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HISTONE DEACETYLASES IN ALCOHOL ASSOCIATED LIVER DISEASE

By

Srineil Bodduluri

B.S. Biology, A.B. History, Duke University, 2012

A Thesis

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DEDICATION

This thesis is dedicated to Drs. Haribabu and Sobha Bodduluri for their unwavering love and support during my educational journey. I am forever grateful for the wonderful opportunities they have offered me and am extremely proud of the example they have set both as scientists and as parents.

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ABSTRACT

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Srineil Bodduluri

July 27, 2021

Alcohol abuse is endemic worldwide and there is no FDA approved treatment for Alcohol-Associated Liver Disease (ALD). Many scholars have posited that targeting Histone Deacetylases (HDAC) may be therapeutic in ALD intervention. In this study, the changes in hepatic gene expression and immune phenotyping of mice undergoing chronic plus binge alcohol exposure was examined. The characterization of relative mRNA levels for all 18 HDAC were performed. Results showed decreased expression of the genes HDAC 1,7,9,10,11 and Sirtuin enzymes (SIRT) 3,4,5,7. Other pathways related to lipid metabolism as well as systemic inflammation and hepatic injury also exhibited significant changes. These altered pathways were normalized with the administration of the HDAC inhibitor Trichostatin A (TSA) in primary mouse hepatocytes. These results suggest the potential importance of HDAC dysregulation in ALD mouse models. Future studies should investigate the interactions between HDAC and the molecular mechanisms that control the gut-liver-brain axis.

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CHAPTER I

INTRODUCTION

Roles for Histone Deacetylases in Alcohol Associated Liver Disease

Ethanol (alcohol) consumption by American adults is widespread and is a major public health concern. The term alcohol use disorder (AUD) refers to the uncontrolled compulsion and preoccupation with alcohol consumption. Ethanol exposure has many negative health outcomes such as Alcohol-Associated Liver Disease (ALD), which has no FDA approved treatment. Clinical presentation of ALD is manifested by a wide spectrum of liver pathologies including steatosis, hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Although prevention through alcohol abstinence is the most effective form of intervention for ALD, it is too often not a realistic clinical pursuit (1). For many of those who reach abstinence, alcohol relapse can range between 22 and 86%, depending on the number of risk factors for relapse present in the subjects life (2).

For the foreseeable future, discovering therapeutic interventions for ALD will undoubtedly be a pressing clinical concern worldwide. Recent findings have documented the promising role of histone deacetylases (HDAC) as therapeutic prospects in ALD. This introduction compiles insights about the role of HDAC and their activity under different conceptualizations of alcohol research. More specifically, the review will highlight the effects of changes in HDAC expression and function on different pathologies in the ALD spectrum.

Histones are proteins found in the nucleus of cells that function to tightly wind and bind DNA into structures called nucleosomes. Like beads on a string of DNA, these nucleosomes serve to compact DNA into tightly bound chromatin. Each nucleosome is comprised of 4 distinct histone proteins (H2A, H2B, H3, H4) made up of a complex of two H2A-H2B dimers combined with a H3-H4 tetramer to form a histone octamer. Histone deacetylases (HDAC) are a family of proteins that remove an acetyl group from histone proteins allowing the DNA to be wrapped more tightly. This acetylation is typically done on an ϵ -amino Lysine (K) residue of the histone. Typically, a histone acetylation event makes DNA more permissive to transcription by the enzyme RNA polymerase whereas a deacetylation event tends to silence gene expression. Multiple studies have identified how the alteration in expression levels of genes involved in ALD etiology or progression are influenced by the acetylation status of the candidate gene's promoter region. HDAC can also deacetylate non-histone, cytosolic, mitochondrial or nuclear proteins on a Lysine residue and for this reason they have also been referred to as lysine deacetylases (KDAC).

HDAC enzymes consist of 18 distinct proteins and are classified into four classes (Class I, Class II, Class III, and Class IV) . This classification is based on their sequence similarity to proteins found in yeast with each HDAC being numbered according to its chronological order of discovery. Class I HDAC are made up of HDAC1, HDAC2, HDAC3, and HDAC8 and have high similarity with the yeast Rpd3 proteins. Class II HDAC (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10) have sequence similarity to the yeast Hda1 protein. The Class III HDAC family consists of enzymes called Sirtuins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, SIRT7) which are homologous to the yeast Sir2 protein. Sirtuins are protein deacetylases that use NAD⁺ as

a cofactor and are inhibited by nicotinamide, a byproduct of Sirtuin activity. HDAC11 is the sole member of Class IV HDAC and has sequence similarities to both Class I and Class II HDAC.

Alcohol Associated Liver Disease shares many characteristics of related non-alcoholic liver pathologies such as non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD). Notably, all three conditions are marked by the initial accumulation of fat droplets inside hepatocytes, a condition known as steatosis. In most liver diseases, and specifically in ALD, the balance of fatty acid metabolism is altered to promote a more lipogenic phenotype as opposed to a more catabolic, or fatty acid β -oxidative one. As liver injury progresses, clinical manifestations of alcoholic hepatitis can be observed. This is characterized by the appearance of Mallory-Denk bodies and hepatic inflammation which is evidenced by the infiltration of neutrophils along with other peripheral immune cells into the liver. After alcoholic hepatitis, the liver may experience fibrosis, a condition marked by excessive collagen deposition on hepatic tissue.

Further along the continuum, the liver advances to become scarred and necrotic in a condition known as cirrhosis of the liver. As cirrhosis becomes worse, it is marked by the arrival of the final pathology in the ALD spectrum, hepatocellular carcinoma (HCC). Much research has been completed on the effect of HDAC in HCC. These studies indicate an important role for HDAC in cellular mechanisms such as survival and replication which are important in the context of cancer. Since HCC is a terminal disease, many experimental therapies such as HDAC inhibitors are routinely tested for this condition. Some of these have shown therapeutic potential. The subsequent sections of this introduction will attempt

to highlight the role of HDAC in each of the four distinct stages of ALD, considering the most recent as well as relevant research being performed in the field.

Hepatic Steatosis

Hepatic steatosis, also called fatty liver, is characterized by the abnormal accumulation of fat and lipids inside hepatocytes. Multiple studies have expounded on the mechanism by which hepatic steatosis occurs. Researchers have proposed therapeutic interventions for fatty liver by specifically modifying genes and proteins related to fatty acid metabolism. There is also an existing body of evidence on how these genes are being modulated by HDAC and Sirtuin proteins to influence intracellular metabolic cascades. For successful reversal of nascent ALD, the paradigm states that the traditionally pro-lipogenic phenotype of ethanol exposed hepatocytes must be attenuated while the catabolic, fatty acid β -oxidative phenotype must be promoted. Some investigators have proposed mechanisms by which HDAC and HDAC inhibition can modulate this lipogenic/ β -oxidative axis with regards to ALD, a few of which will be reviewed below.

One avenue of interest for HDAC intervention in ethanol effects on liver pathology is the alteration in gene expression of the carnitine palmitoyltransferase-1 alpha gene (CPT-1 α). CPT-1 α plays a critical role in the metabolism of fatty acids by promoting β -oxidation within the mitochondria. Specifically, CPT-1 α shuttles long chain fatty acids such as palmitic acid bound to carnitine into the mitochondria where they undergo oxidation and metabolism for the production of energy. Ethanol exposure dramatically reduces hepatic levels of CPT-1 α thus promoting fatty acid accumulation due to impaired catabolism. The increased intracellular free fatty acid levels veer the hepatocytes toward a more steatotic phenotype. One study has documented how the deacetylation of the H3K9

(Histone 3 Lysine 9) residue at the CPT-1 α promoter region caused by HDAC3 binding led to decreased CPT-1 α expression (3). The subsequent blockade of HDAC3 by the Class I and Class II HDAC inhibitor Trichostatin A (TSA) led to a recovery of CPT-1 α expression. Another study found that a selective HDAC2 inhibitor, suberanilohydroxamic acid (SAHA) or vorinostat, can suppress lipogenesis and promote fatty acid β -oxidation by increasing levels of CPT-1 α and decreasing levels of Diacylglycerol O-acyltransferase 2 (DGAT2) (4). In a separate study, it was determined that the repression of the CPT-1 α promoter was mediated by HDAC and that CPT-1 α levels can be rescued in ethanol feeding conditions with the administration of the HDAC inhibitor tributyrin (5). Tributyrin is a triglyceride naturally found in butter that is made up of three butyrate moieties linked via a glycerol backbone. It functions to retain acetylation of the CPT-1 α promoter by inhibiting HDAC1. In addition to tributyrin, a grape seed procyanidin extract not only increases acetylation at the CPT-1 α promoter through HDAC inhibition, but also increased the binding of the transcription factor peroxisome proliferator-activated receptor alpha (PPAR- α) to the CPT-1 α promoter (6). The evidence clearly implicates that the expression levels of the crucial fatty acid metabolism gene CPT-1 α is influenced by HDAC inhibition under conditions of ethanol exposure.

Effects of Sirtuins have also been explored in the context of hepatic steatosis. Numerous studies have described the importance of Sirtuins in promoting fatty acid metabolism and preventing the accumulation of lipids in hepatocytes. Sirtuins are the primary deacetylase for mitochondrial proteins. The most widely studied Sirtuin in the context of hepatic steatosis is SIRT1, which has been implicated in the prevention of fatty liver in both ALD and NAFLD. One study described how SIRT1 promotes β -oxidation by

deacetylating the nuclear receptor and transcription factor PPAR α and transcriptional activator PGC1. Resveratrol, a compound found in berries and grapes, also works to promote β -oxidation of fatty acids by activating SIRT1 (7). Other nutraceuticals have also been shown to ameliorate hepatic steatosis specifically by acting on SIRT1. One study illustrated how Omega-3 Fatty Acids like Docosahexaenoic Acid (DHA) can reduce hepatic lipid accumulation by rescuing depleted SIRT1 levels in mice fed a high-fat diet (HFD) and in HepG2 cells treated with palmitic acid (8). This protective effect of DHA was reversed by SIRT1 inhibitor or adenoviral knockdown of SIRT1 expression. A separate study found that allyl isothiocyanate, a compound found in mustard seeds, reversed HFD induced weight gain and hepatic lipid accumulation in mice by upregulating SIRT1 and 5' AMP activated protein kinase (AMPK), a protein which activates fatty acid oxidation.(9) This effect was also abolished with SIRT1 knockdown.

The mitochondrial Sirtuin SIRT3 has also been implicated to contribute to ethanol and non-ethanol induced hepatic steatosis in multiple ways. One study found that SIRT3 enhanced macroautophagy and chaperon mediated autophagy (CMA) in lipid droplets by activating AMPK. It also determined that SIRT3 deacetylates long-chain acyl-CoA dehydrogenase (LCAD) to promote fatty acid β -oxidation (10). SIRT3 was also demonstrated to be decreased in bovine hepatocytes with fatty liver indicating its role in mammalian steatosis prevention. When SIRT3 was overexpressed, there was a marked increase in genes related to fatty acid β -oxidation such as CPT-1 α (11). SIRT3 is well established as a main mitochondrial deacetylase. In a screen of the liver mitochondrial acetylome, SIRT3 ablation was shown to increase protein hyperacetylation (12). Many of these proteins, when deacetylated by SIRT3, serve to prevent the abnormal accumulation

of lipids in hepatocytes which leads to fatty liver (13). Both HDAC and SIRT function to influence hepatic steatosis mainly by modulating the cellular balance between fatty acid β -oxidation and de-novo lipogenesis. Mechanistically, histone deacetylases have been shown to function at the promoter regions of relevant genes influencing promoter accessibility and transcriptional permissivity. They also deacetylate other targets altering target protein stability and function.

Alcoholic Hepatitis and Liver Fibrosis

Alcoholic hepatitis (AH) is a condition predominantly marked by visible signs of steatosis along with high levels of hepatic inflammation which may or may not be accompanied by systemic inflammation. While the condition is reversible, it paves the way for more permanent and deleterious pathologies associated with ALD. For example, if ethanol exposure is unabated resulting in prolonged and persistent inflammation, hepatic tissue pathology may progress to fibrosis followed by the necrotic and cancerous states of cirrhosis and HCC, respectively. Modeling AH in rodents becomes problematic as protocols for consistent replication of the desired phenotype are not always successful. Hepatic steatosis is easier to induce using established paradigms of feeding and treatment. Even terminal stages of ALD, such as HCC can be modeled using immortalized cell lines, xenografts, and patient biopsies, but transitional stages of ALD such as hepatitis, fibrosis, and cirrhosis remain challenging to study. For this reason, the existing body of evidence on the role of HDAC in the context of transitional stages of ALD remains sparse. However, recent literature has investigated the role of HDAC proteins in the context of AH.

SIRT6 has emerged as a key player in hepatic inflammation in multiple studies with one study highlighting the role of SIRT6 in preventing severe liver injury. This particular study found that mice that overexpressed SIRT6 were protected from ALD as shown by normalization of hepatic lipids, inflammatory response proteins, and oxidative stress markers (14). SIRT6 was also found to increase transcription of the anti-oxidative stress gene MT1, by binding to the MT1 promoter region. Another study demonstrated that high fat diet (HFD) fed SIRT6 KO mice had increased steatohepatitis and inflammation mediated through the upregulation of transcription regulator protein Bach1 and downregulation of antioxidant promoting Nuclear factor erythroid 2-related factor 2 (Nrf2) (15). SIRT6 overexpression corroborated these findings by ameliorating high fat high fructose induced steatosis, inflammation, and fibrosis. Natural compounds can play a role in alleviating hepatitis like symptoms through the modulation of HDAC function. Piceatannol, a metabolite of resveratrol, was shown to increase the amount of SIRT1, 3, and 6 in the liver of C57/BL6 mice and protect them from HFD induced hepatic inflammation (16).

Interfering small nucleotide molecules are another form of epigenetic regulation that shows promising potential for future research. One study described how micro RNAs such as miR-34a can target, silence, and deplete SIRT 1 protein levels (17). Low SIRT1 levels have been attributed to steatosis, inflammation, and fibrosis. This study also illustrates how micro-RNAs, which are long-lived and stable, may be effective epigenetic biomarkers for non-invasively diagnosing ALD via clinical laboratory tests.

HDAC interactions in the gut must also be considered when studying deleterious liver pathologies. One study demonstrated how intestinal specific conditional SIRT1

knockout mice showed protection from ethanol induced liver injury by mitigating hepatic ferroptosis (18). HDAC inhibitors have also been shown to be effective against inflammation and oxidative stress associated with hepatitis. The HDAC inhibitor SAHA (vorinostat) was shown to induce apoptosis in HepG2 cells by enhancing acetylation of histone H4 at the promoter regions of ER-stress associated genes. The subsequent increase in the ER stress pathway is mainly caused by an increase in transcriptional permissiveness of genes like GRP78, ATF4, and CHOP (19).

