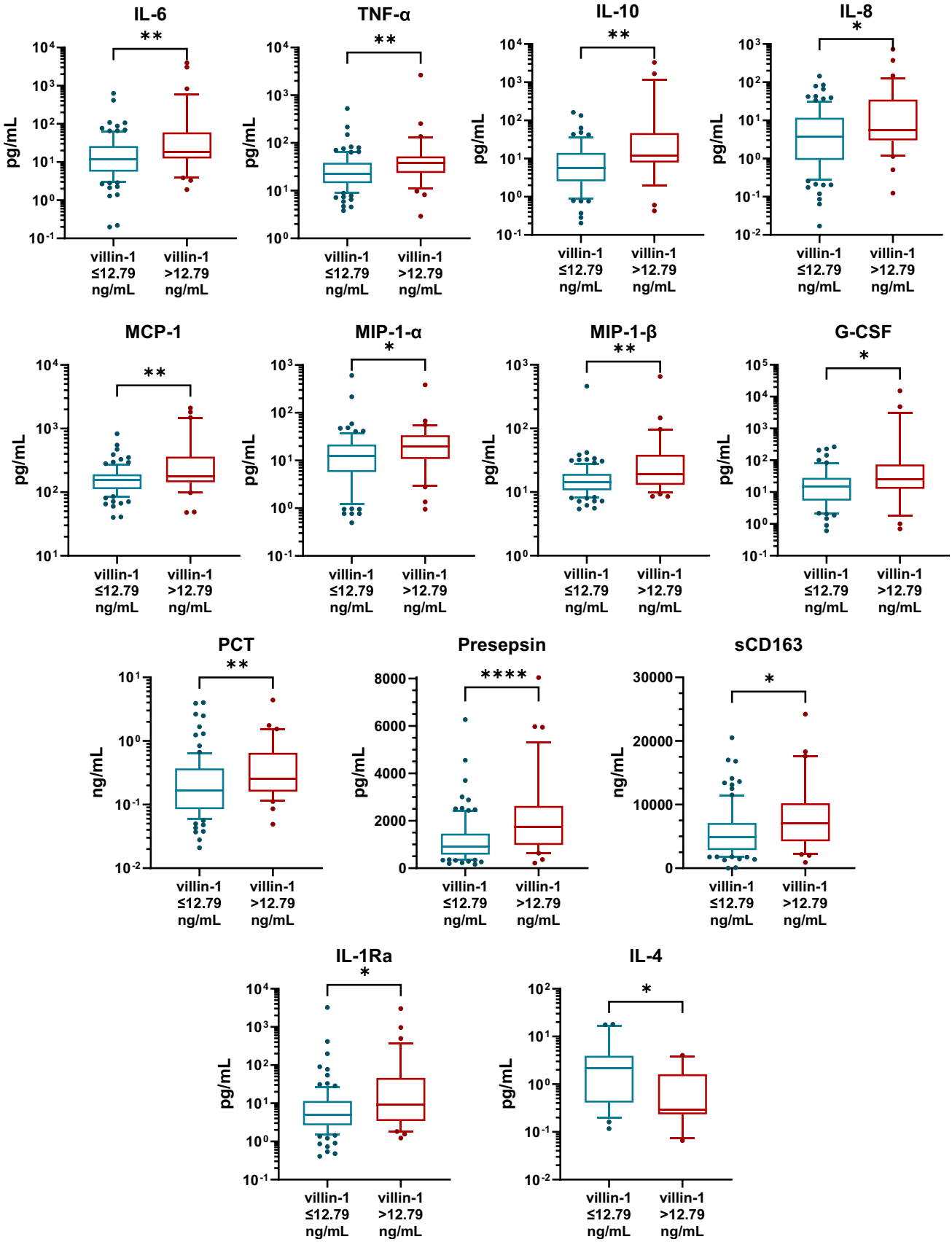

FIGURE 4 | Increased levels of inflammatory molecules are associated with elevated serum villin-1 level in the discovery cohort. Box-and-whiskers graphs represent median with interquartile range (IQR), 10th and 90th percentile. Statistical significance is indicated as: $*p < 0.05$, $**p < 0.01$ and $****p < 0.0001$. G-CSF, granulocyte colony-stimulating factor; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PCT, procalcitonin; sCD, soluble cluster of differentiation; TNF, tumour necrosis factor.

