Reticuloendothelial activation correlates with disease severity and predicts mortality in severe alcoholic hepatitis

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Introduction
Severe alcoholic hepatitis (SAH) continues to have high short-term mortality [1]. Although the prognostic markers for SAH are mainly liver disease severity scores, such as discriminant function and model for end-stage liver disease (MELD) score [2], few other predictors of survival such as markers of macrophage activation [3] and of endothelial activation [4], which are separate from liver disease severity scores, have been identified.

The two cells of the reticuloendothelial system in the liver – Kupffer cells (tissue macrophages) and sinusoidal endothelial cells – reside in the sinusoids close to each other. The term ‘reticulo’ refers to the reticulated (net-like) extensions of phagocytes/tissue-resident macrophages [5]. These two reticuloendothelial cells share common functions, such as innate immune response and scavenging dead or abnormal cells, tissues and foreign substances. CD163, a macrophage-specific protein, is a scavenger receptor for the hemoglobin haptoglobin complex.

The prognostic value of activation of macrophages [6–8] and endothelium [9] have been documented in separate studies in patients with acute-on-chronic liver failure and cirrhosis. However, we are not aware of any single study of the predictive ability of activation of the reticuloendothelial system in SAH patients. If reticuloendothelial activation predicts survival in SAH patients, this would point to its involvement in the pathogenesis of SAH.

This study aimed to analyze the degree of reticuloendothelial system activation, its correlation with disease severity and its ability to predict survival in a cohort of SAH patients.

Materials and methods
Patient recruitment
We prospectively recruited 50 SAH patients admitted to the Department of Hepatology, Christian Medical College, Vellore, India, from December 2018 to November 2019.
The study was approved by the Institutional Review Board. Informed consent was obtained from all patients recruited. Exclusion criteria were age less than 18 years, pregnancy and any preexistent immunological disease. Alcoholic hepatitis was diagnosed using criteria given by the ‘National Institute on Alcohol Abuse and Alcoholism-funded Alcoholic Hepatitis Consortia’. SAH was defined as Maddrey’s discriminant function ≥32 or MELD ≥20 and very severe alcoholic hepatitis was defined as discriminant function ≥60 or MELD ≥30 [10].

For this study, we defined reticuloendothelial system activation as raised levels of markers of macrophage and endothelial activation in circulation.

We analyzed the following study objectives in SAH patients recruited in this study:

1. Degree of reticuloendothelial system activation at baseline.
2. Correlation between reticuloendothelial system activation markers and with systemic inflammatory syndrome (SIRS) and disease severity scores at baseline.
3. Ability of reticuloendothelial system activation markers at baseline to predict short-term survival.

Child score, MELD score [11] and Sequential Organ Failure Assessment (SOFA) score [12] were calculated at baseline. Clinically diagnosed and liver biopsy-proven alcoholic hepatitis was termed definite alcoholic hepatitis, clinically diagnosed and without confounding factors was termed probable alcoholic hepatitis and clinically diagnosed but with potential confounding factors was termed possible alcoholic hepatitis. The presence of SIRS was assessed as per the standard definition [13].

**Measurement of macrophage activation markers: serum ferritin and sCD163**

Serum ferritin was measured using chemiluminescent immunoassay (Siemens immulite 2000 XPi). Serum sCD163 was measured using Human CD163 ELISA (Cat No: DC1630) from R&D systems following the manufacturer’s protocol. For quality control, Quantikine Elisa kit Controls (control set 903) were utilized.

**Measurement of endothelial activation marker: plasma von Willebrand factor antigen**

Whole blood in 0.109-M citrate anticoagulant was collected and centrifuged at 2500 g for 15 min at 4 °C. Commercially available ELISA kit [Zymutest von Willebrand factor (VWF), Hyphen Biomed, France] was used to measure the VWF antigen. The assay was performed as per the manufacturer’s protocol. The normal reference range of plasma VWF antigen is 50 to 150 IU/dL.

**Macrophage activation syndrome criteria**

European League Against Rheumatism/American College of Rheumatology classification criteria for macrophage activation syndrome, 2016 [14]: ferritin >684 ng/mL and 2 or more of the following: platelets ≤181 × 10^9/L, aspartate aminotransferase (AST) >48 U/L, triglycerides >156 mg/dL and fibrinogen ≤360 mg/dL were used.