Hepatic fibrosis, a condition marked by the formation of an abnormally large amount of scar tissue in the liver, is an attempt at wound healing by hepatic tissue. Hepatic stellate cells (HSCs) are the primary cell type in the liver responsible for secreting the extracellular matrix proteins that drive this condition. Mild forms of liver fibrosis are reversible but severe scarring may lead to more severe irreversible pathologies such as cirrhosis. Some recent attempts have documented roles for HDAC in hepatic fibrosis however the field remains underexplored. The novel HDAC inhibitor N-hydroxy-7-(2-naphthylthio) heptanamide (HNHA) reduced activation of HSC in rats with bile duct ligation induced hepatic fibrosis (20). One study described how SIRT6 deacetylates and deactivates the intracellular messenger SMAD3 causing a decrease in TGF- β induced fibrosis. SIRT6 protected hepatocytes from NASH with both liver and HSC conditional SIRT6 knockout mice developing worse NASH while transgenic SIRT6 remained protected (21).

Evidence shows that both AH and hepatic fibrosis are conditions that are influenced by HDAC and could benefit from the development of HDAC related interventions and therapies. Future studies must expand this area of research and bridge the gap between

nascent and terminal stages of ALD to establish a more lucid image of the role of HDAC in the ALD spectrum.

Cirrhosis and Hepatocellular Carcinoma

There is a plethora of literature about the use of HDAC inhibitors and other forms of HDAC modulation in the context of more severe liver diseases such as cirrhosis and hepatocellular carcinoma. While the majority of cirrhosis cases are attributed to excessive ethanol consumption, HCC typically originates from hepatitis and hepatitis like infections with alcohol playing a key but secondary role in oncogenesis. Both cirrhosis and HCC are irreversible and are undoubtedly tragic consequences of chronic alcohol abuse. It is widely accepted that levels of HDAC are elevated in HCC inflicted tissues. The current body of evidence asserts that epigenetic alterations, including elevated HDAC levels, are primary causes of HCC as they lead to dysregulated expression of genes involved in the cell cycle and replication (22). Within the livers of individual HCC patients, the levels of HDAC expression and function are markedly increased in cancerous vs. para-cancerous tissue. Naturally, HDAC inhibition would offer an ideal therapeutic target for such patients, but the lack of selectivity of HDAC inhibitor actions poses substantial difficulty in the design and implementation of such therapies. Most pharmacological attenuation with HDAC inhibitors is not specific to one specific HDAC protein or even a specific class of HDAC. The countless combinations of modulating 18 distinct HDAC enzymes for an effective treatment strategy highlights the need for more work in the field. An expanded investigation will shed light on the complex interplay between these proteins and their synthetic modulation in the context of ALD.

Cirrhosis and HCC are difficult to induce using conventional rodent models of ethanol feeding. The fact that these conditions are largely irreversible also makes them more problematic to treat. The subsequently reviewed papers were not always conducted in the ALD context yet were still included to show the role of HDAC in terminal liver diseases. These diseases share many of the clinical characteristics of severe ALD patients encountered in the clinic and although their etiologies may differ, mechanisms remain similar.

One recent study showed the potential of the HDAC inhibitor mocetinostat (MGCD 0103) to exert an anti-proliferative effect in either Huh7 and HepG2 cell lines as well as mice xenografted with HepG2 cells (23). Mocetinostat strongly inhibits HDAC1 activity, but it also affects HDAC2, HDAC3, and HDAC11. The study showed that this therapy worked by promoting apoptosis and autophagy by arresting the cell cycle and working to increase acetylated residues on H3 and H4. One study found that HDAC3, a protein associated with liver regeneration and repair, was highly upregulated in the cancerous tissues of patients with HCC. The authors found that HDAC3 associates with the transcription factor STAT-3 and works to prevent the deacetylation and transition of acetyl-STAT-3 to activated phospho-STAT-3 (24). It also showed that cancer growth and proliferation was found to be attenuated with the introduction of the FDA approved pan-HDAC inhibitor Panobinostat in tumor cell xenografted mice.

Some proteins are able to directly modulate the levels of HDAC proteins in the cell. The knockdown of the Homeobox A10 protein (HOXA10) suppressed transcription of HDAC1, which is a potential deacetylase for p53 (25). The ability of HDAC to deacetylate and deactivate common tumor suppressor genes such as p53 highlight their importance in

the progression of HCC. Long non-coding RNAs must also be considered when investigating therapeutic possibilities in HCC. A recent study detailed how lncRNA SNHG15 was associated with worse clinical outcomes for HCC. Specifically, SNHG15 acts as a molecular sponge to the HDAC2 inhibiting miR-490 thereby increasing levels of HDAC2 and subsequently aggravating HCC (26).

The potential of HDAC inhibitors to synergize existing modes of cancer chemotherapy is well established. One study has articulated how β -hydroxybutyrate, an inhibitor of HDAC 1, 3, and 4 can augment cisplatin induced apoptosis by inducing cleavage of Caspase -3 and -8 in HCC cells (27). Another endeavor described the role of the Class I HDAC inhibitor resminostat in increasing the efficacy of chemotherapeutics like doxorubicin and the HSP90 inhibitor 17-AAG (28). Curcumin, a natural anti-cancer compound isolated from turmeric was shown to abolish cancer stem cells in the liver. This effect was increased with the HDAC inhibitor TSA and worked by reducing inflammatory NF- κ B signaling (29). Entinostat, an HDAC inhibitor which blocks HDAC1 and HDAC3, showed its ability to combine with a Sonic Hedgehog inhibitor to reduce cell growth and promote apoptosis (30). This study proved successful in HepG2 cells as well as cultured tumor explants. Another study detailed how both SAHA and TSA reduced the proliferation, clonogenicity, and migratory potential of HCC cells. These treatments even enhanced the efficacy of the traditional chemotherapeutic sorafenib (31). In a contrary report, sorafenib was evidenced to be toxic when used in combination with SAHA (vorinostat) alone in patients with unresectable HCC and was not recommended as a combination cocktail for future therapy (32). However, Sorafenib was shown to work synergistically with the Class I and Class II HDAC inhibitor quisinostat to impede HCC

growth (33). A different study found that sorafenib induced apoptosis in HCC cell lines is reduced with SIRT1 overexpression (34). All aforementioned studies illustrate the strong variation in the clinical and laboratory responses of HDAC proteins and HDAC inhibitors in the context of Hepatocellular Carcinoma. These findings clearly highlight the need for more attempts to understand the mechanistic modalities by which HCC arises and the ways that it can be ameliorated using HDAC.

Summary

HDAC activity in the context of ALD remains an exciting yet understudied area of research. Numerous groups have outlined how HDAC influence the various stages and pathologies of the ALD spectrum yet very few practical pharmacological therapies have been devised. The targeting of genes that influence intracellular fatty acid metabolism, specifically the fatty acid β -oxidation/lipogenesis axis has been highlighted in this literature review. HDAC have clearly demonstrated the ability to modulate gene expression through direct histone acetylation/deacetylation, especially at promoter regions of target genes. They have also been shown to post-translationally affect the activities of cytosolic, nuclear, and mitochondrial proteins through their lysine deacetylase activities. The complex molecular circuits that drive inflammation and the role that HDAC play in initiating the inflammatory milieu characteristic of AH remains undefined. A relatively copious amount of research exists on the role of HDAC and HDAC inhibition in the context of HCC, yet any existing FDA approved HDAC inhibitor is typically administered in the clinic with a more conventional chemotherapeutic.

The presence of 18 unique HDAC enzymes in the human genome illustrates the rigor and complexity needed to fully understand the mechanistic intricacies of these

proteins in human disease. Additionally, these enzymes are subject to alternative splicing and are known to exist as multiple isoforms. Aside from varying in their expression levels depending on the tissue, each HDAC also has distinct cellular targets for deacetylation. They must be studied in the context of other intracellular pathways and pharmacological therapies for maximum benefit to the discipline. In addition to identifying cellular targets for deacetylation, researchers must also note the functionality of the HDAC proteins. For various reasons, changes in expression levels may not always translate to changes in cellular function. Overall, HDAC proteins are promising targets for further research and potential drug development. Subsequent studies must consider the years of progress in the field and determine how to best direct future research in ways that will be novel, therapeutic, and clinically translatable.

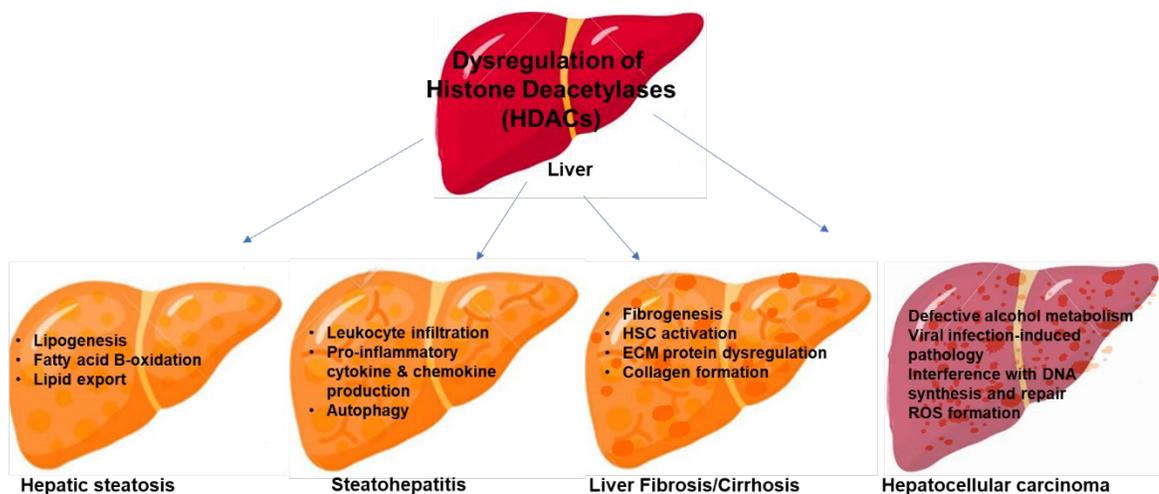


Figure 1. Mechanisms Involved in the Development of Alcohol Associated Liver Disease. Pictorial representation of various HDAC dysregulation induced mechanisms involved in the development of different pathologies of the ALD spectrum.

HDAC Inhibitors/Activators	HDAC Targets	Function	Mechanism of Action in Liver Pathology
Trichostatin A (TSA)	HDAC3	Inhibitor	Rescues Ethanol induced loss of CPT-1a expression (3)
	Pan-HDAC	Inhibitor	Increases Curcumin induced Cancer cell death as well as reduces NF-kB signalling (29)
SAHA (Vorinostat)	HDAC2	Inhibitor	Increases CPT-1a expression and Suppresses DGAT2 (4)
	Pan-HDAC	Inhibitor	Increases Acetylation of H4 leading to apoptosis and expression of ER Stress genes (19)
Tributyryn	HDAC1	Inhibitor	Rescues Ethanol induced loss of CPT-1a expression (5)
B-hydroxybutyrate	HDAC 1,3,4	Inhibitor	Augments Cisplatin Induced Apoptosis by inducing cleavage of Caspases (27)
HNHA	Pan-HDAC	Inhibitor	Reduces activation of HSCs in Fibrogenic Rat Model (20)
Grape Seed Procyanadin		Inhibitor	Increases PPARa binding to CPT-1a promoter and CPT-1a expression (6)
Panobinostat		Inhibitor	Attenuates cancer growth and proliferation in xenografted mice (24)
Resveratrol	SIRT1	Activator	Promotes Fatty Acid B-oxidation (7)
Docosahexanoic Acid (DHA)		Upregulates	Reduces Hepatic Lipid Accumulation (8)
Allyl Isothiocyanate			Reversed HFD induced Weight Gain and Hepatic Lipid Accumulation (9)
miR-34a		Silence/Deplete	Promotes Steatosis, Inflammation, Fibrosis (17)
Piceatannol	SIRT1,3,6	Upregulates	Protects from HFD induced inflammation (16)
Mocetinostat	Class I HDACs	Inhibitor	Exerts anti-proliferative effect on HCC cell lines and xenografts (23)
Resminostat		Inhibitor	Increases efficacy of doxorubicin and the HSP90 inhibitor 17-AAG (28)
SNGH15 (lncRNA)	HDAC2	Increases	Sponges HDAC2 inhibiting miR-490 to aggravate HCC (26)
Entinostat	HDAC 1,3	Inhibitor	Combines with Sonic Hedgehog inhibitor to reduce cell growth and promote apoptosis (30)
Quisinostat	Class I and II HDACs	Inhibitor	Works synergistically with Sorafenib to impede HCC growth (33)

Table 1. Roles for HDAC Inhibitors/Activators in Alcoholic Associated Liver Disease. Tabular representation of HDAC Inhibitors and Activators along with their respective Targets, Functions, and Mechanisms of Action as it relates to Liver Pathologies Associated with ALD

CLASS	HDAC	Cellular Location	Hepatic Targets
Class 1	HDAC 1	Nucleus	CPT-1 promoter (5)
			p53 (25)
	HDAC-2	Nucleus	Promotes Lipogenesis (4)
			Promotes and Aggravates HCC (23)(26)
	HDAC-3	Nucleus and Cytoplasm	CPT-1 Promoter (3)
			Promotes HCC (23)
STAT-3 - prevents STAT-3 activation (24)			
HDAC-8	Nucleus and Cytoplasm	Promotes HCC growth and survival (30)	
Class IIa	HDAC-4	Nucleus and Cytoplasm	
	HDAC-5	Nucleus and Cytoplasm	
	HDAC-7	Nucleus and Cytoplasm	
	HDAC-9	Nucleus and Cytoplasm	
Class IIb	HDAC-6	Mostly in Cytoplasm	
	HDAC-10	Nucleus and Cytoplasm	
Class III	SIRT-1	Nucleus and Cytoplasm	PPAR α and PGC1 to promote Fatty acid B-oxidation (7)
			Reduces Hepatic Lipid Accumulation (8)(9)
			Protects from HFD induced inflammation (16)
			Intestinal SIRT1 deletion mitigates hepatic ferroptosis (18)
	SIRT-2	Nucleus and Cytoplasm	
	SIRT-3	Mitochondria	AMPK - enhances Chaperon Mediated Autophagy and Macroautophagy (10)
			LCAD - promotes Fatty acid B-oxidation (10)
			Overexpression leads to increase in B-oxidation genes like CPT-1a (11)
	SIRT-4	Mitochondria	
	SIRT-5	Mitochondria	
	SIRT-6	Nucleus	Normalizes Hepatic Lipids and Inflammatory Response (14)
Increases transcription of MT1/ Alleviates Oxidative Stress (14)			
Upregulates NRF2 / suppresses oxidative stress (15)			
Downregulates Bach1/ reduces steatohepatitis and inflammation (15)			
Deactivates SMAD-3 / decreases TGF-B induced fibrosis and protects from NASH (21)			
SIRT-7	Nucleolus		
Class IV	HDAC-11	Nucleus and Cytoplasm	Promotes HCC (23)

Table 2. Hepatic Targets of Histone Deacetylases. Tabular representation of Histone Deacetylases along with their classification, cellular location, as well as hepatic targets which influence pathologies associated with Alcoholic Associated Liver Disease.

