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## Diagnostic and Prognostic Significance of Complement in Patients with Alcohol-associated Hepatitis

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## **Diagnostic and Prognostic Significance of Complement in Patients with Alcohol-associated Hepatitis**

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## ABSTRACT

*Background & Aims:* Given the lack of effective therapies and high mortality in acute alcohol-associated hepatitis (AH), it is important to develop rationally-designed biomarkers for effective disease management. Complement, a critical component of the innate immune system, contributes to uncontrolled inflammatory responses leading to liver injury, but is also involved in hepatic regeneration. Here we investigated if a panel of complement proteins and activation products would provide useful biomarkers for severity of AH and aid in predicting 90 days mortality. *Approach & Results:* Plasma samples collected at time of diagnosis from 254 patients with moderate and severe AH recruited from four medical centers and 31 healthy individuals were used to quantify complement proteins by ELISA and Luminex arrays. Components of the classical and lectin pathways, including complement factors C2, C4b and C4d, as well as complement factor I (CFI) and C5, were reduced in AH patients compared to healthy individuals. In contrast, components of the alternative pathway, including complement factor Ba (CFBa) and factor D (CFD), were increased. Markers of complement activation were also differentially evident, with C5a increased and the soluble terminal complement complex (sC5b9) decreased in AH. Mannose binding lectin (MBL), C4b, CFI, C5 and sC5b9 were negatively correlated with model for end-stage liver disease (MELD) score, while CFBa and CFD were positively associated with disease severity. Lower CFI and sC5b9 were associated with increased 90-day mortality in AH. *Conclusions:* Taken together, these data indicate that AH is associated with a profound disruption of complement. Inclusion of complement, especially CFI and sC5b9, along with other laboratory indicators, could improve diagnostic and prognostic indications of disease severity and risk of mortality for AH patients.

**Key words:** alcohol-associated hepatitis; complement pathway; severity, mortality, nomogram.

**Abbreviation:** HC, health control; AH, alcohol-associated hepatitis; MELD, model for end-stage liver disease score; mDF score, maddrey's discriminant function; AST, aspartate aminotransferase;

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ALT, alanine aminotransferase; INR, international normalized ratio; MBL, mannose-binding lectin; C2, complement component 2; C4b, complement component 4b; C4d, complement component 4d; CFBa, complement factor Ba; CFBb, complement factor Bb; CFD, complement factor D; CFI, complement factor I; C3a complement component 3a; C5, complement component 5; C5a, complement component 5a; sC5b9, soluble Complement 5b-9; ND, not done.

## INTRODUCTION

Alcohol-associated hepatitis (AH) is a severe form of alcohol-associated liver disease (ALD), with up to 40% short-term mortality, (1, 2). AH can superimpose in patients at any stage of ALD (steatosis, steatohepatitis, fibrosis or cirrhosis (1, 3). Corticosteroids improve 30-day, but not 90-day or longer survival in only 50% of patients (2, 4). While some centers are successfully performing liver transplantation in patients with first episodes of severe AH not responsive to corticosteroids, patients with AH are not considered suitable candidates for liver transplantation (1, 5). Given these limited treatment options, it is important to develop rationally-designed biomarkers for effective disease management.

Complement is a critical component of the innate immune system. Initially, complement was characterized as an essential system for protection against invading pathogens, but more recent data highlight that complement also contributes to uncontrolled inflammatory responses leading to both liver and kidney injury (6-9). Importantly, complement can also have protective effects, promoting hepatocyte regeneration and wound healing responses (10, 11). Complement is activated by three independent pathways: classical, lectin and alternative. The system involves more than 50 soluble and membrane bound proteins and is under complex regulatory control.

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Making use of genetically modified mice, we and others discovered that complement activation via the classical pathway contributes to the development of hepatic inflammation in murine models of AH (8). Our recent work indicates that activation via the classical vs alternative pathways in response to ethanol may have differential impact on disease progression and resolution. The classical pathway contributes to ethanol-induced inflammation and injury (10, 11). Importantly, ethanol-induced injury in mice can be prevented by treatment with C1INH, a specific inhibitor of the classical pathway (12). In contrast, amplification of complement activation by the alternative pathway is protective, as it contributes to clearance of injured hepatocytes from the liver, promoting wound healing and the resolution of injury (10, 11). Mice deficient in Factor D, an essential mediator of the alternative pathway of complement activation, exhibit increased sensitivity to chronic ethanol-induced liver injury. Hepatic injury was associated with impaired clearance of apoptotic hepatocytes in mice lacking alternative pathway, likely leading to secondary necrosis and fibrosis in the liver (10).

Limited data exist regarding complement activation and disease progression in AH. For example, immunohistochemical analysis of liver biopsies from patients with AH revealed increased expression of C1q, C3, C5, and C5a receptor (C5aR), as well as increased expression of C1q and C5, but not C3, mRNA in AH patients. (13). Evidence of complement activation via both the classical and alternative pathways is detected in livers of patients with AH, and serum C5a is increased in AH patients compared to healthy individuals (10). In the current study, we have quantified circulating complement proteins, complement activation products and complement inhibitory factor, to assess the efficacy of complement factors for disease prediction and the association with disease severity and the risk of mortality in patients with AH.

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## **PATIENTS AND METHODS**

### **Study population**

A total of 285 subjects were included in this study. De-identified plasma samples, along with clinical and demographic data, were obtained from 31 healthy individuals from the Northern Ohio Alcohol Center biorepository and 254 patients diagnosed with AH using clinical and laboratory criteria recommended by the NIAAA Alcoholic Hepatitis consortia (14). AH Patients were recruited from four medical centers participating in the Defeat Alcoholic Steatohepatitis (DASH) consortium (Cleveland Clinic, University of Louisville School of Medicine, University of Massachusetts Medical School, and University of Texas Southwestern Medical Center). A detailed description of patient recruitment, inclusion and exclusion criteria for the DASH consortium has been reported in previous studies (15, 16). Patients with AH were classified as moderate (MELD < 20, N=112) and severe (MELD  $\geq$ 20, N=142) according to the MELD score at admission as part of either of two independent clinical trials (ClinicalTrials.gov identifier # NCT01809132 and NCT03224949). Of the total AH cohort, 88 AH patients were followed for 180 days. This study was approved by the Institutional Review Boards of all 4 participating institutions and all study participants consented prior to collection of data and blood samples.

### **Study design and outcomes**

This study first compared plasma complement factor concentrations in AH and healthy groups at enrollment to evaluate the ability of complement factors to distinguish AH from healthy individuals, to predict disease severity and 90-day mortality. We then developed novel combinatorial models to predict the risk of 90-day mortality.

### **Sample collection and measurement of complement factors**

Blood samples were collected within 48 h of enrollment, plasma was separated and stored at -80°C. Quantification of MBL, C2, C4b, CFBb, CFD, CFI, C3a, C5, and sC5b9 concentration in plasma was performed at Exsera BioLabs in Aurora Colorado, USA. For detailed methodology, see <https://www.exserabiolabs.org>. Plasma C4d, CFBa, and C5a concentrations were quantified by

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ELISA using by the following kits: human C4d enzyme-linked immunosorbent assay (ELISA) kit (MyBiosource, MBS703196); human CFBa ELISA kit (Quidel MicroVue, A033); and human C5a ELISA kit (Hycult Biotech, HK349).

