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Extracellular matrix proteins and the liver-lung axis in disease.

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EXTRACELLULAR MATRIX PROTEINS AND THE

LIVER-LUNG AXIS IN DISEASE

By

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DEDICATION

This dissertation is dedicated to my parents

Dr. Janet Lynch

And

Dr. Evan Massey

for their constant encouragement and support

In all my endeavors.

ACKNOWLEDGEMNETS

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CHAPTER I

INTRODUCTION

A.   <u>Background and rationale for this study</u>

1. The extracellular matrix: more than a scaffold

The extracellular matrix (ECM) forms a complex microenvironment in the

extracellular space and is critical for development, tissue structure, and cellular

response. The ECM consists of a myriad of proteins, glycoproteins, and

proteoglycans that form a cross-linked scaffold that surrounds cells in all solid

organs (1). These fibrous proteins are produced by cells (i.e., fibroblasts) and,

upon secretion into the extracellular environment, crosslink together to create a

meshwork that serves as a scaffold for cells and that provides structure to tissue.

The collagen scaffold to which glycoproteins and proteoglycans adhere is the

main characteristic shared by the ECM of the basement membranes and

interstitial matrices, regardless of physiological domain (1). Physical changes to

the extracellular landscape, including changes in ECM topography, crosslinking,

and biochemistry, can affect cell migration, adhesion, and activation (2). In

addition to their role as structural compounds, these proteins can also serve as

signaling molecules. Indeed, ECM proteins are ligands of cell surface receptors,

including integrins, which bind specific ECM matrix protein domains (e.g., the

RGD motif). The ECM also contributes to cell signaling by binding soluble

signaling molecules including cytokines, chemokines, and growth factors to create signaling gradients required for tissue development and migration of inflammatory cells during acute inflammation (3).

Proteases and protease inhibitors comprise another group of proteins critical in the extracellular space. These enzymes play an important role in the homeostasis of the extracellular environment and contribute to ECM remodeling in disease states (4). Proteases may enhance the deposition of proteins to the ECM (e.g., proteases involved in the complement and coagulation cascades). Proteases can also break down matrix components to allow for rapid changes in the ECM organization, such as matrix metalloproteinases (MMPS), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), and other serine proteases. The effects of ECM degradation by proteases modify both the physical and biochemical environment of the extracellular space. For example, physical dissolution of the ECM by proteases can create space through which cells can migrate (5). Alternatively, cleavage of ECM proteins by proteases can alter cell-ECM interactions by exposing/removing cryptic binding sites; for example, proteolysis of laminin-5 reveals integrin-binding sites which are critical for cell adhesion (6). Proteases can also contribute to ECM dynamics by modulating the activity of other enzymes (e.g., proteinase precursors (7) and soluble mediators (e.g., IL-8, (2)). Because of the wide-reaching effects of proteases, their activity is regulated and maintained via complex, often redundant, mechanisms. Indeed, in addition to regulation of rates of synthesis and secretion, extracellular proteases frequently require activation, often via

cleavage by another protease, and conversely can be inhibited by endogenous inhibitors such as tissue inhibitors of matrix metalloproteinases (TIMPs) (5).

2. The role of the ECM in inflammation and disease.

Tight regulation of the ECM composition is necessary for normal tissue homeostasis, and dysregulation is often a hallmark in tissue pathology. Therefore, the organization, composition, and orientation of the ECM are controlled via multiple mechanisms including protein synthesis, modification, excretion, crosslinking, and enzymatic degradation. Such tight regulation on so many levels also permits rapid changes in the ECM that allow it to play a dynamic and responsive role during inflammation and tissue repair. Perturbations in the ECM during either of these physiological states can contribute to disease initiation and development.

During an acute inflammatory response, the ECM plays a pivotal role by serving as a structural barrier and chemokine reservoir, thereby regulating inflammatory cell migration. Indeed, studies have shown that neutrophil transmigration is greater through vascular areas with lower ECM concentration (8). Additionally, ECM binding of cytokines, including IL-2, TNFα, TGFβ, and RANTES (9-11), have been shown to regulate cell activation, adhesion, and migration. The interaction between the ECM and cell infiltration is bidirectional - as leukocytes integrate structural and biochemical signals from the ECM, they in turn release matrix-degrading proteases (12) which alter the extracellular composition and allow for easier cell migration.

