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Invited Review

Cellular Abnormalities and Emerging Biomarkers in Alcohol-Associated Liver Disease

Ashwani K. Singal* and Shannon M. Bailey†

*Division of Gastroenterology and Hepatology, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, USA
†Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA

Alcohol-associated liver disease (AALD) is the third most common preventable cause for disease burden and mortality in the US. AALD, including alcoholic hepatitis (AH), contributes to half of admissions from decompensated liver disease and 20% of all liver transplants in the US. Peripheral blood cells contribute to systemic inflammation, oxidative stress, mitochondrial dysfunction, and fibrosis in AALD and AH. Alcohol dysregulates function of lymphocytes, neutrophils, monocytes, and tissue macrophages of the innate immune system. These alterations in turn can modulate adaptive immune responses. In this review, we describe these disruptive effects of alcohol on cells of the innate and adaptive immune system and focus on cellular-based emerging biomarkers on diagnosis and prognosis of patients with AALD and AH.

Key words: Liver regeneration; Inflammation; Macrophage; Innate immunity; Hepatic injury; Gut–liver axis

INTRODUCTION

Alcoholic liver disease is due to heavy alcohol consumption, which is typically defined as 3 drinks/day in men and 2 drinks/day in women for 5 years. As this amount of alcohol consumption may often exist with completely normal social and personal functioning of an individual, the word alcoholic may inappropriately stigmatize alcohol consumption behavior. Hence, there is increasing acceptance to change the terminology to alcohol-associated liver disease (AALD). Although, this change is still evolving, the authors in this review will use the term AALD when referring to alcoholic liver disease.

AALD is the third most common cause for chronic liver disease in the US after hepatitis C virus (HCV) infection and nonalcoholic fatty liver disease[1]. About two-thirds of the US population consumes alcohol at some point of their life, with 8%–10% engaging in heavy alcohol use[2,3]. The clinical spectrum of AALD varies from steatosis or fatty liver to more serious presentations such as steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC)[4]. Advanced liver disease occurs in about 15%–20% of people with heavy alcohol use, with potential for cirrhosis and HCC[4]. Alcohol use remains the third most common preventable cause for any disease burden and mortality in the US after smoking and hypertension[5]. It also contributes to about 5% of global mortality and disease-adjusted life years lost, as AALD often occurs among relatively younger populations[3].

A major contributor to this high mortality is from a unique clinical presentation of alcoholic hepatitis (AH), which presents with acute chronic liver failure among people with chronic and active use of alcohol in heavy amounts[2,6]. Disease awareness and better management have resulted in a decrease in AALD-related mortality in the general population over the last century from 20 to 25 to <10 per 100,000 between 1910 and 2006, respectively. However, this is far from 3 to 4 per 100,000, as the projected target for achievement by the WHO[3]. Further, outcome among patients with AH has not improved over the last five decades[7]. One of the reasons for not being

Address correspondence to Ashwani K. Singal, M.D., M.S., FACG, FAASLD, Division of Gastroenterology and Hepatology, University of Alabama Birmingham, AL 35294, USA. Tel: 205-975-9698; Fax: 205-975-0961; E-mail: ashwanisingal.com@gmail.com

able to achieve the targeted or expected decline in AALD mortality is the lack of effective and safe drugs for this potential lethal condition since the recognition of alcohol as a direct hepatotoxicant over six decades ago[8]. Moreover, advancement in the field has been hampered by the lack of animal models that accurately mimic the human phenotype of AALD and AH with potential for multiorgan failure, infections, and mortality[9,10]. However, recent interest in this area of research has led to the development of new models for study[11].

Immune-activated peripheral blood cells contribute to the pathology of AALD and AH, including systemic inflammation, oxidative stress, mitochondrial function, and fibrosis. In the last few years, abnormalities in peripheral and tissue-resident blood cells in AALD have led to interesting and encouraging data on new biomarkers in the management of AALD. In this review, we focus on a) the effect of alcohol on innate and adaptive immunity; b) abnormalities in peripheral blood cells in stable cirrhosis and role of systemic inflammation in AALD and AH; c) mitochondrial dysfunction and cellular bioenergetics in AALD; and d) emerging biomarkers in the AALD field and their clinical utility throughout the course of AALD in diagnosis, predicting response to treatment, early diagnosis of infections, and determining prognosis.

