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A novel score of IL-13 and age predicts 90-day mortality in severe alcohol-associated hepatitis: A multicenter plasma biomarker analysis

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Abstract

Background: Severe alcoholic hepatitis (AH) has a high short-term mortality rate. The MELD assesses disease severity and mortality; however, it is not specific for AH. We screened plasma samples from patients with severe AH for biomarkers of multiple pathological processes and identified predictors of short-term mortality.

Methods: Plasma was collected at baseline from 85 patients with severe AH (MELD $\geq$ 20, Maddrey's discriminant function $\geq$ 32) enrolled in the Defeat Alcoholic Steatohepatitis clinical trial (investigating IL-1 receptor antagonist +pentoxifylline+zinc vs. methylprednisolone+placebo). Samples were analyzed for 43 biomarkers and the markers' association with 28- and 90-day mortalities was assessed.

Results: Thirty-one (36.5%) patients died during the 90-day follow-up with similar ratios in the treatment groups. Eight biomarkers showed an association with mortality. IL-6, IL-22, interferon-α2, soluble TNF receptor 1, lipocalin-2, and α-fetoprotein levels were associated with 28-day mortality, while IL-6, IL-13, and endotoxin levels with 90-day mortality. In multivariable Cox regression, encephalopathy, lipocalin-2, and α-fetoprotein levels were independent predictors of 28-day mortality, and IL-6, IL-13, international normalized ratio levels, and age were independent predictors of 90-day mortality. The combination of IL-13 and age had superior performance in predicting 90-day mortality compared with MELD in the total cohort and the individual treatment groups.

Abbreviations: AH, alcoholic hepatitis; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BAFF, B-cell activating factor; G-CSF, granulocyte colony-stimulating factor; IL-1Ra, IL-1 receptor antagonist; IP, IFN-γ-inducible protein; LBP, lipopolysaccharide binding protein; MCP, monocyte chemoattractant protein; MIF, macrophage migration inhibitory factor; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; RANTES, regulated on activation, normal T-cell expressed and secreted; SAHM-90, severe AH 90-day mortality; sCD, soluble cluster of differentiation; STAT, signal transducers and activators of transcription; sTNF-R, soluble TNF receptor; TWEAK, TNF-like weak inducer of apoptosis.

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Conclusions: We identified predictors of short-term mortality in a cohort exclusively involving patients with severe AH. We created a composite score of IL-13 and age that predicts 90-day mortality regardless of the treatment type with a performance superior to MELD in severe AH.

INTRODUCTION

According to the 2017 report of the joint meeting between the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases,[1] ∼1 million people die each year globally of alcohol-associated liver disease (ALD). The most severe form of ALD is acute alcoholic hepatitis (AH), which often develops in chronic drinkers with liver fibrosis and cirrhosis. AH manifests as rapid jaundice onset, and severe forms lead to liver and other organ failures with a high short-term mortality rate. However, a moderate form of the disease is also recognized with low mortality where patients may be entirely asymptomatic. In these cases, only laboratory tests and liver biopsy reveal the onset of steatohepatitis.[1]

AH is increasingly recognized as a systemic inflammatory condition. Heavy alcohol consumption causes injury in hepatocytes and leads to the release of danger-associated molecular patterns. These, in conjunction with gut-derived pathogen-associated molecular patterns that result from alcohol-induced intestinal dysbiosis and leaky gut, trigger the inflammatory response.[2–4]

Despite extensive research, this deadly disease still lacks sufficient treatment options.[5] Our team demonstrated a crucial role of IL-1β in the pathogenesis of ALD and further showed that anakinra, a recombinant IL-1 receptor antagonist (IL-1Ra) that was previously used in rheumatoid arthritis treatments, ameliorated inflammasome-dependent alcoholic steatohepatitis in mice.[6] Therefore, in the frame of the multicentric Defeat Alcoholic Steatohepatitis trial, we examined anakinra in combination with pentoxifylline (to protect against development of hepatorenal syndrome) and zinc sulfate (to increase gut mucosal integrity) as a potential treatment option for AH. The results of this study indicated the potential benefit of anakinra, but were limited due to sample size.[7]

A major challenge in AH management is the lack of disease-specific biomarkers to predict clinical outcomes and to evaluate response or resistance to therapy.[1]

Comprehensive analysis of parameters indicating systemic inflammation, such as cytokines, chemokines, markers of gut translocation, and potential indicators of liver regeneration, is yet to be completed in AH.

The MELD score universally assesses the severity and mortality of different liver diseases[8] and has been validated in several patient populations. Hence, the MELD score provides a common and objective metric to determine the severity of liver diseases.[9] However, disease-specific scores will likely provide a more accurate prediction for clinical outcomes. Furthermore, since the mortality rate is high only in patients with severe and not mild or moderate AH, studies need to focus on these patients with severe AH (instead of the whole disease spectrum) when designing a new predictive score that aims to maximize accuracy. Indeed, it has been reported that the CLIF-C Acute-on-Chronic Liver Failure[10] score, designed by analyzing patients with the most severe symptoms, provides a better estimation of short-term mortality in acute-on-chronic liver failure compared with a corresponding MELD score.[11–13] Thus, we aimed to identify markers associated with short-term mortality in severe AH and develop a disease-specific score to improve risk stratification of the most vulnerable patients with AH. Therefore, we measured 43 circulating indicators of AH pathology (cytokines, bacterial translocation, liver regeneration, tissue remodeling, and cell activation markers) in a multicenter randomized clinical trial of severe AH upon patients' admission to the hospital. We tested the 28- and 90-day mortality–predicting abilities of these markers and created a new composite score that improves the accuracy of short-term mortality prediction in severe AH.

METHODS

Human subjects' involvement, characterization, and design

The study design and collection of samples for this study were approved by the UMass Medical School Institutional Review Board full committee review and a written informed consent was obtained from all participants. The research was conducted in accordance with both the Declarations of Helsinki and Istanbul.