Knowledge Gaps

Work done by others and our research group has begun to identify dysregulation of HDAC as significant components of ALD pathogenesis as well as potential therapeutic targets for the treatment and prevention of ALD. However, research into the development of HDAC directed therapies requires a detailed understanding of alcohol-mediated pathogenic changes in HDAC expression and function. Presently, the animal models investigating alcohol-mediated pathogenic changes in the liver primarily examine the effects of both chronic and acute (binge) alcohol exposure (35). It is noteworthy that potentially distinct mechanisms underlie binge vs. chronic alcohol exposure mediated pathogenic changes in the liver. Our group has identified significant changes in Class I, II, III, and IV HDACs that occur in response to binge alcohol drinking (36). Moreover, the data obtained also demonstrated that in the binge alcohol drinking model, HDAC could be effectively targeted to mitigate the development of liver disease. However, the impact of chronic ethanol drinking on hepatic HDAC expression and function remains largely undetermined.

Hypothesis and Specific Aims

The current research examines the hypothesis that chronic alcohol-induced alterations in hepatic HDAC as well as histone modifications constitute critical epigenetic mechanisms driving liver disease. To address the proposed hypothesis, the following specific aims were examined:

Specific Aim 1: Determine the effect of chronic ethanol feeding on the expression of different classes of hepatic histone deacetylases (HDACs) and development of liver disease employing the chronic plus binge alcohol feeding mouse model.

Specific Aim 2: Determine the contribution of HDACs in mediating ethanol-induced changes in gene expression, relevant for hepatic ER-stress, metabolism, inflammation, and injury using primary mouse hepatocytes.

Overall, this work aims to perform a comprehensive characterization of all 18 HDAC proteins as well as other important markers of metabolism, inflammation, and injury which will guide future investigations about molecular mechanisms responsible for ALD-like pathologies. The complete elucidation of expression changes of all 18 HDAC in the chronic plus binge feeding model will be compared to other related but distinct mouse models. This work will also illustrate the dysregulation of HDAC and histones under conditions of ethanol exposure as well as the utility of HDAC inhibitors in reversing deleterious phenotypes associated with ALD.

CHAPTER 2

MATERIALS AND METHODS

Experimental Animals

Female mice (C57BL/6) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a specific pathogen-free animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care under a protocol approved by the University of Louisville Institutional Animal Care and Use Committee (Nos. 15251 to Dr. Shirish Barve).. The room was maintained on a 12-hour light/dark cycle.

Experimental Design

A total of 28 female C57BL/6 age-matched (8 weeks) mice were utilized for this study. Until study commencement mice were fed standard rodent chow diet. After beginning the study, mice were then transitioned to the liquid Lieber DeCarli Diet. Mice were given the Lieber DeCarli diet from the start of the study until the end. The liquid diet was the only source of calories and water for mice throughout the course of the study. Diet was acquired from Bio-Serv (Flemington, NJ) and was irradiated before use. Both diets are similar in caloric composition with the pair-fed control diet having matched caloric equivalency with maltodextrin supplementation. Feeding, sacrifice and tissue harvesting was conducted on three separate days to keep mice numbers and post-sacrifice work-up manageable.

In order to mimic human drinking patterns and ALD, the chronic plus binge ethanol feeding model was utilized on the 28 mice selected for the study. All alcohol fed mice were matched to a pair-fed control group which consumed a similar amount of liquid diet to account for any changes in caloric intake between the two groups. Mice were housed in groups of four per cage. There were a total of 7 cages (28 mice). Of these cages, four were treated with ethanol (16 mice) and three were pair-fed controls (12 mice).

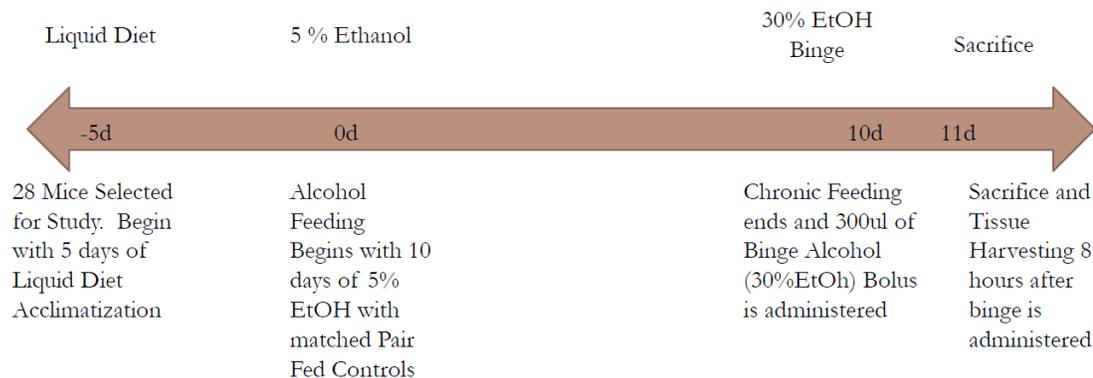


Figure 2. Chronic Plus Binge Feeding Model. A. Timeline representation of the chronic plus binge “10+1” Lieber DeCarli EtOH feeding model.

The chronic plus binge ethanol feeding model was conducted over the course of 16 days. The first five days were used for liquid diet acclimatization where mice were allowed to be fed ad libitum of the pair-fed Lieber DeCarli diet. After the initial five days, mice were then either administered a 5% (weight/volume) ethanol liquid diet or a pair-fed control for 10 days. To most closely mimic caloric feeding amounts for the pair-fed animals, the amount of diet consumed by the ethanol fed groups was recorded after each day, and then an equivalent isocaloric amount was given to the pair-fed controls. The

liquid diet was changed consistently within the same 1 hour window every day for 10 days. After the end of the liquid diet phase, mice were given 5 g/kg of a 30% (weight/volume) ethanol bolus (ddH₂O as diluent) via oral gavage and then sacrificed 8 hours later.

Assessment of Liver Injury

During sacrifice, while mice were deeply anaesthetized with tribromoethanol (Avertin), blood was collected from the inferior vena cava and immediately stored in heparinized tubes. Without freezing, plasma was isolated from whole blood by centrifugation at 2,000 x g for 10 minutes and plasma transaminases (ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase) were measured using the ALT/GPT and AST/GOT reagent (Thermo Fisher, Waltham, MA)

RNA Isolation and Real Time qPCR

After sacrifice small pieces of liver tissue were immediately placed in 1mL TRIzol solution (Invitrogen, Carlsbad, CA) and homogenized using a battery powered pestle. Complementary DNA (cDNA) was prepared using Quanta qScript (Quanta BioSciences, Gaithersburg, MD) using 20ng/μl of input RNA as quantified using a ND-1000 NanoDrop Spectrophotometer (Thermo Fisher, Waltham, MA). Real-Time Polymerase Chain Reaction (RT-PCR) was performed with Quanta Perfecta 7500 SYBR green fast mix and the QuantStudio 6 Flex (Applied Biosystems, Foster City, CA) 384-well Real-Time PCR System using 1μl of cDNA template prepared from an RNA concentration of 20ng/μl and 10μl of total reaction volume. The relative gene expression was normalized to the housekeeping gene, TATA-binding protein (TBP), expression. Results were then

normalized to the average of the pair-fed/untreated control group. Data are presented as fold-change using the delta-delta cycle of threshold ($2^{-\Delta\Delta C_t}$) method.

Gene Name	Forward (5' to 3')	Reverse (3' to 5')
HDAC1	TGGTCTCTACCGAAAAATGGA	TCTCTGCATCTGCTTGCTGT
HDAC2	GGTGGTGCTGCAGTGTGG	CATTCCGGATTGTGTAGCC
HDAC3	TTGAAGATGCTGAACCATGC	GTGCTTCTGGCCTGCTGTA
HDAC4	CTGCATGTGCTGGTGAAAA	CTGGTCTCGGCCAGAAAAT
HDAC5	GCAGCACGTTTTGCTCCT	AGCTTACCCACCGTCTCAT
HDAC6	TGCCAGTTGGTACTGGATTG	GGATCAGTTGCTCTCTGATGG
HDAC7	GCTGGAAACAGAAACCCAAC	TAGCCAGGAGTCTGGACAGG
HDAC8	CCTGATTGACGGGAAGTGTA	AAATTTCCGTCGCAATCGTA
HDAC9	GCTCAGCAAAGAATGCACAG	GATCCACCACAGGCATCATC
HDAC10	GCAGAGCTCAGAGGGTGCT	AGCCACAGAATTCTCCCATC
HDAC11	GCCTATGCAGACATCACACTG	GCTTGTACCCATGAAGTCTC
SIRT 1	CAGACCCTCAAGCCATGTTT	ACACAGAGACGGCTGGAAC
SIRT 2	GCTGCTACACGCAGAACATAG	CCAGCCCATCGTGTATTCTT
SIRT 3	ACAGCTACATGCACGGTCTG	ACACAATGTCGGGTTTCACA
SIRT 4	GAGCAACTGGGAGAGACTGG	CCACAGTTCAGGCACAGGAC
SIRT 5	CCTGTGTCTAGTGGTGGGAAC	GGGGTGGTCTCCATGTAAAA
SIRT 6	CCAAGTGTAAGACGCAGTACG	CTCTACAGGCCCGAAGTCC
SIRT 7	GCTGGAATCAGCACAGCAG	CTTAGGTCGGCAGCACTCAC
CPT-1 α	GCTGCACTCCTGGAAGAAGA	GGAGGGGTCCACTTTGGTAT
Malonyl CoA Decarboxylase	TTCTTCTCCACTGCTCCAC	CCTGTTCCTCTCCTCGGTTT
CXCL2	CAACCACAGGCTACAGGGGC	CTGGGGGCGTCACACTCAAGC
IL-17 Receptor A	GCAGCTGAACCAATGAGC	GCAGCACCAGTGAAACTTGC
CHOP	CCGGAACCTGAGGAGAGAGTGT	AGCTGCCATGACTGCACGTGG
ATF-3	GCGCTGGAGTCAGTTACCGTCA	TTCTTCAGGGGCCCTCAGAC
ATF-4	AAGCCATGGCGCTCTTCACGA	AGTCCCCGCCAACACTTCG

Table 3. Real Time q-PCR Primer Sequences. Tabular representation of Real Time qPCR primers used in the study.

Spleen and Liver Digests for Mononuclear Cell Isolation

Harvested spleens and livers were injected with 1 ml of collagenase medium, containing 1 mg/ml collagenase IV (Roche Diagnostics, Indianapolis, IN), 10 μ g/ml DNase I (Worthington Biochemical Corporation, Lakewood, NJ), 2% FBS in 1X phosphate buffered saline (PBS, Gibco). Spleens were chopped into small fragments and incubated at 37°C for 30 min in 5 ml of collagenase medium. Livers were also chopped, suspended in 5 ml collagenase medium and agitated for 25 minutes at 37°C. EDTA was added to a

final concentration of 10 mM for an additional 5 minutes to foster dissociation of T cell-APC conjugates and the tissue preparations were pushed through a 70 µm cell strainer. Spleen cells had red blood cells lysed using ACK buffer, and the preps were washed, resuspended in PBS-1% FBS, and counted on hemocytometer with trypan blue exclusion. Liver preparations were suspended in 10 ml PBS-1% FBS, centrifuged at 500 x g after gentle layering over a 33% Percoll Plus (Sigma) cushion. Cells were collected from the PBS/33% Percoll interface, RBC lysed and counted as spleen cell preparations.

Flow Cytometric Analysis of Cell Subsets from Liver and Spleen

Liver and spleen cell suspensions were incubated for 5-10 minutes with 1 µg/sample anti-CD16/32 mAb (clone 2.4G2, BioLegend, San Diego, CA) to block non-specific Ab binding to FcR (FcγR III/II), then stained with fluorochrome-labeled antibody panels as detailed in Table 4 in FACS staining buffer (SB: 1X PBS containing 2% FBS and 0.02% sodium azide). Cells were then washed in SB and 1X PBS and fixed in 2% methanol-free formaldehyde (Polysciences, Warrington, PA) for 2-24 hours before acquisition on a BD FACSCanto II flow cytometer (BD Biosciences, CA). In addition to surface staining, cells stained in the T cell nuclear factor panel were fixed overnight and then permeabilized using the eBioscience FoxP3 Transcription Factor Staining buffer set (ThermoFisher). Nuclear factor antibodies were diluted in 1X permeabilization buffer (Perm Buffer) and incubated with the cells for 45 minutes at room temperature. Cells were then washed twice in Perm Buffer and once in SB before re-suspending in SB for acquisition. Intracellular cytokine staining (ICCS) was performed on cells cultured ± activation in RPMI-10% FBS and brefeldin A for 5 hours. After surface marker staining, cells were fixed in 2% formaldehyde overnight and intracellular stains performed in 1X

Perm Buffer. UltraComp eBeads compensation beads (ThermoFisher) incubated with fluorochrome-conjugated antibodies from Table 4 were used as compensation controls. Panel-specific fluorescence minus one (FMO) controls on spleen cell populations were used to define negative events in gating strategies (PMID: 16888771). In select experiments, enumeration of liver cell preparations was performed using comparison to Precision Count beads (BioLegend) per the manufacturer's protocol.

Ab-Fluorochrome	clone	isotype	Vendor	Cat #
CD3 AF488	500.A2	Syr Ham IgG	BioLegend	152322
CD19 PE	1D3	Rt IgG _{2a}	BD Biosciences	553786
Ly6G PerCP-Cy5	1A8	Rt IgG ₁	BD Biosciences	560602
CD8 PE-Cy7	53-6.7	Rt IgG _{2a}	BioLegend	100722
CD4 APC	GK1.5	Rt IgG _{2b}	BioLegend	100412
CD11b APC-Fire/750	M1/70	Rt IgG _{2b}	BioLegend	101262
CD45 V450	30-F11	Rt IgG _{2b}	BD Biosciences	560501
NK1.1 BV510	PK136	ms IgG _{2a}	BioLegend	108738

AF488: Alexa Fluor 488; APC: allophycocyanin; APC-Fire750: fixative-,photo-, temperature-stable allophycocyanin-cyanine7 alternative; PE: R-phytoerythrin; PE-Cy7: R-phytoerythrin-cyanine-7; PerCP-Cy5.5: peridinin chlorophyll protein complex-cyanine 5.5; V450: Violet450, AmCyan alternative with limited spillover into 488 nm detector (FITC)

Table 4. mAb Used for Flow Cytometric Analysis. Tabular representation of monoclonal antibodies used in analysis.