EFFECT OF ALCOHOL ON INNATE AND ADAPTIVE IMMUNITY

Alterations to the normal functioning of the gut–liver axis contribute to the pathology of AALD from multiple insults, including direct effects of alcohol on the innate cells in the gastrointestinal tract[12]. Further, effects of alcohol on innate immunity can disrupt cross-talk between innate and adaptive immunity, resulting in inhibitory effects on adaptive immune responses. For example, alcohol consumption among patients with AALD induces bacterial overgrowth, dysbiosis of the gut microbiome with predominance of harmful bacteria, and increases gut permeability with translocation of gut bacteria across the portal circulation to hepatic parenchyma[13]. Toll-like recptors-4 (TLR-4) or pathogen-associated molecular pattern receptors on hepatic macrophages recognize these pathogens, resulting in inflammatory signaling and immune activation of peripheral blood cells[14]. This inflammatory signaling is mediated by NF- B and, more importantly, IL-1 production from inflammasome activation[13]. The activation of macrophages via lipopolysaccharide (LPS) of the gut bacteria also mediates TLR-4 receptor activation[15]. The resulting liver injury releases danger-associated molecular patterns, which in turn stimulates TLR-4 perpetuating inflammatory signaling and pathology[14].

Alcohol dysregulates key functions of most of the cells of the innate immune system including lymphocytes, neutrophils, monocytes, and tissue macrophages (Fig. 1).

Lymphocytes in AALD

Lymphocytes play an important role in the pathogenesis of AALD. In addition to being components of the innate immune system, these cells also provide adaptive immunity. As we understand, dendritic or antigen-presenting cells (APC) and natural killer (NK) cells are part of the immune system, while subsets of lymphocyte populations such as CD4, CD8, T regulatory cells,

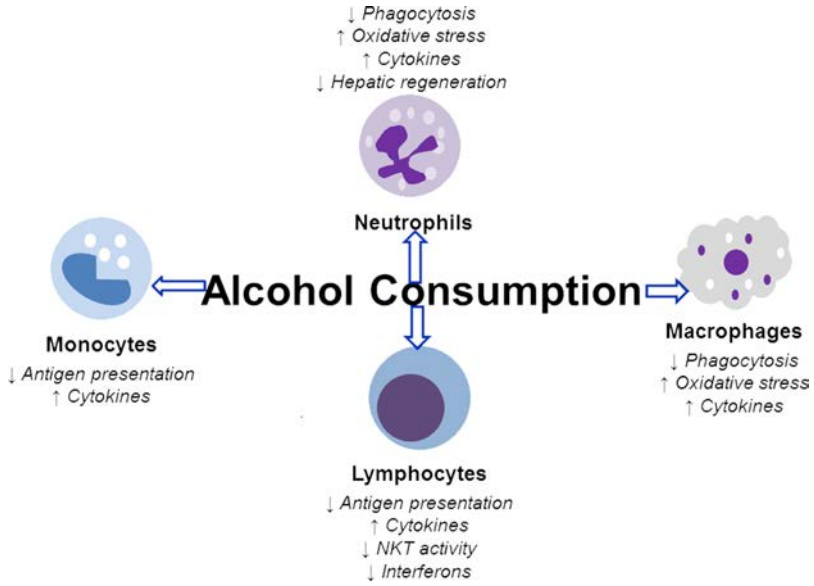

Figure 1. Effects of alcohol consumption on peripheral blood cells.

B lymphocytes, and NK T cells are part of the adaptive immune system. Chronic alcohol consumption inhibits the activity and function of APC or dendritic cells, which is more pronounced in AALD compared to patients with cirrhosis from other etiologies[16]. Alcohol consumption activates T cells with production of cytokines and chemokines including upregulation of NF- B-mediated inflammatory genes[15]. Alcohol inhibits the NK cells, which have anti-HCV and antifibrotic effects. While NK cells do not play much of a role in AALD per se, their inhibition by alcohol may explain a higher propensity and increased severity of disease in the presence of concomitant HCV among chronic alcoholics[15].

NK T cells, a major population of hepatic lymphocytes, produce interferon (IFN) and IL-4 with other cytokines. NK T cells also protect against liver fibrosis by balancing liver cell damage and protective mediators such as IFN[15]. Dendritic cells promote hepatic injury by acting as APC to the T lymphocytes, with subsequent release of cytokines[15]. In another study, chronic alcohol ingestion activated CD4 and CD8 T lymphocytes in mice with AALD and without AALD compared to normal control mice[17]. These changes were associated with upregulation of CD80 and CD86, which are involved in T-cell-mediated APC interaction. These data suggest that chronic alcohol consumption causes immune activation of innate immunity and T cells[17]. Similar findings have been shown in humans with AH. For example, in one translational study, proportion of lymphocytes in the peripheral blood was reported to be increased in AH patients compared to healthy controls (35% vs. 25%), with this alteration persisting and remaining unchanged after 3 months of alcohol abstinence[18]. This increase occurred in T-cell populations with increase in CD4, CD8, and NK cells (CD3 and CD56), while the proportion of B cells (CD19/CD5) decreased[18]. Recent studies have also shown a role of upregulated programmed cell death 1 (PD1) and T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) receptors, which inhibit host protective immunity. Antibodies to these receptors restored the function of cells with production of interferon, suggesting their potential utility in the treatment of AALD and severe AH[19].