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In the setting of chronic inflammation, dysregulation of ECM composition can lead to pathological tissue remodeling and, if not resolved, disease states including hepatic (13, 14) and pulmonary fibrosis (15-17), atherosclerosis (18, 19) and cancer (20, 21). Initiation of these disease states can occur via dysregulation at the level of ECM synthesis and/or degradation, leading to altered matrix accumulation and modification. For example hepatic fibrosis is characterized by a robust increase in collagen Iα1 secretion by hepatic stellate cells (HSCs) (22) combined with a concomitant increase in crosslinking and a decrease in enzymatic degradation (23). Such dynamic changes in extracellular composition can initiate and perpetuate disease via a variety of mechanisms including altered tissue biomechanics (i.e. tissue stiffness) (24, 25), increased integrin-mediated cell adhesion (26) and activation of immune cells (27).

3. Statement of goals

The role of tissue remodeling in advanced stages of liver disease, including fibrosis, is clear. Indeed, the major macroscopic change in hepatic fibrogenesis is characterized by severe collagen deposition and scarring. Currently, hepatic fibrosis is generally considered irreversible, and there is currently no FDA-approved therapy to halt or reverse liver fibrosis. Recent studies have shown that transitional tissue remodeling may occur during inflammatory liver disease, before the development of fibrosis (28). Additionally, protecting against these subtle changes can protect against liver injury and inflammation (29). These findings suggest that targeting the ECM in earlier stages of liver disease could

provide therapeutic benefit by preventing disease progression to later, irreversible stages of fibrosis. The goal of this dissertation is to examine the role of transitional tissue remodeling in fatty liver disease. Furthermore, it aims to identify new extracellular matrix proteins that play a role in early stages of remodeling which could serve as novel therapeutic targets.

4. Natural history of liver disease

The natural history of liver disease is well documented and encompasses a spectrum of disease states including earlier stages of fatty liver disease such as steatosis, steatohepatitis, and later stages of disease including fibrosis and cirrhosis (30, 31). The earliest stage of liver disease, simple steatosis, is characterized by micro- and macro-vesicular fat accumulation and is generally considered reversible if appropriate changes to the lifestyle are made and maintained. In the absence of lifestyle modifications, individuals may develop steatohepatitis, which is characterized by persistence of accumulated fat in addition to chronic hepatic inflammation (hepatitis). Later stages of liver disease, including fibrosis and cirrhosis, are characterized by collagen deposition and regenerative nodules, respectively (31, 32).

Despite the vast amount of money spent on treating alcoholic liver disease, therapeutic options remain limited to palliative care in the late stages of disease. Currently there is no FDA-approved therapy in existence to halt or reverse disease progression. If a rational, targeted therapy is to be developed, currently

accepted mechanisms of disease progression must be better understood and new therapeutic targets identified.

5. Alcoholic liver disease

Alcohol is one of the most commonly recognized causes of liver disease. Alcoholic liver disease (ALD) affects more than 10 million Americans each year at a cost of more than $166 billion annually to treat the medical consequences of this disease (33). After absorption from the small intestine, milli-molar concentrations of ethanol flow through the portal circulation and to the liver for degradation making the liver a prime target of ethanol toxicity. During oxidative metabolism of alcohol in hepatocytes by alcohol dehydrogenase (ADH) and CYP2E1, a robust increase in the NADH/NAD+ ratio lends to a more reduced cellular environment. This reduced microenvironment blocks the beta-oxidation of fatty acids in the liver and increases the esterification of fatty acids. Accumulation of excess fatty acids in the liver leads to storage of these lipids in lipid droplets known as steatosis, which is a hallmark of early liver disease.

Steatosis develops in nearly all individuals who consume moderate amounts of alcohol, and for most people has no health consequences. Indeed, at the organismal level, hepatic steatosis may be viewed as a protective mechanism, as it restricts plasma lipid levels from rising. However chronic alcohol consumption can lead to development of later stages of liver disease, including alcoholic steatohepatitis (ASH) as well as fibrosis and cirrhosis. These more severe stages of ALD develop in only a minority of chronic alcoholics. One

explanation for this discrepancy is the 2-hit hypothesis, which suggests that progression from simple steatosis to later stages of liver disease requires multiple insults, or 'hits' (34, 35). According to this paradigm, a first 'hit,' such as hepatic steatosis, sensitizes the liver to a second hit such as oxidative stress (36) or inflammatory cytokine signaling (37), both of which are also increased by ethanol consumption.

