MONOCYTE SIRT7 AND CYTOKINE EXPRESSION IN ALCOHOLIC HEPATITIS

By

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Abstract

Background: Acute alcoholic hepatitis (AH) is a distinct form of alcoholic liver disease characterized by acute jaundice and hepatic and systemic inflammation. Heavy alcohol consumption is well-tolerated by most individuals. Only a minority of heavy drinkers experience an episode of acute AH. Variation in resident and infiltrating macrophage immune response is believed to underly the variable penetrance observed in acute alcoholic hepatitis. Here we examine the relationship of SIRT7 and inflammatory cytokine expression in peripheral blood monocytes in alcoholic hepatitis.

Objectives: The objectives of this study were to: 1) determine the relationship between p-FOXO3, SIRT7, and inflammatory cytokine response in circulating monocytes in patients with acute AH; 2) assess the association between clinical outcomes, inflammatory cytokine expression levels, and SIRT7; and 3) to compare levels of p-FOXO3, SIRT7, and inflammatory cytokine expression in patients at varying risk for AH.

Methods: Peripheral blood monocytes were isolated from patients with acute AH and various control groups and were treated with 100ng/mL lipopolysaccharide (LPS). Relative expression of SIRT7, TNFα, IL-6, IL-10, ICAM1, and CCL2 were measured using reverse transcriptase real-time PCR. SIRT7 and cytokine expression data were assessed for correlation with model for end-stage liver disease (MELD) score and Maddrey Discriminant Function (MDF) score.

Results: Ninety-nine subjects, including 22 with acute AH were enrolled. There was no significant difference in LPS-stimulated SIRT7 expression among patients with AH, healthy controls, healthy drinkers (alcohol control), and sepsis patients (inflammatory control). In patients with AH, SIRT7 expression is correlated with CCL2 expression ($R=0.689$, $P=0.0004$). SIRT7 expression is not correlated with TNFα, IL6, ICAM1, IL10, MELD score, or MDF score.

LPS-stimulated levels of SIRT7 were not significantly different among patients hypothesized to be at low risk (healthy controls & healthy drinkers), medium risk (alcoholic & non-alcoholic cirrhosis), or high risk (previous history of AH) of developing acute AH.

Conclusions: SIRT7 mRNA expression does not reflect monocyte or clinical phenotype. Further studies assessing SIRT7 and FOXO3 protein levels are needed.

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Chapter 1: Introduction and Review of Literature

Introduction

Globally, over 600,000 people die annually of alcoholic liver disease (ALD). Effective treatments remain elusive, in part due to an incomplete understanding of the underlying disease mechanisms. Among heavy alcohol consumers, 85% remain free of liver disease. The remaining 15% develop alcoholic liver disease which manifests as various forms of liver pathology ranging from steatosis and steatohepatitis to fibrosis, cirrhosis, and ultimately end-stage liver disease.[1] Approximately 20% of patients with alcoholic liver disease will experience one or more episodes of acute alcoholic hepatitis (AH). AH is an acute systemic inflammatory condition characterized by severe liver inflammation and acute jaundice. AH can occur in patients with relatively mild ALD (*i.e.* steatosis or mild steatohepatitis) and no previously recognized clinical signs of liver disease. More often, however, it occurs in the setting of underlying fibrosis or cirrhosis. While cirrhosis is irreversible, the acute inflammation and accompanying hepatic dysfunction seen in alcoholic hepatitis is reversible with abstinence from alcohol. Alcoholic hepatitis can progress to liver failure, multi-organ failure, and death. Current therapies, including glucocorticoids, pentoxifylline, and nutritional support, have limited efficacy. Thirty day mortality is approximately 20-30%.[2,3] Although, for patients who survive the acute inflammatory phase of AH and who remain abstinent from alcohol, the prognosis is good.

AH is a distinct form of ALD and is the focus of this thesis. However, it is important to note that AH exists within the broader category of ALD and shares many of the same epidemiological patterns and risk factors. Furthermore, the pathogenesis of AH and ALD are intertwined, with the existence of ALD increasing one's risk for AH and episodes of AH contributing to the progression of ALD. Thus, the following introductory sections address the

epidemiology, risk factors, and pathogenesis of ALD more broadly before focusing specifically on AH as a distinct disease.

Epidemiology of Alcohol Consumption and Alcoholic Liver Disease

Heavy alcohol consumption is essential for the development of alcoholic liver disease. As such, the geographic distribution of the incidence and prevalence of alcoholic liver disease tends to mirror alcohol consumption patterns. According to the World Health Organization's (WHO) "Global Status Report on Alcohol and Health," alcoholic cirrhosis resulted in 607,000 deaths and 22.2 million disability adjusted life years (DALYs) lost in 2016.[4] 43% of the world's population over age 15 has consumed alcohol within the past year. From 2000 to 2016, the number of current drinkers, defined as those consuming alcohol within the past year, decreased from 63.5% to 54.1% in the Americas and from 70.1% to 59.9% in Europe. However, other areas of the world, especially China, have seen an increase in the number of current drinkers.

The highest alcohol consumption per capita is seen in Russia and Eastern Europe followed by Western Europe and the Americas. The lowest per capita consumption is seen in the Middle East and Southeast Asia in predominantly Islamic populations. Since 2000, per capita alcohol consumption has remained relatively stable in the Americas and Africa. Europe has seen a decline in per capita alcohol consumption while Southeast Asia and the Western Pacific regions have seen increases in consumption. Globally, alcohol consumption has increased slightly from 2000 to 2016. The number of current drinkers has declined in most regions of the world since 2000, yet the number of per capita drinks has remained stable or increased in most areas of the world. Accordingly, the number of drinks per active drinker has increased globally since 2000, with an increase that is quite dramatic in some regions. This trend of increased levels

of alcohol consumption among active drinkers suggests that most regions of the world can anticipate an increase in the incidence of ALD over the next 10-30 years.

In the United States, the National Vital Statistics Reports track ALD-related mortality from death certificates.[5] In 2016, ALD resulted in 21,815 deaths in the United States. The overall death rate for ALD was 6.8 deaths per 100,000 people. The rate was more than twice as high in men (9.5 per 100,000) than women (4.1 per 100,000), which is true in all racial/ethnic categories. Among non-Hispanics, whites (7.7 per 100,000) have higher death rates than blacks (3.8 per 100,000), and American Indian or Alaskan Natives have the highest rates at 28.4 per 100,000. The lowest rates are seen in Asian or Pacific Islanders at 1.4 deaths per 100,000. Hispanics have an intermediate rate at 6.1 deaths per 100,000. In general, the age adjusted rates are slightly lower but tend to mirror the unadjusted rates across the categories of gender, race, and ethnicity. Exceptions to this are Hispanics and American Indian/Alaskan Natives which have higher age adjusted rates (7.7 and 29.2 deaths per 100,000, respectively) which suggests that these minority populations experience mortality due to ALD at a younger age.

Risk Factors for Alcoholic Liver Disease

Alcoholic liver disease tends to affect individuals age 45-64 years old. Men are more likely to be heavy drinkers, and as a result are more likely to develop ALD. However, women are more sensitive to the hepatotoxic effects of alcohol, and for a given level of alcohol consumption, women are more likely than men to develop ALD.[6–8] Genome-wide association studies have associated patatin-like phospholipase domain containing protein 3 (PNPLA3) with an increased risk of ALD and non-alcoholic fatty liver disease.[9] Multiple environmental factors can increase one's risk of developing ALD. Hepatitis C virus infection has been shown to act synergistically with alcohol in causing liver disease. Obesity and insulin resistance, which are

primary risk factors for nonalcoholic fatty liver disease, also increase one's risk for developing ALD.[10] Additionally, dietary patterns and gut microbiome have been associated with ALD.[11] Alcohol consumption leads to intestinal bacterial overgrowth and altered microbial flora including decreased Clostridiales, Akkermansia, and Bacteroides species and increased Enterobacteriaceae, Actinobacteria, Proteobacteria, and others.[12,13] Animal models and early phase human studies have shown promising results in the treatment of AH from altering the gut microbiome through fecal microbiota transfer.[14–16]

Natural History and Pathogenesis

Alcoholic liver disease represents a spectrum of disease with multiple distinct classifications.[17] Excessive alcohol consumption leads to steatosis (fatty deposition) in greater than 90% of drinkers. Approximately 30% of excessive drinkers will develop fibrosis, and with continued drinking, approximately 20% of all heavy drinkers will progress to cirrhosis. Those with cirrhosis are at risk for development of hepatocellular carcinoma which occurs at a rate of 1.5% annually.[1] Alcoholic hepatitis is a distinct entity characterized by acute liver inflammation and jaundice. It occurs in approximately 20% of heavy drinkers and can develop in the setting of steatosis, fibrosis, or cirrhosis. Importantly, steatosis and alcoholic hepatitis are reversible with abstinence. While cirrhosis is not reversible, mortality is much lower in those who remain abstinent.[1]