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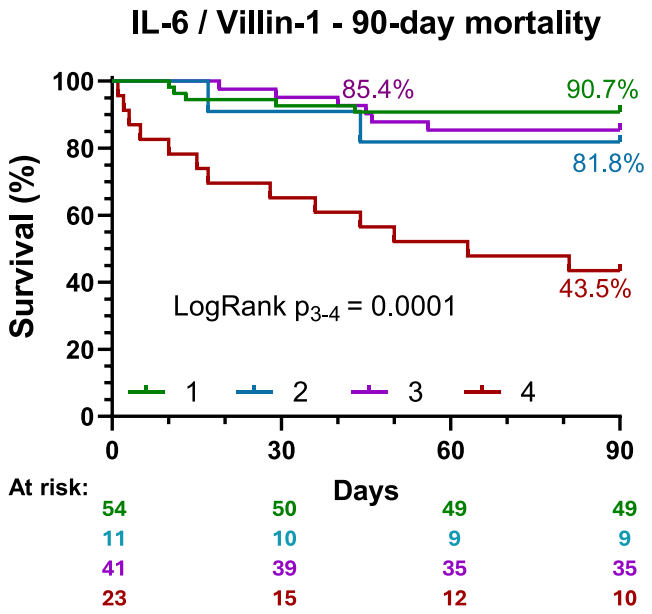

FIGURE 5 | Incremental prognostic value of high serum villin-1 levels over IL-6-indicated systemic inflammation. Patients were divided into the following groups: (1) low systemic inflammation—low serum villin-1; (2) low systemic inflammation—high serum villin-1; (3) high systemic inflammation—low serum villin-1 and (4) high systemic inflammation—high serum villin-1. High systemic inflammation was defined > median IL-6 levels. High serum villin-1 was defined by >12.79 ng/mL. High serum villin-1 levels demonstrated strong prognostic discriminative power in the high IL-6 subgroup.

Next, we performed Cox-regression analysis of logarithmically transformed serum VIL1. We found that VIL1 level increase was a significant predictor of 90-day mortality even after adjusting for the severity of AD and the number of precipitating events in the discovery cohort (Table 2). Since the number of precipitating events was not a significant predictor of mortality in the validation cohort, here we adjusted only for disease severity. Serum VIL1 levels were confirmed as an independent predictor of 90-day mortality also in this cohort (Table 2). Subsequently, we tested VIL1's predictive ability against additional parameters. IL-6 (after logarithmic transformation) had an HR of 1.774 (CI: 1.401–2.246), $p < 0.001$. When it was used alone as well as when added to the previously described model to adjust for the extent of inflammation, VIL1 was still an independent predictor of mortality in both cases. Next, we tested VIL1 levels against MELD and CLIF-C AD scores. Both these analyses confirmed VIL1 as an independent predictor in both cohorts. Finally, we evaluated clinical and laboratory parameters listed in Table 1 using univariate Cox regression. The individual parameters with p values <0.1 were subsequently included in a multivariable regression with a forward selection procedure. In this model, VIL1 remained an independent predictor with the second highest HR value after INR. This model was subsequently tested in the validation cohort that confirmed these results.

3.7 | Serum Villin-1's 90-Day Mortality Predicting Ability Is Independent From the Presence of Kidney Failure

Since our results have raised the possibility that severe acute kidney injury (AKI) may contribute to elevated serum VIL1 levels in AD cirrhosis, we investigated whether the ability of VIL1 to predict mortality is independent of AKI status. If AKI were a major driver of increased serum VIL1 levels, then VIL1 would fail to adequately discriminate between survivors and non-survivors within the subgroups of patients stratified by the presence or absence of AKI. To test this, we classified patients based on AKI status using CLIF-C criteria and further categorised them by serum VIL1 levels. Kaplan–Meier survival curves showed comparable mortality rates between patients with low VIL1 levels, regardless of AKI presence, as well as between those with high VIL1 levels, with or without AKI (Figure 7). This result indicates that the prognostic ability of serum VIL1 is independent of renal failure, thereby reducing the probability that the kidneys have a major impact on serum VIL1 levels, supporting the gut as the primary origin.