6. Non-alcoholic fatty liver disease (NAFLD)

Another common cause of fatty liver disease is obesity. Obesity is a growing problem both nationally and globally, with statistics estimated that more than 78 million U.S adults are clinically obese (38). Obesity is associated with a myriad of health effects including metabolic syndrome, cardiovascular risks, and non-alcoholic fatty liver disease (NAFLD). It has been difficult to estimate the prevalence of NAFLD within populations and figures range depending on the cohort studied, however current estimates indicate that up to 19% of the American population may have some degree of NAFLD (39), with the highest prevalence of NAFLD among the morbidly obese (40). Interestingly, despite a high prevalence of steatosis among obese individuals, it is estimated that less one third of patients with NAFLD will develop nonalcoholic steatohepatitis (NASH) (40-42), the more severe inflammatory stage of the disease. Increased susceptibility to disease progression in some individuals is likely due to a variety of factors, including genetic predisposition, and environmental exposures (43).

Similar to ALD, it is hypothesized that multiple hits are required for the development of later stages of NAFLD (34, 43).

Our lab is particularly interested in the enhanced susceptibility to liver injury caused by low levels of arsenic in the setting of NAFLD. Arsenic is a natural contaminant of the drinking water and at very high concentrations has been shown to cause overt liver damage (44, 45). Arsenic levels found in the United States are generally considered too low to cause toxicity, however even low levels of arsenic exposure have been linked to subtle hepatic changes including capillarization of sinusoidal endothelial cells, a type of remodeling response in the liver (46). Moreover, low levels of arsenic have also been shown to sensitize the liver to inflammatory insult (47). A previous study from our group has implicated an interaction between HFD and subhepatotoxic arsenic exposure, the combination of which caused enhanced liver injury. These data support the hypothesis that HFD may sensitize the liver to otherwise innocuous toxins, including arsenic, resulting in progression of liver disease. As will be demonstrated in this work, a key effect of arsenic appears to be to enhance the deposition of fibrin ECM in response to HFD.

7. ECM in fatty liver disease

As mentioned above, the major part of the study of the role of the ECM in liver disease has focused on the robust increase in collagen deposition which is a hallmark of hepatic fibrosis. However, other studies have shown that other ECM components, including fibronectin, elastin, laminin and hyaluron, are also altered

during hepatic fibrogenesis (48). Global changes in the distribution and concentration of many different ECM components likely contribute to pathological tissue remodeling and the progression of fibrogenesis.

Recent studies suggest that transitional tissue remodeling may occur before fibrosis. For example, Gillis et al showed that ethanol exposure increases the secretion of fibronectin, a structural component of the ECM, by hepatic stellate cells (HSCs) prior to their activation (28). Since activated HSCs play a role in fibrogenesis, these results suggesting that fibronectin secretion is increased prior to fibrogenesis. This group has also shown that enhanced LPS-induced fibrin accumulation caused by acute alcohol exposure is critical to liver injury and inflammation (29, 49, 50). It is likely that there are changes in other ECM proteins contributing to hepatic inflammation. In support of this possibility, a proteomic approach used by Lai et al showed that a myriad of ECM-associated proteins are dysregulated during hepatocarcinogenesis (51) Applying this proteomics technique to changes in the hepatic ECM proteome could identify potential players in transitional tissue remodeling in early stages of fatty liver disease.

8. Alcoholic lung phenotype

It has been estimated that up to 190,000 people suffer from acute lung injury (ALI) annually in the United States (52). The risk of developing more severe forms of ALI such as acute respiratory distress syndrome (ARDS), is significantly higher in chronic alcoholics (53). Furthermore, a history of chronic alcohol

consumption has been shown to increase the risk of mortality in ARDS patients (54). Subsequent rodent studies investigating the possible mechanisms underlying the detrimental effects of alcohol on the lung indicate that chronic alcohol may cause a myriad of pulmonary changes, coined the 'alcoholic lung phenotype,' including increased pro-inflammatory cytokine release (54-57), induction of oxidative stress (58), and tissue remodeling (59, 60). The dysregulated inflammatory response caused by alcohol is dependent on both the amount and duration of alcohol consumption and is suspected to be a key player in alcohol-related lung dysfunction (61). The exact mechanisms that result in the 'alcoholic lung phenotype' remain unclear, but, if identified, would point to potential targets for intervention in alcohol-related lung dysfunction.