Twenty-seven healthy volunteers were recruited for study participation from the University of Massachusetts Medical School by written advertisement. The participating subjects were ethnically diverse, although the

TABLE 1 Clinical and laboratory characteristics of patients with severe alcohol-associated hepatitis at inclusion together and broken down by treatment groups

Patients	All (N = 85)	Steroid (n = 42)	Anakinra (n = 43)
Age	47 (37–53)	46.5 (37–52)	47 (37.5–54)
Sex (male/female)	51 (60)/34 (40)	21 (50)/21 (50)	30 (70)/13 (30)
MELD score	25 (23–28)	24.5 (23–28)	25 (23–27)
Bilirubin (mg/dL)	19.2 (14.0–25.6)	21 (15.8–26.5)	18.5 (13.0–24.5)
Albumin (g/L)	2.5 (2.2–2.7)	2.6 (2.2–2.8)	2.5 (2.2–2.7)
Creatinine (mg/dL)	0.76 (0.58–1.01)	0.73 (0.58–0.97)	0.76 (0.58–1.05)
INR	1.95 (1.60–2.20)	1.90 (1.60–2.27)	2.00 (1.70–2.15)
AST (IU/L)	122 (85–160)	114 (85–160)	125 (85–161)
ALT (IU/L)	38 (27–56)	36 (26–54)	40 (28–58)
ALP (IU/L)	155 (116–203)	156 (112–208)	152 (118–203)
WBC (10^3/mm^3)	9.56 (6.55–14.87)	10.55 (7.04–14.76)	9.30 (6.05–15.11)
Cirrhosis	65 (76.4)	30 (71.4)	35 (81.4)
Ascites	33 (38.8)	15 (35.7)	18 (41.9)
Encephalopathy	21 (24.7)	10 (23.8)	11 (25.6)
Mortality at day			
28	13 (15.3)	8 (19.0)	5 (11.6)
90	31 (36.5)	18 (42.9)	13 (30.2)
180	33 (38.8)	19 (45.2)	14 (32.6)

Note: Data are presented as median (interquartile range) or n (%).
Mortality numbers at days 28, 90, and 180 are also shown.
Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase.

majority were Caucasians. They ranged in ages from 21 to 65 and the ratio of males and females was 15/12.

Four hundred sixteen patients with a history of alcohol abuse were recruited from 4 US clinical centers (the University of Texas Southwestern Medical Center, the University of Massachusetts Medical School, Cleveland Clinic, and the University of Louisville School of Medicine) between September 2013 and October 2018. Disease severity was determined by the MELD score and Maddrey's discriminant function. One hundred four eligible patients with AH with MELD $\geq$20 and Maddrey's discriminant function $\geq$32 were enrolled in the severe arm of the study and randomly assigned to treatment with anakinra [IL-1Ra (100 mg/d subcutaneous injection)] for 14 days or methylprednisolone (32 mg/d) for 28 days. The patients received pentoxifylline (400 mg orally 3 times daily for 1 mo)+zinc sulfate (22 mg orally for 6 mo) in the anakinra group or placebo in the steroid group. One patient later withdrew her consent. A detailed description of patient recruitment, the inclusion and the exclusion criteria, as well as the results and conclusion of the clinical trial have been published.[6,14] Blood samples were collected at baseline. Mortality and cause of death were recorded during the follow-up. In this study, 28- and 90-day (ie, short term) mortalities were assessed. Analyses for 90-day mortality included all patients who died up to the 90th day including patients who died within the first 28 days. By the beginning of this investigation, we had obtained samples from 85 patients with severe AH from all 4 sites. Plasma samples were stored at −80°C until analysis. Supplemental Figure S1 (http://links.lww.com/HC9/A604) illustrates patient selection.

All authors had access to the study data and reviewed and approved the final manuscript.

Biomarker measurements

Plasma levels of 37 cytokines and inflammatory molecules were measured using 2 custom-made Bioplex panels (23-plex human cytokine and 14-plex human inflammation panels; Bio-Rad).

Endotoxin levels were determined by the LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific Pierce; Cat # 88282).

Concentrations of lipopolysaccharide binding protein (LBP), soluble CD14, Sonic Hedgehog, lipocalin-2, and α-fetoprotein were measured by commercially available ELISA kits (Hycult Biotech, Cat # HK315-02; R&D Systems Cat # DC140, Cat # DSHH00, Cat # DY1757, DY1369, respectively).

All assays were carried out according to the manufacturer's instructions in a blinded fashion without prior knowledge of the patient's clinical information. Measurements were performed in duplicates on the

same plate, and the mean values were used. The accepted coefficient of variance was 10%. In cases with higher variance, samples were remeasured.

Statistical analysis

Continuous patient characteristics (Table 1) were summarized as median (interquartile range). Frequencies were examined for categorical variables and presented as n (%). Variables were tested for normality using Shapiro-Wilk *W* test. Mann-Whitney *U* test was used to compare plasma marker levels between 2 groups. Benjamini-Hochberg procedure was used to correct for multiple comparisons. Kruskal-Wallis test or 1-way ANOVA with a post hoc test for multiple comparisons were used to compare 3 groups as appropriate according to the distribution of the variables. The efficiency of different markers to discriminate between survivals and nonsurvivals was estimated by the ROC curve analysis. AUROC with corresponding 95% CIs and *p* values were calculated. The best discriminative thresholds of biomarkers for 28- and 90-day mortalities were defined as the marker levels where sensitivity and specificity reached maximum value. Kaplan-Meier survival curves were plotted to estimate the cumulative probability of mortality. The significance of the observed differences in probabilities was assessed with log-rank tests. The association between clinical variables or plasma levels of the measured markers and mortality during follow-up was assessed with univariable Cox regression. Multivariable analysis was performed with backward elimination procedures and likelihood ratio tests to identify significant independent predictors. Factors that had a *p* value <0.1 in the univariable regression were eligible for the multivariable analysis and included in the 90-day model; however, due to the lower number of events, only factors with significant *p* values were included in the 28-day model. Binary logistic regression was used to build a composite score from identified independent predictors of 90-day mortality. Associations are provided as HR or OR as appropriate with 95% CIs. A 2-sided *p* value < 0.05 was considered statistically significant. For

TABLE 2 Plasma levels of measured chemokines, liver regeneration, cell activation, bacterial translocation, and tissue remodeling markers in healthy controls and patients with severe alcohol-associated hepatitis at inclusion