4 | Discussion

Regardless of the well-established impact of the gut-liver axis on liver diseases, this key aspect is currently absent from severity evaluation and prognostication of AD cirrhosis due to a lack of appropriate biomarkers. We have shown evidence that serum VIL1 level may be suitable to assess gut mucosal barrier injury in AD cirrhosis, providing a reliable and non-invasive approach and validating our results in an independent external cohort. Our work also presents original insights into VIL1's role in the progression and outcome of AD cirrhosis.

VIL1 is the major actin-binding protein in microvilli. Its expression in VIL1-null cells reorganises the actin cytoskeleton and promotes the formation of microvilli-like structures, while VIL1 knockdown in intestinal epithelial cells reduces brush border assembly [27, 36]. Therefore, VIL1's redistribution from the brush border to the basolateral membrane may destabilise actin filaments in microvilli. Elevated intracellular calcium levels, in response to cellular injury or stress, activate VIL1's actin-severing function [27]. These potentially lead to microvilli disassembly or shortening. Microvilli radically increase enterocyte surface area, aiding nutrient absorption and hosting enzymes essential for final digestion [37]. The dense array of microvilli also forms a physical barrier, shielding the body of epithelial cells from pathogens [27]. Thus, microvilli loss may weaken barrier functions and impair energy homeostasis through malabsorption. Indeed, histological analysis revealed increased lymphocytic and plasma cell infiltration in association with decreased VIL1 tissue expression, consistent with compromised barrier integrity. Additionally, calcium-activated VIL1's cytoplasmic relocation may cause broader actin disorganisation, potentially affecting cell shape and intracellular transport. Since tight junctions are connected to and regulated by the actin cytoskeleton [38], actin disruption could also weaken these structures, increasing paracellular permeability.

While VIL1 is downregulated in the tissue in response to injury [29], it also leaks into the circulation during an acute hit [31, 32]. Consequently, we observed that serum VIL1 levels in AD cirrhosis exhibit bimodal dynamics, with a decrease in SDC patients compared to HC and a re-elevation due to intensified tissue leakage upon superimposed acute injury [31, 32]. Thus, it reflects the extent of acute mucosal damage, leading to and indicated by increased serum levels of inflammatory markers, and is associated with short-term mortality in these patients.