The pathogenesis of alcoholic liver disease is incompletely understood. It is known that alcohol contributes to the development and progression of liver disease through both direct toxicity to hepatocytes and through indirect mechanisms.[18] The major pathway of ethanol metabolism is via alcohol dehydrogenase which produces acetaldehyde via reduction of NAD+ to NADH. Acetaldehyde is further metabolized by aldehyde dehydrogenase to acetate; this

reaction also requires reduction of NAD+. The toxic intermediate, acetaldehyde results in microtubule dysfunction and production of reactive oxygen species. The increased NADH/NAD+ ratio results in oxidative mitochondrial damage resulting in decreased ATP production. The minor pathway of hepatic ethanol metabolism by cytochrome P450-2E1 also contributes to the production of acetaldehyde and ROS. Ethanol also results in damage to cellular membranes through interaction with and oxidation of membrane phospholipids.[19]

In addition to the direct toxic effects of alcohol and its metabolites, the pathogenesis of alcohol in liver disease is known to involve immune mechanisms. Alcohol consumption disrupts the tight junctions between epithelial cells in the intestine, leading to bacterial translocation from the gut and release of bacterial lipopolysaccharide (LPS).[20,21] This LPS enters the liver via the portal venous circulation where it can trigger an inflammatory immune response through interaction with hepatic macrophages. The pro-inflammatory effect of LPS is mediated through activation of toll-like receptor 4 which leads to induction of nuclear factor-kappaB (NF-κB) culminating in transcription of proinflammatory cytokines including TNFα, IL-1β, and IL-6. The mechanism of alcohol induced inflammation in AH is further expanded upon in Chapter 3, Introduction of Thesis.

Chapter 2: Retrospective Analysis of Monocyte Count in Alcoholic Hepatitis

Introduction

As discussed in Chapter 1 and Chapter 3, resident macrophages play a key role in the pathogenesis and resolution of inflammation in alcoholic hepatitis. Additionally, studies in animal models of alcoholic hepatitis (AH) have demonstrated that circulating monocytes infiltrate the liver and contribute to the pathogenesis of AH.[22] In a small cohort of 12 patients, McKeever *et.al.* demonstrated that patients with AH have elevated absolute monocyte counts (AMC) compared to healthy controls.[23] Shi and Thomas also report a single case of monocytosis in AH.[24] To our knowledge, these two reports represent the only studies of AMC in AH. Given the small sample size contained in these previous studies, we sought to confirm these findings in a larger retrospective cohort. Additionally, we sought to evaluate the relationship between AMC in patients with alcoholic hepatitis and disease severity and prognosis.

AH is characterized by an acute rise in bilirubin to greater than 3 mg/dL, AST 50-400 IU/mL, and AST/ALT ratio of >1.5 in the setting of heavy alcohol consumption (>40 g/day for females, >60 g/day for males) for greater than 6 months.[25] When AH presents classically, without any potentially confounding conditions, the diagnosis can be made clinically. However, in 10-20% of cases, an accurate diagnosis cannot be made without liver biopsy which is the gold standard for confirming the diagnosing AH.[25] Liver biopsy is rarely performed in cases of AH due the associated risk of complications. For this reason, we also sought to evaluate whether AMC might be useful in aiding in the clinical diagnosis of AH. To assess the diagnostic utility of AMC in AH, we compare the AMC in cases of clinically and histologically diagnosed AH to a group of controls with histologically diagnosed non-AH alcoholic liver disease.

Methods

Study Population. Patients age 18-70 admitted to a large tertiary care academic medical center hospital with an ICD-9/10 diagnosis code associated with alcoholic liver disease were identified retrospectively. Patients with ICD codes for infection (sepsis, spontaneous bacterial peritonitis, urinary tract infection, pneumonia), immunodeficiency, HIV, or hematologic malignancy were excluded. The full listing of ICD codes used for inclusion and exclusion can be found in Appendix A. Based on recommendations from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) Alcoholic Hepatitis Consortia, the following laboratory inclusion criteria which are consistent with a probable diagnosis of AH were applied: AST >50, AST/ALT > 1.5, AST & ALT < 400, total bilirubin >3mg/dL.[25] Patients meeting the inclusion/exclusion criteria were then divided into two groups: AH (cases) and ALD (controls) based on the following criteria. Patients with a clinical diagnosis of AH and no recent liver biopsy were classified as AH. Patients with a liver biopsy occurring within the 14 days prior or 30 after the inpatient admission were classified as either AH or ALD based on the results of the liver biopsy. Patients without either a clinical (ICD-9/10) diagnosis of acute AH or a history of ALD plus a liver biopsy were not considered for inclusion in this study.

Only the patients' first recorded admission for AH was included in the study. Subsequent readmissions for AH were excluded. Initial and follow up absolute monocyte count (AMC), absolute lymphocyte count (ALC), absolute neutrophil count (ANC), and white blood cell (WBC) count, were determined. Clinical outcomes including model for end-stage liver disease (MELD) score, Maddrey discriminant function (MDF), and 30-day, 90-day, 180-day, and 1-year mortality were assessed. MELD and MDF scores were calculated using the component lab values from a patient's first labs obtained after admission. MDF score was estimated from the international normalized ratio (INR) value assuming a prothrombin control time of 12.0 seconds

and an international sensitivity index of 1.0. Data were automatically extracted through the center's I2B2-based electronic health record research data repository.[26,27] Data cleaning was conducting using SQLiteStudio version 3.2.1. Pathology reports were manually reviewed for patients receiving a liver biopsy. A histologic diagnosis of alcoholic hepatitis was defined as macrovesicular steatosis plus one or more of the following: neutrophil infiltration, hepatocyte ballooning, or Mallory-Denk bodies. This definition is consistent with recommendations from the National Institute on Alcohol Abuse and Alcoholism Alcoholic Hepatitis Consortia.[25]

Statistical Analysis. Continuous variables were evaluated for normality using the Shapiro-Wilk test and quantile-quantile plots. One sample Student's T test and the Wilcoxon signed-rank tests were used to compare the absolute blood counts to the reference ranges. Two-sample T tests, Wilcoxon-Mann-Whitney test, and Fisher's exact test were used to compare the AH group with the ALD control group. Spearman's correlation coefficient was used to evaluate relationships between MELD, MDF, and AMC. AMC was compared between cases stratified as mild (MDF $\leq$ 32) or severe (MDF > 32) using the two-sample T test and the nonparametric Wilcoxon Mann-Whitney test. Logistic regression was used to evaluate the effect of AMC on 30-day, 90-day 180-day, and 1-year mortality. The diagnostic utility of AMC was evaluated using receiver operating characteristic analysis to assess the ability of AMC to distinguish between cases of AH and ALD controls. All statistical analyses were conducted using SAS version 9.4, Cary, NC.

Results

Table 1 compares the demographic and study characteristics between the AH (cases) and the ALD (control) groups. The median age for patients in the AH cohort was 48 years which was slightly younger than patients in the ALD cohort (53 years, $P = 0.0054$). The distribution of race

and ethnicity was similar in the 2 cohorts. The two groups had similar MELD and MDF scores suggesting similar severity of liver disease. The ALD group had a significantly higher percentage of patients with HCV infection compared to the AH group (52% vs 20%, *P* = 0.0019).

Table 1. Comparison of demographics, comorbidities, and clinical laboratory data between AH (cases) and ALD (controls).

	AH (n = 164)		**ALD (n = 21)**		***P* value[a]**
Age	48	(38 – 54)	53	(49 – 58)	0.0054
Female	60	(37)	4	(19)	0.1117
Race					
White	126	(77)	18	(86)	0.5735
Black	14	(9)	3	(14)	
Other	22	(13)	0	(0)	
Unknown	2	(1)	0	(0)	
Ethnicity					0.7021
Non Hispanic, Latino or Spanish Origin	144	(78)	20	(95)	
Hispanic, Latino or Spanish Origin	18	(11)	1	(5)	
Not Recorded	1	(1)	0	(0)	
HCV infection	33	(20)	11	(52)	0.0019
HBV infection	5	(3)	1	(5)	0.5198
MELD	26.2	± 6.8	25.5	± 6.7	0.6223
MDF	53	(32 – 84)	47	(37 – 78)	0.6654
Total Bilirubin	10.3	(5.89 – 18.2)	4.6	(3.8 – 9.1)	0.0022
AST	139	(96 – 200)	108	(83 – 158)	0.0334
ALT	56	(32 – 68)	48	(31 – 72)	0.8813

Table Values are n (%) or mean ± SD or median (interquartile range). [a]*P* value calculated from *t* test, Wilcoxon-Mann-Whitney, or Fisher exact test as appropriate. AH = Alcoholic Hepatitis, ALD = alcoholic hepatitis, HCV = hepatitis C virus, HBV = hepatitis B virus MELD = model for end-stage liver disease, MDF = Maddrey discriminant function, AST = aspartate aminotransferase, ALT = alanine aminotransferase.