Monocytes and Macrophages in AALD

Like dendritic cells, monocytes also act as APC. Among patients with advanced liver disease and severe AH, monocytes fail to respond with production of TNF- , which has been shown to be due to reduced human leucocyte antigen-D related (HLA-DR) and increased MER tyrosine kinase (MERTK) expression on cell surface[20–22]. A potential role of prostaglandin E2 has been postulated as inhibitory to the function of these cells[23]. These changes are associated with the inability of the monocytes and tissue-resident macrophages to mount inflammatory responses against invading pathogens and antigens, resulting in immune paralysis with development of liver failure and secondary infections.

Kupffer cells comprise the majority of tissue-resident macrophages in the body and are activated by gut-derived endotoxin in response to alcohol. This activation in turn results in the release of cytokines and chemokines and sets the stage for inflammatory signaling, as well as reactive oxygen species production with oxidative stress[24]. The protective function of these cells is to clear the bacterial load and endotoxins and prevent infections. With the development of structural damage of the liver tissue in AALD and loss of tissue macrophages and Kupffer cells, patients remain at risk for bacterial infections, uncontrolled bacteremia, and sepsis[25].

Neutrophils in AALD

Neutrophils play an important role against invading microorganisms, tissue trauma, or inflammatory signals. Recruitment of neutrophils occurs in response to acute liver injury or any systemic inflammatory signaling. Neutrophils accumulated in the hepatic microvasculature and sinusoids can transmigrate into the hepatic parenchyma if they receive a signal from distressed cells[26]. The main function of neutrophils recruited into the liver is to remove dead cells as a basis for regeneration, but neutrophils may also be involved in mediating hepatic inflammation and cell injury[26]. For example, recent studies demonstrate significant neutrophil accumulation in the liver following chronic plus binge ethanol feeding in mice with in vivo depletion of neutrophils by administration of anti-Ly6G antisera reducing liver injury[27,28].

Neutrophil recruitment and activation are associated with increased proinflammatory cytokines such as IL-8 and TNF- with decreased levels of anti-inflammatory cytokines such as IL-10[29]. After transmigration, neutrophils adhere to distressed hepatocytes through (2) integrins and ICAM-1 expressed on hepatocytes[30]. Neutrophil contact with hepatocytes mediates apoptosis[31], as well as by oxidative killing via reactive oxygen species, leading to hepatocellular oncotic necrosis[32–35]. In one study of 35 AH patients, hepatocyte apoptotic index as measured by TUNEL staining was associated with severity of AH and Maddrey's discriminant function score, but not with the degree of neutrophilic infiltration on the liver tissue[36].

Defects in phagocytosis and oxidative burst in neutrophils are transmissible by patient plasma to normal neutrophils, suggesting a role of soluble factors such as endotoxin and LPS[37]. There is a suggestion that administration of TLR-4 inhibitors may restore defects in oxidative burst, however not the phagocytic dysfunction of neutrophils, suggesting that TLR overexpression may be the result and not the cause of neutrophil activation[37].

Recently, these defects in neutrophils have been shown to be due to a defect in myeloperoxidase release and the AKT/P38-MAPK pathway, which is salvageable with TLR-7/8 agonists, suggesting their potential therapeutic role[38]. Further, these defects have been linked to outcomes and with onset of infections and multiorgan failure[39]. Patients with cirrhosis do decompensate with infections as a precipitating factor, suggesting the presence of these neutrophil defects in stable cirrhosis patients[40]. In a study on 108 cirrhosis patients, neutrophil phagocytic dysfunction was associated with increased expression of TLR-4 receptors, resting respiratory burst, inflammatory cytokine levels, and phagocytic impairment. These defects were associated with the severity of cirrhosis and Child's class[40]. Although patients with compensated cirrhosis apparently had normal neutrophil function, a translational in vivo study showed phagocytic defect at the site of inflammation[40].