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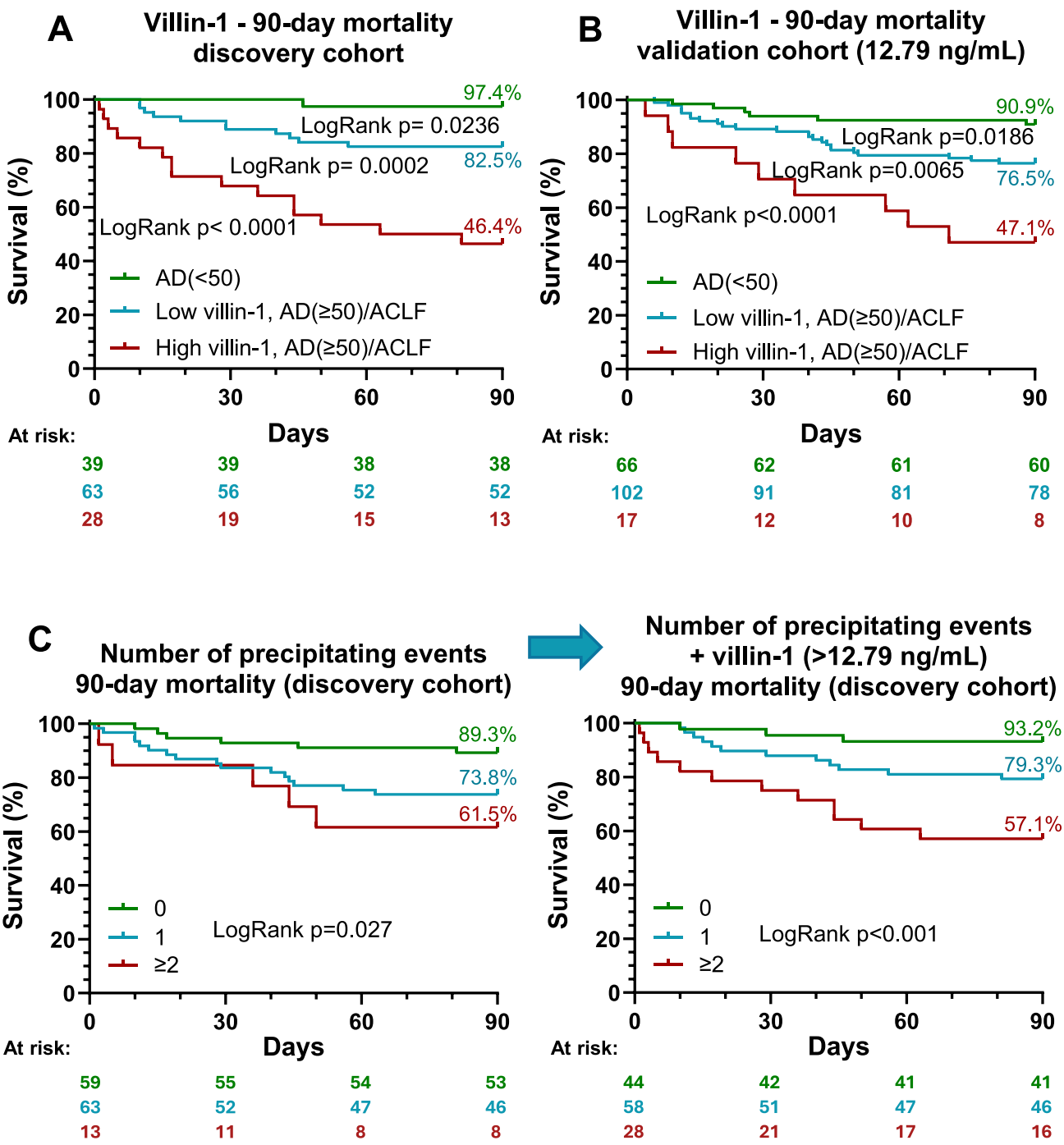

FIGURE 6 | The addition of high villin-1 level (> 12.79 ng/mL) to currently used severity measures improves the accuracy of disease outcome prediction. Patients with high villin-1 level (> 12.79 ng/mL) and severe acute decompensation (AD) defined by chronic liver failure consortium (CLIF-C) AD score ≥ 50 or acute-on-chronic liver failure (ACLF) have significantly increased 90-day mortality rate compared to other groups (A, B). Adding high villin-1 level to the number of precipitating events results in the recategorisation of patients, improving 90-day mortality prediction. Overall log-rank *p* values are shown (C). Numbers of patients at risk are displayed at every 30 days.

This is perfectly in line with the ACLF paradigm, which defines organ failure as organ-specific impairment identified by established markers exceeding mortality-associated threshold values [16]. Importantly, in the context of ACLF, organ failure does not imply a complete molecular collapse but rather a significant functional decline that impacts survival. While organ injury progresses gradually, the cut-off levels in ACLF are set arbitrarily, based on their association with mortality. Similarly, serum VIL1 aligns with established organ failure criteria by demonstrating the key characteristics: it serves as an indicator of gut mucosal damage and functions as a predictor of short-term mortality. As a single marker, its discriminative power for survival (AUROC: 0.721) is comparable to the composite MELD score (0.722) and surpasses that of

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TABLE 2 | In Cox regression analysis, serum villin-1 predicts 90-day mortality independently of severity measures in both the discovery and validation cohorts.