The comparison of white blood cell counts to the normal reference ranges is summarized in Table 2. For the AH cohort, the mean WBC was 11.33 thousand cells per µL, which is not significantly higher than the normal reference range. The mean AMC for the AH cohort was 0.95

thousand cells per µL which is significantly higher than the upper limit of normal (*P* = 0.0015). The follow up AMC on day 10 of admission was also significantly elevated (mean=1.11, *P* = 0.0039). The day 10 AMC was only available for 37 of 164 patients in the AH cohort and 6 of 21 patients in the ALD cohort. Mean ANC was also elevated in the AH cohort (8.26 thousand cells per µL) (*P* = 0.0903); although this did not reach statistical significance. ALC was within the normal reference range. For the ALD cohort, AMC was similarly elevated (0.97 thousand cells per µL) (*P* = 0.0511). The mean WBC, ANC, and ALC were all within the reference range for the ALD cohort.

Table 2. Comparison of white blood cell counts in each cohort to the normal reference ranges.

		AH Cohort (n = 164)			ALD Cohort (n = 21)			AH vs ALD
	Reference Range[a]	Mean	95% CI	*P* Value[b]	Mean	95% CI	*P* Value[b]	*P Value*[c]
WBC	4.5 – 11.0	11.33	(10.29 – 12.36)	0.3350	7.82	(6.66 – 8.99)		0.0432
AMC	0.0 – 0.8	0.95	(0.87 – 1.02)	0.0015	0.97	(0.799 – 1.15)	0.0511	0.5749
ANC	1.8 – 7.0	8.26	(7.48 – 9.04)	0.0903	5.42	(4.26 – 6.58)		0.0127
ALC	1.0 – 4.8	1.32	(1.19 – 1.45)		1.29	(1.03 – 1.55)		0.9396
AMC Day 10	0.0 – 0.8	1.11	(0.91 – 1.31) n = 37	0.0039	0.78	(0.37 – 1.18) n = 6		0.2844

Units are in thousand cells per µL. [a]Reference range refers to the normal laboratory reference range for the specified blood count. [b]*P* values for each cohort are obtained from one-sample *t* test or Wilcoxon signed rank test and are in comparison to the upper limit of the normal reference range. *P* values are omitted for measures falling within the normal reference ranges. [c]*P* value obtained from Wilcoxon-Mann-Whitney test comparing the mean blood count from the AH cohort to the ALD cohort. WBC = white blood cell count, AMC = absolute monocyte count, ANC = absolute neutrophil count, ALC = absolute lymphocyte count.

In comparing the AH cohort and the ALD cohort (Table 2), the AH group had significantly higher white blood cell count (WBC) and absolute lymphocyte count (ALC). There was no significant difference in absolute monocyte count (AMC) or absolute lymphocyte count (ALC) between the two groups. When the analysis of AMC was limited to only those patients

with biopsy confirmed AH (n = 9) there remained no significant difference in AMC between the AH group (median AMC 0.70, IQR 0.60-1.10) and the ALD group (median AMC 1.00, IQR 0.78 – 1.19) (P = 0.4545). The receiver operating characteristic analysis which was used to assess the ability of AMC to distinguish between cases of AH and ALD controls indicates that AMC is neither sufficiently sensitive nor specific. The area under the ROC curve was 0.5377 (Figure 1).

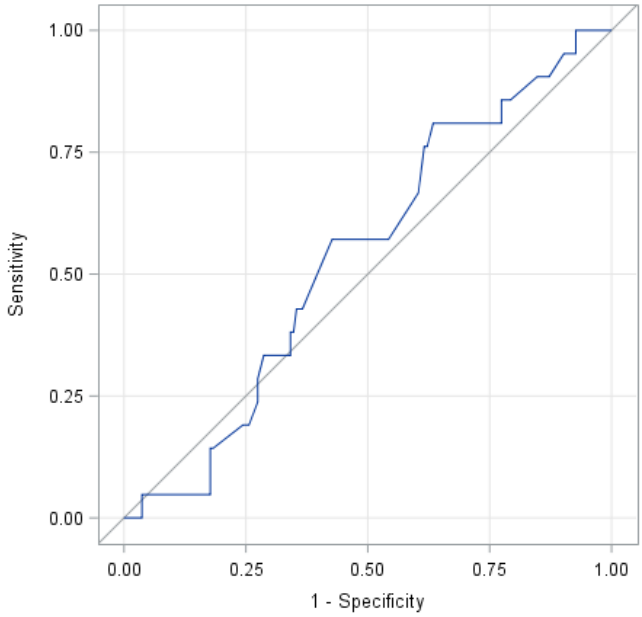

Figure 1. ROC Curve for absolute monocyte count.
AMC is neither sensitive nor specific for distinguishing cases of AH from ALD controls. Area Under the Curve = 0.537.

In patients with alcoholic hepatitis, AMC was found to be positively correlated with MELD score with a Spearman's correlation coefficient (*R*) of 0.400, $P < 0.0001$ (Figure 2). AMC was similarly correlated with discriminant function score ($R = 0.330$, $P < 0.0001$) (Figure 3). Patients with severe AH defined as MDF ≥ 32 have a mean monocyte count of 1.01 thousand cells per µL which is significantly higher than patients with mild AH (MDF < 32) who have a mean monocyte count of 0.75 ($P = 0.0011$). Similarly, patients with a MELD score of greater

than 20 have a higher mean AMC (1.02 thousand cells per µL) compared to patients with a MELD score ≤ 20 (mean AMC 0.71 thousand cells per µL) ($P = 0.0002$).

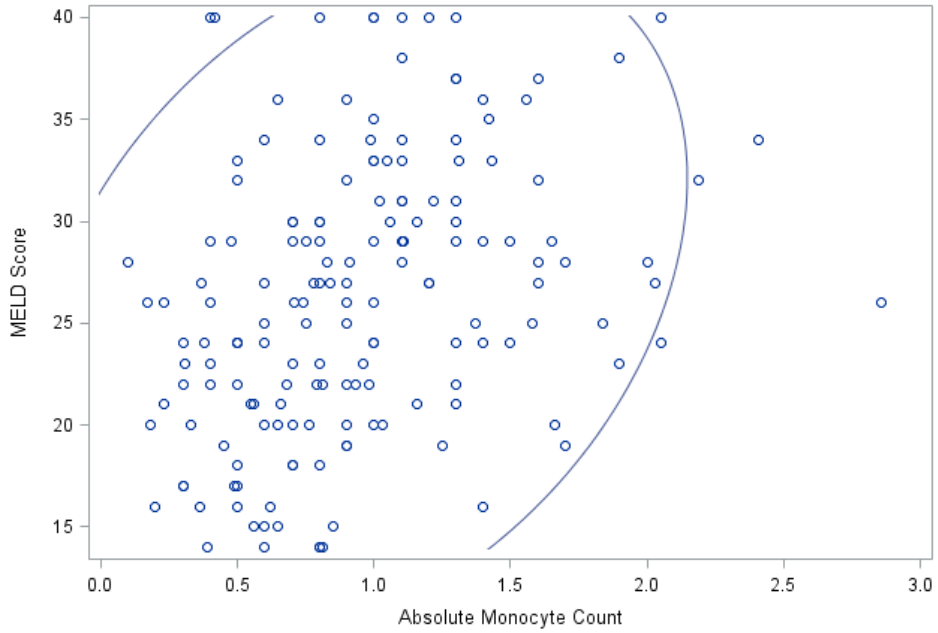

Figure 2. Correlation between MELD score and absolute monocyte count.
n = 163, Spearmans $R = 0.400$, $P < 0.0001$. Shown with 95% prediction ellipse.

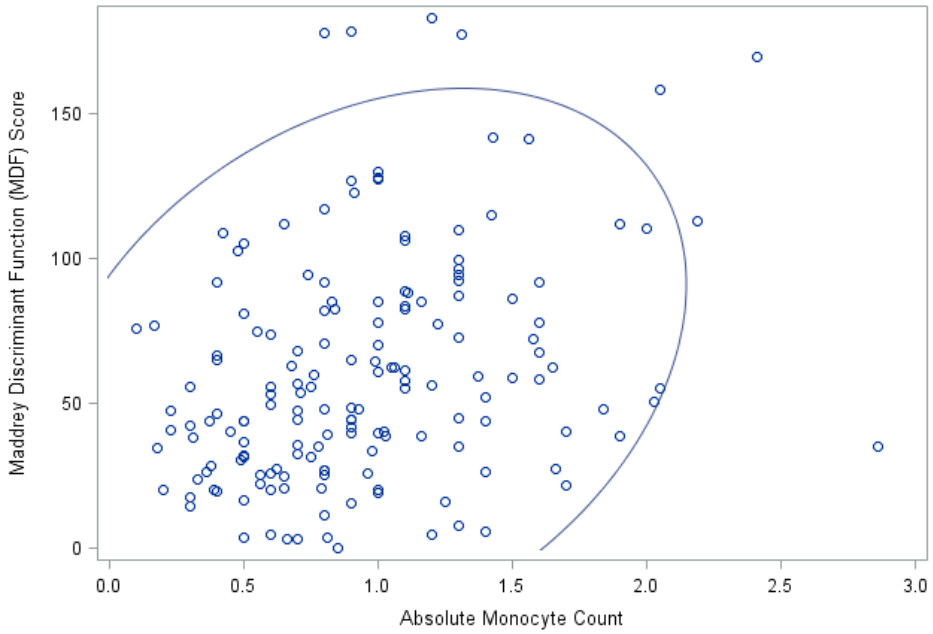

Figure 3. Correlation between MDF and absolute monocyte count.
n = 163, Spearman's $R = 0.330$, $P < 0.0001$. Shown with 95% prediction ellipse.