### **Statistical analysis**

Continuous variables were expressed as means + standard error. Two group comparisons were made using unpaired t-test or the Mann-Whitney test for continuous variables, based on the results of Kolmogorov-Smirnov normality test. The Welch's analysis of variance (ANOVA) and Games-Howell post-hoc test was applied to compare continuous variables across healthy individuals, patients with moderate and severe AH. Categorical variables are reported as counts and percentages; Chi-square or Fisher's exact tests were used for comparing categorical factors. Spearman correlation coefficient was used to estimate the association of plasma complement factors and variables of interest. Logistic regression analysis was used to assess the association between plasma complement factors and the likelihood of having AH and severity of AH.

For assessment of independent associations with 90-day mortality, the univariate and multivariate Cox regression hazard model was used. Kaplan-Meier survival curves were plotted to estimate the cumulative probability of mortality for complement factors and laboratory indices related to mortality (17). In order to select the suitable indicators to establish the prognostic model, feature selection was performed using the information gain attribute ranking method (18). Information gain was measured by comparing the entropy of the data before and after transformation. Only attributes of variables  $>0.01$  were selected for logistic regression model.

In order to assess predictive potential and to limit model over-fitting, the leave-one-out cross-validation method (19) was implemented. The diagnostic accuracy for the diagnosis of AH and prognostic accuracy of 90-day mortality of AH patients was evaluated using receiver operating characteristic (ROC) curves. Comparisons of the area under the ROC were performed by the integrated discriminating improvement statistics test (20). Multiple testing was controlled



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using a false discovery rate (FDR) approach and FDR p values  $<0.05$  were considered to be statistically significant (21). All statistical analyses were performed with STATA (Version 16.0, IBM, New York, USA), R (Version R-3.6.2, USA), and Orange (Version 3.24.1, USA).

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## RESULTS

### Characteristics of the study population

Patient demographics and clinical characteristics are summarized in Table 1. Average age and sex distributions were different between healthy individuals and patients with AH (Table 1). However, these differences in age and sex did not appear to affect overall complement concentrations in the plasma, as shown by principal component analysis (PCA) (Supplementary Figure 1). MELD score, Maddrey's discriminant function score, Child-Pugh Score, aspartate aminotransferase (AST), total bilirubin, creatinine, and international normalized ratio (INR) were higher in patients with severe AH compared to moderate AH patients, while serum albumin was lower in severe AH compared to moderate AH patients.

### Multiple Plasma Complement Factors Distinguish AH and Healthy Subjects

A total of 12 complement factors were measured in plasma samples from healthy individuals and patients with AH including complement proteins (MBL, C2, CFD, C5), complement activation products (C4b, C4d, CFBa, CFBb, C3a, C5a), the complement protein complex sC5b9 and the complement inhibitor CFI. AH patients had lower complement proteins C2 and C5, higher CFD and no difference in MBL (Figure 1A). Despite lower concentrations of individual complement proteins, some complement activation products were increased in AH, including CFBa and C5a (Figure 1A). In contrast, the terminal complement protein complex sC5b9, as well as C4b, C4d, and CFI, were decreased in AH relative to healthy individuals (Figure 1A).

In order to better understand the relationship between complement and AH, we performed correlation analysis between complement factors and AH (Supplemental Figure 1C). This analysis combined both moderate and severe AH patients. C2, C4b, C4d, CFI, C5 and sC5b9 were negatively correlated with AH, while CFBa and CFD concentrations were positively correlated with AH. Multiple logistic regression revealed that C2, C4b, C4d, CFBa, CFD, CFI, C5 and sC5b9 were significantly associated with AH in comparison with healthy subjects after adjusting for age and sex, respectively (FDR  $p < 0.05$ , Table S1). ROC curves were constructed and

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demonstrated that multiple complement factors were able to distinguish AH patients from healthy individuals (Figure 1B and Supplemental Table 2): C4d (AUC 0.953; sensitivity 95.7% and specificity 99%), C4b (AUC 0.918; sensitivity 90% and specificity 85%), CFD (AUC 0.908; sensitivity 86.9% and specificity 83%), sC5b9 (AUC 0.896; sensitivity 86.7% and specificity 85.0%), and CFI (AUC 0.825; sensitivity 90% and specificity 76%).

### **Expression of mRNA for Complement Proteins in Liver of AH patients**

Complement factors are produced by many cells and tissues in the body, but the liver is an important source of circulating complement proteins (22, 23). Indeed, the liver is the sole source of MASP2, C8A, and C9 (24). In order to investigate if expression of complement genes differed between patients with AH and healthy individuals, we made use of a publicly available transcriptomics data from Affo, et al. in livers of 15 patients with AH compared to 7 healthy controls (GSE28619 (25)) and RNA sequencing data from livers from 5 AH patients and 5 healthy individuals from Peiffer, et al (GSE143318 (26)). PCA of the expression of complement genes separated AH and healthy individuals in both datasets (Supplemental Figure 2A and 2B). A total of 25 differentially expressed complement genes (DEGs) were detected in GSE28619 and 34 complement DEGs were detected in GSE143318 (Supplemental Figure 2 and Supplementary Table 7). The volcano plot and Venn diagram (Supplemental Figure 2C) show that 20 complement DEGs were present in both datasets. Sixteen of these overlapping complement DEGs were down-regulated, including CFI and components of the C5b9 (C6, C8ab and C9), consistent with the decrease in plasma CFI and sC5b9 (Figure 1A).

### **Classical and Lectin pathway negatively correlated to clinical disease severity in AH, while the Alternative Pathway was positively correlated**

Since changes in the quantity of multiple complement factors were associated with AH, we next investigated whether concentrations of individual complement factors in circulation were also correlated with disease severity. As shown in Table 1 and Figure 1A, MBL, C4b, CFI, and sC5b9 were lower and CFBa was higher in the severe AH patients compared to patients with moderate

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AH (FDR  $p < 0.05$ ). Correlation analysis revealed that components of the classical and lectin pathways, MBL and C4b, were negatively associated with MELD score, while members of the alternative pathway, CFb<sub>a</sub> and CFD, were positively associated with MELD (Figure 2A). CFI, C5 and sC5b9 were also negatively associated with MELD score (Figure 2A). Additional univariate logistic regression analysis (Supplemental Table 3) showed that plasma MBL (unadjusted OR = 0.580, 95%CI 0.418-0.806;  $p = 0.006$ ), C4b (unadjusted OR = 0.900, 95%CI 0.845-0.958;  $p = 0.004$ ), CFI (unadjusted OR = 0.871, 95%CI 0.828-0.916;  $p < 0.001$ ), and sC5b9 (unadjusted OR = 0.997, 95%CI 0.994-1.000;  $p = 0.0057$ ) were negatively associated and CFB<sub>a</sub> (unadjusted OR = 1.001, 95%CI 1.000-1.002;  $p = 0.030$ ) was positively associated with severity of AH. We also established ROC curves to show the ability of complement factors to distinguish patients with moderate from severe AH (Figure 1C). CFI had moderate ability to distinguish patients with severe AH from moderate AH (AUC 0.710, sensitivity 79% and specificity 57%). sC5b9 showed an AUC equal to 0.613, with sensitivity 50% and specificity 74%. Interestingly, CFI was more sensitive and sC5b9 was more specific in distinguishing disease severity. Taken together, these data suggest that complement factors may be useful biomarkers in predicting disease severity in AH and that complement pathways may differentially contribute to disease severity (Figure 2B).