	Univariable analysis			Multivariable analysis		
	HR	**95% CI**	***p***	**HR**	**95% CI**	***p***
Discovery cohort						
(ln) villin-1	2.405	1.636–3.535	**< 0.001**	2.518	1.648–3.848	**< 0.001**
Severity of AD						
AD < 50		Reference			Reference	
AD ≥ 50	10.117	1.341–76.298	**0.025**	4.731	0.561–39.903	0.153
ACLF	24.161	3.090–188.927	**0.002**	16.616	2.047–133.220	**0.008**
Number of PE						
0		Reference			Reference	
1	2.693	1.054–6.884	**0.039**	2.040	0.743–5.599	0.166
2	4.266	1.301–13.986	**0.017**	4.344	1.117–16.896	**0.034**
Validation cohort						
(ln) villin-1	1.540	1.074–2.209	**0.019**	1.491	1.033–2.150	**0.033**
Severity of AD						
AD < 50		Reference			Reference	
AD ≥ 50	3.302	1.337–7.917	**0.007**	3.212	1.339–7.705	**0.009**
ACLF	5.437	1.097–26.953	**0.038**	4.444	0.890–22.188	0.069
Number of PE						
0		Reference				
1	1.256	0.614–2.569	0.533			
2	1.600	0.374–6.845	0.526			

Abbreviations: (ln), logarithmically transformed; ACLF, acute-on-chronic liver failure; AD < 50, CLIF-C AD score < 50; AD, acute decompensation; CI, confidence interval; HR, hazard ratio; PE, precipitating event.

creatinine (0.656) and bilirubin (0.658), markers of kidney and liver failure, respectively. Furthermore, when combined with existing severity estimation methods (CLIF-C AD score and presence of ACLF), it further enhances prognostic accuracy in advanced disease stages. This suggests that VIL1 provides insight into an aspect of the disease that has been previously overlooked in these scores, which is in line with the neglected role of acute gut mucosal barrier injury. Moreover, serum VIL1 levels demonstrate synergistic effects also with IL-6 in predicting poor outcomes.

To date, there is no clear or widely accepted definition of gut failure in the literature. The intestinal tract, similar to the liver, has multiple functions. In liver diseases, the barrier function is of key importance. Our study lays down the concept of gut mucosal barrier failure in AD cirrhosis as an additional organ failure to the six currently included in the CLIF-SOFA score. Furthermore, we are proposing that serum VIL1 is a promising marker candidate to address the hitherto unmet clinical need to incorporate acute gut mucosal barrier injury in the risk stratification of these patients.

Based on our results, patients with severe AD (CLIF-C AD score ≥ 50) and high VIL1 levels (considered as an indicator for gut mucosal barrier failure) could be reclassified as ACLF, since these two groups had similar 90-day mortality rates (52.3% vs. 54.5%, respectively). Moreover, serum VIL1 was able to improve risk stratification provided by ACLF grade in our discovery cohort.

Importantly, improved risk assessment could help correctly allocate clinical attention and resources making newly identified ACLF patients eligible for intensive monitoring and treatment ultimately contributing to improved outcomes. An established gut barrier failure marker might also open avenues for optimised antibiotics or other gut-targeted therapies, thereby personalising management beyond current models. For example, in a patient with elevated inflammatory markers and/or systemic inflammatory response syndrome (SIRS) but no clear infectious source, elevated VIL1 levels would strongly suggest bacterial translocation instead of an overt infection, which might be treated by non-absorbing antibiotics rather than broad-spectrum systemic ones. Similarly, an elevated gut injury marker level might serve as an indication for antibiotic

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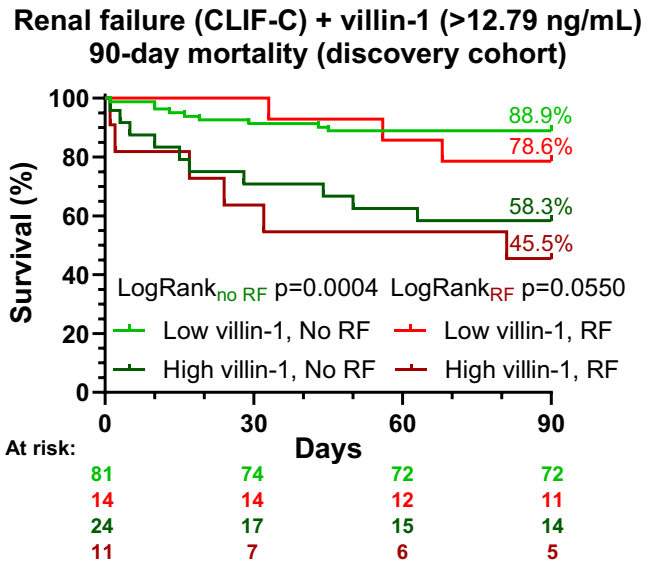