In the AH cohort, 30-day, 90-day, 180-day and 1-year mortality were 9%, 14%, 17%, 24%, respectively. Initial AMC following admission for AH was not associated with an increased odds of 30-day, 90-day, 180-day, or 1-year mortality. Follow up AMC on day 10 (+/- 2 days) was associated with increased odds of mortality at 30 days (OR 5.30, 95% CI 1.00-28.19), 90 days (OR 2.55, 95% CI 0.69 – 9.50), 180 days (OR 3.92, 95% CI 1.01-15.31), and 1 year (OR 4.33, 95% CI 1.10-17.05). Due to the small number of cases with a follow up AMC on day 10 of admission these odds ratios contain a large degree of uncertainty as seen in the wide confidence intervals. Additionally, the effect of day 10 AMC on odds of mortality did not reach the traditional threshold for statistical significance for 30 and 90-day mortality.

Discussion

This retrospective cohort study supports the findings from McKeever *et.al.* of an elevated absolute monocyte count in alcoholic hepatitis.[23] We also find that absolute monocyte count is positively correlated with disease severity as determined by MELD and MDF scores. Monocyte count at the time of admission was not associated with an increased odds of mortality. However, follow up monocyte count on day 10 (± 2 days) of hospital admission was associated with an increased odds of mortality. The day 10 monocyte count was only available for 37 out of 164 patients in the AH cohort. The availability of a day 10 monocyte count indicates that these patients were hospitalized for at least 8 days and were still receiving regular blood counts. As such, these 37 patients likely represent a more severely ill group and the association of AMC with increased odds of mortality may not generalize to all patients with AH. Additionally, this relatively small sample size leads to wide confidence intervals for the odds ratio. For these reasons our findings of increasing odds of mortality with increasing day 10 monocyte count should be interpreted with caution.

One of the objectives of this study was to determine the diagnostic utility of AMC in alcoholic hepatitis. It is uncommon for patients with suspected AH to receive a confirmatory liver biopsy. For this reason, we opted to compare patients with clinically or histologically diagnosed AH to a cohort of ALD controls. The ALD control group was defined as patients who were admitted to the hospital with a primary diagnosis of alcohol-related liver disease (*e.g.* alcoholic cirrhosis, fibrosis, decompensated cirrhosis) and who met the clinical laboratory criteria for a probable diagnosis of AH (AST > 50, AST/ALT > 1.5, AST & ALT < 400, total bilirubin > 3mg/dL). The control patients were required to have had a liver biopsy confirming the absence of alcoholic hepatitis on histology within the 14 days prior or 30 days after their hospital admission. Thus, we were able to define a control cohort of patients that had a clinically similar presentation to patients with alcoholic hepatitis (*i.e.* hospitalization with jaundice, elevated AST, and a history of alcohol use) who did not have histological findings consistent with AH. We then compared this ALD control cohort to patients with a clinical (non-histological) diagnosis of AH. In doing so we found that AMC is elevated to a similar degree in both the AH cases and ALD controls. This finding was confirmed in subgroup analysis consisting only of patients with histologically confirmed AH. Future studies should evaluate whether AMC is elevated in patients with other causes of cirrhosis, such as viral hepatitis, autoimmune diseases, and non-alcoholic steatohepatitis. This would help determine if elevated AMC is a feature of chronic liver disease in general or if elevated AMC is unique to alcohol-related liver disease.

The findings of elevated AMC in patients with AH and ALD are important in that they provide evidence that circulating monocytes play a role in the pathogenesis of alcoholic liver diseases. Previous studies have demonstrated hepatic infiltration of circulating monocytes in animal models of AH. In combination with these previous studies, the findings presented here

suggest that peripheral monocytes can be utilized to provide insight into the pathogenesis of alcoholic hepatitis. This approach is utilized in the remainder of this thesis, where we evaluate SIRT7 and cytokine expression in peripheral blood monocytes from patients with alcoholic hepatitis.

Chapter 3: Rational for Studying FOXO3 and SIRT7 in Alcoholic Hepatitis

Background

Acute alcoholic hepatitis (AH) is characterized by prolonged hepatic and systemic inflammation and can progress to liver failure, multi-organ system failure, and death. Disease penetrance for AH is highly variable among heavy drinkers.[7] Approximately 15% experience AH, while the other 85% appear to be protected. Variation in resident and infiltrating macrophage immune response is believed to underlie the variable penetrance observed in acute AH. Although the underlying mechanisms are incompletely understood, it is known that alcohol causes release of lipopolysaccharide (LPS) from the gut which interacts with hepatic macrophages leading to inflammatory cytokine production in alcoholic hepatitis. In Chapter 2, we demonstrate that AH patients have elevated numbers of circulating monocytes. Further, it has previously been shown that monocytes from patients with AH produce increased levels of TNFα, IL6, and MCP-1 in response to stimulation with LPS.[28–31]

The forkhead box transcription factor FOXO3 appears to play a key role in the susceptibility to and pathogenesis of alcoholic hepatitis. FOXO3 has been shown to have hepatoprotective effects in alcohol fed mice, and suppression of FOXO3 by the combination of alcohol and hepatitis C virus (HCV) has been shown to lead to more severe liver injury.[32] The transcriptional activity of FOXO3 is highly variable and is dependent upon post-translational modifications. LPS has been shown to induce JNK-dependent phosphorylation of FOXO3 at serine-574.[33] S-574 phospho-FOXO3 (pFOXO3) has altered promoter specificity which leads to apoptosis of monocytes and macrophages and downregulation of inflammation through suppression of NF-κB and inflammatory cytokines.[33–36] The phosphorylation of FOXO3 at serine-574 by JNK is largely limited to the acetylated version of FOXO3.[37] The acetylation status

of FOXO3 is regulated by the histone deacetylases SIRT1 and SIRT7. It has also been shown that in patients with AH, SIRT7 is upregulated which leads to decreased acetylation of FOXO3 and thus prevents phosphorylation of FOXO3 and suppression of the inflammatory phenotype observed in AH.[37]

Objectives

The objectives of this study were to 1) determine the relationship between p-FOXO3, SIRT7 and inflammatory cytokine response in circulating monocytes in patients with acute alcoholic hepatitis; 2) assess the association between clinical outcomes, inflammatory cytokine expression levels, and SIRT7; and 3) to compare levels p-FOXO3, SIRT7, and inflammatory cytokine expression in patients at varying risk for alcoholic hepatitis.

We hypothesized that patients with alcoholic hepatitis have elevated levels of SIRT7 compared to controls, which leads to decreased formation of anti-inflammatory pFOXO3 and thereby produces an enhanced inflammatory phenotype in monocytes and a more severe clinical phenotype. Additionally, we hypothesized that abnormalities in SIRT7 and LPS induced cytokine expression would exist in patients at high risk for alcoholic hepatitis, including those with a history of alcoholic hepatitis and alcoholic cirrhosis, compared to controls.

Chapter 4: Methods

Study Approval

This study was approved by the institutional review board at the University of Kansas Medical Center. All subjects provided informed consent.