In 20 healthy volunteers, we tested serum sCD163 levels.

**Follow-up**

The study cohort was followed up for 90 days.

**Statistical analysis**

Discrete variables were shown as percentages and continuous variables were expressed as median with interquartile range (IQR). The Mann–Whitney test was used for continuous variables. Statistical analysis of the data was carried out using the software Statistical Package for Social sciences, version 16, and R version 3.6.0. Correlation of markers of macrophage activation and endothelial activation at baseline was done with liver disease severity scores using the Spearman correlation test. To evaluate reticuloendothelial activation markers to predict mortality, a sample size of 50 was calculated, assuming short-term mortality in SAH to be more than 60% [1]. The Kaplan–Meier survival curve was plotted and the log-rank test was used to compare survival curves of patients with low and high sCD163 and VWF values (cut off was based on median values). Cox proportional regression was used to assess predictors of survival. The level of significance was set at \( P < 0.05 \).

**Results**

**Patient characteristics**

Fifty SAH patients (discriminant function, 76.2, 54.5–106.6; median, IQR; MELD score, 30, 26.2–36 and SOFA score, 7, 5–10) were recruited into the study.

Two had definite SAH [biopsy suggesting alcoholic steatohepatitis in both with underlying mild portal fibrosis (1) and bridging fibrosis with incomplete nodularity (1)], 28 probable SAH and 20 possible SAH [probable sepsis, 7; concomitant potentially hepatotoxic drug, 4; gastrointestinal bleed, 2; atypical laboratory tests, 7 (AST, 102 IU/L, 40–438 IU/L; median, range; serum bilirubin, 12 mg/dL; 8–32 mg/dL, AST:ALT ratio, 1.74, 0.8–3.43)]. Baseline characteristics of the SAH cohort are given in Table 1.

A total of 36 (72%) patients satisfied the SIRS criteria. Hepatic encephalopathy and acute kidney injury were seen in 18 and 30 patients, respectively.

Of the 50 SAH patients, 43 patients met the criteria for very severe alcoholic hepatitis [discriminant function, 76.23 (55.06–104.35)].

Patients were treated with standard medical treatment. In addition, 12 patients were treated with plasma

**Table 1. Baseline demographics and laboratory parameters in severe alcoholic hepatitis patients studied**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SAH(n = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>45 (37–49) years</td>
</tr>
<tr>
<td>Sex</td>
<td>Male:49, female:1</td>
</tr>
<tr>
<td>Total white blood count (cells/mm^3)</td>
<td>13200 (9575–18625)</td>
</tr>
<tr>
<td>Platelet count (10^9/mm^3)</td>
<td>1.15 (0.68–1.61)</td>
</tr>
<tr>
<td>Serum total bilirubin (mg/dL)</td>
<td>21.48 (11.33–27.69)</td>
</tr>
<tr>
<td>Serum albumin (g/L)</td>
<td>2.4 (2.17–2.72)</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.56 (0.91–3.0)</td>
</tr>
<tr>
<td>International normalized ratio</td>
<td>1.95 (1.73–2.54)</td>
</tr>
<tr>
<td>Plasma VWF level (IU/dL)</td>
<td>748 (519.3–976.2)</td>
</tr>
<tr>
<td>Serum ferritin (ng/mL)</td>
<td>1410 (873.1–2116.2)</td>
</tr>
</tbody>
</table>

Continuous values are expressed as median (IQR). SAH, severe alcoholic hepatitis; VWF, von Willebrand factor.
exchange and steroid, 6 patients were treated with only steroids and 6 patients with pentoxifylline.

Degree of reticuloendothelial system activation at baseline in severe alcoholic hepatitis patients

Of the 50 SAH patients, serum ferritin >500 ng/mL was seen in 41 patients (82%). All 50 SAH patients had raised serum sCD163. Serum sCD163 levels in SAH patients were higher [5.39 (4.02–6.7) μg/mL] compared to healthy controls [0.53 (0.48–0.74) µg/mL, P < 0.001]. Plasma VWF levels were raised in all 48 SAH patients tested [VWF levels, 748 (519.2–976.2) IU/dL]. In total 37 patients (74%) fulfilled the macrophage activation syndrome criteria – serum ferritin > 684 ng/mL in 37 patients, platelets <181 × 10⁹/L in 37 patients, AST >48 U/L in 47 patients, triglycerides >156 mg/dL in 23 patients and fibrinogen ≤360 mg/dL in 43 patients.