	Controls		Patients			
	Median	IQR	Median	IQR	*p*	Corrected *p*
IL-1Ra	86.07	71.76–118.02	311.71	162.99–975.76	< **0.001**	< **0.001**
IL-1α	5.11	3.69–9.71	9.92	5.46–13.39	**0.001**	**0.001**
IL-1β	0.82	0.47–1.46	1.64	1.09–2.54	< **0.001**	< **0.001**
IL-6	1.20	0.57–1.92	20.98	13.70–34.39	< **0.001**	< **0.001**
IL-8	3.48	1.66–4.67	293.71	178.73–541.71	< **0.001**	< **0.001**
IL-9	154.53	120.58–189.84	154.22	104.54–205.71	0.981	0.981
IL-10	0.00	0.00–0.69	3.20	1.10–5.04	< **0.001**	< **0.001**
IL-12p40	0.00	0.00–12.76	277.84	206.15–351.42	< **0.001**	< **0.001**
IL-12p70	5.96	1.45–11.92	3.92	1.98–5.61	0.056	0.062
IL-13	1.07	0.55–2.89	0.77	0.50–1.54	0.174	0.187
IL-17A	1.40	0.32–4.20	6.26	3.93–9.60	< **0.001**	< **0.001**
IL-18	48.42	39.19–72.35	183.32	121.70–261.40	< **0.001**	< **0.001**
IL-20	14.01	9.64–20.90	22.96	18.95–28.52	< **0.001**	< **0.001**
IL-22	41.32	26.17–52.51	49.47	37.38–63.33	**0.012**	**0.015**
TNF-α	43.50	34.92–52.84	75.26	60.46–93.56	< **0.001**	< **0.001**
sTNF-R1	866.26	786.32–1116.80	4199.20	3144.19–5608.07	< **0.001**	< **0.001**
sTNF-R2	474.57	402.94–542.07	1845.80	1339.70–2375.59	< **0.001**	< **0.001**
TRAIL	52.89	42.47–64.13	40.26	30.93–51.69	**0.002**	**0.002**
TWEAK	277.21	230.70–319.64	193.39	159.24–255.89	< **0.001**	< **0.001**
BAFF[a]	7.31	5.86–8.49	47.33	30.86–72.52	< **0.001**	< **0.001**
MCP-1	25.63	21.78–31.96	40.37	23.39–56.75	**0.002**	**0.002**
MIF	630.66	501.10–915.95	1335.52	1018.42–1681.06	< **0.001**	< **0.001**
MIP-1α	0.80	0.49–1.28	4.74	3.20–7.37	< **0.001**	< **0.001**
MIP-1β	127.80	115.46–162.16	142.50	105.04–188.77	0.608	0.622
GRO-α	364.29	297.14–435.51	445.38	282.69–695.14	**0.040**	**0.046**

TABLE 2. (*continued*)

	Controls		Patients			
	Median	IQR	Median	IQR	*p*	Corrected *p*
sCD163[a]	104.65	84.80–124.75	639.21	548.03–737.95	**< 0.001**	**< 0.001**
sCD14[b]	1.51	1.41–1.81	2.52	2.09–2.98	**< 0.001**	**< 0.001**
LBP[b]	12.88	10.32–16.22	28.84	19.99–40.33	**< 0.001**	**< 0.001**
Endotoxin[c]	0.96	0.82–1.14	2.26	1.90–2.97	**< 0.001**	**< 0.001**
Osteopontin[a]	63.68	57.11–84.38	206.11	173.36–287.36	**< 0.001**	**< 0.001**
Lipocalin-2[a]	72.91	58.83–87.70	235.30	136.28–369.59	**< 0.001**	**< 0.001**
HGF[a]	0.28	0.23–0.31	4.27	3.25–5.55	**< 0.001**	**< 0.001**
G-CSF	60.36	31.90–97.14	130.00	88.06–173.68	**< 0.001**	**< 0.001**
Sonic Hedgehog	233.54	163.77–337.49	208.97	132.11–281.61	0.249	0.261
α-fetoprotein[a]	1.00	0.74–1.55	1.55	1.02–1.99	**0.005**	**0.007**
MMP-1	151.21	130.05–167.06	456.04	314.59–857.19	**< 0.001**	**< 0.001**
MMP-2[a]	44.66	38.30–53.37	198.76	155.63–295.03	**< 0.001**	**< 0.001**
Chitinase 3-l.1[a]	7.69	6.59–9.52	17.54	15.68–19.29	**< 0.001**	**< 0.001**
IFN-α2	2.13	1.64–2.56	5.39	4.32–6.40	**< 0.001**	**< 0.001**
IFN-β	0.00	0.00–2.71	23.62	18.79–29.27	**< 0.001**	**< 0.001**
IP-10	351.62	262.54–435.57	1131.86	736.85–1759.92	**< 0.001**	**< 0.001**
Pentraxin-3[a]	9.38	6.91–16.37	52.18	34.36–70.21	**< 0.001**	**< 0.001**
RANTES[a]	4.54	2.61–9.69	3.17	1.09–6.07	**0.023**	**0.027**

Note: Data are presented as pg/mL, unless they are marked with:
p values were corrected for multiple comparisons using the Benjamini-Hochberg method.
Numbers in bold highlight significant *p* values.
[a]ng/mL.
[b]μg/mL.
[c]EU/mL.
Abbreviations: BAFF, B-cell activating factor; G-CSF, granulocyte colony-stimulating factor; IL-1Ra, IL-1 receptor antagonist; IP, IFN-γ-inducible protein; LBP, lipopolysaccharide binding protein; MCP, monocyte chemoattractant protein; MIF, macrophage migration inhibitory factor; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; RANTES, regulated on activation, normal T-cell expressed and secreted; sCD, soluble cluster of differentiation; sTNF-R, soluble TNF receptor; TWEAK, TNF-like weak inducer of apoptosis.

statistical analyses and graphical presentation, SPSS 25.0 and GraphPad Prism 8.3 programs were used.

RESULTS

Significantly increased levels of circulating immune mediators suggest systemic overactivation of inflammatory pathways in acute AH

First, we aimed to characterize the activation of the inflammatory cascade in severe AH by measuring a broad range of inflammatory and immunoregulatory cytokines, chemokines, as well as markers of gut translocation and liver regeneration (Table 2). Plasma samples obtained at study enrollment before initiation of study drugs were analyzed and compared with those of healthy controls. Active infection was an exclusion criterion, thus cytokine levels most likely reflect AH-associated changes.

Circulating levels of the IL-1 family members, IL-1α, IL-1β, IL-1Ra, and IL-18, were significantly higher in AH compared with controls. In the TNF family of cytokines, TNF-α, soluble TNF receptor (sTNF-R) 1, and sTNF-R2 were significantly upregulated, while TWEAK and TRAIL levels were decreased in AH compared with controls. Of the immunoregulatory cytokines, IL-6, 1L-10, IL-12p40, IL-17a, and IL-20 were upregulated, but IL-12p70 and IL-13 were not significantly altered. Type I interferons, IFN-α2, IFN-β, as well as IFN-γ-inducible protein-10 were significantly increased in AH. These changes indicate a generalized activation of innate immune activation pathways. In line with this, the levels of circulating chemokines including monocyte chemoattractant protein 1, macrophage inflammatory protein (MIP)-1α, MIP-1β, macrophage migration inhibitory factor, IL-8, Gro, and petraxin-3 were all significantly increased, while RANTES (regulated on activation, normal T-cell expressed and secreted) was decreased in severe AH.