Patient Selection and Definition of Study Groups

Subjects age 18-70 years old from the following seven groups were included in this study:

1. Acute Alcoholic Hepatitis: Subjects with severe acute alcoholic hepatitis were recruited from the inpatient hepatology consult service at a large tertiary academic medical center. A diagnosis of severe acute alcoholic hepatitis was defined as a consulting hepatologist's clinical diagnosis of acute alcoholic hepatitis with supporting evidence including elevated aspartate aminotransferase (AST), AST/alanine aminotransferase (ALT) ratio of greater than 1, elevated total bilirubin, a history of alcohol consumption consistent with alcoholic hepatitis, and no other identified etiology of liver disease.
2. Sepsis: Patients with sepsis were recruited in the inpatient setting of the same hospital through monitoring of the institution's positive blood culture log which is updated daily by the clinical lab. The diagnosis of sepsis was based on positive blood cultures and two or more systemic inflammatory response syndrome (SIRS) criteria (Temperature > 38 C or < 36 C, heart rate > 90 beats/min, respiratory rate > 20 breaths/min, white blood cell count greater than 12,000/μL or less than 4,000/μL with 10% band neutrophils) at the time blood cultures were drawn.
3. Alcohol Controls: Individuals with current, regular alcohol consumption and no known current or previous liver disease. The Substance Abuse and Mental Health Services Administration (SAMHSA) defines heavy alcohol consumption as binge drinking (4 or

more drinks for females or 5 or more drinks for males on the same occasion) on 5 or more days per month. The National Institute on Alcohol Abuse and Alcoholism (NIAAA) defines low risk drinking as no more than 7 drinks per week for women and no more than 14 drinks per week for men. Thus, for the purpose of this study, regular alcohol consumption was defined as individuals either meeting the definition of heavy alcohol consumption or exceeding 14 drinks per week for men or exceeding 7 drinks per week for women These subjects were recruited from the community. Subjects with a history of liver disease or viral hepatitis were excluded from this group.

4. Alcoholic Cirrhosis: Patients with a histologic, radiographic, or clinical diagnosis of alcoholic cirrhosis were recruited from outpatient hepatology clinics. This group includes patients on the liver transplant waiting list. Patients with alcohol consumption within the last three months were excluded from this group, as were patients with other etiologies of cirrhosis.
5. Cirrhotic Controls (non-alcohol related cirrhosis): Patients with a histologic, radiographic, or clinical diagnosis of cirrhosis and no history of regular heavy alcohol consumption were recruited from outpatient hepatology clinics.
6. History of Alcoholic Hepatitis: this group includes patients who were hospitalized within the past 5 years for an episode of severe alcoholic hepatitis. Patients with ongoing alcohol consumption within the previous 3 months were excluded. Patients with evidence of ongoing systemic inflammation as evidenced by a temperature > 37.8, elevated white blood cell count, or recent changes in bilirubin or INR were also excluded.
7. Healthy Controls: Healthy adults with no history of heavy alcohol use and no history or evidence of liver disease were recruited from the community.

The following exclusion criteria were applied to all of the study groups. Subjects with immune deficiencies, HIV, HBV, active malignancy, or current infection (except the sepsis group) were excluded. Patients taking immunosuppressive medications, other than glucocorticoids for the treatment of alcoholic hepatitis, were excluded.

Outpatient subjects (groups 4, 5, & 6 above) were identified through hepatology clinic schedules and were recruited during their regularly scheduled outpatient visits. Community volunteers (groups 3 & 7) were recruited through distribution of recruitment flyers on the medical center campus and in the community. Community volunteer subjects (groups 3 & 7) were compensated twenty dollars for their participation.

Monocyte Isolation

5-20 milliliters of whole blood was collected from subjects through venipuncture or through existing arterial or intravenous catheters into EDTA tubes. Whole blood samples were stored on ice or at 4 degrees Celsius until further processing which occurred within eight hours of sample collection. Whole blood components were separated by centrifugation at 350 x g for 15 minutes. The buffy layer (3-4 mL) containing the white blood cells was extracted and diluted with an equal volume of phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were then isolated using a density gradient method. The PBS/blood mixture was layered on top of a density gradient solution (Histopaque-1077, Sigma-Aldrich) and centrifuged at 150 x g for 30 minutes. The PBMCs were removed from the interphase and washed with PBS. Peripheral blood monocytes were then isolated from the PBMC fraction using anti-CD14 antibody conjugated microbeads and magnetic column separation (MACS Cell Separation, Miltenyi Biotec). Freshly isolated monocytes were resuspended in 2 mL of RPMI 1640 medium (Gibco) and divided into four fractions in a 24 well culture plate. After approximately 12-24

hours in culture, two of the four wells for each sample were treated with 500 mL of 200 ng/mL Escherichia coli lipopolysaccharide (LPS) in RPMI for a final LPS concentration of 100 ng/mL. An additional 500 mL of RPMI (without LPS) was added to the untreated samples at the same time as LPS treatment to maintain an equal volume of 1mL in both the untreated and LPS treated samples. After 6 hours, cells were collected for protein and RNA analysis.

RNA Analysis

RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method (TRIzol Reagent, Invitrogen). cDNA was prepared from total RNA using a cDNA reverse transcription kit with random primers (Applied Biosystems). Quantitative real-time PCR was performed using a CFX96 real-time system (Bio-Rad, Hercules, CA, USA). 20 microliter reaction volumes utilizing 10 µL of 2x iQ SYBR Green Supermix (Bio-Rad), 3 µL of cDNA (diluted 1:4) and 7 µL of 1 µmol/L forward and reverse primer solution. Gene expression normalized to GAPDH was determined for interleukin 6 (IL-6), interleukin 10 (IL-10), intracellular adhesion molecule 1 (ICAM1), tumor necrosis factor alpha (TNFα), chemokine (C-C motif) ligand 2 (CCL2), and SIRT7. PCR primer sequences are available in Appendix B.

Statistical Analysis

The mRNA expression data was not normally distributed and had frequent outliers. For this reason, nonparametric tests were exclusively utilized in the analysis. The Kruskal-Wallis test was utilized to compare relative mRNA expression for SIRT7 and cytokines among different patient groups with the null hypothesis of equivalency among groups. In cases where the null hypothesis was rejected at a type I error rate (alpha) of 5%, post-hoc pairwise comparisons were conducted for all groups using the Dwass, Stell, Critchlow-Flinger Method. The effect of treatment with LPS on each of the six mRNA targets within each of the seven patient groups was

assessed using a Wilcoxon-signed rank test for each comparison. SIRT7 expression, cytokine expression, and clinical severity (MELD score) were assessed for simple correlation using Spearman's rank correlation. Statistical analysis and generation of plots were conducted using SAS version 9.4, Cary, NC.

Chapter 5: Results

Subject Recruitment and Demographics

A total of 99 subjects meeting inclusion/exclusion criteria were enrolled between July 2017 and February 2019. The number of subjects in each study group as well as the distribution of age and sex within each group is summarized in Table 3. Fifty-four percent of subjects were male. The distribution of sex was not equal within the 7 patient groups ($P = 0.0351$, Pearson Chi-Square). A higher proportion of males was seen in the acute AH group (77%) and the healthy control group (78%). The overall median age was 51 (IQR 36-57). The distribution of age was not equivalent across the 7 study groups ($P < 0.0001$). The cirrhotic control group was significantly older than the acute AH group ($P = 0.0159$) and the alcohol control group ($P = 0.0029$). The alcoholic cirrhosis group was also significantly older than the alcohol control group ($P = 0.0118$). Table 4 summarizes the clinical laboratory data for the acute alcoholic hepatitis group. Prior to initiation of recruitment for this study, samples from healthy controls were collected under a separate biobanking protocol. For a portion of the healthy control samples collected under this protocol, the subject age was not collected. We are therefore unable to report the exact distribution of age in the healthy control group. We estimate that the healthy controls range in age from 25 to 60 with the distribution skewed towards a younger population compared to the overall study population.

Table 3. Demographics

Study Group	N	Age Median (IQR)	Sex (% M)	MELD Median (IQR)
Acute Alcoholic Hepatitis	22	45 (32-56)	77	27 (21-38)
History of Alcoholic Hepatitis	14	46 (36-53)	43	12 (10-15)
Alcoholic Cirrhosis	13	55 (46-60)	54	14 (10-18)
Cirrhotic Controls	14	61 (56-66)	36	9 (8-12)
Sepsis	14	54 (30-59)	57	N/A
Alcohol Controls	12	28 (27-43)	25	N/A
Healthy Controls	10	unknown*	78	N/A
Total	99	51 (36-57)	54	16 (10-24)

Table 4. Clinical Laboratory Data for the Acute Alcoholic Hepatitis Group

Measure	Median	IQR
MDF	58	(42-91)
AST	133	(77-178)
ALT	62	(35-76)
Alkaline Phosphatase	153	(121-232)
Total Bilirubin	16.6	(10-30)
White Blood Cell Count	13.6	(9.4-17.7)
Absolute Monocyte Count	1.2	(0.8-1.4)

SIRT7 and Inflammatory Cytokine Response in Acute Alcoholic Hepatitis

To determine the relationship between SIRT7 and inflammatory cytokine expression we measured basal and LPS-stimulated mRNA levels (normalized to GAPDH) for SIRT7, IL6, IL10, TNFα, CCL2, and ICAM-1 in peripheral blood monocytes from patients with acute alcoholic hepatitis and the following control groups: healthy controls, alcohol controls, and sepsis. These results are summarized in Figure 4. There was no significant increase in SIRT7 mRNA expression after treatment with LPS for 6 hours ($P > 0.05$ for all four groups, Wilcoxon Signed-Rank test). With regards to basal SIRT7 expression levels, the alcohol control group had significantly higher SIRT7 expression compared to the acute AH group ($P = 0.0039$), healthy control group ($P = 0.0008$), and sepsis group ($P = 0.0177$). The sepsis group had significantly higher SIRT7 expression compared to healthy controls ($P = 0.0419$). There was no significant difference in SIRT7 expression between the acute AH group and the sepsis group ($P = 0.6875$) or the healthy control group ($P = 0.3868$). There were no significant differences in SIRT7 expression after treatment with LPS or in fold-change of SIRT7 expression among the 4 groups (Figure 4 A).