### **Complement factors CFI and sC5b9 were negatively associated with 90-day mortality in patients with AH**

Of the 254 AH patients in our cohort, 88 AH patients were followed up for 180 days (Table 2). Overall, 10 of 88 AH patients died within the 28 days follow up in our study (28 days mortality was 11.4%), another 23 patients died within the 90 days (90 days mortality was 26.1%), and additional 25 patients died within the 180 days (180 days mortality was 28.4%) (Table 2 and Supplemental Table 3). Here we present 90-day mortality data, and interestingly, correlation analysis found that CFI and sC5b9 were the only two complement factors negatively correlated with the risk of 90-day mortality in AH patients (Supplemental Figure 3). Both plasma CFI and sC5b9 concentrations were lower in patients who died during the 90-day follow-up (Figure 3A) (FDR  $p < 0.05$ ). As expected, non-survivors had higher MELD, Maddrey's discriminant function

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and Child-Pugh scores, as well as serum creatinine, INR and total bilirubin and lower serum albumin concentrations (Table 2 and Figure 3A).

Based on these correlations, we then investigated whether complement factors, especially CFI and sC5b9, were independent prognostic indicators for AH patients. Using Cox regression analysis, only sC5b9 (adjusted HR=0.992, 95%CI 0.985-0.999; p=0.034) was an independently associated with 90-day mortality of AH patients after adjustment for age, sex, race and MELD score (Table 3). The continuous variables, lower CFI, sC5b9 and albumin, and higher MELD score, Maddrey's discriminant function and Child-Pugh scores, creatinine, INR, and total bilirubin were associated with increased 90-day mortality (FDR p<0.05). The optimal cut-off values for these continuous variables were calculated using the Youden-Index obtained with from the ROC analysis for the prediction of 90-day mortality from the withheld samples in the leave-one-out cross-validation procedure (Supplemental Table 5). Kaplan-Meier survival plots found that CFI < 8.7 mcg/mL, sC5b9 <163.5 ng/mL, albumin < 2.68 g/L, total bilirubin  $\geq$  11.35 mg/dL, creatinine  $\geq$  0.82 mg/dL, INR  $\geq$  1.85, MELD score  $\geq$  20, Maddrey's discriminant function score  $\geq$  32, and Child-Pugh score  $\geq$  7 were associated with increased 90-day mortality (Figure 3B).

### **Models integrating complement factors with current models for the prediction of 90-day mortality**

We next investigated whether the addition of complement factors to current predictive models, MELD and Maddrey's discriminant function, could improve prognostics for 90-day mortality in AH. Feature selection identified 10 significant variables (p>0.01). MELD score, Maddrey's discriminant function score, and Child-Pugh score as the top 3 variables for prediction of 90-day mortality of AH patients, as expected (Supplemental Figure 4). The next 7 of the top 10 ranked features were albumin, CFI, total bilirubin, sC5b9, INR, creatinine, and age (Supplemental Figure 4). However, in order not to over-represent any one clinical measure, we did not include total bilirubin (used in both MELD and Maddrey's discriminant function scores) or INR and creatinine (used in MELD scores) when building the new integrated models. When used individually, AUC

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of CFI was estimated to be 0.621 (95%CI 0.481-0.762) and sC5b9 was 0.652 (95%CI 0.517-0.788). These complement factor AUCs were similar to those of individual clinical values: creatinine (AUC=0.623, 95%CI 0.484-0.762), INR (AUC=0.689, 95%CI 0.570-0.808) and albumin (AUC=0.643, 95%CI 0.527-0.759). However, two of the integrated models: MELD-Complement score, combining albumin, age, CFI and sC5b9 with MELD score (AUC 0.847; sensitivity 78% and specificity 86%) and the Maddrey's discriminant function-Complement score, combining albumin, age, CFI, sC5b9, creatinine, INR with Maddrey's discriminant function score (AUC 0.858; sensitivity 91% and specificity 77%) trended towards being a better predictor of 90 days mortality than MELD (AUC 0.814; sensitivity 70% and specificity 85%) or Maddrey's discriminant function score (AUC 0.773; sensitivity 74% and specificity 71%) alone; however, these differences did not reach statistical significance (FDR  $p > 0.05$ ) (Supplemental Table 5).

Importantly, in severe AH patients, the combined MELD-Complement score had a better predictive ability to assess the risk of 90-day mortality in severe AH patients (AUC 0.760; sensitivity 83% and specificity 70%; Supplemental Table 6) compared to MELD alone (13.4% better) and Maddrey's discriminant function (26.8% better). These improvements in predicting the risk of 90-day mortality were primarily driven by the male patients in the cohort, with the MELD-Complement score (AUC 0.890; sensitivity 81% and specificity 90%) improving over Maddrey's discriminant function score (AUC 0.742; sensitivity 75% and specificity 70%,  $p < 0.016$ ) and MELD score (AUC 0.785; sensitivity 69% and specificity 83%,  $p < 0.020$ ) at predicting the 90-day mortality in male, but not female, AH patients (Table 4). This sex difference may be due to the enhanced predictability in males since males were more likely to have severe AH.

In order to allow users to easily calculate the risk of 90-day mortality using the new MELD-Complement combined model, we created a nomogram to provide graphical depictions of all variables in the MELD-Complement integrated model (Figure 4). The calibration curve of the nomogram showed good agreement between the predictive risk and the observed mortality probability in the total AH cohort (Supplemental Figure 5). The Hosmer-Lemeshow test was not

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significant ( $P= 0.575$ , Supplemental Table 5), suggesting the new model was correctly specified for the prediction of 90 day mortality.

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## DISCUSSION

In this study, using 254 patients with AH from four medical centers enrolled as part of the NIAAA UO1 DASH consortium, we explored the relationship between plasma complement factors and AH. Complement factors (C4d, C4b, CFD, CFI, C5 and sC5b9) have good diagnostic abilities for the identification of AH from healthy individuals and were correlated with disease severity. Of these complement factors, both CFI and sC5b9 were negatively associated with 90-day mortality in AH patients and sC5b9 was an independent predictor for 90-day mortality of AH patients after adjustment for age, sex, race and MELD score. Models integrating complement factors CFI and sC5b9 with current models for the prediction of 90-day mortality, including MELD and Maddrey's discriminant function, improved the ability to predict 90-day mortality compared to current models alone. Taken together, these data suggest that complement factors are valuable diagnostic and prognostic biomarkers in patients with AH.

Our analysis revealed that plasma complement factors (C2, C4b, and C4d), activated by the classical and lectin pathways, were decreased in AH patients compared to healthy individuals, whereas complement factors (CFBa and CFD), components of the alternative pathway, were increased. Further evidence of differential activation of complement in AH is provided by contrasting increase in the anaphylatoxin C5a compared to the decrease in sC5b9, both down-stream complement activation products. Complement activity is regulated by multiple inhibitory factors and regulators; expression of CFI, an important complement inhibitor, was decreased in liver and circulation in AH patients while expression of CD59, a potent inhibitor of the complement membrane attack complex, was also decreased. Overall, these data suggest that AH differentially impacts complement activation pathways (Figure 2B).