Consistent with gut leakiness, endotoxin and LBP were significantly increased in AH, and this was paralleled with increased circulating levels of both M1 and M2 monocyte/macrophage activation (soluble CD14, soluble CD163, respectively).

Circulating growth factors that affect B cell, myeloid cell, and/or hepatocyte proliferation were significantly increased including B-cell activating factor, granulocyte colony-stimulating factor, HGF, IL-22, lipocalin-2 (also called neutrophil gelatinase-associated lipocalin), and osteopontin, while Sonic Hedgehog levels were not different. α-fetoprotein, an indicator of dedifferentiated hepatocytes and liver regeneration, was also significantly increased in AH compared with controls. Finally, increased levels of matrix metalloproteinase (MMP)-1, MMP-2, and chitinase 3-like protein indicated upregulation of tissue remodeling (Table 2).

In addition, Sonic Hedgehog, IFN-B, IL-20, and MMP-2 levels were found to be significantly increased, while LBP, IL-1Ra, IL-9, and osteopontin levels were decreased in patients with cirrhosis compared with patients without it (Supplemental Table S1, http://links.lww.com/HC9/A604).

Baseline biomarker levels at hospital admission are associated with 28- and 90-day mortalities in severe AH

Of the 85 patients with severe AH, 13 (15.3%) died during the first 28 days. During the total 90-day follow-up, 31 patients (36.5%) were deceased; 18 (42.9%) in the steroid and 13 (30.2%) in the anakinra group (Table 1). No patient has undergone liver transplantation during the first 90 days after admission. Nine patients were lost to follow-up (3 steroid-treated and 6 anakinra-treated patients).

Eight biomarkers (lipocalin-2, α-fetoprotein, endotoxin, IL-6, IL-13, IL-22, IFN-α2, and sTNF-R1) showed statistically significant association with short-term mortality (Table 3). Seven of these molecules were increased in patients with severe AH compared with healthy controls, while 1 was not significantly different (IL-13). The most robust increase was detected in the levels of IL-6 with about a 17.5-fold increase in median value compared with controls (Table 2). Of these markers, the following ones correlated with MELD score: IL-6 ($r = 0.218$, $p = 0.044$), IL-13 ($r = -0.272$, $p = 0.011$), endotoxin ($r = 0.262$, $p = 0.015$), lipocalin-2 ($r = 0.328$, $p = 0.002$).

At day 28, IL-6 [median (interquartile range): 40.68 (23.61–56.77) vs. 19.77 (13.33–30.77) pg/mL], IL-22 [65.15 (36.43–58.88) vs. 47.88 (51.33–80.33) pg/mL], IFN-α2 [6.70 (4.91–8.36) vs. 5.12 (4.26–6.11) pg/mL], sTNF-R1 [5464 (3767–6982) vs. 4064 (3080–5471) pg/mL], and lipocalin-2 [318.4 (200.6–692.4) vs. 218.6 (132.4–350.4) ng/mL] levels were significantly elevated in nonsurvivor patients with AH compared with survivors. In contrast, α-fetoprotein [1.07 (0.65–1.43) vs. 1.68 (1.09–2.12) ng/mL] levels were significantly decreased in nonsurvivors compared with survivors (Table 3). However, at day 90, besides IL-6 [29.34 (17.26–40.68) vs. 18.47 (13.11–30.51) pg/mL], only IL-13 [0.53 (0.23–0.90) vs. 0.90 (0.56–1.83) pg/mL] and endotoxin [2.52 (1.99–3.22) vs. 2.18 (1.79–2.65) EU/mL] were associated with mortality (Table 3).

To seek an explanation for the observed differences between markers associated with 28- and 90-day mortalities, we divided nonsurvivor patients according to the time of mortality (Supplemental Figure S2, http://links.lww.com/HC9/A604). We found that the levels of those markers that were associated with 28-day mortality (lipocalin-2, IFN-a2, sTNF-R1, α-fetoprotein, IL-22, IL-6) showed a different trend of change compared with the markers that were associated only with 90-day mortality (IL-13 and endotoxin). In the former set, patients who died within the first 28 days had altered marker levels compared with both survivors and patients who died later (between days 28 and 90). In the latter set, the 2 nonsurvivor groups had similar levels regardless of the time of death (Supplemental Figure S2, http://links.lww.com/HC9/A604).

Markers predicting 28-day mortality

ROC curves were used to assess the discriminative power of markers associated with 28-day mortality and to identify the associated best discriminatory cutoffs (Table 4). Based on the AUROC values, IL-6 (0.740), IL-22 (0.733), and α-fetoprotein (0.741) provided better discrimination than IFN-α2 (0.699), sTNF-R1 (0.686), and lipocalin-2 (0.696). Kaplan-Meier curves were plotted using the identified cutoffs (Figure 1A), which showed significantly reduced survival in patients with increased (or in the case of α-fetoprotein: decreased) levels of these markers (log-rank $p < 0.001$ for all).

We also used univariable Cox regression analysis to assess 28-day mortality, in which (Ln)IL-6, (Ln)α-fetoprotein, (Ln)lipocalin-2, (Ln)sTNF-R1, presence of encephalopathy, and age were found to be predictors (Supplemental Table S2, http://links.lww.com/HC9/A604), therefore, included in the multivariable model. In the multivariable analysis, (Ln)α-fetoprotein [HR (95% CI): 0.589 (0.431–0.804), $p = 0.001$], (Ln)lipocalin-2 [2.226 (1.012–4.899), $p = 0.047$], and presence of encephalopathy [5.417 (1.486–19.747), $p = 0.010$] were found to be independently associated with 28-day mortality (Table 5).