Basal TNFα expression was not significantly different among the 4 groups (Figure 4 B). LPS treatment resulted in a significant increase in TNFα expression for the acute AH group ($P = 0.0003$), the alcohol control group ($P = 0.0005$), and the sepsis group ($P < 0.0001$); the increase in TNFα expression after LPS for healthy controls did not reach statistical significance ($P = 0.0625$). After treatment with LPS, the alcohol control group had significantly higher TNFα expression compared to the acute AH group ($P = 0.0283$). The remaining groups had similar levels of TNFα expression.

Basal IL6 levels were low for all four groups (Figure 4 C). Treatment with LPS resulted in increased IL6 expression ($P < 0.001$ for all groups except healthy control, $P = 0.0625$). After treatment with LPS, the alcohol control group had significantly higher IL6 expression compared to the acute AH group and the sepsis group ($P = 0.0004$ and $P = 0.0325$, respectively). The fold change in IL6 expression after LPS treatment was greatest for the alcohol control group.

Basal ICAM1 expression was low in all 4 groups but was significantly lower in the alcohol control group than in the other 3 groups ($P < 0.01$ for all). ICAM1 expression was increased by treatment with LPS ($P < 0.001$ for all groups except healthy control, $P = 0.125$). Following treatment with LPS, all 4 groups had similar levels of ICAM1 expression ($P = 0.0751$) (Figure 4 D).

Basal CCL2 expression was significantly lower in the healthy control group compared to the other 3 groups ($P < 0.01$ for all). Treatment with LPS increased the level of CCL2 expression in the acute AH group ($P = 0.0105$) and the sepsis group ($P = 0.0203$) but not in the alcohol control group ($P = 0.6221$) or the healthy control group ($P = 0.4316$). After treatment with LPS, CCL2 expression was not significantly different among the 4 groups ($P = 0.0773$) (Figure 4 E).

Basal expression of the anti-inflammatory cytokine IL10 was low in all groups but was significantly lower in the alcohol control group compared to acute AH ($P = 0.0073$) and sepsis ($P = 0.0432$). Treatment with LPS resulted in a significant increase in IL10 expression in acute AH ($P < 0.0001$), alcohol controls ($P = 0.0005$), healthy controls ($P = 0.0371$), and sepsis ($P = 0.0002$). Treatment with LPS produced the largest IL10 increase in the alcohol control group. Post-LPS levels of IL10 were significantly higher in the alcohol control group compared to healthy controls ($P = 0.0105$). All other groups had similar post-LPS levels of IL10 (Figure 4 F).

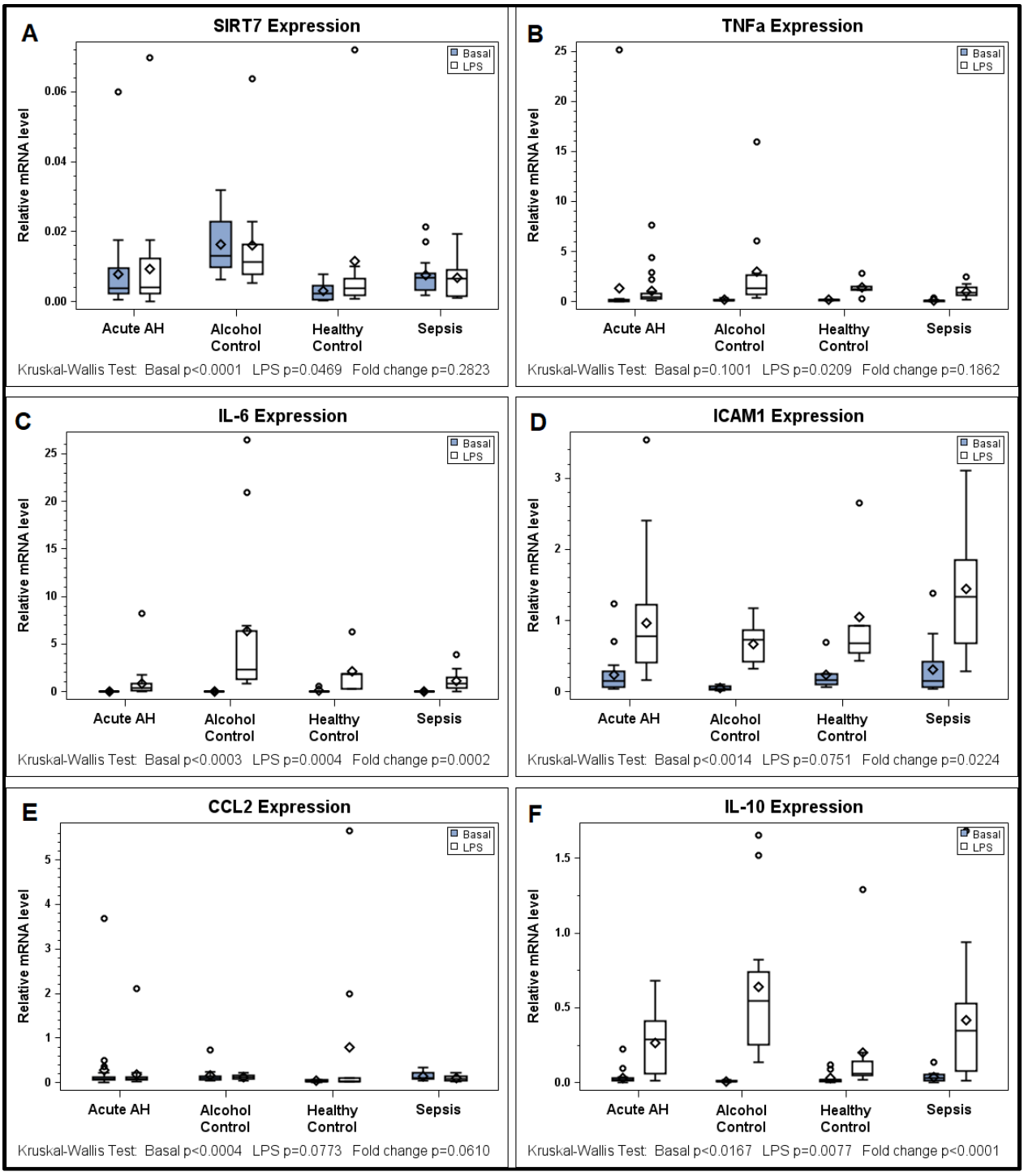

Figure 4. SIRT7 and Cytokine mRNA Expression in Alcoholic Hepatitis Compared to Alcohol Controls, Healthy Controls, and Sepsis Controls

Relationship Between SIRT7, Cytokine Expression, and Clinical Severity

Spearman's correlation coefficient was used to assess the relationship between SIRT7 mRNA expression, cytokine expression and clinical severity. Correlations were assessed for all subject groups combined and for the acute AH group only. For all subject groups combined, basal SIRT7 levels were correlated with basal CCL2 levels ($R = 0.619$, $P < 0.0001$) but not with basal levels of TNFα, IL6, ICAM1, IL10, or MELD score. Basal SIRT7 levels were correlated with the following cytokines levels after stimulation with LPS: IL10 level ($R = 0.447$, $P < 0.0001$), IL6 level ($R = 0.282$, $P = 0.0089$), TNFα level ($R = 0.430$, $P < 0.0001$), and CCL2 ($R = 0.470$, $P < 0.0001$). LPS-stimulated levels of SIRT7 were correlated with LPS-stimulated levels of IL10 ($R = 0.347$, $P = 0.0006$), TNFα ($R = 0.425$, $P < 0.0001$), and CCL2 ($R = 0.724$, $P < 0.0001$). LPS-stimulated levels of SIRT7 were not correlated with MELD score, ICAM1, or IL6. MELD score was inversely correlated with basal ICAM levels ($R = -0.305$, $P = 0.0224$), post-LPS IL-10 levels ($R = -0.413$, $P = 0.0009$), basal IL6 levels ($R = -0.473$, $P = 0.0002$), and basal TNFα levels ($R = -0.363$, $P = 0.0059$).