FIGURE 7 | High villin-1 level (>12.79 ng/mL) can predict 90-day mortality independently of the presence of renal failure. In this analysis, we used the data and samples of pre-ACLF patients from the time of ACLF development when available to boost the numbers in the kidney failure group. Renal failure (RF) is based on chronic liver failure consortium (CLIF-C) renal failure score. Numbers of patients at risk are displayed at every 30 days.

prophylaxis in the future to prohibit the development of SIRS. Nonetheless, these possibilities will need careful evaluation by future studies.

Additionally, an established gut failure marker might be able to identify patients most likely to benefit from therapies aimed at enhancing the gut barrier (e.g., tight junction modulators, mucus secretagogues, and so forth) for future clinical trials and serve as a primary surrogate endpoint to demonstrate target engagement and biological efficacy.

The concept that gut barrier failure could be considered a novel organ failure in the ACLF framework is further supported by the fact that serum VIL1 showed the highest median level in patients with ACLF and showed a gradual increase by ACLF grade. High levels were also detected prior to the development of ACLF (i.e., in pre-ACLF patients) suggesting VIL1 is an early biomarker in the development of ACLF. All of these observations suggest a close and direct link between VIL1 and AD-related systemic injury leading to organ failures.

In accordance with the proposed triggering role of gut barrier injury in AD, high VIL1 levels were also able to improve the mortality prediction provided by the number of precipitating events [39].

However, VIL1 is not a perfect marker. The severity-dependent bimodal behaviour and the possible contribution of the kidneys to increased serum levels warrant caution when interpreting VIL1 results. Future experimental research should clarify the precise quantitative contributions of individual organ systems to serum VIL1 concentrations during AD. Nonetheless, we synthesised existing literature data and indirect evidence to explore this question within our patient cohorts. Of the potential cellular sources of serum VIL1 [28], the biliary duct epithelium was excluded due to the lack of associations between liver damage and function-related parameters and VIL1 levels. Considering that the liver is the main site of damage in cirrhosis, this finding is of particular importance.

The molecular size of VIL1 (92.5 kDa) suggests resistance to renal elimination, implying that reduced kidney excretion due to kidney failure should not significantly alter serum VIL1 levels. However, given the observed association between (creatinine-based) CLIF-C renal failure scores [16] and serum VIL1 levels, the partial contribution of VIL1-expressing proximal tubule epithelial cells to increased serum VIL1 concentration could not be excluded in the presence of severe AKI. On the other hand, the creatinine-VIL1 correlation plot revealed that isolated increases in either marker are substantially more frequent than concurrent elevations, arguing against renal origin as the primary source of serum VIL1. Moreover, the mild correlation may reflect concurrent intestinal and renal injury rather than a direct causal relationship. Indeed, portal hypertension and inflammation-related hemodynamic disturbance in AD cirrhosis may simultaneously induce renal ischemia leading to AKI [40], and may cause circulatory congestion, oedema, and consequent hypoxia with disturbed mucosal integrity in the intestines [41, 42]. Consistently, serum VIL1 demonstrates proportional elevation with systemic injury—of which kidney function is a sensitive indicator—as evidenced by the additive impact of multiple organ failures on VIL1 levels. Critically, the prognostic utility of serum VIL1 remained independent of renal failure status, further supporting an alternative origin for circulating VIL1.

On the other hand, we demonstrated a direct link between intestinal tissue and serum levels of VIL1, namely, decreased values in gut tissue were associated with decreased serum baseline levels (i.e., patients with the least severe acute injury). Furthermore, we have shown that patients with VIL1 levels above the optimal threshold have increased inflammation supporting an association with gut barrier injury and BT. We believe that all these results together, also considering the extensive surface size of the gut compared to other VIL1-containing locations, sufficiently support the intestinal origin of serum VIL1, which therefore can be considered the first non-invasive marker assessing acute gut mucosal barrier injury in AD cirrhosis. However, to establish VIL1 as a gut barrier failure marker, a prospective validation study will be required with sequential VIL1 measurements in patients with unstable decompensated cirrhosis to map its trajectory during the development and resolution of acute decompensation. Frequent blood sample collection might be needed during the AD episode to clarify VIL1's kinetics. Furthermore, these results should be correlated with direct markers of intestinal permeability and microbial translocation to mechanistically link VIL1 levels to the biological consequences of gut barrier dysfunction.