9. Parallel mechanisms of injury in the liver and lung

Currently, the effects of chronic ethanol exposure on the liver and lung are considered to be independent of each other and thus are often studied separately. However, independent studies investigating the effects of ethanol on the liver and lung individually have revealed that similar mechanisms are at play in each organ and, more specifically, that these organs have similar responses to alcohol exposure. For example, alcohol enhances LPS-induced organ damage in both the liver and lung (58, 62). Additionally, oxidative stress, a well-known mediator of alcohol-induced liver injury (63) is also involved in increased susceptibility to lung injury caused by alcohol (64). Furthermore, both organs possess resident macrophages that can play a critical role in dysregulated

inflammation after alcohol exposure (65) and both exhibit subtle changes in the composition of the extracellular matrix (ECM) following alcohol exposure. The deleterious effects of tissue remodeling are well understood with regards to hepatic (66) and pulmonary fibrosis (67), however recent work from both this lab and Roman's group have implicated more subtle ECM changes to be a key step in disease progression in the liver and lung, respectively. Specifically, the Roman lab has shown that chronic alcohol exposure increases pulmonary fibronectin deposition via alterations in both expression and degradation of ECM components (68). Similarly, our lab has shown that ethanol enhances increased hepatic fibrin deposition caused by LPS and that subsequent inflammatory damage to the liver is a direct effect of fibrin accumulation (29, 50, 69-71).

10. The gut-liver-lung axis

Chronic alcohol abuse can act as a first 'hit' that leads to alterations in tissue architecture without causing detectible organ damage. This alcoholic first 'hit' can render the host susceptible to injury in the presence of a second stimulus, such as endotoxemia. Circulating levels of endotoxin (i.e. LPS) are increased in chronic alcoholics due to changes in intestinal permeability caused by alcohol exposure. This increase in circulating endotoxin appears to be a common second 'hit' that drives progression of liver and lung injury. While the role of the gut:liver axis in alcohol induced liver disease is now well-established (72, 73), we propose that organ-organ interactions after alcohol exposure are not limited to the gut:liver axis but may also include the lung. For example, more than 30

years ago one cohort study showed that end-stage liver disease increased

mortality associated with ARDS from 40-60% to 100% (74), suggesting that liver

injury may directly impact susceptibility to lung injury. Other studies investigating

the multi-organ effects of endotoxemia indicate that interaction between the liver

and lung is not only likely, but may occur via LPS-induced mediators (75)

including TNFα (76), an inflammatory cytokine markedly increased in both end-

stage liver patients (77) and experimental models of liver injury. The role of

TNFα in pulmonary injury is also well established (55). As mentioned above, the

effects of chronic ethanol exposure on the liver and lung have most often been

studied independently. In order to study the possibility of interdependence

between these two organs, a model of concomitant liver and lung injury must be

established. Once established, additional studies investigating possible links

between liver and lung injury will allow a better understanding of inter-organ

interactions in this model.

B. <u>Aims and proposals</u>

1. Protective effects of prebiotics in the setting of non-alcoholic liver disease

Our lab has already shown that arsenic can sensitize the liver to non-alcoholic

fatty liver disease caused by a high fat diet.   In this model, enhanced damage

correlated with an increase in hepatic inflammation and in sinusoidal fibrin

deposition in the liver. Hepatic inflammation and injury caused by a HFD is

mediated by multiple mechanisms, including gut dysbiosis. Indeed, HFD feeding

increases gut leakiness, causing endotoxemia and systemic inflammation (78)

and liver steatosis (79). Protecting against endotoxemia by altering the microbiota caused by HFD exposure can also protect against inflammation and metabolic syndrome in mice (80). Furthermore, the benefits of pre- and probiotics on liver injury have been established in models of alcoholic and non-alcoholic liver disease (81-83). Enhanced liver injury caused by the combination of HFD and arsenic in this model may be mediated by interactions between HFD and arsenic on the gut microbiome. Therefore the goals of this study were to 1) determine the protective effects of oligofructose (OFC) and 2) to identify interactions between HFD, arsenic, and OFC on the gut microbiome in a model of enhanced liver injury caused by concomitant exposure to HFD and arsenic. Importantly, fibrin ECM deposition may play a key role in this response.