Markers predicting 90-day mortality

Next, we assessed 90-day mortality using the same approach. Of the 3 markers, IL-13 had the highest AUROC (0.714) with an optimal cutoff level of <0.61 pg/mL. The predictive power of IL-6 (0.632) was reduced compared with that observed for 28-day mortality. The optimal cutoff level was >26.82 pg/mL (Table 4). The ROC curve of endotoxin did not reach statistical

TABLE 3 Plasma levels of measured biomarkers associated with short-term (28 and 90 d) mortality in patients with severe AH

	Survivors		Nonsurvivors			
	Median	IQR	Median	IQR	*p*	Corrected *p*
28 d						
Lipocalin-2[a]	218.56	132.37–350.35	318.43	200.60–692.38	**0.025**	**0.042**
α-fetoprotein[a]	1.68	1.09–2.12	1.07	0.65–1.43	**0.005**	**0.023**
Endotoxin[b]	2.20	1.81–2.94	2.52	2.20–3.14	0.082	0.103
IL-6	19.77	13.33–30.77	40.68	23.61–56.33	**0.005**	**0.023**
IL-13	0.81	0.50–1.63	0.54	0.3–0.88	0.068	0.097
IL-22	47.88	36.43–58.88	65.15	51.33–80.33	**0.007**	**0.023**
IFN-α2	5.12	4.26–6.11	6.70	4.91–8.36	**0.022**	**0.042**
sTNF-R1	4063.79	3079.81–5470.80	5464.46	3766.58–6981.86	**0.011**	**0.027**
90 d						
Lipocalin-2[a]	213.89	119.92–345.87	247.37	188.09–404.88	0.056	0.140
α-fetoprotein[a]	1.62	1.02–2.12	1.27	1.025–1.979	0.269	0.374
Endotoxin[b]	2.18	1.79–2.65	2.52	1.99–3.22	**0.020**	0.101
IL-6	18.47	13.11–30.51	29.34	17.26–40.68	**0.043**	0.140
IL-13	0.90	0.56–1.83	0.53	0.23–0.90	**< 0.001**	**0.009**
IL-22	47.88	37.67–56.44	56.13	37.09–74.81	0.096	0.192
IFN-α2	5.12	4.26–6.15	5.39	4.42–6.83	0.432	0.480
sTNF-R1	4148.81	3253.79–5472.76	4357.85	3004.06–6485.32	0.602	0.602

Note: Data are presented as pg/mL, unless they are marked with:
p values were corrected for multiple comparisons using the Benjamini-Hochberg method.
Numbers in bold highlight significant *p* values.
[a]ng/mL.
[b]EU/mL.
Abbreviations: AH, alcoholic hepatitis; sTNF-R1, soluble TNF receptor 1.

significance, thus, it was not included in the calculation of predictive models.

Kaplan-Meier curves demonstrated a higher mortality rate in patients with low IL-13 and high IL-6 levels during the 90-day follow-up compared with patients with opposite levels of these biomarkers (64.6% vs. 22.4%, log-rank $p < 0.001$ and 52.4% vs. 31.6%, log-rank $p = 0.024$, respectively) (Figure 1B).

Univariable Cox regression analysis revealed that (Ln) IL-13, (Ln)INR, (Ln)creatinine, age, and presence of ascites were significantly associated with 90-day mortality, while the *p* value of (Ln)IL-6, sex, and presence of encephalopathy was slightly above the level of significance (Supplemental Table S3, http://links.lww.com/HC9/A604). In multivariable Cox regression analysis of these factors, (Ln)IL-13 [HR (95% CI): 0.746

TABLE 4 Short-term (28 and 90 d) mortality-predicting capacity of the measured plasma biomarkers in patients with severe AH assessed by receiver operating characteristic analysis

	AUROC	95% CI	*p*	Best cutoff	Sensitivity (%)	Specificity (%)
28-d mortality						
IL-6	0.740	0.562–0.918	**0.006**	> 29.3 pg/mL	76.92	71.23
IL-22	0.733	0.568–0.899	**0.008**	> 51 pg/mL	84.62	61.64
α-fetoprotein	0.741	0.621–0.860	**0.006**	< 1.84 ng/mL	100.00	43.84
IFN-α2	0.699	0.519–0.879	**0.023**	> 6 pg/mL	69.23	73.97
sTNF-R1	0.686	0.518–0.855	**0.033**	> 6532 pg/mL	46.15	90.41
Lipocalin-2	0.696	0.542–0.849	**0.025**	> 510.2 ng/mL	38.46	93.15
90-d mortality						
IL-6	0.632	0.521–0.733	**0.043**	> 26.82 pg/mL	54.84	70.91
IL-13	0.714	0.604–0.823	**< 0.001**	≤ 0.61 pg/mL	67.74	74.55
Endotoxin	0.622	0.495–0.749	0.063	—	—	—

Note: Best discriminatory cutoff values were identified as the marker levels, where sensitivity plus specificity reached the maximum value.
Numbers in bold highlight significant *p* values.
Abbreviations: AH, alcoholic hepatitis; sTNF-R1, soluble TNF receptor 1.