The general purpose of early neutrophil infiltration is to remove dead or dying cells as a prerequisite for wound repair and regeneration. In fact, the degree of neutrophilic leucocytosis and the density of neutrophil infiltration on the hepatic tissue predict the outcome from an episode of AH among patients with alcohol-related cirrhosis and AALD[41,42]. In one study, levels of hepatic growth factor derived from recruited neutrophils into the liver were higher among 25 patients with AH compared to 20 nondrinker healthy controls and 20 patients with alcohol-related cirrhosis without AH[43]. Factors determining the protective role of neutrophils mediating hepatic regeneration versus its damaging effects on the liver tissue remain unknown. It is most likely that these defects are acquired during peripheral circulation of these cells through the bone marrow and reticuloendothelial system. Another speculation is that younger cells recruited from the bone marrow have better function and hepatic regenerative capacity. For example, use of growth factors such as granulocyte colony-stimulating factor in patients with acute-on-chronic liver failure and AH increases hepatic density of bone marrow-derived young $CD34^+$ cells, with benefits on hepatic regeneration, decreased occurrence of infections, improved liver disease, and better survival[44].

OXIDATIVE STRESS AND MITOCHONDRIAL DYSFUNCTION

Hepatic inflammation, oxidative stress, and mitochondrial dysfunction also contribute to the pathophysiology of AALD and AH[26]. Typically, mitochondrial dysfunction is defined as a partial loss of activity in the enzymatic components of oxidative phosphorylation, increases in generation of reactive oxygen species, and/or mutations and damage to mitochondrial DNA[38,45,46]. Mitochondrial dysfunction in AALD can be caused by numerous factors following alcohol exposure, including inflammation from gut-derived bacterial endotoxin[12], hepatic inflammation and apoptosis releasing damage-associated molecular patterns[47], activation of Kupffer cells with nitric oxide synthase induction and nitrative stress[48–51], and mitochondrial-mediated metabolism of alcohol via the CYP2E1 pathway of alcohol resulting in tissue hypoxia and enhanced oxidative stress[50,52,53]. The central role of mitochondrial dysfunction in AALD is also underscored by the recent finding that a mitochondrial-targeted antioxidant, MitoQ, prevents steatosis in an animal model of ethanol hepatotoxicity[50]. Similarly, treatment with S-adenosylmethionine and betaine prevents chronic alcohol-mediated impairments in mitochondrial function at multiple levels, including mtDNA damage, oxidant production, the proteome, and bioenergetics[54]. In addition, liver mitochondria after chronic alcohol administration are much more sensitive to nitrative stress from nitric oxide and reactive oxygen species-dependent protein inhibition[49,55,56].

Bioenergetics in Peripheral Blood Cells as a Marker of Disease State and Mitochondrial Health

Peripheral blood cells activated in a disease state can mediate processes involved in inflammation and crosstalk with body tissues. It is becoming clear that bioenergetics in peripheral blood cells may also reflect the disease state of peripheral organs and may be useful as markers of disease severity and outcomes in many diseases, including sepsis, diabetes mellitus, and neurodegenerative disorders[57]. In animal models, use of MitoQ and other mitochondrial-targeted antioxidants has been shown to be associated with reduction in steatosis, decreased nitrative stress, improved tissue hypoxia, and decrease in serum aminotransferase levels[50,58]. Thus, peripheral blood cells undergoing immune activation by the systemic inflammatory state in AALD and AH may be an appropriate setting to study mitochondrial health as biomarkers of disease state and prognosis.

The traditional approach to assess bioenergetics has been to isolate mitochondria from tissues or cells, and then assess biochemical function using measurement of oxygen consumption with added substrates or by performing individual enzymatic assays for each respiratory chain complex. The limitation of this strategy is that in vivo, extramitochondrial factors and compartments regulate the supply of substrates for oxidative phosphorylation, thus regulating mitochondrial metabolism[59]. With the advent of new high-throughput methods for the measurement of oxygen consumption in intact cells, bioenergetics can more readily be determined in clinical samples[45,59]. The resulting bioenergetics profile may then be useful for detecting subtle and early changes in bioenergetics function, which may then be predictive of

mitochondrial failure and development of disease[25,57,59,60]. It is also becoming evident that mitochondrial function in leukocytes is essential for normal immune responses[27,50,56,61]. This concept has precedent in other systemic and inflammatory disease states, where mitochondrial defects in monocytes and platelets have been described, such as diabetes mellitus, Alzheimer's disease, and septic shock[44,61–64].