Based on pre-clinical studies in murine models of ethanol-induced liver injury, it is likely that this differential activation of complement in AH is of potential importance to the progression of AH. For example, activation of the classical pathway, via C1q, contributes to inflammation and injury in murine models of ethanol-induced liver injury (8), while CFD, in the alternative pathway, is



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protective (10). CFD is also known to be protective in models of carbon-tetrachloride induced liver fibrosis, likely facilitating the clearance of injured hepatocytes to promote recovery (11).

Therefore, it is possible that activation of the alternative pathway, as indicated by CFBa and CFD, is a protective response to injury, rather than a process contributing to progression of injury in AH. As with many innate immune pathways, complement is involved in both injury and repair.

Complement can be activated in response to pathogens and/or tissue injury (6, 27). While pre-clinical studies indicate that complement contributes to both ethanol-induced injury and repair (8, 9, 11), there is also a likely contribution of complement to defend from bacterial and/or viral infections in patients with AH. Reduced expression of multiple complement proteins likely contributes to the impaired ability of patients with AH to mount defenses against pathogens (3, 28-30) and may contribute to the increased risk of infection observed in AH patients treated with glucocorticoids and immunosuppressive therapies (3, 31).

The liver is an important, but not the sole, source of most circulating complement proteins (22, 23); only MASP2, C8A, and C9 are exclusively produced by the liver (24). In cirrhosis, circulating ficolins-1-3, C3 and C4, as well as MASP2 were decreased with increasing disease severity (32, 33), suggesting that reduced circulating complement may be due to decreased expression in the liver. Using 2 independent data sets that are publicly available (GSE28619 (25) and GSE143318 (26)), we find that expression of complement genes in liver could also distinguish patients with AH from healthy individuals. Interestingly, the majority of complement genes with reduced expression in liver in patients with AH were concentrated in the classical and lectin pathways, similar to the changes in plasma complement factors. Previous reports found evidence of complement activation by both the classical and alternative pathways in livers of patients with AH (10). Collectively, these data indicate that expression of complement proteins by the liver is impaired in AH patients. However, that expression and/or activation of alternative pathway complement factors are likely not primarily regulated by the liver as complement pathway shown in Figure 2B (22, 23). Of note, adipose tissue is an important source for expression of complement

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factors in the alternative pathway (34). Given recent evidence that adipose-liver interactions are important for the progression of ethanol-induced liver injury in mice, future studies into the contribution of adipose tissue to whole body complement activation in AH will be of potential importance.

Limited effective treatment options are available for patients with either moderate or severe AH (1-3). Current clinical trials rely on 90-day mortality as their primary endpoint (16, 31) and improved 90-day mortality prognostics may be particularly important for selecting patients for early liver transplants (35). However, current diagnostic criteria, including MELD and Maddrey's discriminant function are not optimal for prognostic evaluation of 90-day mortality. Therefore, identification of additional biomarkers that can improve prognostic evaluation are of importance to the field (36). Here we have developed novel combined models including complement factors CFI and sC5b9 with either MELD or Maddrey's discriminant function score that result in improved predictions of 90-day survival in severe AH (Table 4). These models appear to be particularly effective in male patients (Table 4). We have developed a convenient nomogram for clinical practice incorporating the complement factors CFI and sC5b9 with MELD score, albumin and age for providing a more sensitive and specific prognostic indicator of 90-day mortality in AH (Figure 4).

The current study has several strengths and limitations. Strengths include using well characterized patients and prospectively collected clinical data from a large multi-center AH cohort to evaluate the relationship between plasma complement factors with both disease severity and 90-day mortality in AH for the first time. We selected key complement factors reflecting quantity and activation of complement factors in plasma as well as analysis of liver gene expression data (25) and used assays with a rapid turnaround time. Limitations include the need for validation cohorts, given the highly innovative observations, to confirm the predictive potential of the models, to establish reliable threshold values, and to further evaluate the sex specific prognostic value of

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complement factors in predicting 90-day mortality. As the majority of participants in this study are Caucasians, the impact of racial and ethnic diversity on complement in AH could not be investigated. It will also be important to determine whether complement factors can predict response to therapies and/or risk of infection in AH. Future studies dissecting the differential contribution of the classical/lectin pathways vs alternative pathways to disease progression or resolution will be of particular importance in the development of complement factors as biomarkers or as targets of therapeutic intervention.

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## Figure legends

**Figure 1. Changes of baseline complement factors in plasma and the ability of plasma complement factors to distinguish between AH and healthy individuals.** (A) Concentrations of MBL, C2, C4b, C4d, CFBa, CFBb, CFD, CFI, C3a, C5, C5a, and sC5b9 were quantified in the plasma of AH patients and healthy individuals by ELISA and Luminex arrays. For most endpoints, a total of 243 plasma samples were analyzed: AH patients: n=213 (98 moderate AH and 115 severe AH) and healthy individuals n=30. Plasma C4d: AH patients: n=201 (94 moderate AH and 107 severe AH) and healthy individuals n=23. Plasma CFBa and C5a: 242 AH patient: n=242 (104 moderate AH and 138 severe AH) and healthy individuals n=23. All data are represented, showing minimum to maximum, with means  $\pm$  SEM superimposed. Values with different superscripts are significantly different,  $p < 0.05$ . (B) ROC curves for distinguishing AH patients from healthy individuals based on plasma complement factors. AUROC of top 6 complement factors (C4d, C4b, CFD, sC5b9, CFI, and C5) are illustrated. (C) ROC curves for distinguishing patients with moderate AH from patients with severe AH based on plasma complement factors. AUROC of top 5 complement factors (CFI, CFBa, C4b, sC5b9 and MBL) are illustrated.

**Figure 2. Correlation analyses of complement factors and clinical laboratory values in AH patients according to disease severity.** (A) Spearman's correlation analyses of plasma complement factor concentrations and clinical variables of interest. R coefficients are given within the blocks. Correlations with  $p < 0.05$  are shown in color (blue for positive correlations and red for negative correlations). (B) Schematic summary of dysregulation of expression and activation of complement in AH. Analysis of circulating complement factors and gene expression data from patients with AH indicates that expression and activation of complement is dysregulated in AH patients. Analysis of complement factors in the plasma suggest that activation of the classical and lectin pathways is compromised in AH, with the alternative pathway enhanced. These changes in the concentration of plasma complement factors are in accord with the liver transcriptome analysis

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which revealed that expression of complement genes in the classical and lectin pathways (C1r, MASP1, and MASP2) as well as the inhibitor of complement (CFI), and multiple components of the terminal complement complex (C6, C8a, C8b, and C9) were decreased in AH patients compared to healthy individuals.

**Figure 3. CFI and sC5b9 are decreased in non-survivor AH patients at 90-days and predict 90-day mortality.** (A) CFI and sC5b9 were analyzed as in Figure 1. Concentrations of CFI and sC5b9, as well as clinical laboratory values, are illustrated. All data are represented, showing minimum to maximum, with means  $\pm$  SEM superimposed. P values are illustrated. (B) Kaplan-Meier plots are illustrated for CFI, sC5b9, albumin, creatinine, INR and total bilirubin, P values are provided. Non-survivors all had MELD score  $\geq$  20, Maddrey's discriminant function score  $\geq$  32, or Child-Pugh score  $\geq$  7 groups, so these Kaplan-Meier plots are not illustrated.