2. Characterization of a model of concomitant liver and lung injury

A number of studies independently investigating the liver and lung response to chronic alcohol exposure have indicated that similar mechanisms contribute to alcohol-induced changes in these two organs including oxidative stress, dysregulated inflammatory response, and tissue remodeling. An important function of the liver is as a host-defense system; thus liver injury and the resultant reduction in hepatic function could enhance susceptibility of other organs to injury, including the lungs. For example, mortality due to ARDS was increased to nearly 100% in individuals with end-stage liver disease (74). This as well as work by Siore et al (84) have indirectly suggested that liver injury and/or hepatic-derived mediators may drive injury in the lung. One potential link

between liver and lung injury is the pro-inflammatory cytokine TNFα. TNFα

levels in the systemic circulation are increased after alcohol exposure in both

experimental models of alcohol exposure (85, 86) and in patients with ALD (77).

The resident hepatic macrophages, Kupffer cells, are known to be a major

source of pro-inflammatory mediators into the systemic blood (65, 87) and likely

contribute to the robust increase in pro-inflammatory signaling after alcohol

exposure. Moreover, TNFα has also been linked to pulmonary injury in

experimental models (88, 89). Therefore, the purpose of this study was to

characterize a model of concomitant liver and lung injury and to investigate the

potential role of TNFα as link between liver and lung injury after chronic alcohol

exposure. These studies lay the groundwork for future studies on the interaction

of the role of the liver in lung ECM remodeling in the 'alcoholic lung phenotype.'

3. Can we characterize the hepatic extracellular proteome?

As mentioned above, the role of the ECM in later stages of liver disease (i.e.,

fibrosis) is well recognized. Previous studies from this lab and other groups (28,

90) have shown that changes in the ECM also occur in early pre-fibrotic stages of

liver disease. These transitional changes in the ECM appear to play a critical

role in the sensitization of the liver to insult, particularly in the context of fatty

liver. While several matrix proteins have been implicated as players in liver injury

and inflammation (i.e., fibrin, fibronectin) there are likely other ECM proteins that

also contribute to hepatic injury. Identification of new ECM proteins that are

altered by ethanol exposure would provide new insights into mechanisms of

hepatic tissue remodeling that contribute to progression of ALD. There are likely

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changes in the hepatic ECM that have not yet been identified due to the insolubility of matrix proteins making them difficult to extract and analyze. Additionally, the study of alterations to the hepatic ECM has primarily been hypothesis-driven which can hamper the discovery of new potential players. The purpose of this study was to use a serial extraction technique which would allow extraction and enrichment of tightly cross-linked proteins coupled with LC-MS analysis. When paired together, these methods will allow extraction of proteins with low expression levels and poor solubility and analysis of global changes in the hepatic ECM.

Overall aim of this dissertation.

The overall aim of this dissertation is to build on the unifying hypothesis that ECM dyshomeostasis contributes to hepatic pathology, as well as to pathology in other organs, such as the lung. Indeed, as described above, the enhancement of liver injury caused by arsenic exposure in the context of HFD (Aim 1) is characterized by a robust increase in fibrin deposition; the intervention strategies that protect against this pathology will shed new insight into future studies. The development of a new model to characterize the interaction between liver and lung in the alcoholic lung phenotype (Aim 2), coupled with the new ECM proteomic strategies (Aim 3), lay the foundation for testing the hypothesis that liver injury is critical in transitional ECM remodeling that occurs with alcohol exposure (i.e., the 'alcoholic lung phenotype'). Taken together, this work will

shed new mechanistic insight into the complex interplay between injury, matrix

dyshomeostasis and disease progression.

CHAPTER II

EXPERIMENTAL PROCEDURES

A. **Animals and treatments**

Mice were housed in a pathogen-free barrier facility accredited by the

Association for Assessment and Accreditation of Laboratory Animal Care, and

procedures were approved by the University of Louisville's Institutional Animal

Care and Use Committee.

At time of sacrifice, animals were anesthetized with ketamine/xylazine

(100/15 mg/kg, i.p.). Blood was collected from the vena cava just prior to

sacrifice by exsanguination and citrated plasma was stored for further analysis.