Assessment of Cellular Bioenergetics in Peripheral Blood Cells

To determine cellular bioenergetics, we and others use the XFe96 analyzer from Agilent Technologies (previously Seahorse Biosciences), which measures oxygen and protons (pH) in the extracellular environment of cell populations prepared using the techniques recently described[45,57,65,66]. For example, the system is capable of measuring 92 samples at a time (5 technical replicates and 18 different conditions; cell types or patients) and is equipped with four injection ports per well to allow for injection of a compound of interest or to add inhibitors that can aid in the elucidation of defects in individual cellular respiration pathways or enzymes[57,65]. Overall oxygen consumption rate (OCR) related to six key aspects of mitochondrial function can be measured as shown in Figure 2: a) basal mitochondrial respiration: this serves as baseline respiration; b) the proportion of basal activity that is linked to ATP production and level of proton leak (after injecting oligomycin in the medium): an increase in the proton leak is consistent with the mitochondria becoming less efficient; c) the maximal oxygen respiration (after injecting FCCP in the medium): a decrease in this parameter would be consistent with a deficit in mitochondrial biogenesis, damaged mitochondrial DNA, and/or oxidative phosphorylation proteins; d) the reserve capacity (maximal minus basal respiration): determines the threshold at which bioenergetics dysfunction occurs (e.g., when reserve capacity is zero or negative, the cell cannot meet the bioenergetics demand through mitochondrial respiration and the bioenergetics threshold is exceeded[66]; and e) the level of nonmitochondrial oxygen consumption (after injecting antimycin in the medium): represents other oxygen-consuming processes in the cells, such as proinflammatory enzymes cyclooxygenases and lipoxygenases.

EMERGING BIOMARKERS IN AALD

Diagnosis of alcohol-related cirrhosis can be made accurately in most cases without liver biopsy and using clinical, biochemical, and imaging criteria[67]. However, some of the clinically unmet needs in the field of AALD are a) identifying subjects engaging in heavy alcohol consumption who are at risk for advanced liver disease, b) diagnosing AH without a liver biopsy, c) assessing response to specific treatment and personalized medicine, d) early diagnosis of infections, and e) prognosis and predicting outcomes of the disease state. These biomarkers can originate from blood or serum or plasma, urine, stool, breath, and cell-based assays. In the following sections, we highlight several potential new biomarkers in AALD with a summary provided in Table 1.

Biomarkers for Risk of Advanced Liver Disease

As advanced liver disease with cirrhosis and HCC develops in 15%–20% of people with heavy alcohol

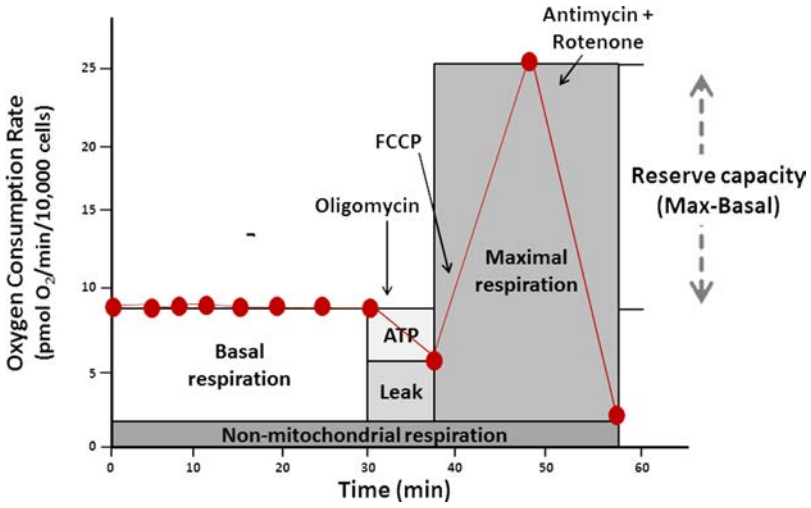

Figure 2. Scheme of a typical extracellular flux (XF) experiment in which basal, ATP-linked, proton leak, nonmitochondrial, and maximal respiration, as well as reserve capacity, can be determined in cells following serial injections of oligomycin, FCCP, and antimycin + rotenone.