**Figure 4. Nomogram for a new combined model (MELD-Complement score) to predict 90 days mortality.** (A) Based on the results of feature selection, MELD score, as well as albumin, sC5b9, age, and CFI (4 of the top 10 ranked features) were selected for logistic regression analysis to build the prognostic model. Each independent factor was assigned a score on the points scale. The sum of each special score was obtained as total weighted point. The sum of points corresponds to the estimated probability of 90 days death of AH patients.



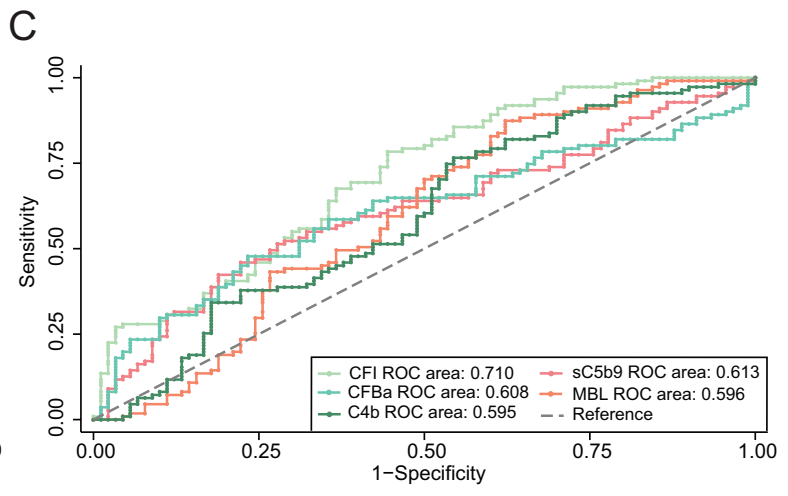
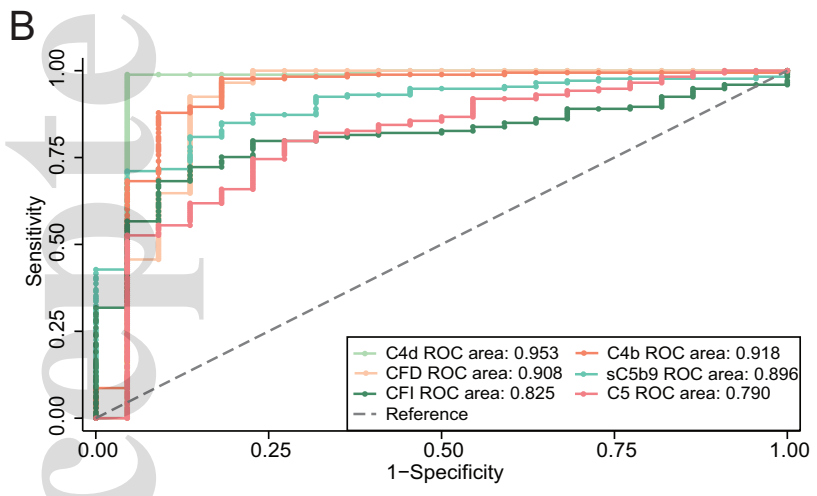
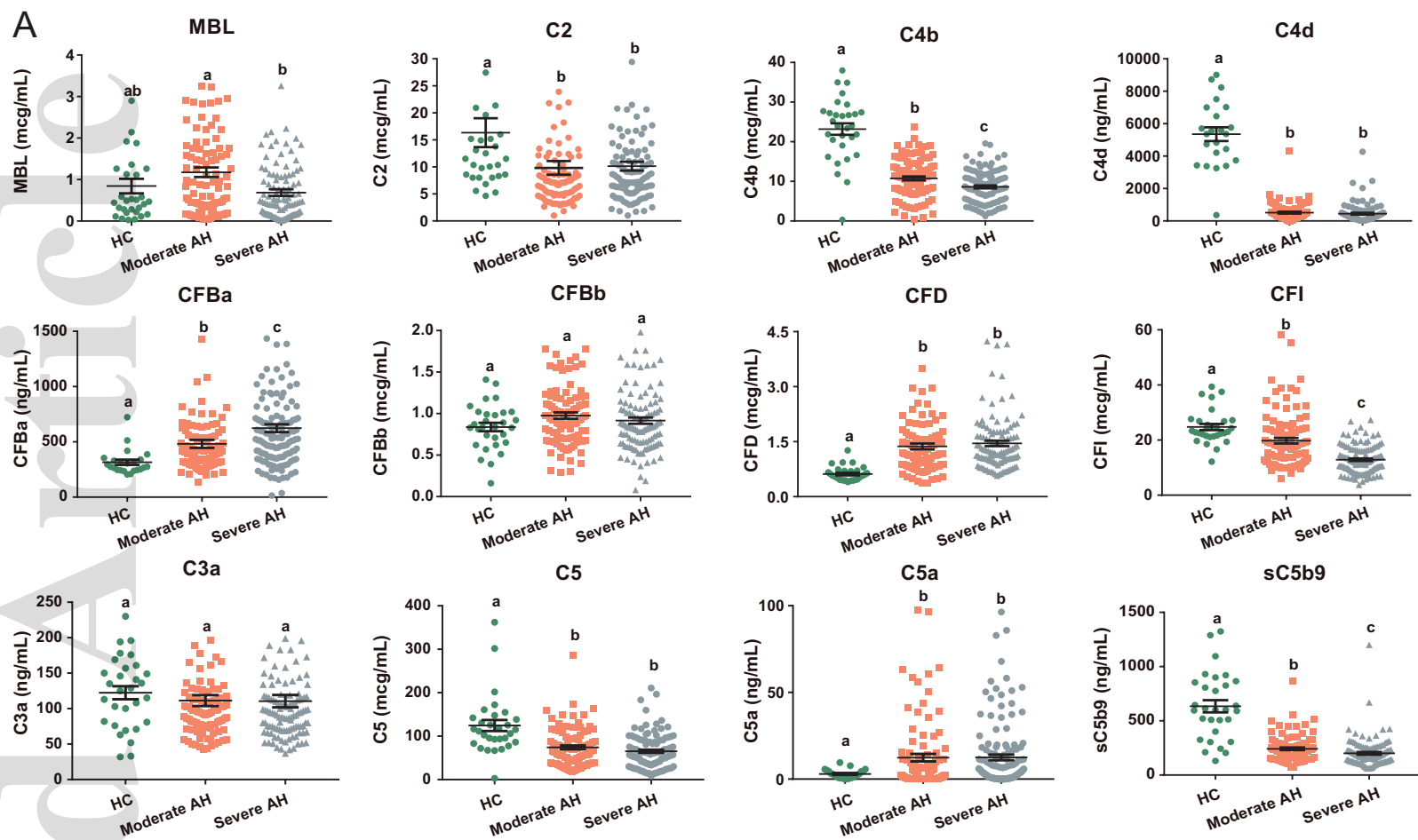
**Table 1. Demographic, clinical characteristics and plasma complement concentrations of study participants**