Prior to lavage of the lungs, 10 mL of PBS was perfused through the heart to

flush the pulmonary vasculature of erythrocytes. Bronchioalveolar lavage (BAL)

was performed by flushing the lung two times with 400 µL sterile PBS. Cells in

the BAL fluid (BALF) were separated by centrifugation and removed from

remaining BALF and fixed on slides for further analysis (91). Portions of liver

tissue and lung tissue were snap-frozen in liquid nitrogen for later analysis or

fixed in 10% neutral buffered formalin for subsequent sectioning and mounting on

microscope slides. A portion of liver tissue was frozen-fixed in Tissue Tek OCT-

Compound (Satura Finetek, Torrance, CA). Total RNA was immediately

extracted from fresh liver and lung tissue using RNA-stat (Tel-Test, Austin, TX) and chloroform: phenol separation (see RNA isolation Methods).

1. Arsenic, high fat diet, and oligofructose feeding

Four week old male C57Bl6/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Food and tap water were allowed ad libitum. Mice were fed AIN-76 Purified diet (Harlan Laboratories) for one week to reduce potential confounding factors of arsenic present in standard laboratory chow (92). Mice were exposed to sodium arsenite (4.9 ppm in drinking water) or tap water for one week prior to initiating feeding with either commercially available experimental diets (Table 2.1; Harlan Laboratories, Madison WI) including a low fat diet (LFD; 13% fat in calories), a high fat diet (HFD; 42% fat in calories), or a HFD containing the prebiotic oligofructose (5% w/w OFC) for 10 weeks. This exposure level of arsenic was determined by preliminary range-finding experiments to cause no overt liver damage in mice fed low-fat diet. Food and water consumption were measured twice a week. Body weight was measured once a week

2. Chronic model of alcohol exposure

Eight week old male C57BL6/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and exposed to either ethanol-containing Lieber-DeCarli diet (Dyets, Inc.) or iso-caloric control diet. During the exposure period, animals were housed in pairs in shoebox cages in a room held at 75 ºF. Diet was provided in vacuum tubes and replaced between 4 and 5 pm daily. Both ethanol-fed animals and their pair-fed counterparts received control diet for the

Table 2.1. Diet Composition

	Low Fat Diet		High Fat Diet	
	% kCal	Source (% by weight)	% kCal	Source (% by weight)
Protein	19.2	Casein	15.2	Casein
Carbohydrate	67.9	Sucrose/Corn starch	42.7	Sucrose/Corn starch
Fat	13.0	Milkfat (3.7%)/ soybean oil (1.3%)	42.0	Milkfat (21.2%)
kCal/g	3.6		4.5	

first two days of liquid diet feeding to allow acclimation to the liquid diet feeders. After 2 days of acclimation, ethanol concentrations increased incrementally over the course of three weeks before reaching the highest ethanol concentration, 6% (vol/vol) for the final three weeks of exposure. Ethanol concentrations in the ethanol-containing diets were as follows: 0% for two days of acclimation, 1% for two days, 2% for two days, 4% for one week, 5% for one week, and 6% for three weeks. Ethanol-containing diet was provided *ad libitum* for the entire course of the study. Because of the relatively high caloric content of ethanol, pair-fed control animals received an iso-caloric control diet; the calories in the iso-caloric diet were matched by adding a calorie-equivalent of maltose-dextrin. To account for the reduced food consumption of ethanol-fed mice, pair-fed mice were given the volume of diet consumed by their ethanol-fed counterparts the night before. At the conclusion of the feeding period, the two diet groups were further separated into additional groups that received either LPS (*E. coli*; 10 mg/kg i.p Sigma, St. Louis, MO) or vehicle (saline). Animals were euthanized 4 or 24 h after LPS (or vehicle) injection.

a. Etanercept administration

Etanercept (Etan; Wyeth) is an FDA-approved TNFα blocker. Etanercept is a fusion protein of the human p75 TNF receptor attached to the Fc portion of human IgG1. In this study, some animals were administered etanercept (10 mg/kg in PBS) by i.p. injection two times per week during ethanol administration, as described by others (93, 94). Control animals received PBS vehicle, (1 μl/g; i.p) twice per week.