Table 1. Emerging Biomarkers in Alcohol-Associated Liver Disease (AALD) and Alcoholic Hepatitis (AH)

Biomarker	Comment
Biomarkers for diagnosis of AH and severe spectrum of AALD	
PNPLA3 gene polymorphisms	*PNPLA3 p*olymorphisms associated with severe AH in AALD patients[68,69]
M1 macrophages	Transformation of hepatic and circulating macrophages to M1 phenotype in AH[70,78,79]
WBC and platelets	WBC and platelet counts >11 and >148,000, respectively, predict diagnosis of AH[71]
Trimethylamine (TMA)	Breath trimethylamine (TMA) levels to diagnose AH[74]
CCL-20	Higher CCL-20 in AH patients[97] (Fig. 3)
CK-18 fragments—M65 and M30	Higher CK-18 fragments and lower M30:M65 ratio in AH patients[78,79,97]
Monocyte bioenergetics	Decreased basal, ATP, and proton leak-linked oxygen consumption in AH patients[57]
Biomarkers to assess treatment response and disease prognosis	
Neutrophils	Neutrophil infiltration and neutrophilic leukocytosis predict better outcome in AH patients[41]
MELD and Lille score	Dynamic model with MELD at baseline and Lille score at 1 week of corticosteroid treatment is a better predictor of disease outcome from severe AH than either score alone[82,90,92]
SIRS and serum LPS	Multiorgan failure and mortality higher in AH patients with SIRS; LPS predictive of mortality[93,94]
Procalcitonin	Predictive of AH patients with SIRS and infection[94]
CK-18 fragments—M65 and M30	M30:M65 ratio predictive of 30-day outcome[79]
Gene signaling pathways	Upregulation of hepatic regenerative pathway genes associated with steroid response and good outcome; upregulation of inflammation pathway genes associated with nonresponse to steroids, progressive liver failure, and bad outcome[86]
Hepatic venous pressure gradient (HVPG)	Hepatic venous pressure gradient (HVPG) greater in nonsurvivors among severe AH patients (HVPG > 22 mmHg)[98]
Monocyte bioenergetics	Improved bioenergetics parameters in five of eight AH patients treated with corticosteroids[57]
Biomarkers to predict development of infection in AALD and AH	
Bacterial DNA	High circulating levels of bacterial DNA predictive of infection and infection-related mortality[106]
Oxidative burst	Decreased oxidative burst and reduced NADPH oxidase predictive of infection in AH[106,109]

consumption, other disease-modifying factors likely play a role in predisposing an individual to advanced liver disease[4]. There are emerging data on the *PNPLA3* GC and GG polymorphisms association with AH phenotype among patients with AALD[68,69]. For example, GC and GG polymorphisms of the *PNPLA3* gene compared to CC or wild-type gene predispose an individual to susceptibility of AALD and advanced and severe spectrum of the disease[68]. There is emerging data to show that the transformation of hepatic and peripheral circulating macrophages to proinflammatory phenotype or M1 macrophages with decreased immune activation and cytokine signaling characterizes the disease pathology of AH[62,70]. In this regard, studies designed to characterize the phenotype of peripheral macrophages are attractive as they might also serve as unique biomarkers to identify individuals likely to develop AH.

Biomarkers for Noninvasive Diagnosis of AH

As the clinical diagnosis of AH may be inaccurate in 20–30% of cases, either due to questionable history of alcohol consumption or confounding by the presence of liver disease etiologies other than alcohol, liver biopsy remains the gold standard for its definitive diagnosis[67]. Liver biopsy being invasive is not without complications in these sick patients with liver and multiorgan failure with coagulopathy. Moreover, leucocytes and platelets respond to systemic inflammation with increases in their peripheral counts. For example, in a clinical study of suspected AH patients undergoing liver biopsy, a white cell count >11 and a platelet count of >148,000 had an accuracy of about 85%–90% for diagnosis of AH[71]. Interestingly, gut bacteria convert phosphatidylcholine, a component of dietary lipids to trimethylamine (or TMA), which further gets converted to TMA oxide by hepatic flavin monooxygenases[72]. Importantly, patients with chronic liver disease have reduced conversion of TMA to its oxide, which is augmented by intestinal bacterial overgrowth and dysbiosis induced by alcohol consumption[73]. In one study, breath levels of TMA were useful in stratifying AH diagnosis among patients presenting with decompensated AALD[74]. Alcohol exposure of the HepG2 cells also stimulates secretion of extracellular vesicles by hepatocytes, which then leads to