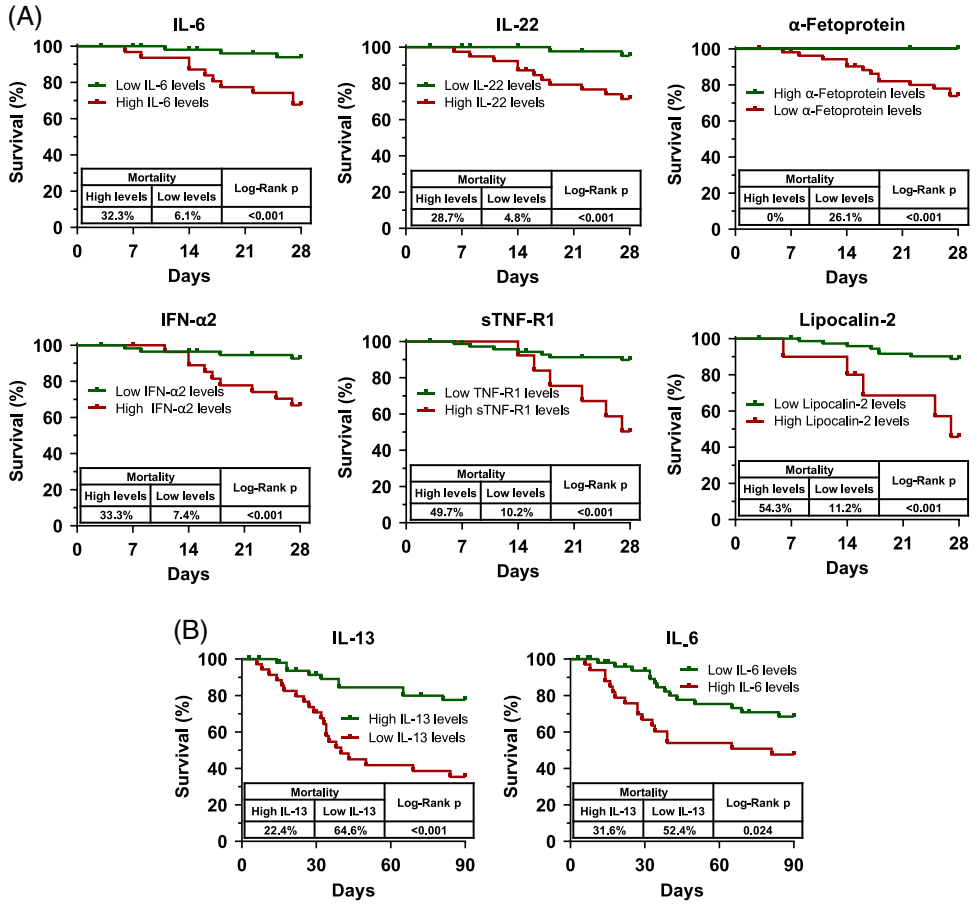


FIGURE 1 Short-term (A: 28 d and B: 90 d) mortality–predicting capacity of the measured plasma biomarkers in patients with severe AH assessed by Kaplan-Meier curves. Best discriminatory cutoff values identified by receiver operating characteristic curve analyses were used to plot Kaplan-Meier survival curves. Abbreviations: AH, alcoholic hepatitis; sTNF-R, soluble TNF receptor.

(0.599–0.929), $p = 0.009$], (Ln)IL-6 [1.732 (1.068–2.807), $p = 0.026$], (Ln)INR [4.658 (1.097–19.782), $p = 0.038$], and age [1.061 (1.018–1.105), $p = 0.005$] were independent predictors of mortality during the 90-day follow-up (Table 6).

TABLE 5 Multivariable Cox regression analysis for identifying independent predictors of 28-day mortality

	Multivariable Cox regression analysis		
28-d mortality	**HR**	**95% CI**	***p***
Age	1.078	0.100–1.162	0.051
Encephalopathy	**5.417**	**1.486–19.747**	**0.010**
Lipocalin-2 (Ln)	**2.226**	**1.012–4.899**	**0.047**
α-fetoprotein (Ln)	**0.589**	**0.431–0.804**	**0.001**
IL-6 (Ln)	1.151	0.470–2.816	0.759
sTNF-R1 (Ln)	0.659	0.120–3.610	0.631

Note: Only variables that had significant *p* values in the univariable Cox regression analysis were included in this multivariable model. Laboratory parameters and biomarker levels were logarithmically transformed (Ln) before the analysis.
Numbers in bold highlight significant independent predictors: presence of encephalopathy, (Ln)lipocalin-2, and (Ln)α-fetoprotein.
Abbreviation: sTNF-R1, soluble TNF receptor 1.

A novel composite, severe AH 90-day mortality score (SAHM-90 score) based on the combination of IL-13 levels, and age has superior 90-day mortality predictive performance compared with MELD score in patients with severe AH

We used binary logistic regression to combine independent predictors identified by multivariable Cox regression. The number of events allowed us to create such a composite score only for 90-day mortality, where we had the opportunity to combine a maximum of 3 predictors (of the identified 4: IL-6, IL-13, INR, and age). Finally, the logistic model included age and IL-13 (Figure 2A). There was neither correlation nor collinearity between IL-13 levels and age (data not shown). The computed equation was $= (\text{Age} \times 0.081) - (\text{IL-13} \times 1.365) - 3.165$.

Next, we compared the 90-day mortality-predicting efficiency of this novel composite score that we named SAHM-90 score to the MELD score using ROC analyses and Cox regression. The SAHM-90 score had a higher AUROC value than the MELD score (AUROC: 0.780, $p < 0.001$ vs. AUROC: 0.675, $p = 0.008$, respectively) in our

TABLE 6 Multivariable Cox regression analysis for identifying independent predictors of 90-day mortality among clinical factors, laboratory parameters, and biomarkers

	Multivariable Cox regression analysis		
90-d mortality	**HR**	**95% CI**	***p***
Sex	0.499	0.223–1.115	0.090
Age	**1.061**	**1.018–1.105**	**0.005**
Ascites	1.971	0.951–4.085	0.068
Encephalopathy	0.891	0.344–2.307	0.812
AST/ALT ratio	0.895	0.637–1.257	0.522
INR (Ln)	**4.658**	**1.097–19.782**	**0.037**
Creatinine (Ln)	1.218	0.547–2.713	0.629
IL-6 (Ln)	**1.732**	**1.068–2.807**	**0.026**
IL-13 (Ln)	**0.746**	**0.599–0.929**	**0.009**

Note: Only variables that had p values <0.1 in the univariable Cox regression analysis were included in this multivariable model. Laboratory parameters and biomarker levels were logarithmically transformed (Ln) before the analysis.
Numbers in bold highlight significant independent predictors: age, (Ln)INR, (Ln) IL-6, and (Ln)IL-13 levels.
Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase.

patient population with severe AH (Figure 2B). Univariable and multivariable Cox regression analyses confirmed superior 90-day mortality–predicting performance of SAHM-90 score compared with MELD in a time-dependent manner [multivariable: MELD: HR (95% CI): 1.024 (0.937–1.118), $p = 0.604$; SAHM-90: 2.491 (1.687–3.678), $p < 0.001$] (Figure 2C).

Finally, we applied the SAHM-90 score in the 2 treatment groups separately. Both ROC curves were statistically significant (steroid: AUROC: 0.745, $p = 0.007$ and anakinra: AUROC: 0.826, $p = 0.001$) (Figure 2D). Univariable Cox regression analyses (Figure 2E) confirmed the 90-day mortality–predicting capacity of the new score in each treatment group [steroid: HR (95% CI): 2.306 (1.238–4.296), $p = 0.008$ and anakinra: 2.976 (1.629–5.437), $p < 0.001$].

DISCUSSION

In this study, we show remarkable changes in 43 cytokines, inflammatory molecules, bacterial translocation, liver regeneration, and tissue remodeling–related molecules observed in 85 patients with severe AH. To the best of our knowledge, this is the largest-scale explorative biomarker study conducted to date in patients with severe AH. Consistent with previous reports, we found significant increases in circulating levels of key proinflammatory cytokines including those in the IL-1 family (IL-1α, IL-1β, IL-1Ra, and IL-18), TNF family (TNF-α, sTNF-R1, and sTNF-R2), immunoregulatory cytokines (IL-6, 1L-10, IL-12p40, IL-17a, and IL-20), as well as chemokines (monocyte chemoattractant protein 1, MIP-1α, MIP-1β, migration inhibitory factor, IL-8, Gro, and Petraxin-3). Markers of liver regeneration (HGF, IL-22, α-fetoprotein) and tissue remodeling (MMP-1, MMP-2, and chitinase 3-like protein) were also increased in the circulation of patients with AH. Increased circulating levels of endotoxin and LBP indicated enhanced gut microbial translocation.