<b>Variables</b>	<b>HC</b>	<b>Moderate AH</b>	<b>Severe AH</b>	<b>P value<sup>1,2</sup></b>
No. of cases	31	112	142	
<b>Age, mean (SE)</b>	40.42(2.70)	48.92(1.01)	47.15(0.79)	0.014
<b>Male, n (%)</b>	13(41.9)	67(59.8)	95(66.9)	0.032
<b>Race, n (%)</b>				0.453
White	31(100)	102(91.1)	128(90.1)	
African American	0	7(6.3)	11(7.7)	
Asian American	0	1(0.9)	0	
American Indian	0	1(0.9)	1(0.7)	
Other	0	1(0.9)	2(1.4)	
<b>Liver cirrhosis, n (%)</b>	ND	11(71.3)	114(84.4)	0.013
<b>Liver function score, mean (SE)</b>				
MELD	ND	13.36(0.41)	26.08(0.43)	<0.001
Maddrey's discriminant function score	ND	12.65(1.40)	57.71(2.28)	<0.001
Child-pugh score	ND	7.81(0.17)	10.56(0.11)	<0.001
<b>90-day mortality, n (%)</b>	ND	0/28(0)	23/60(38.3)	<0.001
<b>Laboratory tests, mean (SE)<sup>3</sup></b>				
AST (IU/L)	ND	97.69(7.12)	119.62(5.63)	0.015
ALT (IU/L)	ND	44.34(3.16)	46.95(2.81)	0.537
Total bilirubin (mg/dL)	ND	4.73(0.50)	18.80(0.76)	<0.001
Albumin (g/L)	ND	3.17(0.07)	2.64(0.05)	<0.001
Creatinine (mg/dL)	ND	0.79(0.04)	1.15(0.09)	0.001
INR	ND	1.32(0.03)	1.93(0.04)	<0.001
<b>Complement factors, mean (SE)</b>				
MBL (mcg/mL)	0.84(0.17) <sup>ab</sup>	1.18(0.12) <sup>a</sup>	0.69(0.08) <sup>b</sup>	<0.001
C2 (mcg/mL)	16.34(2.67) <sup>a</sup>	9.82(1.26) <sup>b</sup>	10.14(0.82) <sup>b</sup>	0.015
C4b (mcg/mL)	23.20(1.46) <sup>a</sup>	10.75(0.50) <sup>b</sup>	8.60(0.38) <sup>c</sup>	<0.001

C4d (ng/mL)	5350(424) <sup>a</sup>	513(56) <sup>b</sup>	446(55) <sup>b</sup>	<0.001
CFBa (ng/mL)	315(23) <sup>a</sup>	482(36) <sup>b</sup>	623(35) <sup>c</sup>	<0.001
CFBb (mcg/mL)	0.84(0.05) <sup>a</sup>	0.98(0.04) <sup>a</sup>	1.01(0.10) <sup>a</sup>	0.579
CFD (mcg/mL)	0.62(0.04) <sup>a</sup>	1.37(0.08) <sup>b</sup>	1.62(0.18) <sup>b</sup>	0.003
CFI (mcg/mL)	24.77(1.12) <sup>a</sup>	19.78(1.01) <sup>b</sup>	12.88(0.47) <sup>c</sup>	<0.001
C3a (ng/mL)	122(9) <sup>a</sup>	111(7) <sup>a</sup>	130(20) <sup>a</sup>	0.683
C5 (mcg/mL)	124(12.54) <sup>a</sup>	74(4) <sup>b</sup>	65(4) <sup>b</sup>	<0.001
C5a (ng/mL)	2.97(0.53) <sup>a</sup>	16.36(4.75) <sup>b</sup>	14.01(2.24) <sup>b</sup>	<0.001
sC5b9 (ng/mL)	634(56) <sup>a</sup>	242(12) <sup>b</sup>	200(12) <sup>c</sup>	<0.001

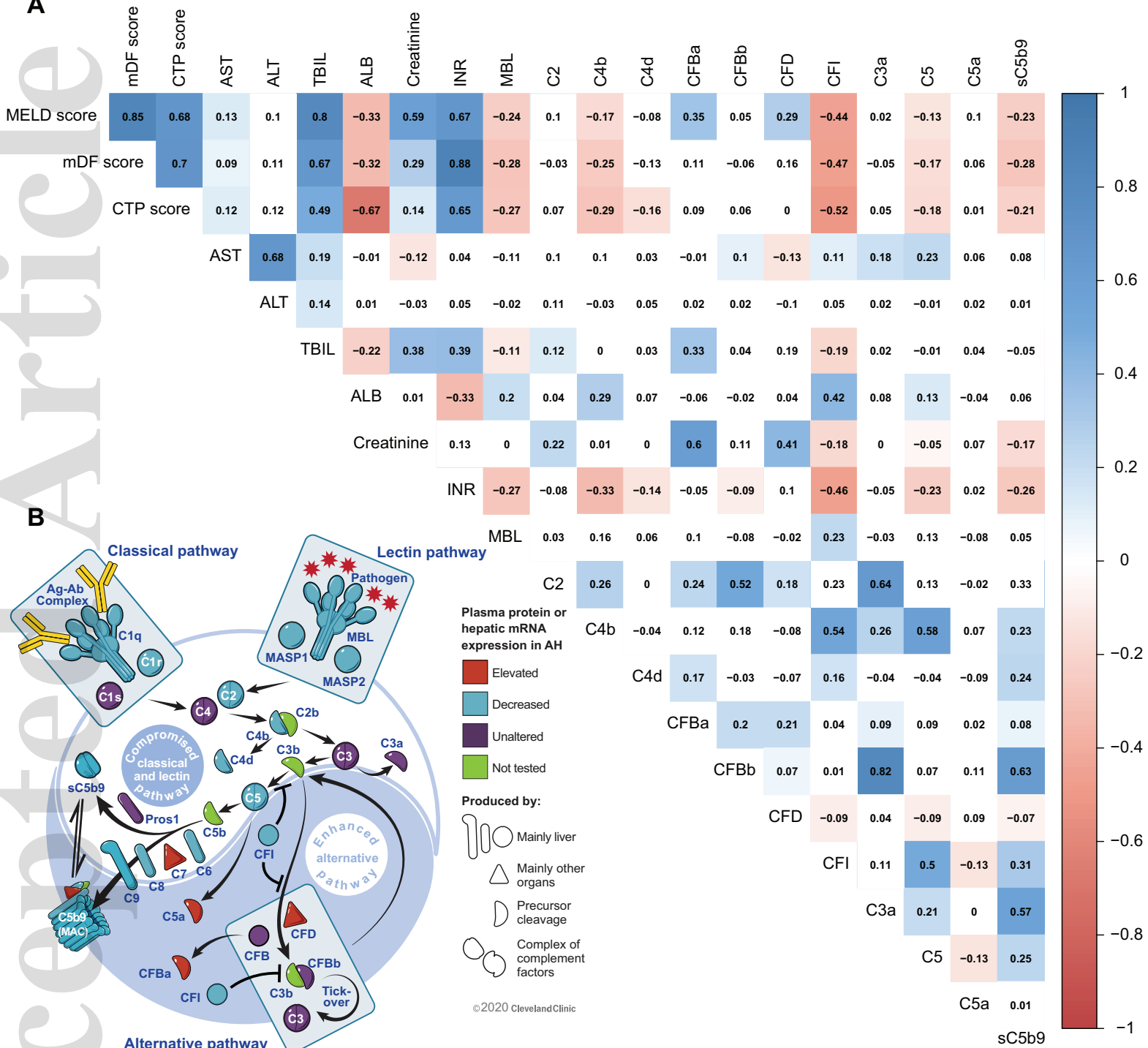
Abbreviation: HC, healthy control; AH, alcohol-associated hepatitis; MELD, model for end-stage liver disease score; AST, aspartate aminotransferase; ALT, alanine aminotransferase; INR, international normalized ratio; MBL, mannose-binding lectin; C2, complement component 2; C4b, complement component 4b; C4d, complement component 4d; CFBa, complement factor Ba; CFBb, complement factor Bb; CFD, complement factor D; CFI, complement factor I; C3a complement component 3a; C5, complement component 5; C5a, complement component 5a; sC5b9, soluble Complement 5b-9; ND, not done.