Immunohistochemical Staining and Hematoxylin and Eosin Staining

For histological analysis, liver sections were fixed in 10% buffered formalin for 24 hours and embedded in paraffin. Tissue sections were stained with hematoxylin-eosin (H&E) and examined under light microscopy at 40× magnification. For Malonyl CoA Decarboxylase stain, the protocol for Immunohistochemistry on formalin embedded tissue sections was followed from manufacturer's instructions for the MLYCD Rabbit Polyclonal Antibody (ProteinTech, Rosemont, IL). Images were imaged using a BZ810 Analyzer (Keyence, Osaka, Japan) and quantified using accompanying software.

Western Blot Analysis

Protein extracts were prepared by adding approximately 75mg of liver tissue to 0.5mL 1x MSD Tris Lysis Buffer (Meso Scale Discovery, Rockville, MD) with protease inhibitors. The tissue buffer mixture was sonicated at 40% amplitude in three 30 second bursts while being kept on ice. Samples were then centrifuged at 4°C for 10 minutes at 20,000 x g. The supernatant was removed and transferred to tubes for protein quantification or storage at -80°C.

After quantification, an equivalent amount of protein (30µg) was mixed with SDS loading buffer and protein samples were separated by electrophoresis on pre-cast 4-15% gradient polyacrylamide gels and transferred onto a PVDF membrane. The blots were blocked with 5% (w/v) nonfat dry milk constituted in 1x TBS-T (Tris buffered saline, 10 mM Tris HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 1 hour at room temperature. Membranes were incubated overnight at 4°C with the primary antibody, Acetyl-Histone H3 (Rabbit Anti-Mouse) (MilliporeSigma, Burlington, MA) diluted 1:5000 in 5 % BSA. After incubation, membranes were washed in 1x TBS-T and then incubated for one hour

in anti-Rabbit HRP secondary antibody (Cell Signaling, Danvers, MA) diluted 1:2000 in 5% nonfat dry milk. The membranes were subsequently imaged using enhanced chemiluminescence light (ECL) detection reagents (Amersham, Arlington Heights, IL).

After imaging the blot was then stripped using Restore Plus Western Blot Stripping Buffer (ThermoFisher, Waltham, MA) for 15 minutes. After washing stripping buffer, membranes were then blocked in 5% nonfat dry milk in 1x TBS-T for one hour. Blots were then washed and incubated overnight at 4°C with the primary antibody GAPDH Rabbit mAb (Cell Signaling, Danvers, MA) diluted 1:2000 in 5% nonfat dry milk. The membrane was then imaged using the HRP and detection reagent as mentioned above.

Isolation, Culture, and Treatment of Primary Hepatocytes

Mice were deeply anesthetized using a ketamine/xylazine solution and sprayed with alcohol to keep surfaces and mice sterile. Mice were then mounted on a dissecting board and laparatomized to expose the liver and the inferior vena cava (IVC). The IVC was then cannulated with a 22-gauge catheter which was connected to a perfusion line. The mouse liver was then perfused with an EGTA solution for 5 minutes at a rate of 5 ml/min followed by cutting of the portal vein. The SVC above the liver was clamped off. After EGTA perfusion, the liver was then perfused with collagenase for 10 minutes under the exposure of a heat lamp.

After collagenase perfusion, the liver was removed and minced in a petri dish with cold 5% FBS DMEM media. The digested liver was filtered through a cell strainer and then centrifuged at 4°C at 400rpm for 5 minutes. The cells were subsequently washed with 20mL HBSS and then spun down at 400 rpm for 5 minutes. After checking viability, cells

were then resuspended in 30mL HBSS solution with 18mL of Percoll mixed with 2mL of HBSS. Tubes were inverted to mix and then centrifuged for 20 minutes at 400rpm with no to low brakes. The cells were then washed twice with HBSS and then counted and checked for appropriate viability. Cells were then subsequently plated onto 6 well plates in 2 mL of 5% DMEM at a density of 0.25 million cells/mL. Cells were allowed to incubate and attach overnight and then treated the next morning.

Alcohol treated cells were exposed to 50mM of ethanol diluted in fresh 5% FBS DMEM. TSA treated cells were exposed to TSA at a concentration of 5 μ M. Wells not treated with TSA were exposed to the TSA vehicle DMSO at equivalent amounts. After 24 hours of treatment, fresh 5% FBS DMEM was added and the cells were then re-exposed to ethanol, TSA, or DMSO for an additional 5 hours before harvest. RNA from cells was harvested using TRIZol and subsequently analyzed using qRT-PCR as mentioned above.

Statistical Analysis

One mouse from the original cohort of 44 was lost during oral ethanol gavage and was therefore was unable to yield any data in this experiment. The data represented are a compilation of the surviving 27 mice. GraphPad Prism 8 software (GraphPad Software, San Diego, CA) was used to perform all statistical tests in the study. Student T-test (Unpaired T-test) was performed to analyze data for two group comparison with parametric distribution. One-Way ANOVA was conducted on parametrically distributed data for comparison between multiple groups. Mann-Whitney U-Test was performed on non-parametrically distributed data for two group comparisons. Unpaired Student T-Test was performed on parametrically distributed data for two group comparisons. Image intensity was quantified using ImageJ software and the band intensity ratios of Acetyl-Histone H3

was compared and normalized to GAPDH values. All data were considered significant if $p \leq 0.05$.

CHAPTER 3

RESULTS

Mouse Body and Liver Weights

A total of 27 mice subjected to chronic plus binge ethanol or pair fed treatment were sacrificed approximately 8 hours post binge ethanol bolus and the total body weight as well as the total liver weight of each individual mouse was recorded. Body weights were documented to show unremarkable changes with alcohol treatment. Alcohol feeding slightly increased mean liver weight but this was deemed to be insignificant. Overall, no significant change in mean body and liver weights with ethanol exposure were observed.

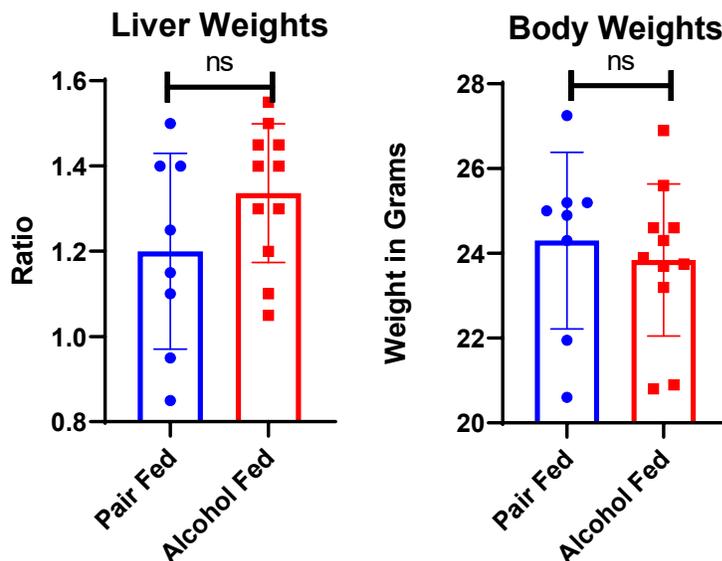


Figure 3. Mean Body and Liver Weight by Treatment Group. Graphical Representation of Mean Body and Liver Weights from Chronic Plus Binge Ethanol Feeding Study. Mice were weighed post-anesthesia and before sacrifice. Livers were removed after sacrifice and sectioned for subsequent analyses. Mann Whitney U-Test performed for significance.

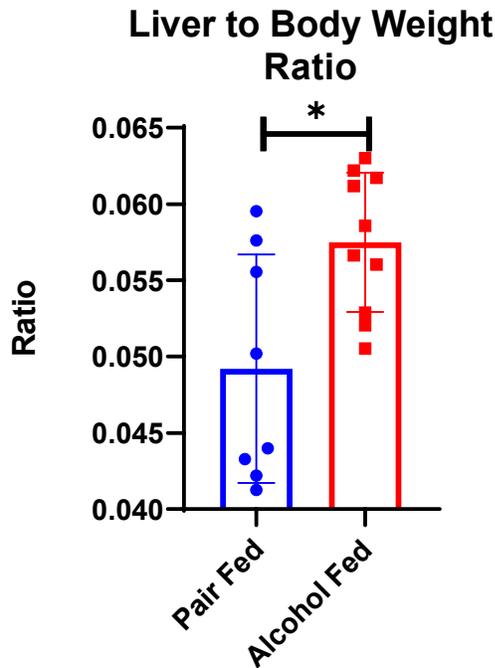


Figure 4. Liver to Body Weight Ratio by Treatment Group. Graphical Representation of Liver to Body Weight Ratio from Chronic Plus Binge Ethanol Feeding Study. Mann Whitney U-Test performed for significance. ($p \leq 0.05$)

However, there was a significant increase in liver to body weight ratio in mice exposed to alcohol indicating alcohol induced hepatic injury and hepatomegaly combined with weight loss.

Hepatic Gene Expression of Histone Deacetylases

The level of mRNA expression of all 18 HDAC enzymes including all Sirtuin enzymes in the livers of PF and AF mice were interrogated using their respective primers by qPCR. Results indicated variable levels of gene expression for these enzymes. Of the 11 HDAC histone deacetylase genes, 6 significantly changed their hepatic gene expression with alcohol as assessed by Mann Whitney U-Test.

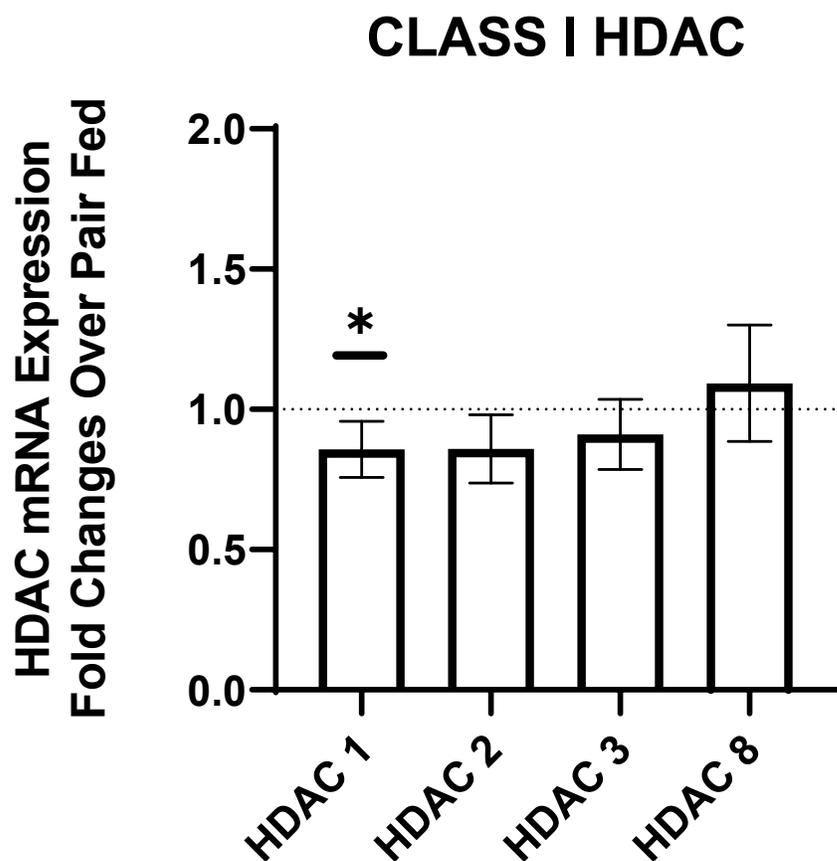


Figure 5. Hepatic Gene Expression Level Changes of Class I HDACs with Chronic Plus Binge Alcohol Treatment. HDAC 1 ($p \leq 0.05$). Results are exhibited as fold changes in Alcohol Fed group ($n=11$) normalized to average of Pair Fed group ($n=8$) which was normalized to 1. Mann-Whitney U-Test performed for significance.

HDAC1 was the only Class I HDAC to significantly downregulate its expression with alcohol treatment and only did so with 20% reduction in fold change. The Class II HDAC enzymes were more broadly affected with three members (HDAC 7,9,10) showing decreased expression levels between 30%-80% fold change. The sole of member of the Class IV HDAC enzyme family HDAC11 dramatically reduced its expression level at 60%-75% . HDAC6 showed a small upregulation while HDAC 5 showed a small downregulation. The other members of the HDAC Class I, II, and IV families showed unremarkable changes in expression levels with values deemed to be either insignificant or slightly changed.

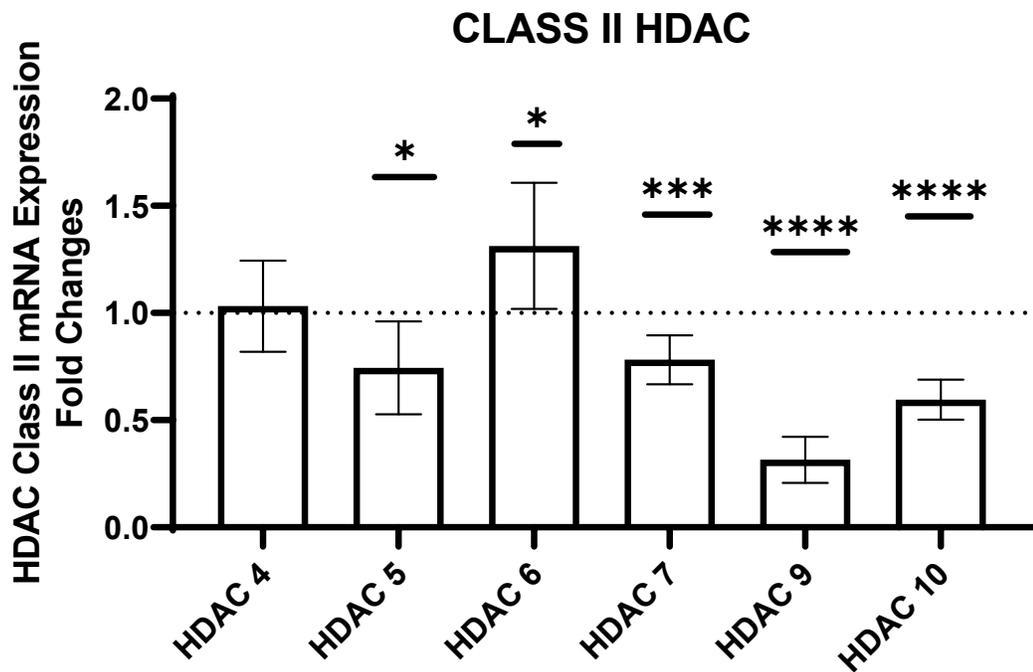


Figure 6. Hepatic Gene Expression Level Changes of Class II HDACs with Chronic Plus Binge Alcohol Treatment. HDAC 5 ($p \leq 0.05$) HDAC 6 ($p \leq 0.05$) HDAC 7 ($p \leq 0.0005$) HDAC 9 ($p \leq 0.0001$) HDAC 10 ($p < 0.0001$). Results are exhibited as fold changes in Alcohol Fed group (n=11) normalized to average of Pair Fed group (n=8) which was normalized to 1. Mann-Whitney U-Test performed for significance.