In clinical practice, well-defined, easy-to-use cut-off levels can assist the application of biomarkers. However, we measured lower VIL1 levels in the validation cohort compared to the discovery cohort which resulted in a lower optimal cut-off level in the former. This could be caused by multiple factors. First, simple inter-individual variances might explain the observed

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differences. Notably, the discovery cohort was a single-centre cohort while the validation cohort was a multi-centre one. The composition of the two cohorts regarding the number of patients in the different severity groups (especially the proportion of ACLF patients) was also different. At last, different lot numbers of the kits utilised might also contribute to differences. Regardless, to enter clinical practice—after further validation including cohorts beyond the MICROB-PREDICT consortium—a routine laboratory method will need to be developed for VIL1 measurement, and the exact threshold will need to be re-determined using this technique. The cut-off will need to be defined specifically for AD cirrhosis as VIL1 levels can be influenced by the presence of liver disease and its severity. Importantly, the trends and the observed associations were identical between the two cohorts and both cut-off values were approximately at the second tertile of the corresponding cohort highlighting the reliability of our results.

5 | Conclusion

Acute gut mucosal barrier damage plays a central role in the development of AD and ACLF. Yet, it has not been acknowledged among the traditional severity measures of AD and ACLF due to the lack of available non-invasive biomarkers. Serum VIL1 is a promising marker to indicate gut barrier damage and—after further validation—has the potential to define a 'new' organ failure, refining current risk estimation and clinical classification methods, thereby improving the efficiency of therapy planning and ultimately patient survival.

Author Contributions

David Tornai: conceptualization, writing – original draft, writing – review and editing, formal analysis, visualization. **Boglarka Balogh:** writing – original draft, project administration, writing – review and editing. **Aniko Csillag:** investigation, methodology, writing – review and editing. **Andras Budai:** investigation, methodology, writing – review and editing, visualization. **Andras Kiss:** writing – review and editing, methodology, supervision. **Peter Antal-Szalmas:** writing – review and editing, investigation, methodology, supervision. **Gabor Mehes:** writing – review and editing, methodology, supervision. **Lukacs Barath:** investigation, writing – review and editing, methodology. **Tamas Tornai:** conceptualization, writing – review and editing, investigation. **Istvan Tornai:** investigation, writing – review and editing. **Zsuzsanna Vitalis:** investigation, writing – review and editing. **Nora Sipeki:** investigation, writing – review and editing. **Tamas Dinya:** investigation, writing – review and editing. **Attila Enyedi:** investigation, writing – review and editing. **Florian A. Rosenberger:** investigation, writing – review and editing. **Wim Laleman:** investigation, writing – review and editing. **Minneke J. Coenraad:** investigation, writing – review and editing. **Ferran Aguilar:** investigation, writing – review and editing, methodology. **Joan Claria:** investigation, writing – review and editing. **Richard Moreau:** investigation, writing – review and editing. **Jonel Trebicka:** investigation, writing – review and editing, supervision. **Maria Papp:** investigation, writing – original draft, writing – review and editing, supervision, conceptualization, funding acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Clinical and laboratory characteristics of a subpopulation of the discovery cohort with duodenal biopsy samples. **Table S2:** Baseline clinical and biochemical characteristics of discovery cohort stratified by severity groups. **Table S3:** Baseline clinical and biochemical characteristics of validation cohort stratified by severity groups. **Table S4:** Organ failures in ACLF patients. **Table S5:** Multivariable Cox regression analyses of 90-day mortality adjusting for the impact of systemic inflammation in the discovery cohort. **Table S6:** Multivariable Cox regression analyses of 90-day mortality adjusting for MELD and CLIF-C AD scores. **Table S7:** Univariable Cox regression analyses of 90-day mortality evaluating clinical and laboratory parameters. **Table S8:** Multivariable Cox regression model of individual parameters derived from univariable analysis for 90-day mortality.

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