1. P values for clinical parameters compare moderate to severe AH
2. Complement factors with different superscripts (a, b, or c) are significantly different from each other across HC, moderate AH and severe AH,  $p < 0.05$ .
3. Normal ranges of AST, ALT, total bilirubin, albumin, creatinine may vary slightly depending on the different machines and methods used at the 4 medical centers enrolling patients. In general, the normal range of AST or ALT is lower than 40 U/, total bilirubin is lower than 1.2 mg/dL, albumin is approximately 3.5 to 5.5 g/dL, and creatinine approximately 0.6 to 1.2 mg/dL.

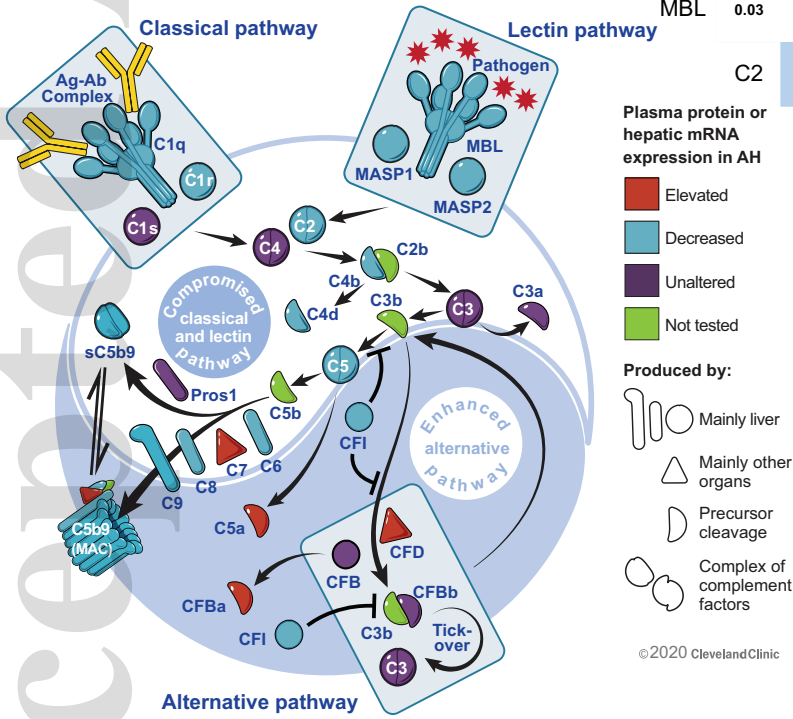


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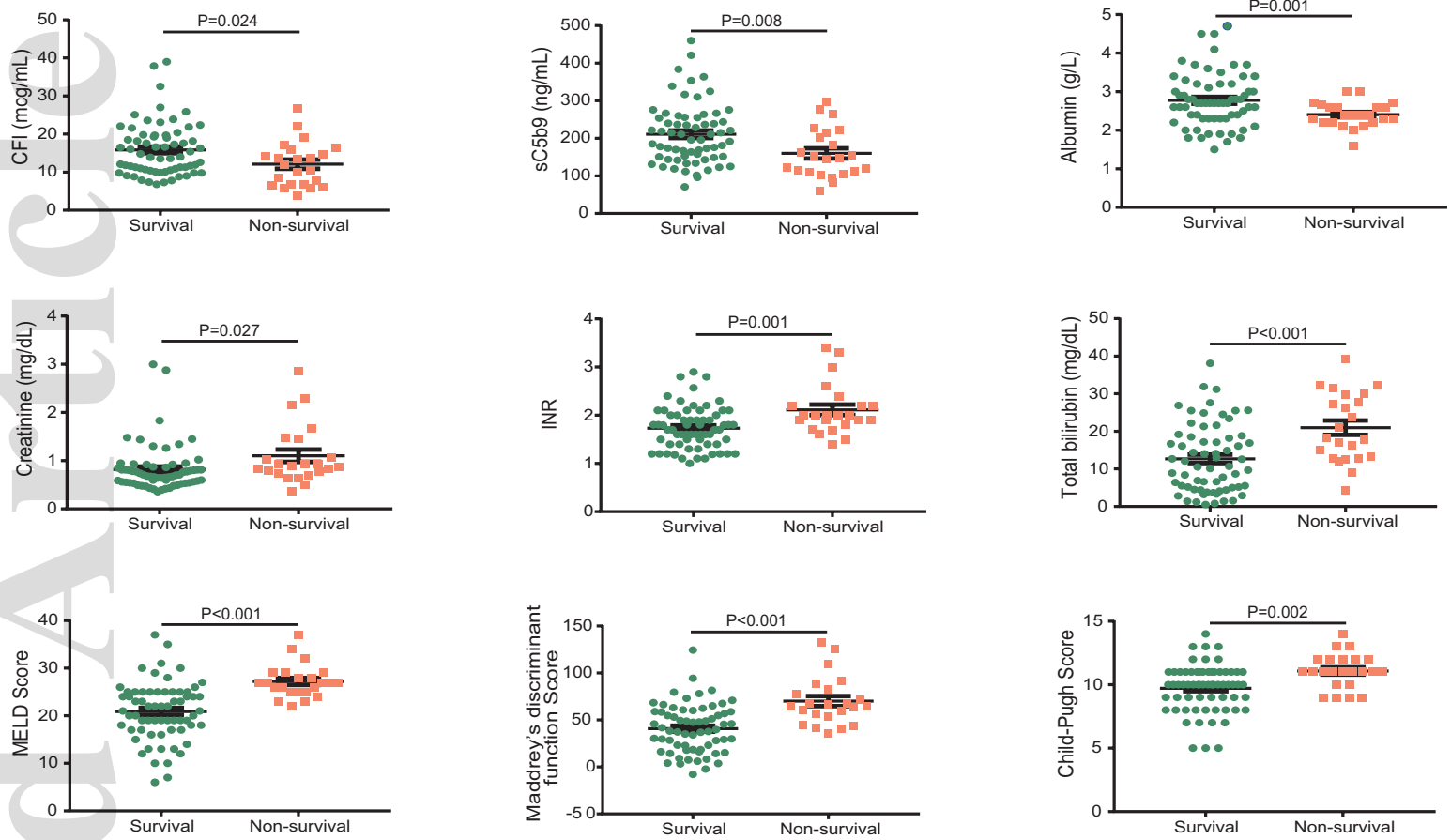
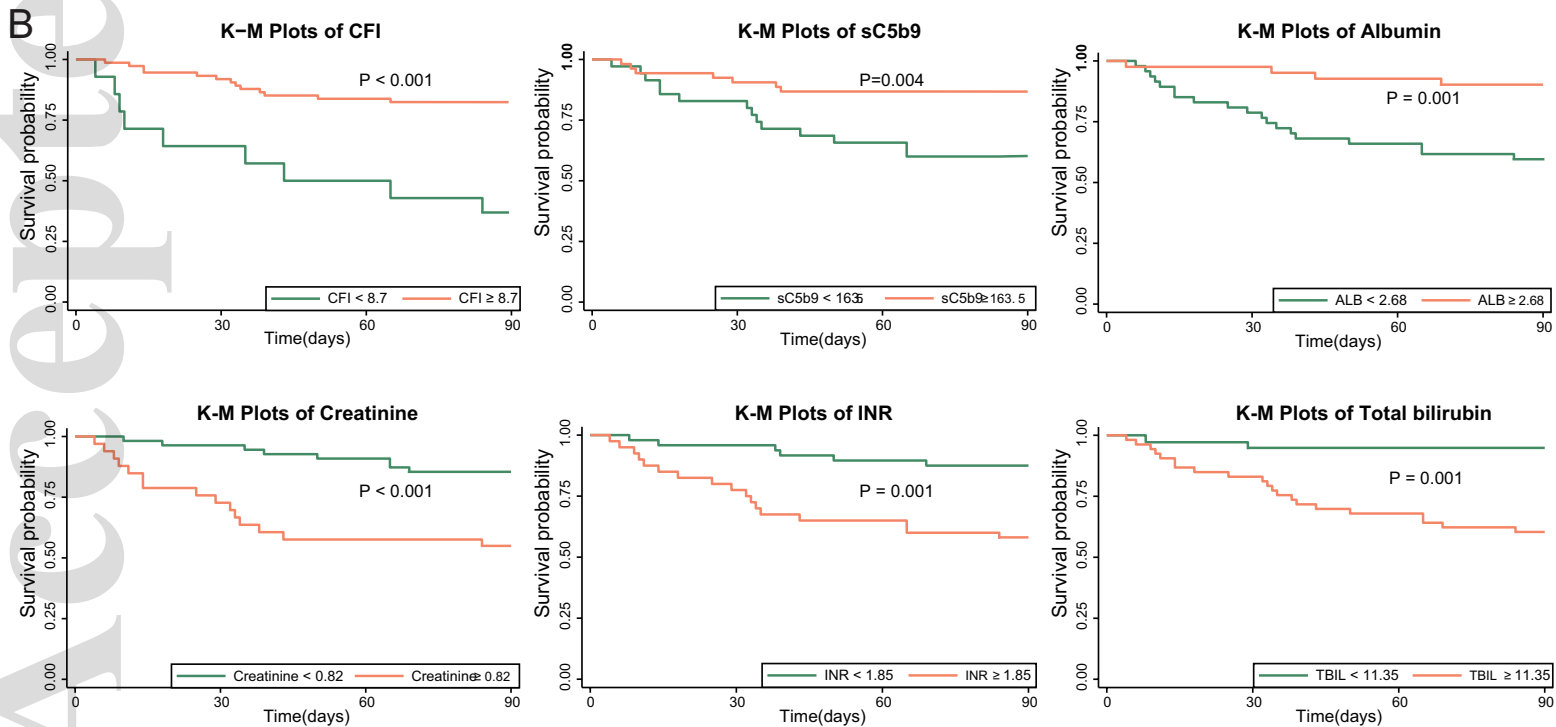
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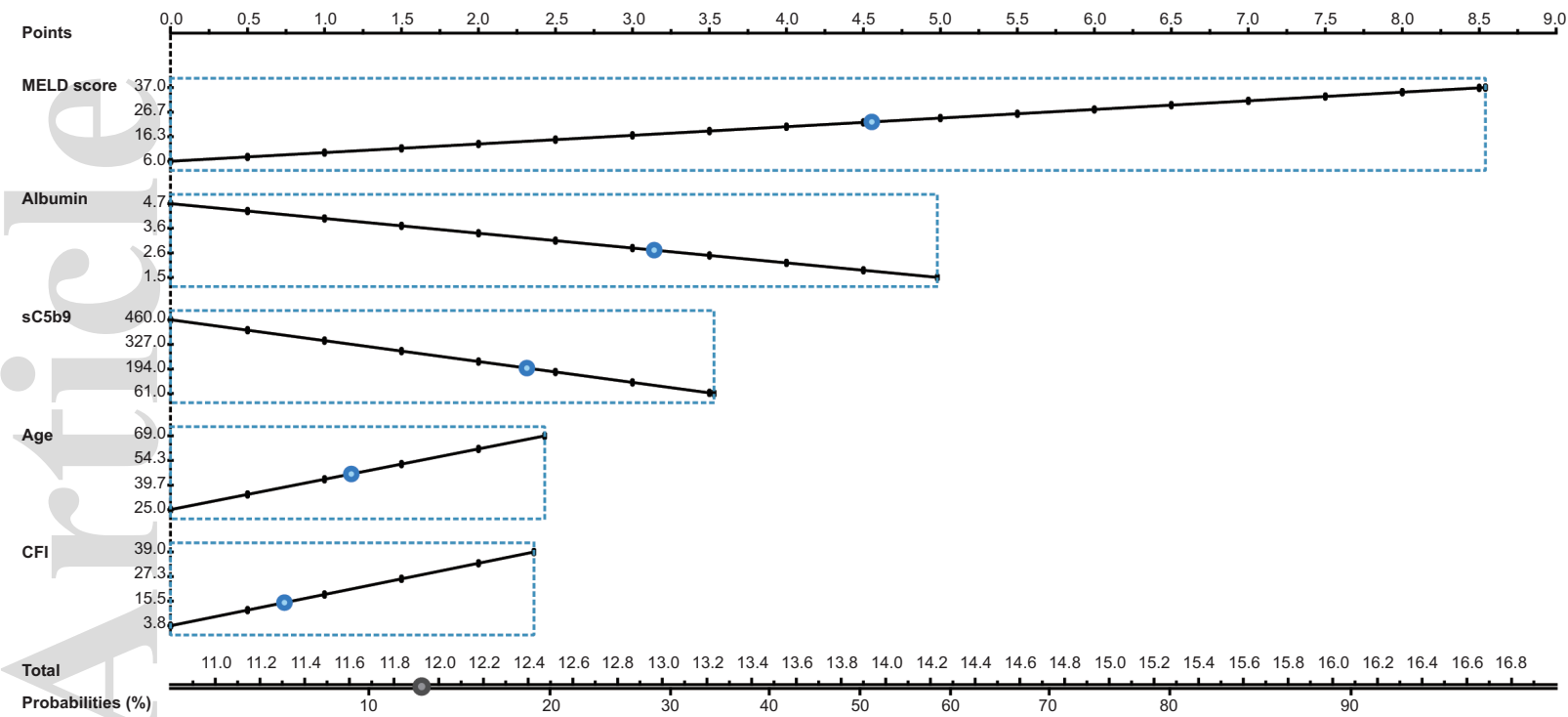
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