We assessed both 28- and 90-day mortalities and identified 8 biomarkers that were associated with mortality at these time points.

Our present paper focused exclusively on patients with severe disease (both Maddrey's discriminant function ≥ 32 and MELD score ≥ 20 were required) because mortality is highest in this population of patients with ALD.[1] The recruitment of such patients without any other ongoing inflammatory or chronic diseases is extremely challenging—about 80% of the screened patients were excluded (did not meet the strict criteria, did not consent, etc.).[14] Because high short-term mortality in patients with severe AH is a remaining clinical challenge, we aimed to identify new biomarkers with sufficient power to discriminate among severely ill patients and more precisely predict survival by focusing exclusively on this population. The fact that immune activation and immune exhaustion are parallelly present in AH with a dynamically changing ratio according to disease severity[15–17] underlines the need to investigate biomarkers in a disease severity–specific manner.

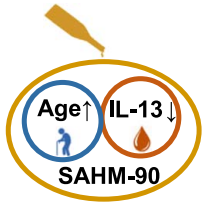
We found that IL-6, IL-22, IFN-α2, sTNF-R1, lipocalin-2, and α-fetoprotein levels were associated with mortality in the first 28 days after hospital admission. While each one of these molecules affects different biological pathways (IL-6 has proinflammatory and regenerative effects, IL-22 regulates epithelial cell and hepatocyte functions, lipocalin-2 is a product and activator of neutrophil leukocytes and also a surrogate marker of acute kidney injury, and α-fetoprotein is a product of hepatocyte proliferation), orchestrated changes in biological processes regulated by these key molecules are pivotal in AH.[18]

Ninety-day mortality was associated with changes in IL-6, IL-13, and endotoxin levels. Although IL-6 and endotoxin were not confirmed by the multiple comparison test, plenty of literature data suggest the association of these molecules with the outcome in different liver diseases, so we did not discard them based on this analysis.

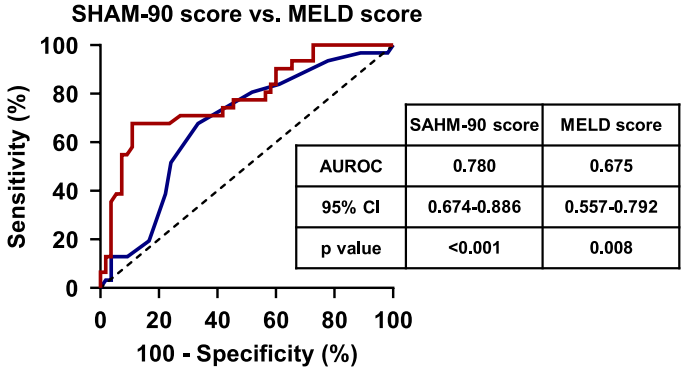
We can speculate a few reasons to provide an explanation for the observed time-dependent association between different biomarkers and mortality. First, the production rate and half-life (ie, turnover) of the markers might influence the range of the provided prediction. Therefore, markers having rapidly changing concentrations are expected to reliably predict outcomes in a shorter term than those with more stable levels when measured at one point in time. Second, the different molecular mechanisms, whose functional states are indicated by the marker levels (eg, inflammation, regeneration, etc.), might influence mortality to different extents at different times during the disease

(A)

Binary logistic regression			
	OR	95% CI	p value
Age	1.084	1.024-1.148	0.005
IL-13	0.255	0.104-0.626	0.003
Constant	0.042		0.021
Computed equation	*=(Age*0.081)-(IL-13*1.365)-3.165*		



(B)

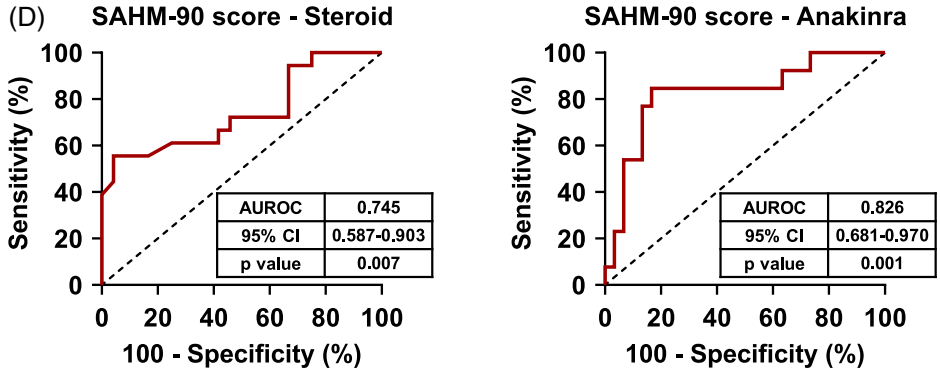


	SAHM-90 score	MELD score
AUROC	0.780	0.675
95% CI	0.674-0.886	0.557-0.792
p value	<0.001	0.008

(C)

90-day mortality	Univariate Cox regression analysis			Multivariate Cox regression analysis		
	HR	95.0% CI	p value	HR	95.0% CI	p value
MELD score	1.099	1.016-1.188	0.018	1.023	0.937-1.117	0.610
SAHM-90 score	2.491	1.687-3.678	<0.001	2.385	1.568-3.627	<0.001

(D)



(E)

90-day mortality	Univariate Cox regression analysis		
	HR	95.0% CI	p value
In Steroid group	2.306	1.238-4.296	0.008
In Anakinra group	2.976	1.629-5.437	<0.001

FIGURE 2 IL-13 levels and age combined to a new composite score (SAHM-90 score) predicts 90-day mortality in patients with AH regardless of treatment with higher performance than MELD score. Binary logistic regression (A) was used to combine IL-13 and age into a composite score (SAHM-90 score). The computed equation is shown. A comparison of ROC analyses (B) of the SAHM-90 score and MELD score indicated that the SAHM-90 score had a significantly higher AUROC value than the MELD score in patients with severe alcohol-associated hepatitis. Univariable and multivariable Cox regression analyses (C) demonstrated superior 90-day mortality–predicting performance of the SAHM-90 score compared with the MELD score. (D) SAHM-90 score was applied in the 2 treatment groups [methylprednisolone (aka. steroid) and anakinra+pentoxifylline+zinc (aka. anakinra)] separately; both ROC curves were statistically significant. Univariable Cox regression analyses (E) confirmed the 90-day mortality-predicting capacity of the SAHM-90 score in each treatment group. Abbreviations: AH, alcoholic hepatitis; SAHM-90, severe AH 90-day mortality.