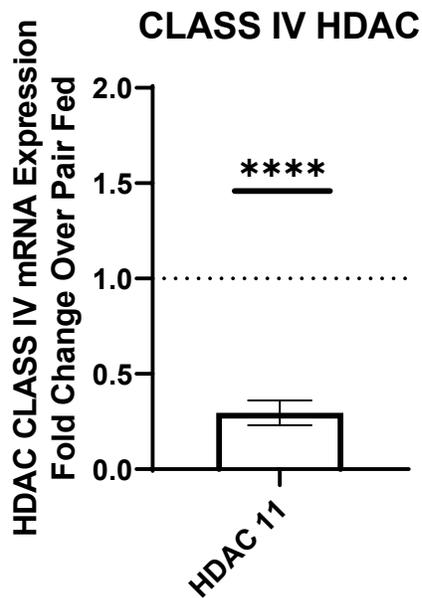


Figure 7. Hepatic Gene Expression Level Changes of Class IV HDACs with Chronic Plus Binge Alcohol Treatment. HDAC 11 ($p \leq 0.0001$). Results are exhibited as fold changes in Alcohol Fed group ($n=11$) normalized to average of Pair Fed group ($n=8$) which was normalized to 1. Mann-Whitney U-Test performed for significance.

The expression levels of the 7 Class III family of HDAC proteins also called the Sirtuins were also examined in this study. There was a noticeable and significant decrease in the expression levels of 4 Sirtuin genes (SIRT 3, 4, 5, 7) with alcohol treatment. These genes exhibited a significant (Mann-Whitney U-Test) decrease of between 25-60% in fold change. The other 3 Sirtuin genes did not show a remarkable change in expression patterns with alcohol treatment with significance levels being either insignificant or slightly significant. This comprehensive expression profile of all 7 Sirtuin enzymes in the chronic plus binge alcohol feeding model has yet to be characterized in the literature.

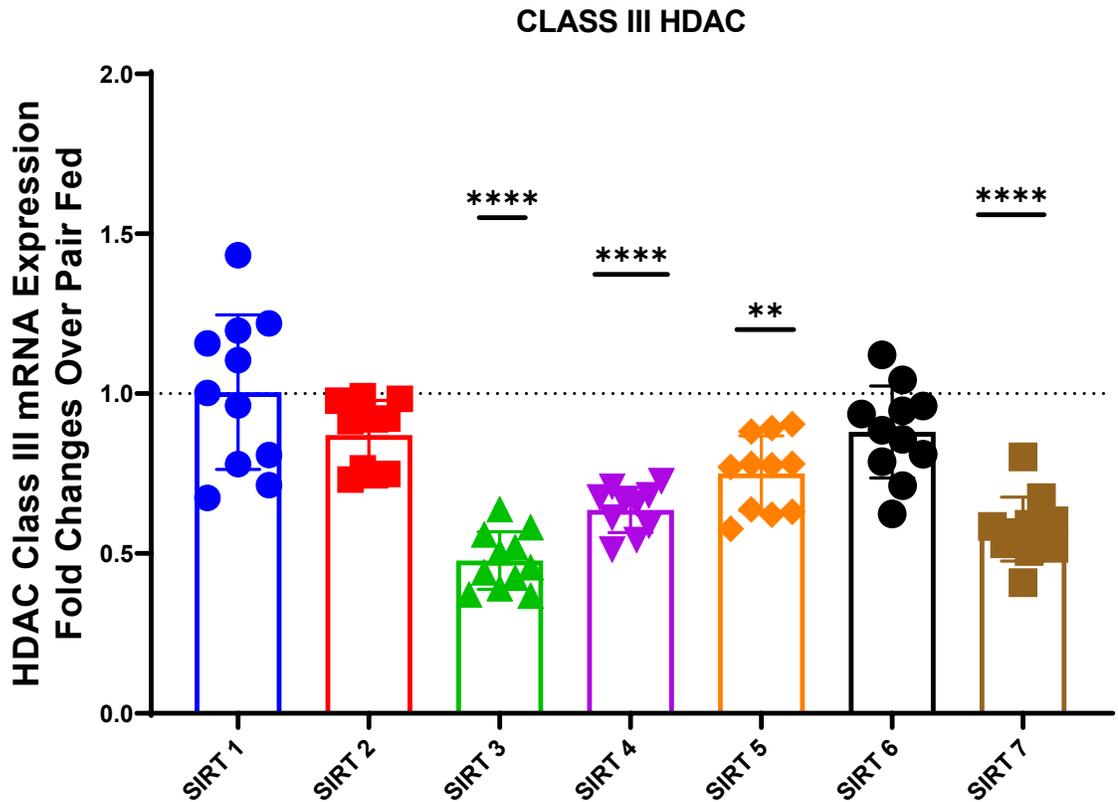


Figure 8. Hepatic Gene Expression Level Changes of Class III HDACs (Sirtuins) with Chronic Plus Binge Alcohol Treatment. SIRT 3 ($p \leq 0.0001$) SIRT 4 ($p \leq 0.0001$) SIRT 5 ($p \leq 0.001$) SIRT 7 ($p \leq 0.0001$) Results are exhibited as fold changes in Alcohol Fed group ($n=11$) normalized to average of Pair Fed group ($n=8$) which was normalized to 1. Mann-Whitney U-Test performed for significance.

Induction of Endoplasmic Reticulum Stress

Alcohol feeding is known to induce Endoplasmic Reticular (ER) stress in hepatic tissue as a response to liver injury. Three markers for ER stress were examined in this study, CHOP, ATF-3, and ATF-4. These markers are transcription factors that initiate a complex response to liver injury and ER stress and are markedly increased with chronic plus binge alcohol administration.

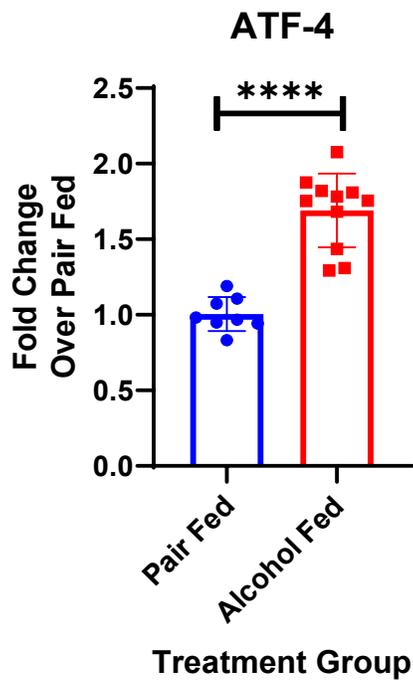
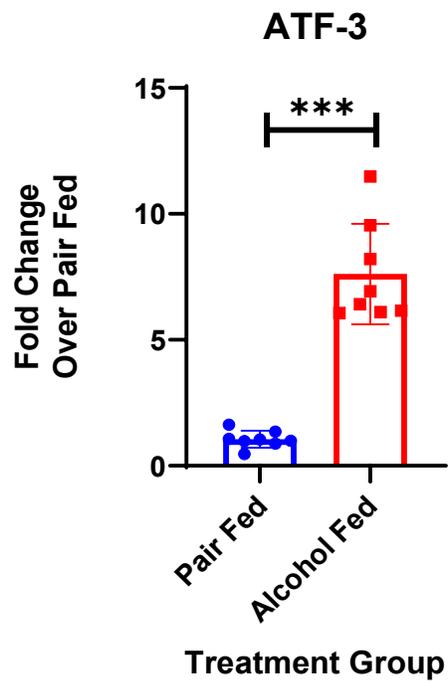
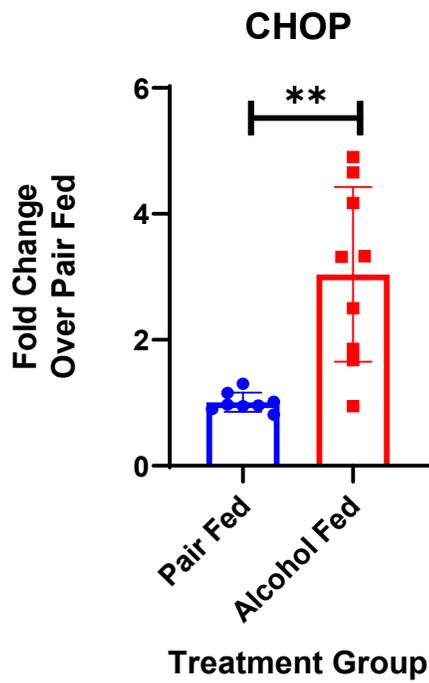
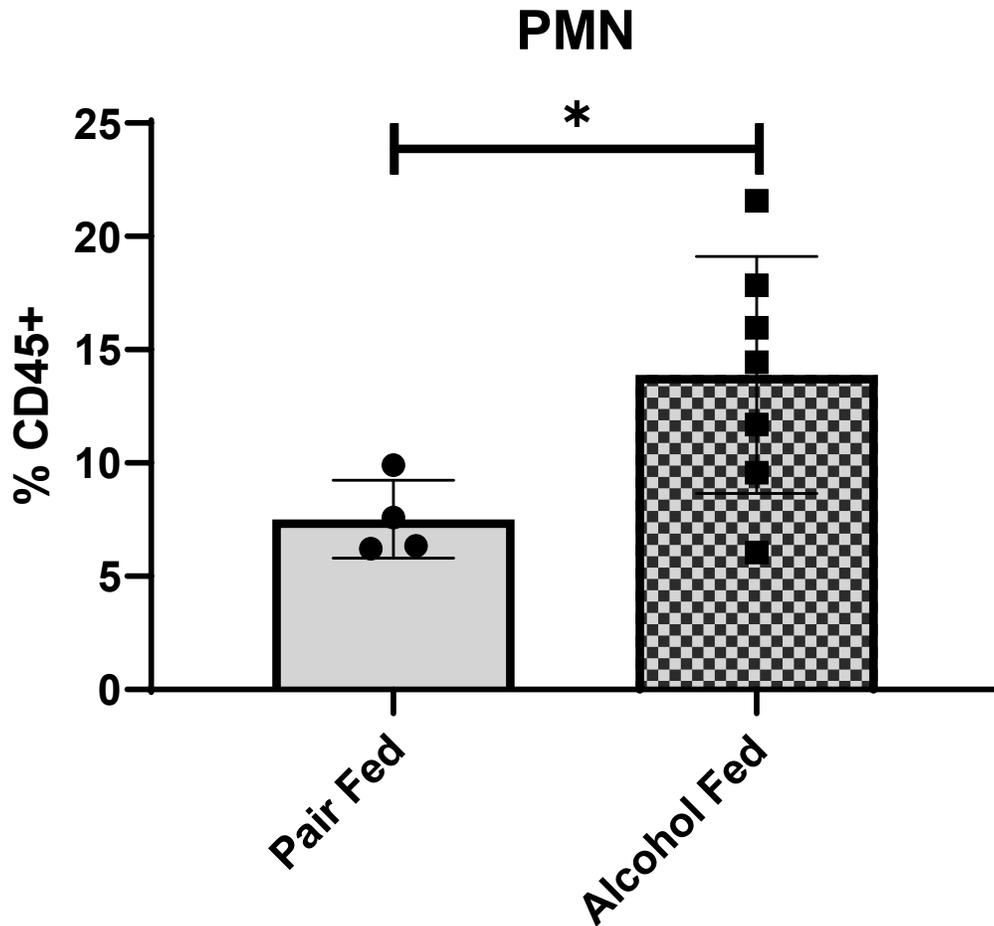


Figure 9. Hepatic Gene Expression Changes of ER Stress Genes in Chronic Plus Binge Alcohol Feeding Study. A. CHOP ($p \leq 0.01$) B. ATF-3 ($p \leq 0.001$) C. ATF-4 ($p \leq 0.0001$) Mann-Whitney U-Test performed for significance.

Increased Immune Cell Infiltration into Liver Lysates

Mononuclear cells isolated from collagenase/DNase I digests of livers from PF or AF mice were obtained and surface stained with a general immune cell phenotyping panel and assessed by flow cytometry as described in Materials and Methods. As shown in Figure 10, lymphocytes were gated using a typical forward scatter/side scatter light scatter pattern, and then further gated for T cell subsets, B cells, CD11b+ cells and neutrophils. When comparing the frequency of the cell population of liver isolates, neutrophil and natural killer (NK) T cells showed a marked increase between pair-fed and alcohol fed mice.



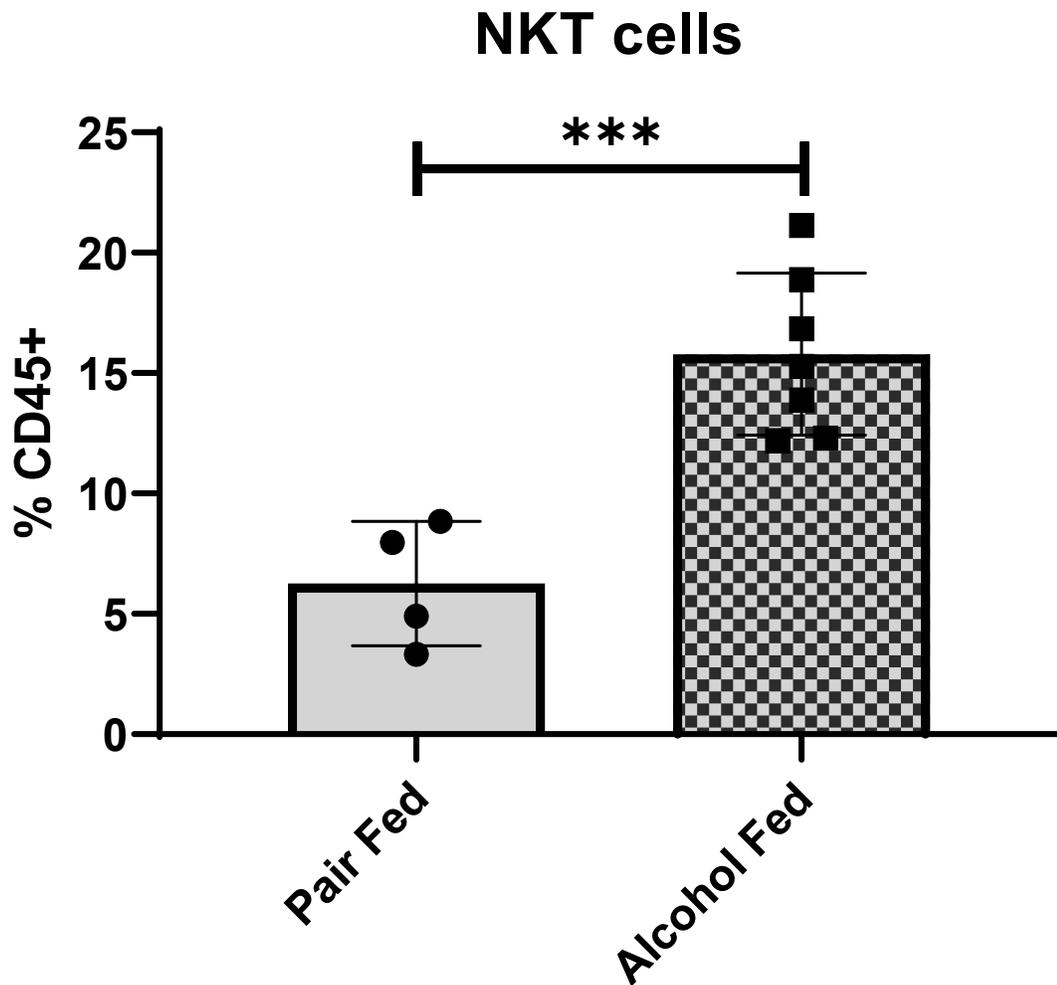


Figure 10. Immune Cell Infiltration of Polymorphonuclear Leukocytes and Natural Killer T Cells into Liver Digests in Chronic Plus Binge Ethanol Feeding.
A. PMN or Polymorphonuclear Leukocytes ($p < 0.05$) **B.** NKT or Natural Killer T-Cells ($p < 0.001$) Unpaired Student's T-Test performed for significance.