Correlation between reticuloendothelial system activation markers and with systemic inflammatory syndrome and disease severity scores at baseline in severe alcoholic hepatitis patients

sCD163 levels showed weak correlation with ferritin (r = 0.39; P = 0.005). There was no significant correlation between VWF levels and the macrophage activation markers.

In total, 75% (27/36) of SAH patients with SIRS and 71.2% (10/14) of SAH patients without SIRS satisfied the macrophage activation syndrome criteria. There was no significant difference in the reticuloendothelial system activation markers (ferritin, sCD163 and VWF levels) between the patients with SIRS and those without SIRS (data not shown).

Ferritin levels showed a weak correlation with disease severity (MELD and SOFA) scores. sCD163 showed a moderate correlation with the MELD score and a weak correlation with the SOFA score. VWF levels moderately correlated with MELD and SOFA scores (Fig. 1).

Ability of baseline reticuloendothelial system activation markers to predict survival in severe alcoholic hepatitis patients

A total of 29 patients in the SAH cohort died during the median follow-up of 25 (9–90) days, with a maximum follow-up of 90 days. The Kaplan–Meier survival curve for the SAH cohort is shown in Fig. 2. The median survival time was 28 days (95% confidence interval (CI), 19.1–36.8 days) for the whole cohort. The hazard ratios for reticuloendothelial activation markers are given in
Table 2. The baseline plasma VWF level predicted short-term mortality independent of sCD163 and MELD score in the study patients [adjusted hazard ratio, 1.002 (95% CI, 1–1.004)].

The SAH cohort was divided into patients having high and low sCD163 and VWF values at baseline, using median values as the cutoff. The survival was significantly reduced in patients with sCD163 >5.4 μg/mL ($P = 0.035$) and VWF >748 IU/L ($P = 0.032$) (Fig 3).

Discussion

To our knowledge, this is the first study analyzing the prognostic value of activation of the reticuloendothelial system in SAH patients. All SAH patients studied had marked activation of the reticuloendothelial system (the median serum sCD163 level was 10-fold higher than healthy controls and the median plasma VWF level was 5-fold above the upper limit of normal). In our study, at baseline, the levels of reticuloendothelial system activation markers correlated with liver disease severity scores. Plasma VWF level independently predicted mortality in SAH patients.

On analysis of macrophage activation markers in SAH patients: ferritin >500 ng/mL was found in 82% patients, raised sCD163 were found in 100% of patients and macrophage activation syndrome criteria were met in 74% of patients in our study.

Although the markers of the reticuloendothelial system were activated in all SAH patients studied, only 72% of patients had SIRS. The reticuloendothelial system activation markers were not significantly different in SIRS patients compared to patients who did not satisfy the SIRS criteria.

Both the macrophage activation markers (ferritin and sCD163) and endothelial activation marker (VWF) correlated with MELD and SOFA scores at baseline. However, the baseline VWF level did not correlate with sCD163 levels. How to explain this? The raised plasma VWF levels may be due to increased VWF release (from endothelial cells or platelets) or due to reduced VWF clearance or both [15]. VWF is cleared by macrophages [16]. It is also possible that the specific factors which trigger endothelial activation are different from those which trigger macrophage activation.

Is the marked rise in reticuloendothelial system activation markers a consequence of liver injury or do they also contribute to the pathogenesis or worsening of SAH? The markers of macrophage activation and endothelial activation correlated with disease severity scores and independently predicted mortality in SAH patients studied. This suggests that overactivation of the reticuloendothelial system probably has an important role in SAH pathogenesis. Thus, the reticuloendothelial system overactivation maybe a potential new therapeutic target in SAH. Studies of treatment to reduce reticuloendothelial system overactivation are needed in SAH patients to see if this treatment improves survival.

Macrophage activation correlates with liver disease severity and short-term survival in acute-on-chronic liver failure (ACLF) patients [17]. Raised VWF levels are shown to predict survival in cirrhosis [18], ACLF [9] and acute hepatotoxicity [19]. Treatment aimed at VWF reduction is being explored in liver failure [15,19]. Amelioration of macrophage activation is yet another target to treat liver failure.