For the acute AH group, basal SIRT7 levels were correlated with basal CCL2 levels ($R = 0.534$, $P = 0.0105$), and LPS-stimulated SIRT7 level was correlated with LPS-stimulated CCL2 ($R = 0.689$, $P = 0.0004$). SIRT7 expression was not correlated with TNFα, IL6, ICAM1, IL10, MELD score, or MDF score. Neither MELD score nor MDF were correlated with basal or LPS-stimulated levels of SIRT7, ICAM1, TNFα, IL6, CCL2, or IL10 in the acute alcoholic hepatitis group.

Comparison of SIRT7 and Cytokine Expression in Patients at Varying Risk for AH

We hypothesized that SIRT7, as a mediator of inflammation, would have relatively higher expression in patients at high risk compared to patients at low risk for acute alcoholic

hepatitis. This hypothesis was based on the assumption that alcohol abstainers (healthy controls) and regular alcohol consumers without a history of liver disease (alcohol controls) would represent a groups of individuals at low risk of developing acute AH, patients with non-alcohol related cirrhosis would represent an intermediate risk group, and patients with alcoholic cirrhosis or a previous history of AH would represent the highest risk of developing acute AH. To test this hypothesis, we measured basal and LPS-stimulated levels of SIRT7 and inflammatory cytokines in monocytes from the following five patient groups: healthy controls, alcohol controls, cirrhotic controls (non-alcohol related cirrhosis), alcoholic cirrhosis, and patients with a history of cirrhosis. The findings from this experiment are summarized in Figure 5.

SIRT7 expression at baseline was significantly lower in the healthy control group than the alcohol control group ($P = 0.0012$), the cirrhotic control group ($P = 0.0468$), and the alcoholic cirrhosis group ($P = 0.0047$). The group with the highest hypothesized risk of developing acute AH (those with a previous history of AH) did not have significantly different SIRT7 mRNA levels than any of the other groups. None of the five groups had a significant increase in SIRT7 expression after treatment with LPS, and there was no significant difference in LPS-stimulated levels of SIRT7 mRNA among any of the five groups (Figure 5 A). Basal TNFα expression was low for all five groups and increased with LPS. After treatment with LPS, TNFα expression was similar for all five groups (Figure 5 B).

Basal IL-6 expression was low for all five groups and increased post-LPS treatment ($P < 0.01$ for all groups except healthy controls $P = 0.0625$). Following treatment with LPS both the cirrhotic control group ($P = 0.0178$) and the alcohol control group ($P = 0.0041$) had significantly higher IL-6 mRNA levels than the history of AH group. All other groups had similar levels of IL-6 (Figure 5 C). Basal ICAM1 expression was low in all five groups and increased

significantly in all groups ($P < 0.01$ for all) except the healthy control group ($P < 0.1250$). Post-LPS ICAM1 level was similar across all groups except for cirrhotic controls which were significantly higher than alcohol controls ($P = 0.0027$) (Figure 5 D).

Basal CCL2 expression was low and did not increase with LPS in any of the five groups ($P > 0.05$ for all). Post-LPS CCL2 expression was similar among the five groups ($P = 0.1113$) (Figure 5 E). Basal expression of the anti-inflammatory cytokine IL-10 was low in all groups and increased significantly following stimulation with LPS ($P < 0.05$ for all). Following treatment with LPS, the healthy control group had significantly lower IL-10 expression than the alcohol control group ($P = 0.0166$), the cirrhotic control group ($P = 0.0030$), the alcoholic cirrhosis group ($P = 0.0090$), and the history of AH group ($P = 0.0135$). Additionally, the history of AH group had significantly lower IL-10 expression than the cirrhotic control group ($P = 0.0118$) (Figure 5 F).

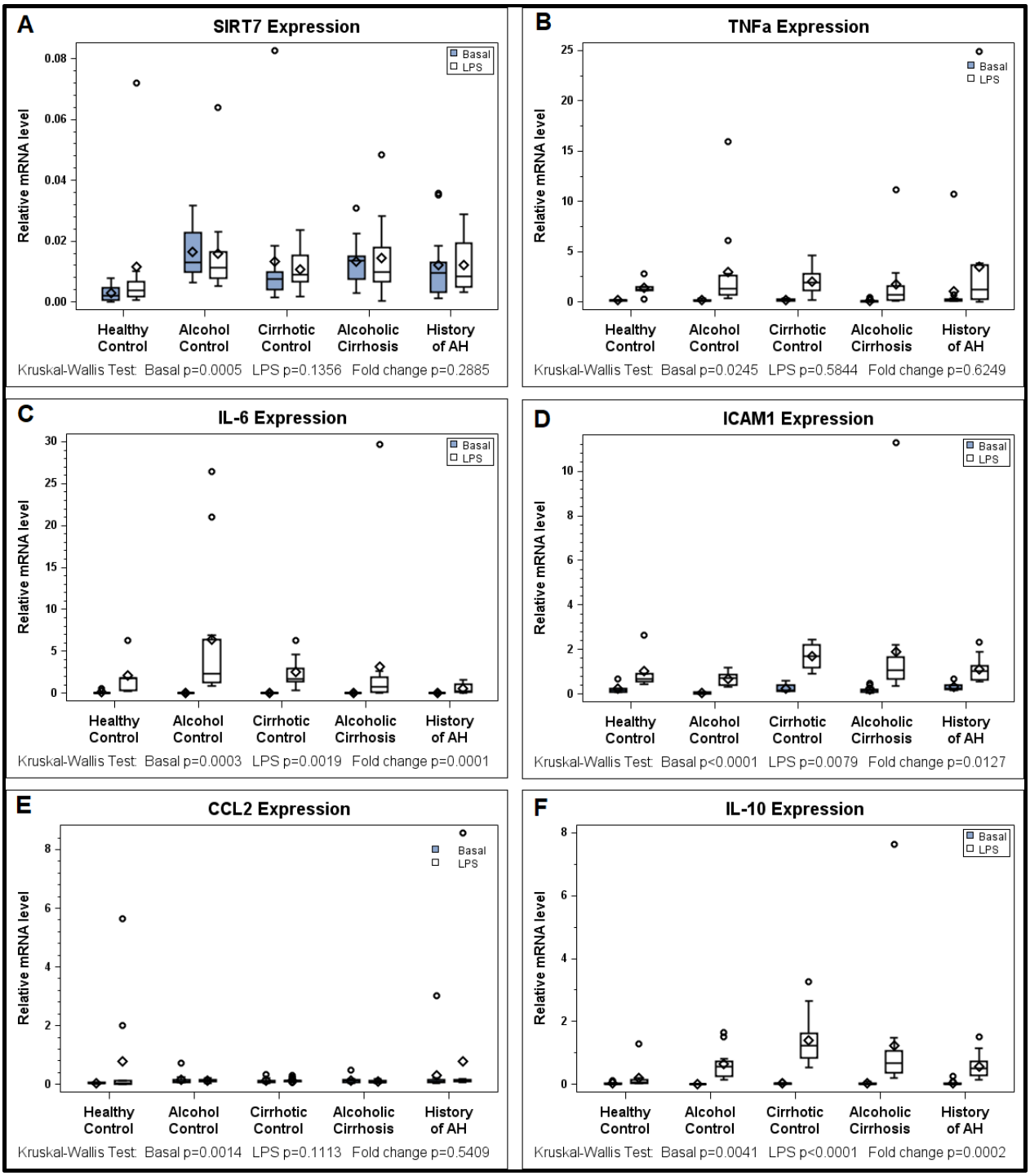

Figure 5. SIRT7 and Cytokine mRNA Expression in Patients at Varying Risk for Acute Alcoholic Hepatitis

Chapter 6: Discussion

This cross-sectional study of SIRT7 expression in peripheral blood monocytes from patients with acute alcoholic hepatitis and various control populations is the first known study of its kind. Based on previously published findings demonstrating the protective role of pFOXO3 in alcohol induced liver injury and the regulation of pFOXO3 by SIRT7, we hypothesized that SIRT7 would be elevated in patients with acute AH. We also hypothesized that through deacetylation of FOXO3, elevated SIRT7 would be associated with increased inflammatory cytokine production and therefore a more severe clinical phenotype. The results presented here are unanticipated and are not consistent with these hypotheses.

In our analysis of SIRT7 mRNA expression we found that basal and LPS-stimulated levels of SIRT7 mRNA in monocytes from patients with acute AH were not significantly different from either healthy controls or inflammatory controls (sepsis) and were about 3-fold lower than in the alcohol control group. This finding seems to suggest that SIRT7 is not a unique regulator of monocyte phenotype in alcoholic hepatitis. However, it may be the case that total mRNA levels do not reflect the SIRT7 protein levels or SIRT7 activity which may be controlled by other mechanisms. For example, SIRT7 expression is translationally regulated by various micro RNAs (including miR-125a-5p, miR-125b, miR-93, miR-3666, and miR-340) which have been shown to downregulate SIRT7 activity.[38–42] SIRT7 has also been shown to be post-translationally regulated through phosphorylation by cyclin-dependent kinase 1 and AMP-activated protein kinase as well as polyubiquitination. Immunoblots for SIRT7 are needed to confirm the relationship between SIRT7 mRNA and protein levels before we can draw definitive conclusions regarding relative SIRT7 levels in monocytes in alcoholic hepatitis.