Hepatic Inflammation

The expression changes of genes related to hepatic inflammation was also examined. Results show a significant increase in the expression levels of the chemokine and neutrophil chemoattractant CXCL2 by hepatocytes indicating liver injury and inflammation. The signal for infiltrating neutrophils is one clinical characteristic of ALD that is replicated successfully under this chronic plus binge study. Other genes such as IL-17 Receptor A also showed significant upregulation in whole liver lysates indicating an increase in inflammatory signal received by the liver in response to injury and inflammation. IL-17A is one of the primary pro-inflammatory cytokines released in response to infection. It is primarily released by immune cells, but its receptor is present on many organs and tissues to react during the course of an IL-17A ligand stimulus.

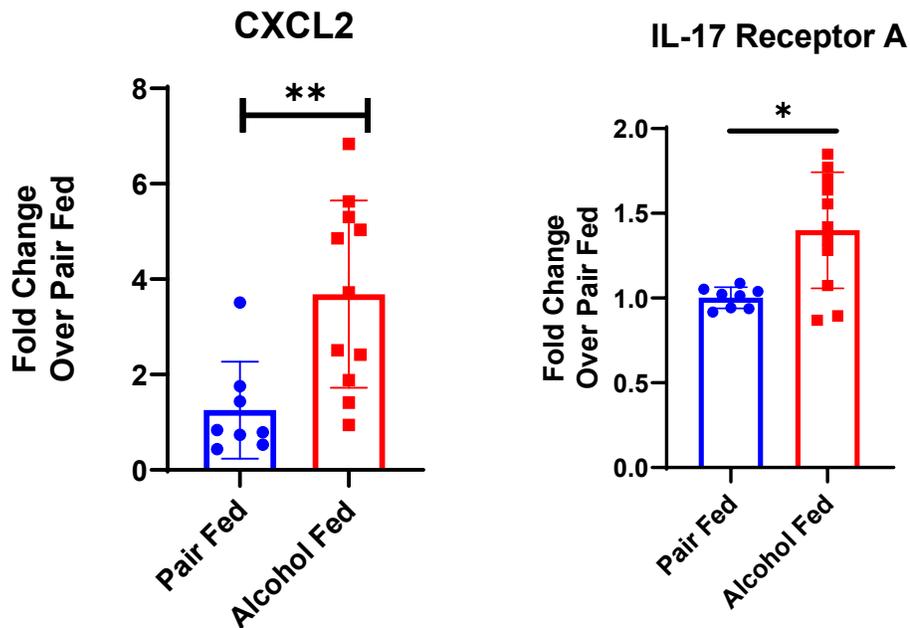


Figure 11. Expression Changes of Genes Related to Hepatic Inflammation in Chronic Plus Binge Alcohol Feeding Study. A. CXCL2 ($p \leq 0.01$) B. IL-17 Receptor A ($p \leq 0.05$) Mann-Whitney U-Test performed for significance.

Plasma Transaminases as a Marker for Liver Injury

The chronic plus binge model is known for its ability to cause the characteristic liver injury associated with Alcohol-Associated Liver Disease. Plasma transaminase (Alanine Aminotransferase ALT and Aspartate Aminotransferase AST) levels show a clear and significant increase in the alcohol treated group as compared to pair-fed. As expected, there was no dramatic change or increase in transaminase levels in any individual mouse in the pair-fed groups indicating small intragroup variability.

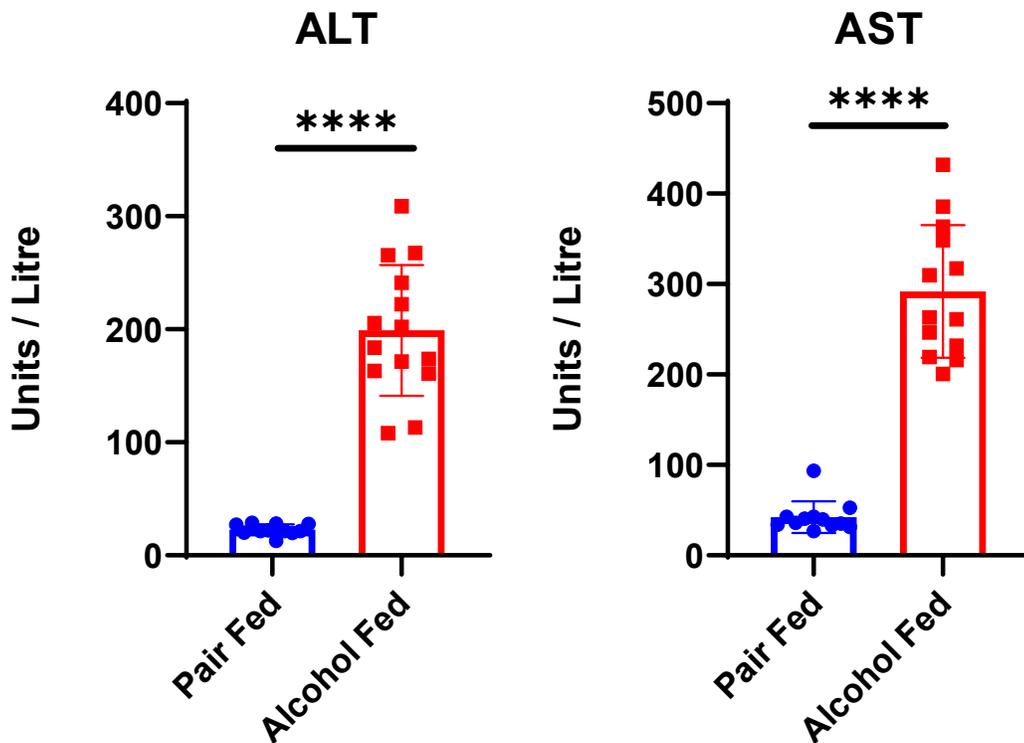


Figure 12. Plasma Transaminase (ALT/AST) Levels in Chronic Plus Binge Alcohol Feeding Study. Graphical Representation of Plasma Transaminase (ALT/AST) Levels measured fresh on day of sacrifice. One Way ANOVA Test Performed for Significance. ($p \leq 0.0001$)

Hematoxylin and Eosin Histological Examination of Liver Tissue

Histological staining of mouse liver tissue revealed characteristic hepatic fat accumulation with ethanol feeding. Visible signs of hepatic macrovesicular steatosis are illustrated in the representative images of ethanol fed mice.

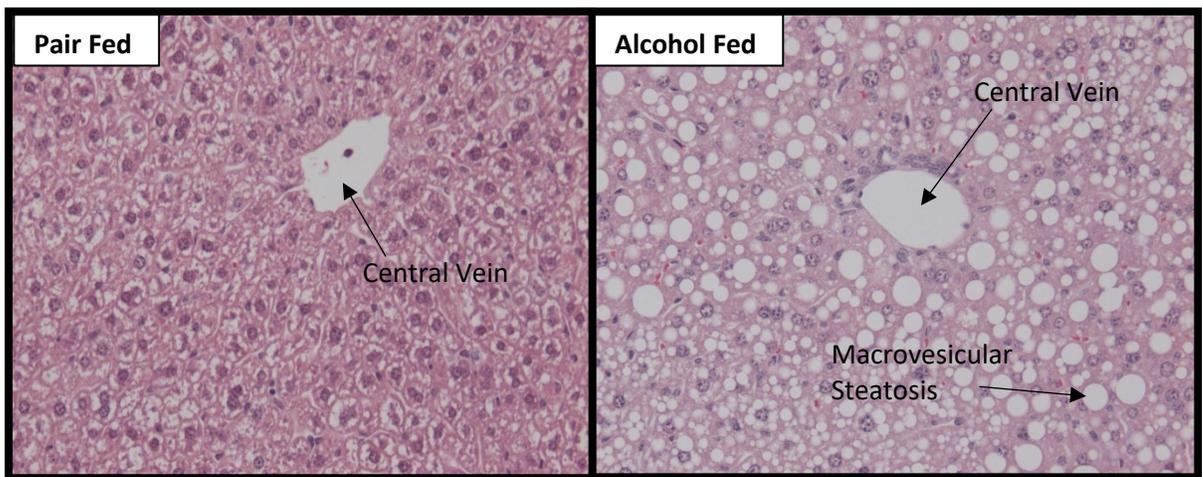


Figure 13. Representative Hematoxylin and Eosin (H&E) Stain of Pair Fed and Alcohol Fed Mice From Chronic Plus Binge Alcohol Feeding Study.

Induction of Hepatic Fat Accumulation

In addition to the histone deacetylases, the expression levels of other key metabolic genes were assessed. Ethanol treatment showed a decrease in expression of genes related to fatty acid catabolism and fatty acid β -oxidation. There was a significant decrease in the gene carnitine palmitoyl transferase I alpha (CPT-1 α). This gene is responsible for shuttling long chain fatty acids into the mitochondria where they undergo β -oxidation and produce energy. In addition to CPT-1 α , the gene malonyl CoA decarboxylase is also significantly attenuated with alcohol treatment. Malonyl CoA decarboxylase takes a

carboxyl group off from malonyl CoA forming acetyl CoA which can then be utilized by the citric acid cycle to generate energy in the form of ATP.

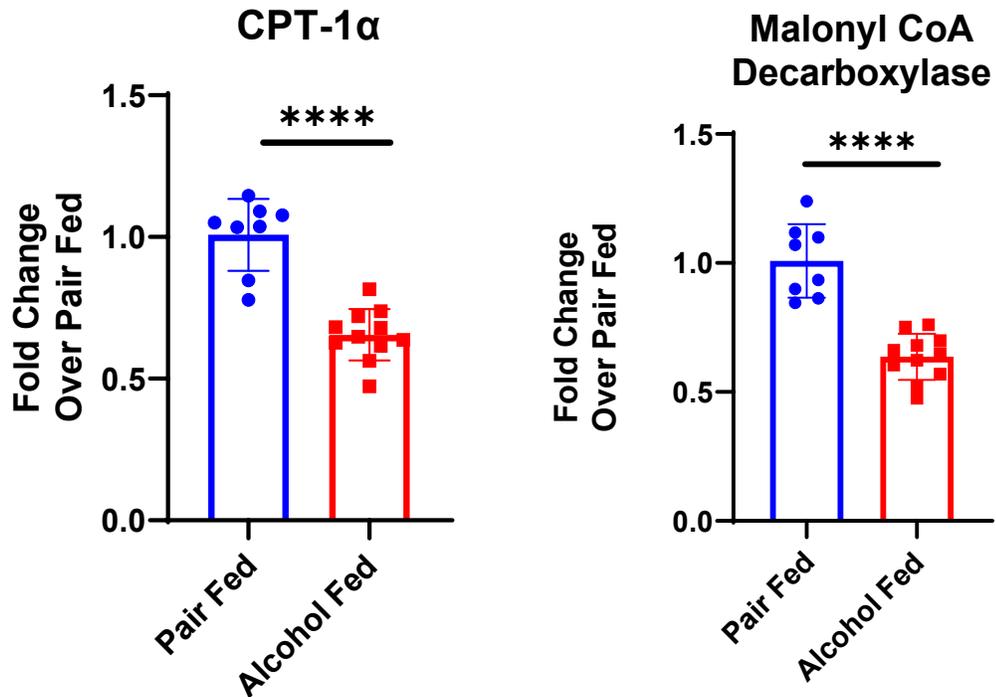


Figure 14. Hepatic Gene Expression Changes of Key Metabolic Genes in Chronic Plus Binge Alcohol Feeding Study. A. CPT1 α ($p \leq 0.0001$) B. Malonyl CoA Decarboxylase ($p \leq 0.0001$) Mann-Whitney U-Test performed for significance.

Histological Quantification of Malonyl CoA Decarboxylase Protein Attenuation

To see if decreased mRNA expression levels in the malonyl CoA decarboxylase gene corresponded to a visible change in protein expression levels, an Immunohistochemical stain on liver tissue from both alcohol fed and pair fed mice was performed. Results indicated an expected attenuation of malonyl CoA decarboxylase protein levels. Representative images are displayed and quantified levels of the protein as measured by percent area positive are illustrated in the following figure.