B. Proteomics

1. 3-step ECM extraction (95-97)

Sample preparation and wash. Snap frozen liver tissue (75-100 mg) was
immediately added to ice-cold phosphate-buffered saline (pH 7.4) wash buffer
containing commercially available protease and phosphatase inhibitors (Sigma
Aldrich) and 25 mM EDTA to inhibit proteinase and metalloproteinase activity,
respectively. While immersed in wash buffer, liver tissue was diced into small
fragments using a scalpel. The diced sample was washed 5 times to remove
contaminants. Between washes, samples were pelleted by centrifugation at
10,000×g for 5 min and wash buffer was decanted. NaCl extraction. Diced
samples were incubated in 10 volumes of 0.5 M NaCl buffer, containing 10 mM
Tris HCl (pH 7.5), proteinase/phosphatase inhibitors, and 25 mM EDTA. The
samples were mildly mixed on a plate shaker (800 rpm) overnight at room
temperature. The following day, the remaining tissue pieces were pelleted by
centrifugation at 10,000×g for 10 min. The supernatant was saved and labeled
as the NaCl fraction. SDS extraction. The pellet from the NaCl extraction was
subsequently incubated in 10 volumes (based on original weight) of a 1% SDS
solution, containing proteinase/phosphatase inhibitors and 25 mM EDTA. The
samples were mildly mixed on a plate shaker (800 rpm) overnight at room
temperature. The following day, the remaining tissue pieces were pelleted by
centrifugation at 10,000×g for 10 min. The supernatant was saved and labeled
as the SDS extract. Guanidine HCl extraction. The pellet from the SDS
extraction was incubated with 5 volumes (based on original weight) of a

denaturing guanidine buffer containing 4 M guanidine HCl (pH 5.8), 50 mM

sodium acetate, 25 mM EDTA, and proteinase/phosphatase inhibitors. The

samples were vigorously mixed on a plate shaker at 1200 rpm for 48 hours at

room temperature; vigorous shaking is necessary at this step to aid in the

mechanical disruption of ECM components. The remaining insoluble

components were pelleted by centrifugation at 10,000×g for 10 minutes. This

insoluble pellet was retained and solubilized as described below. The

supernatant was saved and labeled as the GnHCl fraction. Deglycosylation and

solubilization. The supernatants from each extraction were desalted using

ZebaSpin columns (Pierce) according to manufacturer's instructions. The

desalted extracts were then mixed with 5 volumes of 100% acetone and stored at

-20^{0}C overnight to precipitate proteins. The precipitated proteins were pelleted

by centrifugation at 16,000×g for 45 minutes. Acetone was evaporated by

vacuum drying in a RotoVap for 1 hour. Dried protein pellets were resuspended

in 500 uL deglycosylation buffer (150 mM NaCl, 50 mM sodium acetate, pH 6.8,

10 mM EDTA, and proteinase/phosphatase inhibitors) that contained

chondroitinase ABC (*P. vulgaris*; 0.025 U/sample), endo-beta-galactosidase (*B.

fragilis*; 0.01 U/sample) and heparitinase II (*F. heparinum*; 0.025 U/sample).

Samples were incubated overnight at 37^{0}C. Samples containing the pellet

remaining after the guanidine HCl step received 20 uL DMSO to aide in

solubilization. Protein concentrations were estimated by absorbance at 280 nm

using BSA in deglycosylation buffer for reference standards.

2. Sample cleanup and preparation for liquid chromatography

Pooled samples in deglycosylation buffer were provided to and subsequently analyzed by the University of Louisville Proteomics Biomarkers Discovery Core (PBDC). At the PBDC, samples in deglycosylation buffer were thawed to room temperature and clarified by centrifugation at 5,000xg at 4°C for 5 min. Samples were reduced by adding 5.55 µL of 1 M DTT to 50 µL (25 µg) of each sample and then incubating at 60°C for 30 min after which 144.45 µL of 8 M urea in 0.1 M Tris-HCl pH 8.5 was added to each sample. Each reduced and diluted sample was digested with a modified Filter-Aided Sample Preparation (FASP) method. Recovered material was dried in a SpeedVac and redissolved in 200 µL of 2% (v/v) acetonitrile (ACN)/0.4% formic acid (FA). The samples were then trap-cleaned with a C18 PROTO™ 300Å Ultra MicroSpin Column (The Nest Group, Southborough, MA). The sample eluates were stored at -80°C for 30 min, dried in a SpeedVac, and stored at -80°C until analysis. Before liquid chromatography, dried samples were warmed to room temperature and dissolved in 2% (v/v) ACN/0.1% FA to a final concentration of 0.25 µg/µL. For analysis 16 µL (4 µg) of sample was injected into the Orbitrap Elite.