We did not see increased TNFα, IL-6, IL-10, CCL2, or ICAM mRNA expression in AH patients. This finding is inconsistent with previous reports. McClain *et.al.* report elevated basal and LPS-stimulated levels of TNFα in monocytes from 16 AH patients compared to healthy controls.[28] Schafer *et.al.* report increased TNFα in LPS-stimulated monocytes from patients with alcoholic fatty liver, alcoholic cirrhosis, and alcoholic hepatitis compared to healthy controls; increased IL-6 production was only seen in monocytes from patients with alcoholic fatty liver.[29] Devalaraja *et.al.* report increased basal and LPS-stimulated CCL2 in monocytes from a cohort of 15 patients with alcoholic hepatitis compared to healthy controls.[30] Importantly, these three studies all measured cytokine levels in the cytosol and/or culture media rather than using cytokine mRNA levels as we have utilized in this study. As discussed above for SIRT7, mRNA levels at the time of cell collection may not reflect protein expression. Thus, it would be preferable to measure cytosolic and/or media cytokine levels in addition to mRNA levels. Furthermore, we utilized a 6-hour duration of LPS treatment which may not have been optimal for identifying changes in mRNA expression for each the various cytokines measured. Schafer *et.al.* and Hill *et.al.* utilized a 3-hour LPS treatment for stimulating TNFα and Devalaraja *et.al* utilized a 20-hour LPS incubation prior to measuring CCL2. Thus, differences in both the method of measuring cytokine expression and duration of LPS treatment may explain the discrepancy in our findings.

Our study has several limitations, including the limitation of using mRNA expression as an indicator of SIRT7 and cytokine levels, as discussed above. Additionally, the distribution of age and sex was not equivalent in each of our seven study groups which may confound the results.

Future Directions

The results presented here represent one component our proposed research plan. We plan to extend our analysis of cytokine production by using enzyme-linked immunosorbent assay (ELISA) to measure cytokine concentrations in monocyte culture medium. If this cytokine analysis reveals a difference in inflammatory phenotype between AH and control monocytes, we will evaluate whether the difference can be explained by SIRT7 or pFOXO3 protein levels.

A central component to our hypothesis is the role of FOXO3 in regulating inflammatory phenotype through transcriptional activation. The experiments presented here did not measure FOXO3. Rather, we evaluated the inflammatory phenotype of AH monocytes and the association with SIRT7, which we believed to be a key regulator of FOXO3 activity. In order to directly characterize the relative levels of pFOXO3 in alcoholic hepatitis, we are planning to conduct immunoblots from patients with acute AH. To date, we have been unsuccessful in conducting pFOXO3 immunoblots due to the low protein concentration obtained from cultured monocytes. As an alternative, if our attempts at pFOXO3 immunoblots remain unsuccessful we will use an ELISA to quantify levels of pFOXO3 in AH patients relative to controls. If pFOXO3 is decreased in AH as we hypothesize, we will be able to determine if the decrease in pFOXO3 is related to increased SIRT7 (as assessed by immunoblot). If SIRT7 does not prove to be a primary regulator of pFOXO3, other members of the sirtuin family such as SIRT1 could be evaluated. If pFOXO3 levels in AH monocytes are found to be equivalent to controls, we could then utilize PCR arrays to identify new targets for further investigation.

The relationship between circulating monocytes and liver macrophages in the pathogenesis of AH also deserves further attention. Mouse models have confirmed that circulating monocytes infiltrate the liver and differentiate into hepatic macrophages in response

to acute alcohol ingestion.[20,22] Furthermore, Li *et.al.* have shown that pFOXO3 promotes hepatic macrophage apoptosis and differentiation of infiltrating macrophages to an anti-inflammatory M2 phenotype.[22] It remains unknown whether the phenotype of circulating monocytes (and their response to LPS) is reflective of the phenotype of liver macrophages. As such, our findings of no increased cytokine or SIRT7 mRNA expression in AH monocytes may not reflect the phenotype assumed by these cells upon liver infiltration and differentiation to tissue macrophages which is likely influenced by the hepatic cytokine environment. This is not easily studied in humans due to practical issues with obtaining liver tissue samples from acute AH patients.

One finding that is of interest going forward is the relative difference in cytokine expression between healthy controls and alcohol controls. Prior to this study, we hypothesized that there would be no difference in cytokine expression between these two groups. However, in the alcohol control group we observed increased expression of SIRT7 as well as increased production of IL-6 and IL-10 in response to LPS compared to healthy controls. IL-10 is an anti-inflammatory cytokine with inhibitory effects on myeloid cells. Thus, its increased expression in healthy drinkers (alcohol control group) as compared to healthy non-drinkers (healthy control group) fits the growing hypothesis that alcohol consumption is well tolerated in the majority of individuals due to an adaptive, anti-inflammatory immune process. This hypothesis is illustrated by Li *et.al.* who demonstrated that FOXO3-dependent macrophage apoptosis leads to differentiation of infiltrating macrophages to an anti-inflammatory alcohol-tolerant phenotype in alcohol fed mice.[22] The observed differences in circulating monocyte cytokine expression and how these relate to FOXO3 expression should be confirmed in a larger sample of healthy alcohol drinkers and abstainers.

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Appendix A: ICD-9/10 Inclusion & Exclusion Criteria for Retrospective Study

Inclusion Criteria: one or more of the following diagnoses

1) Alcoholic Hepatitis

571.1 Acute alcoholic hepatitis

K70.1 Alcoholic hepatitis

2) Other Alcohol Related Liver Disease

571.0 Alcoholic fatty liver

571.2 Alcoholic cirrhosis of liver

571.3 Alcoholic liver damage, unspecified

572.8 Alcoholic liver failure

572.8 Hepatic failure due to alcoholism

K70.2 Alcoholic fibrosis and sclerosis of liver

K70.3 Alcoholic cirrhosis of liver

K70.4 Alcoholic hepatic failure

K70.9 Alcoholic liver disease, unspecified

Exclusion Criteria

1) Infection

038 Septicemia

481 Pneumococcal pneumonia

482 Other bacterial pneumonia

483 Pneumonia due to other specified organism

484 Pneumonia in infectious diseases classified elsewhere

485 Bronchopneumonia, organism unspecified

486 Pneumonia, organism unspecified

567.23 Spontaneous bacterial peritonitis

590 Infections of kidney

599.0 Urinary tract infection, site not specified

A40 Streptococcal sepsis

A41 Other sepsis

J13 Pneumonia due to Streptococcus pneumoniae

J14 Pneumonia due to Hemophilus influenzae

J15 Bacterial pneumonia, not elsewhere classified

J16 Pneumonia due to other infectious organisms, not elsewhere classified

J17 Pneumonia in diseases classified elsewhere

J18 Pneumonia, unspecified organism

K65.2 Spontaneous bacterial peritonitis

N39.0 Urinary tract infection, site not specified

2) Immune Deficiency

279 Disorders involving the immune mechanism

B20-B20 Human immunodeficiency virus

D80-D89 Certain disorders involving the immune mechanism

3) Hematologic Malignancy

200-208.99 Malignant neoplasm of lymphatic and hematopoietic tissue

C81-C96 Malignant neoplasms of lymphoid, hematopoietic and related tissue

<u>Covariates:</u>

070.2 Viral hepatitis B with hepatic coma

070.3 Viral hepatitis B without mention of hepatic coma

070.7 Unspecified viral hepatitis C

B18 Chronic viral hepatitis

Appendix B: RT-PCR Primer Sequences

SIRT7 forward: 5’-GACCTGGTAACGGAGCTGC-3’

SIRT7 reverse: 5’-CGACCAAGTATTTGGCGTTCC-3’

TNFα forward: 5’-CAGCAGGCACAGGCTCC-3’

TNFα reverse: 5’-CCTGCAACAAGATGATCGTGA-3’

IL-6 forward: 5’-TGAGGAGACTTGCCTGGTGA-3’

IL-6 reverse: 5’-CACAGCTCTGGCTTGTTCCT-3’

IL-10 forward: 5’-TCACATGCGCCTTGATGTCT-3’

IL-10 reverse: 5’-TGCCAAGCCTTGTCTGACAT-3’

CCL2 forward: 5’-GGTGAGACCTGCCTGAATG-3’

CCL2 reverse: 5’-GTTGGGGTCCTGGCATC-3’

ICAM1 forward: 5’-CCATGGTACCTGCACACCTA-3’

ICAM1 reverse: 5’-TGTCTTGAGTCTTGCTCCTTCC-3’

GAPDH forward: 5’-GAAGGTGAAGGTCGGAGTC-3’

GAPDH reverse: 5’-GAAGATGGTGATGGGATTTC-3’