course. Finally, the markers themselves are often indicators of multiple pathological processes. For instance, IL-6, a highly studied pleiotropic molecule, well-knowingly plays a dual role in the pathogenesis of AH by inducing inflammation and injury but also promoting liver regeneration.[19] IL-22 is a molecule akin to IL-6. Both molecules induce the activation of signal transducers and activators of transcription

(STAT) 3, mediated by Janus kinases, but the effects of IL-22 are restricted to cells of epithelial origin. In liver diseases, IL-22 was reported to display hepatoprotective properties, while it also plays a major part in the induction of acute phase reaction.[20] However, increased levels of both IL-6 and IL-22 have been reported to be associated with poor survival in various liver diseases.[21–24]

One of the acute phase molecules induced by STAT3 activation is lipocalin-2. Many cell types can produce it, but hepatocytes have been shown to be the main source of increased serum lipocalin-2 after injury, and the induction depends highly on the effect of IL-6. Lipocalin-2 has antibacterial and neutrophil-attracting properties, but its role in apoptosis, cell survival, and regeneration has also been proposed.[25] A small study reported the predictive potential of increased lipocalin-2 in patients with cirrhosis for hepatorenal syndrome–related and all-cause mortalities.[26]

Increased α-fetoprotein is a marker for the appearance of dedifferentiated/immature hepatocytes, therefore indicating ongoing liver regeneration after injury. However, in contrast to IL-6 and IL-22, α-fetoprotein is not an inducer but a "consequence" of liver regeneration. Therefore, higher levels favor better outcomes,[27] which is in line with our observation of reduced α-fetoprotein in nonsurvivors. Importantly, α-fetoprotein is already available for routine laboratory practice as a tumor marker; therefore, incorporating it also into risk assessment approaches in AH could be done without difficulties.

Our study identified the first-time low IL-13 level as a predictor of mortality in severe AH. Receptor binding of IL-13 leads to the activation of STAT6.[28] IL-13 was reported to display anti-inflammatory properties by suppressing proinflammatory mediators and is also involved in wound repair after injury.[29] In contrast, chronically increased IL-13 contributes to fibrosis development.[30] IL-13 levels have been reported to be higher in patients with stable chronic alcoholism compared with controls.[31] Interestingly, in our severe acute AH population, baseline IL-13 concentrations were not elevated and showed a trend to be lower than those measured in healthy controls (although this finding was not statistically significant). The suppressive effect of extensive acute alcohol consumption on the secretion of certain cytokines has been described.[32] However, reduced levels of IL-13 in nonsurvivors compared with survivors may also indicate immune exhaustion of anti-inflammatory regulation in AH.

In a study of type I diabetes, IL-6 was reported to enhance cytotoxic insults in pancreatic β cells, while IL-13 antagonized this effect.[33] Our findings that both increased IL-6 and decreased IL-13 are determinants of prognosis in acute severe AH raise the possibility that similar events might also take place in the liver during extensive alcohol abuse. The role of IL-13 in this hypothesis is further supported by a recent study that reported that eosinophil-driven IL-4/IL-13 mediated hepatoprotective function in a mouse model of acetaminophen-induced liver injury.[34] Moreover, direct tissue damage is not the only consequence of the imbalance of proinflammatory and anti-inflammatory mechanisms in liver diseases. The splanchnic vasodilation and systemic vasoconstriction can lead to the increase of portal pressure and renal hypoperfusion,[35] contributing to the progression and development of complications. IL-6 is a highly recognized marker of systemic inflammation and its association with mortality in liver diseases has been reported by multiple studies.[21–23,35] In our study, IL-6 showed the most robust elevation among the inflammatory cytokines in patients with AH compared with controls with a further increase in nonsurvivors. Age has also been recognized as an independent predictor of mortality and has been incorporated into several predictive models [Glasgow Alcoholic Hepatitis Score; Age, Bilirubin, INR, and Creatinine score; Lille score; Beclere model; and CLIF-C AD (Acute Decompensation) score]. However, the strong 90-day mortality predictive power of IL-13 in severe AH is a novel finding. Indeed, the combination of IL-13 and age had a superior 90-day mortality prediction efficiency compared with the gold standard MELD score and sustained good performance when tested in the 2 treatment groups separately. Importantly, no statistically significant difference was detected in survival between the treatment groups neither at day 28 nor at day 90.

INR, the fourth independent predictor in the Cox model, was also tested, but could not be incorporated in the composite score.

While our study has some limitations, the investigated markers were tested both as continuous and categorical variables by multiple statistical methods (ROC, log-rank, Cox regression, and logistic regression). Furthermore, we tested the SAHM-90 score in the anakinra and steroid treatment groups separately as an internal validation and obtained consistent results. This supports the reliability of our findings and also indicates that the predictive capacity of the SAHM-90 score is independent of the administered treatment. We also note that cytokine levels in our study were measured by a multiplex assay system; therefore, the displayed concentration values may numerically differ from the values measured by other techniques. However, the trends should be the same, and therefore, the observed differences and associations are supposed to be independent of the measuring method. Therefore, the concept of the SAHM-90 score has the potential to help clinical risk stratification in severe AH in the future, while the downstream pathways of the identified markers may serve as targets for therapeutic research in severe AH.

DATA AVAILABILITY STATEMENT

Data will be made available upon reasonable request from interested principal investigators.

AUTHOR CONTRIBUTIONS

Gyongyi Szabo and David Tornai designed the study and wrote the manuscript. David Tornai performed the measurements and analyzed the data. Aimee Kroll-Desrosiers assisted with preliminary statistical analyses. Bruce Barton, Srinivasan Dasarathy, Craig J. McClain, Arthur McCullough, Mack Mitchell, and Svetlana Radaeva contributed to the collection of human samples and clinical data. JungAe Lee and Bruce Barton supervised and advised on the proper use of statistical analyses. All authors read the manuscript, provided feedback, and approved it before submission.

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CONFLICTS OF INTEREST

Gyongyi Szabo consults for Evive Bio, Merck, Novartis, Durect Corporation, Terra Firma, Cyto Therapeutics, Pfizer, and Surrozen. She owns stock in Glympse, Zomagen, and Bioscience/Ventyx. She has other interests with Springer Nature Group and UpToDate. Mack Mitchell advises HepaTX and Prodigy Biotech. Craig J. McClain consults for Intercept. He received grants from Target. The remaining authors have no conflicts to report.

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