activation of macrophages, resulting in caspase 3 activation[75]. Cytokeratin-18 (CK-18), a main component of Mallory–Denk bodies, is released in response to hepatocyte injury in AH and is carried within the vesicles released from hepatocytes. Two components can be detected in the serum: one with total and the fragmented form (or M65) released in response to total cell death or necrosis, and the cleaved fraction (or M30), which is released in response to apoptosis[76]. Patients confirmed to have AH on transjugular liver biopsy have been shown to have higher levels of both the fractions of CK-18 and levels of chemokine CCL-20[77,78]. In another study, patients with AH compared to healthy controls and decompensated cirrhosis due to alcohol had lower M30:M65 ratio[79]. As systemic inflammation in AALD and AH suppresses mitochondrial function and bioenergetics, we examined the importance of studying this in peripheral monocytes in our prospective cohort consisting of 63 decompensated AALD patients, 28 with severe AH. Our preliminary data showed reduced basal, ATP linked, and proton leak-linked mitochondrial OCR in AH patients. In a logistic regression model, basal and ATP-mediated OCR predicted diagnosis of AH among decompensated AALD patients, after controlling for serum bilirubin and white blood cell count[7]. Further, in a subset of 32 (14 with severe AH) patients from this cohort, cytokine profile measured using the multiplex approach showed CCL-20 levels (mean ± SEM) to be higher in AH patients compared to 18 patients with decompensated AALD without AH (1,256 ± 57 vs. 434 ± 36 pg/ml, $p = 0.032$) (Fig. 3).

Biomarkers for Predicting Response to Treatment

Currently, corticosteroids are the only available therapeutic option for the treatment of severe AH[80,81]. However, these drugs are limited in a major way due to noneligibility in about 40% of AH patients, unpredictable nonresponse in about 40%, which can only be determined at 1 week after administration of these drugs, and risk of complications and infections[82]. Further, the mortality benefit is limited to only about a month from the presentation, with no long-term survival benefit from these drugs[83]. Patients with hepatorenal syndrome also do not respond to corticosteroids, and it is recommended that the hepatorenal syndrome be treated first before initiation of corticosteroids[80]. Response to corticosteroids is inherently based on the immune activation in a patient with AH. This was shown in an in vitro study, where isolated lymphocytes from AH patients compared to those from healthy controls were less sensitive to inhibition of their proliferation by corticosteroids, and this was further reduced in nonresponders to corticosteroids compared to responders[84]. Thus, there remains a knowledge gap and critical need for simple biomarkers that will aid in predicting responsiveness to corticosteroids and also as a way to better personalize the medical therapy of severe AH patients.

Further, we have learned about the important beneficial aspects of hepatic and macrophage-derived TNF on the hepatic regenerative capacity from studies showing the detrimental effects of anti-TNF agents in patients with severe AH[85]. Further, neutrophil infiltration on the liver biopsy and neutrophilic leukocytosis in these patients are favorable markers for better prognosis and survival in severe AH[41]. In a recently reported study on severe AH patients from India and France, gene expression studies performed on the hepatic tissue showed that upregulation of genes involved in hepatic regenerative pathways is associated with responsiveness to steroids and better outcome[86]. Until these biomarkers are validated in multicenter studies with larger sample sizes, steroid responsiveness will continue to be determined using Lille score at 1 week or at least 4 days of steroid therapy[87].

In our prospective cohort, of the eight AH patients treated with corticosteroids, repeat bioenergetics measurements on basal, ATP linked, and maximal mitochondrial OCR in the peripheral monocytes tended to improve in five responders (Lille score: <0.45); however, this

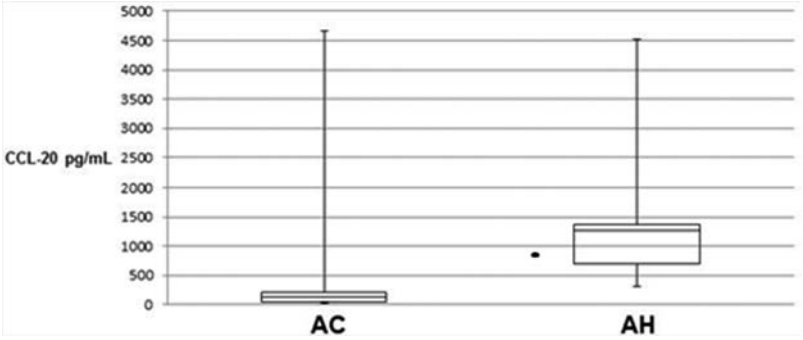

Figure 3. Levels of chemokine CCL-20 in serum comparing 18 patients with alcoholic cirrhosis (AC) versus 14 patients with alcoholic hepatitis (AH).

tended to decrease or remain unchanged among three nonresponders[7]. Although corticosteroids predominantly have anti-inflammatory effects[88], there is some evidence that these drugs may stimulate synthesis of mitochondrial proteins[28,73]. These data support the hypothesis that circulating leukocytes may serve as real-time sensors of mitochondrial function and liver disease pathology, as well as be sensitive to therapeutic intervention.