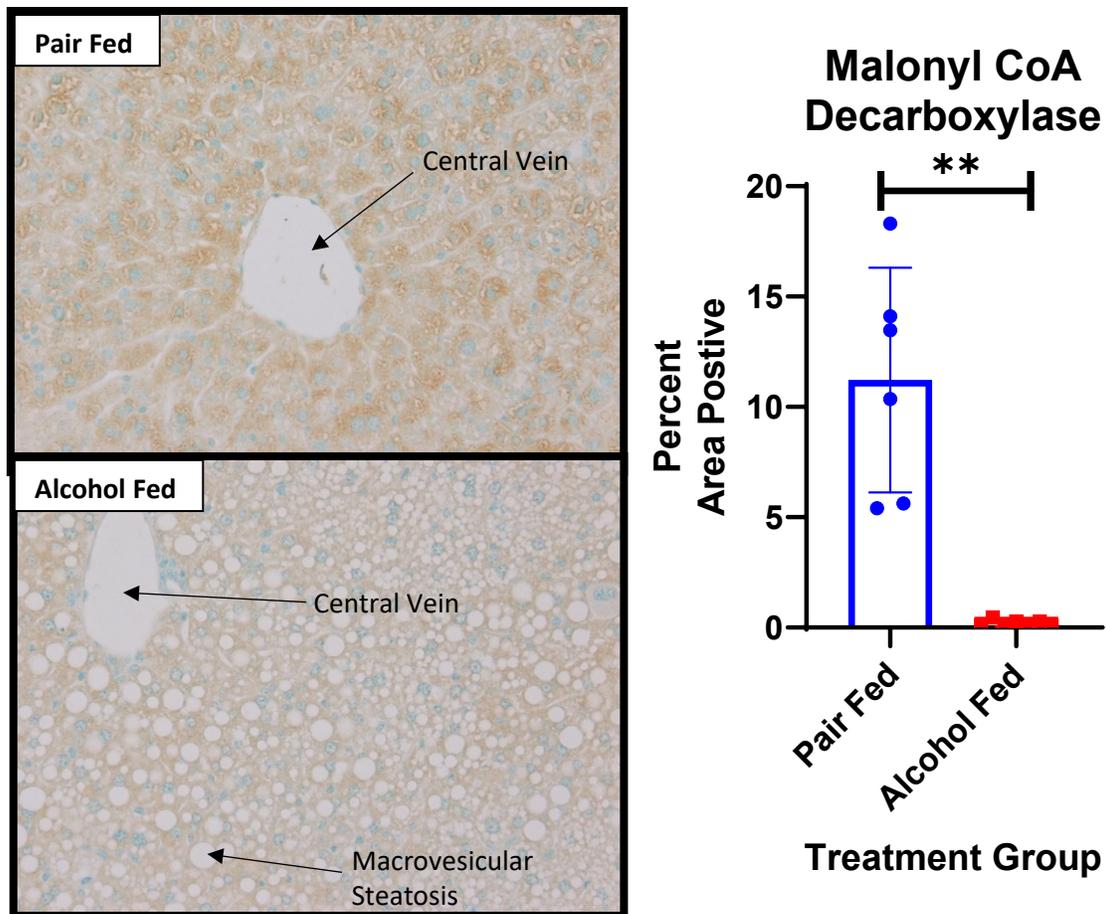


Figure 15. Immunohistochemical Staining Showing Attenuation of Malonyl CoA Decarboxylase Protein Levels with Chronic Plus Binge Alcohol Treatment. Quantification of percent area positive of representative images performed. ($p \leq 0.005$) Mann-Whitney U-Test Performed for Significance. Images taken on BZ810 Analyzer and quantified on the same.

Ethanol Exposure Increases Global Histone Acetylation

Western Blotting was performed on hepatic protein lysates isolated from chronic plus binge ethanol exposed mice. The overall global levels of histone acetylation were assessed by quantifying protein expression of Acetyl Histone H3. Results indicated a greater than 2-fold increase in hepatic Acetyl H3 ratio levels when normalized to the housekeeping gene GAPDH. These results implicate the potential effects of ethanol on

HDAC expression and function that are involved in the management of histone acetylation status. Global changes (increase) in histone acetylation is also indicative of the influence of ethanol in altering epigenetic regulation of hepatic gene expression.

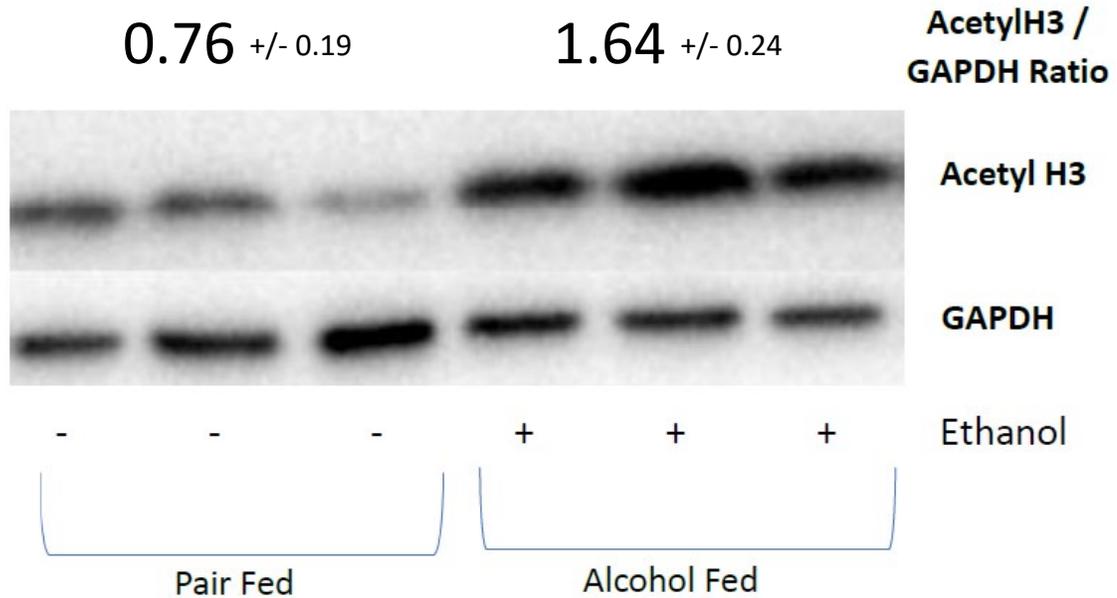


Figure 16. Chronic Plus Binge Ethanol Feeding Results in Increased Global Histone Acetylation. Hepatic Liver Lysates were probed with anti-Acetyl H3 antibody. Results indicate increased acetyl H3 levels when normalized to housekeeping protein GAPDH.

HDAC Inhibitor TSA Ameliorates Alcohol Induced ER Stress

To test to see if the HDAC inhibitor Trichostatin A is capable of attenuating ER stress as a result of alcohol exposure, primary mouse hepatocytes were exposed to ethanol and TSA or ethanol alone for 48 hours. Results indicate that TSA is capable of ameliorating alcohol induced ER stress as evidenced by decreased expression of CHOP and ATF-3 with alcohol and TSA co-treatment. Possible mechanisms include preserving acetylation on lysine residues of these transcription factors, thereby promoting stability.

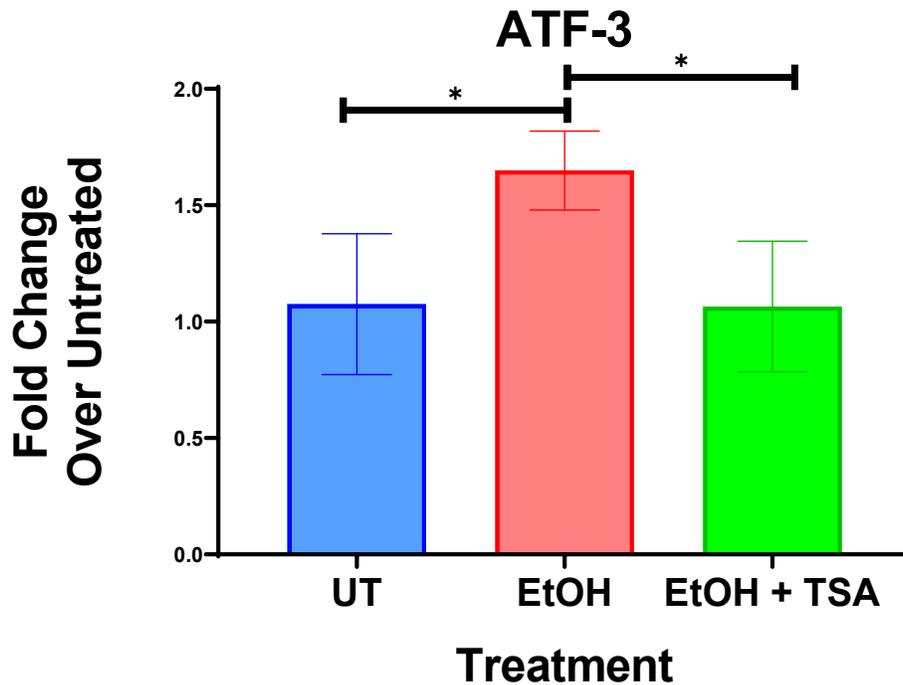
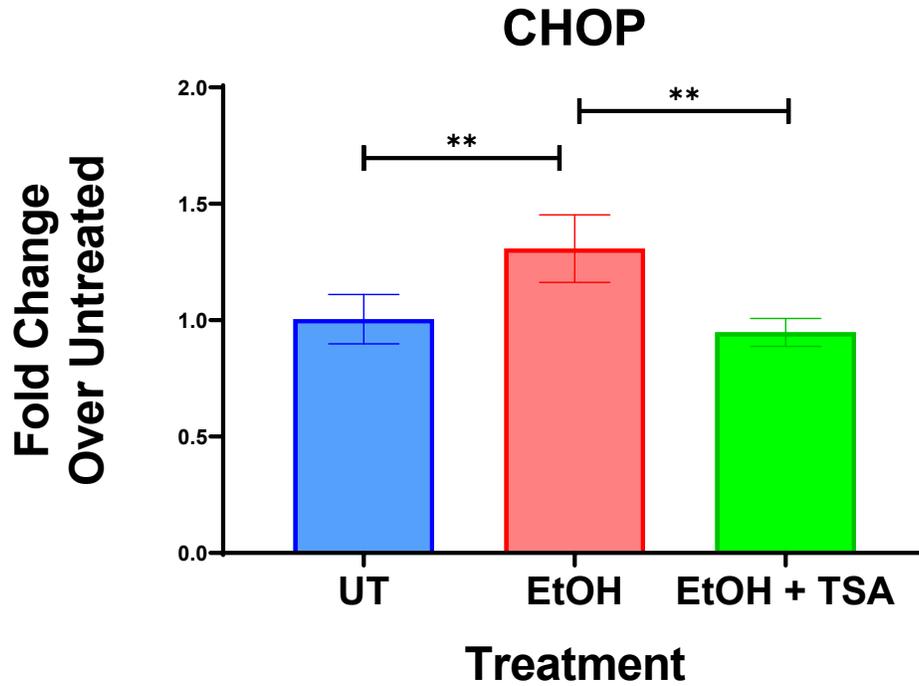


Figure 17. The HDAC Inhibitor Trichostatin A (TSA) Ameliorates Ethanol Induced ER Stress in Primary Hepatocytes cultured for 48hrs. A. CHOP ($p \leq 0.01$) for UT vs EtOH and EtOH vs. EtOH + TSA. B. ATF-3 ($p \leq 0.05$) for UT vs. EtOH and EtOH vs. EtOH + TSA. One-Way ANOVA performed for significance.

HDAC Inhibitor TSA Rescues Ethanol Induced Attenuation of Malonyl CoA Decarboxylase and CPT-1 α

It is hypothesized that the alcohol induced attenuation of genes involved in fatty acid β -oxidation are a driving mechanism behind the hepatic steatosis characteristic of ALD. The fatty acid β -oxidation/lipogenesis balance inside the cell is clearly dysregulated with alcohol treatment. It is believed that alterations in histone acetylation at the promoter regions of these genes are directly responsible for changes in gene expression. A previous study documented how treatment with the HDAC inhibitor and nutraceutical tributyrin rescues CPT-1 α expression by preserving acetylation at the CPT-1 α promoter (5). Here we show that treatment with the synthetic HDAC inhibitor Trichostatin A (TSA) rescues alcohol induced attenuation of the gene malonyl CoA decarboxylase and CPT-1 α in primary mouse hepatocytes. It is hypothesized that TSA exerts its effect in a similar manner to tributyrin, specifically by altering histone acetylation at the malonyl CoA decarboxylase promoter region. Future studies using Chromatin Immunoprecipitation (ChIP) will delineate exact molecular mechanisms responsible for malonyl CoA decarboxylase promoter instability.

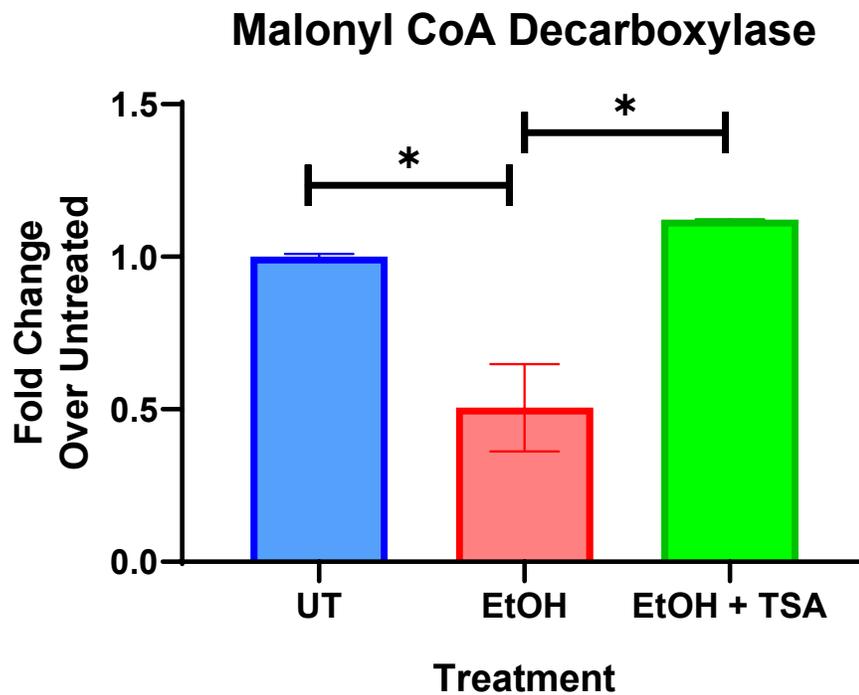
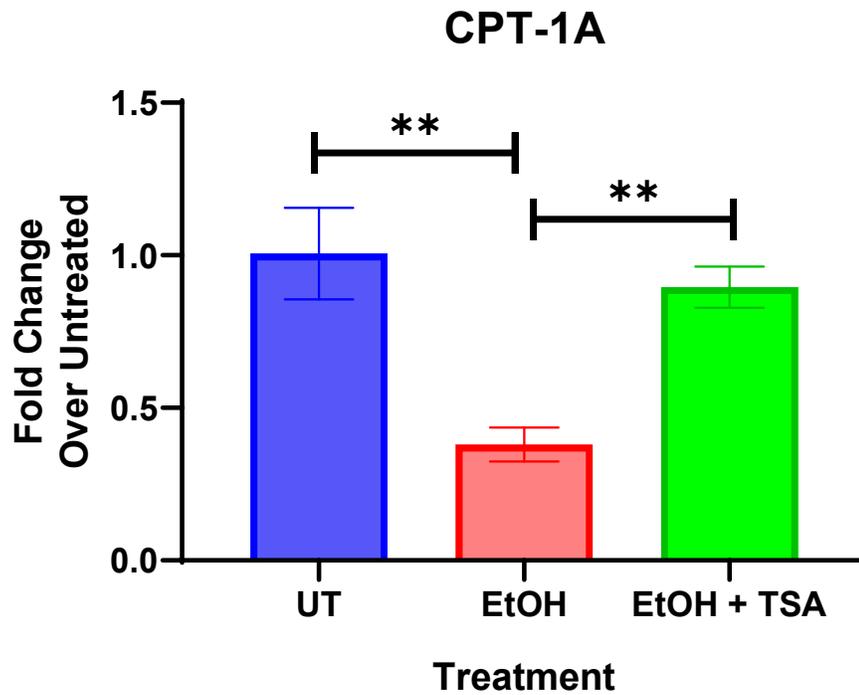


Figure 18. The HDAC Inhibitor Trichostatin A (TSA) rescues Ethanol Induced Attenuation of CPT-1 α and Malonyl CoA Decarboxylase Gene Expression in Primary Hepatocytes cultured for 24hrs. A. CPT-1 α ($p \leq 0.01$) for UT vs EtOH and EtOH vs. EtOH + TSA. B. Malonyl CoA Decarboxylase ($p \leq 0.05$) for UT vs. EtOH and EtOH vs. EtOH + TSA. One-Way ANOVA performed for significance.