The inflammatory milieu in SAH patients necessitates innate immune response [20,21] as well as increases the need for scavenging function [22]; both these are functions of the reticuloendothelial system and would trigger its activation.

Table 2. Adjusted hazard ratio using cox regression analysis for short-term mortality in severe alcoholic hepatitis patients

<table>
<thead>
<tr>
<th>Hazard ratio (95% CI)</th>
<th>P value</th>
<th>Adjusted hazard ratioa</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin 1.000 (1.000–1.000)</td>
<td>0.276</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sCD163 1.240 (1.081–1.422)</td>
<td>0.002</td>
<td>1.180 (0.994–1.401)</td>
<td>0.059</td>
</tr>
<tr>
<td>VWF 1.002 (1.001–1.004)</td>
<td>0.002</td>
<td>1.002 (1.000–1.004)</td>
<td>0.019</td>
</tr>
</tbody>
</table>

CI, confidence interval; VWF, von Willebrand factor.
aAdjusted for MELD and VWF/sCD163.

Fig. 3. (a) Survival curves for severe alcoholic hepatitis patients with high and low sCD163 values. (b) Survival curves for severe alcoholic hepatitis patients with high and low von Willebrand factor values.
VWF is a glycoprotein released from endothelial cells and platelets. Raised plasma VWF levels predict survival over 25.6 (23.6–33) months in patients with chronic liver disease [18], over 8 days in patients with ACLF [9] and over 7 days in patients with acute hepatotoxicity [19]. VWF multimers are the largest sized molecules in normal human plasma [23]. The liver sinusoids appear to be VWF-free zones in health [24]. It is likely the raised plasma VWF levels may clog the liver sinusoids, impair the perfusion and contribute to liver dysfunction/failure.

The tissue macrophages are abundant in liver sinusoids, probably as part of the innate immune defense to counter invading pathogens/foreign substances entering the body from the gastrointestinal tract [24]. Activation of innate immune response leads to an increase in the size of the activated macrophages as well as the recruitment of monocytes from the bone marrow, which differentiates into macrophages in the liver.

Activation of macrophages and endothelial cells (i.e. reticuloendothelial system activation) in the liver is an important defense mechanism in response to an invader. Its uncontrolled activation may exacerbate acute tissue injury or promote chronic disease [25]. The activation of the reticuloendothelial system in response to an invading virus is mostly not recognized by the human host. An overactive response by macrophages will manifest as fever, organ failures, enlarged spleen (features of macrophage activation syndrome) and can lead to short-term mortality [14].

Reticuloendothelial system overactivation may occur in other syndromes of liver failure (such as other causes of ACLF, acute liver injury and acute liver failure) and failure of other organs [26]. The impaired perfusion in hepatic microcirculation as the reticuloendothelial cells enlarge and compromise the sinusoidal lumen may result in liver failure. Treatment to ameliorate reticuloendothelial system activation may improve the microcirculatory impedance in these situations, may reverse organ failure and improve survival [15]. Dynamic changes in reticulo-endothelial activation, with specific treatment strategies, such as plasma exchange, need to be further explored.

Our study has a few limitations. Our pilot study has a small number of patients enrolled. We studied only one marker of endothelial activation (VWF antigen). In addition to endothelial activation, the other causes of raised plasma VWF levels in SAH patients may be increased VWF release from platelets and reduced VWF degradation/clearance. We have not explored this question in the present study. The lack of controls with other liver diseases is another study limitation. Is the reticuloendothelial system overactivation, organ-specific or is it a systemic process? In our dataset, we have not addressed this question in detail. However, correlation of overactivation of the reticuloendothelial system with liver disease severity scores and its prognostic ability in SAH patients in our study suggest that either part of reticuloendothelial overactivation is liver-specific or some of the consequences of this process are liver-specific or both.

In conclusion, we demonstrate marked activation of the reticuloendothelial system in SAH patients. VWF levels predict short-term mortality in SAH patients. Studies in a larger number of SAH patients are needed to validate these findings and also to see if treatment to ameliorate reticuloendothelial system activation improves organ failure and survival.

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Conflicts of interest
There are no conflicts of interest.

References