Biomarkers for Assessing Prognosis of Disease

Of the various scoring systems available to gauge disease severity and prognosis of patients with AH, traditionally modified discriminant function (mDF) index and model for end-stage disease (MELD) score are used in clinical practice[89–91]. Dynamic modeling using the MELD at baseline and Lille score at 1 week of corticosteroid treatment also appears to provide better prediction of outcomes than either of the two scores alone[82,90,92]. Of the clinical variables, hepatic encephalopathy and presence of systemic inflammatory response syndrome (SIRS) are the most consistent and reliable markers for disease severity and predicting outcome[93,94]. For example, SIRS is an accurate predictor for onset of renal failure in these patients, a major determinant of patient mortality[95]. Liver biopsy performed in about 15%–20% of these patients in the US mainly for diagnostic uncertainty also provides prognostic information[41]. It must be recognized that these scoring systems provide prognosis on a short-term basis, and the long-term outcomes in these patients are mainly dependent on their ability to remain sober after recovery from the episode of AH[96], as about 30%–35% and 40%–50% of severe AH patients relapse to alcohol use within 6 months and 1 year, respectively[83]. Currently, there are no factors or variables that can accurately predict risk of relapse to alcohol use in this population.

Data are also emerging on the role of LPS and procalcitonin levels in predicting response to treatment and onset of multiorgan failure, a major cause of death in patients with severe AH[94]. Serum biomarkers of systemic inflammation especially extracellular vesicles, CCL-20, and CK-18 M65:M30 levels have shown promise in estimating disease outcome and prognosis in AH patients[77–79]. In one study, M30:M65 ratio was better than traditional scores of estimating disease severity of AH and its outcome at day 30 from presentation[79]. Progression of liver failure in AH and other liver diseases is a balance between progression of sterile inflammation and regenerative capacity of liver parenchyma. In this regard, levels of anti-inflammatory cytokines such as IL-10 or IL-22 and inflammatory cytokines and chemokines including CCL-20 have emerged as predictors of patient outcome[97]. Further, gene expression studies performed on liver tissue have shown upregulation of genes involved in hepatic regeneration among patients responsive to corticosteroids and good outcome, and genes involved in inflammation signaling pathways upregulated in patients nonresponsive to treatment with progressive liver failure and bad outcome[86]. Invasive technique like hepatic venous pressure gradient measurement has also been shown to predict outcomes and disease course; however, this has not been used for this sole purpose in practice in this population[98].

Biomarkers for Early Diagnosis of Infections

Infection is a common occurrence and follows patients with liver failure including those with severe AH[99]. Infections occur in about 30%–50% among hospitalized cirrhotic compared to 5%–7% of hospitalized patients from causes other than liver cirrhosis[100,101]. Abnormalities of innate and adaptive immunity especially in neutrophils among AALD patients make them susceptible to bacterial infections[102]. Also, immunocompromised state of cirrhosis and portal hypertension bypassing of gut bacteria and endotoxins to systemic circulation set the stage for development of infections in these patients[103]. Further, cytokine storm of ongoing inflammation and pathology in AH can also cause immune paralysis with susceptibility of these patients for multidrug-resistant and fungal nosocomial and hospital-acquired infections, which are less responsive to antibiotics, and may progress to development of septic shock and high mortality[104,105]. This risk increases further with treatment of these patients, especially with corticosteroids[99,106]. The accurate diagnosis of infections in these patients often becomes difficult, as features of SIRS are not accurate predictors of infection given the frequent presence of sterile inflammatory state in these patients[107]. Further, blood cultures can be negative in about 30%–40% of patients with bacterial sepsis, and there is often a lag time of many weeks before fungal culture results are available[108]. Over the last few years, biomarkers have emerged to predict bacterial infections in these patients, such as procalcitonin, levels of bacterial DNA, oxidative burst in monocyte and neutrophils, and extracellular vesicles in peripheral blood samples[39,94,106,109].

Limitations of the Existing Biomarkers

Over the last decade or so, the field is widening and expanding on the identification of biomarkers at every stage of the disease of AH, including early diagnosis, estimating prognosis, identification of infections, and determining responsiveness to treatment. However, all these studies are from single centers with small sample size. Further, many of these tests and assays are expensive, involve a complex technique for measurement, and are unavailable at many centers and are not commercially available. Clearly, these encouraging data need further validation on larger samples from multicenter recruitment, before their routine application in routine clinical practice. The ongoing studies in the NIAAA-funded

consortia in the US are an ideal opportunity for joining hands with other centers and investigators to collect samples on well-characterized patients as a basis for the development of accurate, simple, validated, and reliable biomarkers for management of patients with AALD and severe AH.

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