CHAPTER 4

DISCUSSION

As indicated by the literature the results from our chronic plus binge ethanol feeding study shared many of the characteristics of clinical ALD phenotypes. There was clear and distinct liver injury with ethanol treatment as evidenced by elevated ALT/AST. In addition, there was a significant increase in the liver to body weight ratio in mice exposed to alcohol indicating alcohol induced hepatic injury and hepatomegaly combined with weight loss.

The hepatic gene expression of all 18 HDAC enzymes in the chronic plus binge alcohol feeding model has yet to be characterized in the literature. Results indicate marked attenuation of 10 HDAC in RNA extracted from whole liver lysates 8-9 hours after terminal binge administration. A similar study looked at the expression of HDAC levels in mice treated with 3 consecutive binges (4.5g EtOH/kg) at 12 hour intervals without chronic exposure to Ethanol. The study reported similar findings with decreases in HDAC 1, 7, 9, 10, 11 but also reported an increase in HDAC3 expression levels which was not observed in this study (36). This is the first study to examine the comprehensive gene expression profile of all the Sirtuin enzymes in the chronic plus binge mouse model. One recent study documented the upregulation of SIRT3 with ethanol exposure but this study indicates a dramatic decrease in SIRT3 expression levels (38). In addition to SIRT3; SIRT4, SIRT5 and SIRT7 were also significantly decreased. SIRT 3, 4, and 5 are enzymes that localize

to the mitochondria so results would indicate that alcohol consumption would impair mitochondrial related processes such as fatty acid β -oxidation and oxidative phosphorylation for ATP generation. All 18 HDAC enzymes exhibited relatively condensed fold change levels (0.25 to 1.5) indicative of strong basal expression of these genes. Rather than being turned on or off, these genes are constitutively expressed and ethanol treatment serves to only slightly modulate their expression.

Further research into the promoter region activity of these genes is needed to elucidate epigenetic explanations for the varying levels of gene expression with ethanol treatment. While this study only described hepatic gene expression levels, further studies must identify changes in the function and activity of these enzymes. Changes in expression level may not always correlate to increased or decreased intracellular activity or function.

Alcohol is known to alter the expression of key metabolic genes, most notably ones involved in the regulation of fatty acid metabolism. The modified balance of fatty acid β -oxidation with de-novo lipogenesis is one of the key distinguishing factors for the initiation of ALD. The expression of the gene responsible for shuttling long chain fatty acids into the mitochondria where they undergo β -oxidation, carnitine palmitoyl-transferase 1 alpha (CPT-1 α) was markedly decreased in the ethanol treated mice. Attenuated CPT-1 α production would theoretically shift the hepatocytes to a more lipogenic phenotype, increasing the amount of free fatty acids in the cell by blocking one of the main routes of metabolism. This result would match the expected phenotype of steatosis that is commonly witnessed in patients undergoing early stage symptoms of ALD.

In addition to CPT-1 α , the gene malonyl CoA decarboxylase is also significantly attenuated with alcohol treatment. Malonyl CoA decarboxylase takes a carboxyl group off

of malonyl CoA forming acetyl CoA which can then be utilized by the citric acid cycle to generate energy in the form of ATP. Malonyl CoA is primarily responsible for de-novo lipogenesis as it is an input and precursor for the synthesis of new fatty acids. When malonyl CoA decarboxylase is downregulated the cellular flux will be altered to promote a more lipogenic phenotype as there will be a buildup of malonyl CoA in the cell. It becomes clear how exactly hepatic steatosis can occur with ethanol treatment by witnessing the changes in expression level of these two genes. Decreased CPT-1 α will promote an impairment in fatty acid β -oxidation while attenuated malonyl CoA decarboxylase will promote more de-novo lipogenesis. These results provide mechanistic proof for the ability of the chronic plus binge ethanol feeding model to induce one of the hallmark phenotypes associated with ALD, hepatic steatosis.

Ethanol feeding is known to cause gut leakiness and gut permeability (39). Leakage of gut microbial products into systemic circulation promotes hepatitis and hepatic neutrophil infiltration (40-42). These are characteristics of ALD patients in the clinic and were successfully observed in the current study as evidenced by increased expression of the chemokine CXCL2 and hepatic IL-17 receptor A. Hepatocytes as well as resident immune cells in the liver such as Kupffer cells release chemokines in response to liver injury (43). Chemokine secretion can lead to inflammatory cell infiltration, angiogenesis, and hepatocyte activation and survival (44).

IL-17A is also extremely important in the progression of human ALD however the exact mechanism remains underexplored. IL-17A is produced by numerous cell types but most notably peripheral blood mononuclear cells (PBMCs) in response to an inflammatory signal (45). The leakage of pro-inflammatory products from the gut such as bacterial

endotoxin is a primary driver of hepatic inflammation. The study indicates there is an increase in IL-17 receptor A expression in the liver in response to alcohol treatment indicating that resident liver cells are more primed to respond to a pro-inflammatory signal. Future studies must examine each individual cell type both in the liver and in systemic circulation to outline exact ways by which IL-17A and other inflammatory signals are modulated in terms of gene expression. Even deeper studies into the epigenetic regulation of these genes will be extremely valuable to identify druggable targets and therapeutic potentialities.

Increased immune cell infiltration to the liver as evidenced by upregulation of PMN and NKT cell populations. NKT cells are essential to hepatic neutrophil recruitment. These cells migrate to the liver in response to injury and secrete many proinflammatory cytokines and chemokines. One study documented how animals deficient in NKT cell populations were able to resist chronic plus binge alcohol induced hepatic recruitment of neutrophils (46). PMN infiltration to the liver is direct evidence of the neutrophilia indicated by upregulated CXCL2.

Results demonstrated that in the chronic plus binge ethanol feeding model there is a global increase in hepatic histone acetylation. Ethanol feeding causes this phenotype in potentially two ways. Primarily, ethanol feeding as demonstrated earlier in the binge ethanol feeding model can decrease overall HDAC histone deacetylating function by affecting the gene expression of multiple HDAC (36). Additionally, ethanol via its metabolism, can increase the levels of acetyl CoA, a key substrate for HATs enhancing their histone acetylating function (47). Taken together ethanol can affect the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs), shifting

the balance more towards histone acetylation causing a global increase in the status of histone H3 acetylation. These data also suggest that ethanol can alter the epigenetic regulation of multiple genes by influencing histone acetylation patterns at their respective promoter regions. It is noteworthy that the global increase observed in histone acetylation, may or may not complement histone modifications occurring regionally, at the gene-specific level (5). Accordingly, ethanol-induced changes in gene promoter histone acetylation status and accompanying transcriptional activity has to be assessed specifically for each relevant gene under consideration.

There is direct evidence from earlier studies that HDAC inhibitors (tributylin) can serve to modulate the promoter region of a gene such as CPT-1 α to make it more permissive to transcription (5). This is accomplished by preserving acetylation at the CPT-1 α promoter. HDAC inhibitors can potentially be employed as pharmacologic interventions to rescue alcohol induced attenuation of beneficial genes. Earlier we have demonstrated that ethanol feeding decreases levels of two genes (CPT-1 α and Malonyl CoA Decarboxylase) responsible for inhibiting hepatic steatosis. Here we demonstrate that another HDAC inhibitor, Trichostatin A (TSA), can rescue expression levels of these genes in primary mouse hepatocytes. Mechanistically, it is believed that TSA is directly interacting with the promoter regions of these genes to increase transcriptional permissivity. Future chromatin analysis will be useful for outlining underlying mechanisms behind this observed phenotype.

The HDAC inhibitor Trichostatin A was also shown to ameliorate alcohol induced ER stress in primary mouse hepatocytes cultured for 48 hours with alcohol. It is widely accepted that alcohol does induce ER stress, however the exact mechanisms have not been

completely elucidated. Some have attributed ER stress due to toxic metabolites produced by ethanol metabolism such as acrolein (48). It is likely that HDAC proteins play an important role in the induction of ER stress. Potential mechanisms include the HDAC mediated preservation of acetyl moieties on lysine residues of transcription factors such as CHOP and ATF-3. By deacetylating proteins, HDAC enzymes may expose lysine residues to ubiquitination which ultimately destines the protein for degradation. Attenuation of HDAC by inhibitors such as TSA may ameliorate alcohol induced ER stress.

Detailed mechanisms by which HDAC mediate changes in physiology must be outlined in order to truly benefit the discipline. Techniques such as chromatin immunoprecipitation (ChIP), co-immunoprecipitation, and gain of function/loss of function studies may be crucial to identifying the target substrates for each individual HDAC and how they may influence intracellular molecular cascades. There is clear evidence that HDAC are altered under alcohol feeding conditions. The pursuit of therapies for ALD should investigate HDAC enzymes and their substrates as potential leads for clinical investigation. More detailed explanations of molecular mechanisms by which HDACs influence ALD pathology is surely needed to advance the field.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Alcohol abuse continues to be a major worldwide public health concern and there is a pressing need for relevant pharmaceuticals in the market to treat Alcohol-Associated Liver Disease (ALD). The possibility of targeting Histone Deacetylases (HDAC) has shown promise as a therapeutic avenue in ALD intervention. The current study examined the changes in hepatic gene expression of all 18 HDAC in mice subjected to the chronic plus binge ethanol feeding model.

Results showed decreased expression of the genes HDAC 1,7,9,10,11 and SIRT 3,4,5,7 in alcohol fed mice. Genes related to fatty acid metabolism such as CPT-1 α and malonyl CoA decarboxylase also showed significant attenuation with ethanol treatment. The expression levels of these genes were rescued with administration of the HDAC inhibitor Trichostatin A (TSA) in cultured primary hepatocytes.

In addition, chronic plus binge feeding upregulated pro-inflammatory genes such as the chemokine CXCL2 as well as IL-17 receptor A. Also, an increase in immune cell infiltration was observed as measured by increased PMN and NKT cell populations in the liver. Alcohol was also found to induce ER stress as evidenced by upregulated CHOP, ATF-3, and ATF-4. TSA was exhibited to ameliorate ethanol induced upregulation of CHOP and ATF-3.

The etiology of ALD may originate from dysregulation of HDAC proteins. Ethanol feeding clearly attenuates the gene expression of multiple HDAC proteins in addition to increasing global histone acetylation. Future studies should aim to elucidate molecular mechanisms between HDAC and liver diseases that originate from the gut or systemic circulation as HDAC serve as promising targets for future therapies.

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CURRICULUM VITAE

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Education

University of Louisville School of Medicine, Louisville, KY

2019 - Present

Department of Pharmacology and Toxicology

Graduate Research Assistant (MS/PhD)

Cumulative GPA 3.77/4.0

Duke University, Durham NC

2008-2012

B.S. Biology (Major)

A.B. History (Major)

GPA: 3.37/4.0

DuPont Manual High School, Louisville KY

2004-2008

Math, Science, & Technology Magnet

GPA: 3.86/4.0

13 Advanced Placement Courses

National AP Scholar

Experience

Research Technologist

October 2018 – July 2019

University of Louisville School of Medicine

Department of Gastroenterology, Hepatology, & Nutrition

Responsibilities: Performed Cell Culture Experiments on colon cancer cells treated with Urolithin A and other synthetic derivatives and assessed levels of epithelial tight junction proteins using Western Blotting. Participated in Large Fecal Matter Transfer Studies (FMT) where stool samples from alcoholic hepatitis patients were transferred to mice in combination with chronic alcohol feeding. The subsequent effects on gut microbiota composition, system endotoxemia, as well as hepatic and neuroinflammation are currently being studied.

Research Technologist

October 2017 – September 2018

University of Louisville School of Medicine

Department of Medicine

Responsibilities: genotyping, mouse colony maintenance, laboratory maintenance

NCI R25 Cancer Research Intern

May 2013 – August 2013

University of Louisville School of Medicine

Department of Pharmacology and Toxicology

Responsibilities: Pharmacogenomics work in the Hein Laboratory examining the toxicity of the anti-tuberculosis drug Isoniazid on human hepatocytes with variable NAT2 acetylator genotypes

Awards and Honors

1. IPIBS Fellow 2019
2. 3rd Place in Research! Louisville Undergraduate Poster Competition 2013
3. National AP Scholar, DuPont Manual High School 2008
4. Eagle Scout, Troop 317, Christ Church United Methodist 2008
5. Royal Conservatory of Music Level 7 Piano Examination with Honors, 2005

Scientific Outreach

1. Hillandale Elementary Science Club, Durham, NC

Publications

Peer-Reviewed Publications

1. Doll, M.A.; Salazar-Gonzalez, R.A.; **Bodduluri, S.**; Hein, D.W. Arylamine N-acetyltransferase 2 genotype-dependent N-acetylation of isoniazid in cryopreserved human hepatocytes. *Acta Pharmaceutica Sinica B*, 2013; Jul;7(4):517-522. Doi: 10.1016/j.apsb.2017.05.003

Presentations (Abstracts)

1. Barker, David F.; **Bodduluri, Srineil**; Walraven, Jason M.; Hein, David W. 5' RACE Analysis of Rat N-acetyltransferase Genes, Nat1 and Nat2, Reveals Utilization of Conserved Promoters and mRNA Splicing Patterns. Research! Louisville 2009
2. **Bodduluri, Srineil**; Doll Mark A.; Salazar-Gonzalez, Raul A.; Hein, David W. Acetylase Genotype Dependent N-acetylation of Isoniazid in Human Hepatocytes In Situ. Research! Louisville 2013

Manuscripts in Preparation

1. **Bodduluri, S**; Ghare, S; Joshi-Barve, S; McClain, CJ; Barve, SS. Recent Roles for Histone Deacetylases (HDACs) in Pathologies Associated with Alcohol Associated Liver Disease (ALD). *Manuscript in preparation.*