3. Liquid Chromatography

Dionex Acclaim PepMap 100 75 µm x 2 cm nanoViper (C18, 3 µm, 100 Å) trap and Dionex Acclaim PepMap RSLC 50 µM x 15 cm nanoViper (C18, 2 µm, 100 Å) separating columns were used. An EASY n-LC (Thermo, Waltham, MA) UHPLC system was used with buffer A = 2% (v/v) acetonitrile / 0.1% (v/v) formic acid and buffer B = 80% (v/v) acetonitrile / 0.1% (v/v) formic acid as mobile

phases. Following injection of the sample onto the trap, separation was

accomplished with a 140 min linear gradient from 0% B to 50% B, followed by a

30 min linear gradient from 50% B to 95% B, and lastly a 10 min wash with 95%

B. A 40 mm stainless steel emitter (Thermo, Waltham, MA; P/N ES542) was

coupled to the outlet of the separating column. A Nanospray Flex source

(Thermo, Waltham, MA) was used to position the end of the emitter near the ion

transfer capillary of the mass spectrometer. The ion transfer capillary

temperature of the mass spectrometer was set at 225°C, and the spray voltage

was set at 1.6 kV.

4. Mass Spectroscopy

An Orbitrap Elite – ETD mass spectrometer (Thermo) was used to collect

data from the LC eluate. An Nth Order Double Play with ETD Decision Tree

method was created in Xcalibur v2.2. Scan event one of the method obtained an

FTMS MS1 scan for the range 300-2000 m/z. Scan event two obtained ITMS

MS2 scans on up to ten peaks that had a minimum signal threshold of 10,000

counts from scan event one. A decision tree was used to determine whether

collision induced dissociation (CID) or electron transfer dissociation (ETD)

activation was used. An ETD scan was triggered if any of the following held: an

ion had charge state 3 and m/z less than 650, an ion had charge state 4 and m/z

less than 900, an ion had charge state 5 and m/z less than 950, or an ion had

charge state greater than 5; a CID scan was triggered in all other cases. The

lock mass option was enabled (0% lock mass abundance) using the 371.101236

m/z polysiloxane peak as an internal calibrant.

5. Data Analysis

Proteome Discoverer v1.4.0.288 was used to analyze the data collected by the mass spectrometer. The database used in Mascot v2.4 and SequestHT searches was the 6/2/2014 version of the UniprotKB *Mus musculus* reference proteome canonical and isoform sequences. In order to estimate the false discovery rate, a Target Decoy PSM Validator node was included in the Proteome Discoverer workflow.

The Proteome Discoverer analysis workflow allows for extraction of MS2 scan data from the Xcalibur RAW file, separate searches of CID and ETD MS2 scans in Mascot and Sequest, and collection of the results into a single file (.msf extension). The resulting .msf files from Proteome Discoverer were loaded into Scaffold Q+S v4.3.2. Scaffold was used to calculate the false discovery rate using the Peptide and Protein Prophet algorithms. The results were annotated with mouse gene ontology information from the Gene Ontology Annotations Database

C. <u>Clinical chemistry</u>

Levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically using standard kits (Thermotrace, Melbourne, Australia).

D. <u>Hepatic lipid measurements</u>

Total lipid was extracted from mouse livers as previously described (98, 99). Briefly, mouse liver (25-50 mg) was pulverized in a liquid-nitrogen cooled mortar and pestle. Pulverized liver tissue was incubated in a methanol:chloroform (2:1)

mixture on ice for thirty minutes. At the end of incubation, extracts were centrifuged at 900 x g for 10 min at 15°C. After centrifugation, lipids are partitioned into the chloroform (bottom) layer. This bottom layer was removed using a glass syringe and dried in an evaporating centrifuge. The resulting pellet was resuspended 5% fatty acid-free bovine serum albumin then frozen at -80°C overnight. Prior to analysis, samples were vortexed then sonicated for 10 minutes at 37°C in a bath sonicator. Hepatic triglyceride (Thermo Scientific, Middletown, VA) non-esterified fatty acids (Sigma Aldrich, St. Louis MO) were determined using standard kits. Values were normalized to initial liver tissue weight.

E. Histology

1. General morphology

Formalin fixed, paraffin embedded liver and lung tissue were cut at 5 µm and mounted on charged glass slides. Sections were deparaffinized with Citrisolv (Fisher, Waltham, MA) and rehydrated through graded ethanol. Sections were then stained with hematoxylin and eosin (H&E). After staining, samples were dehydrated through graded alcohol, washed in Citrisolv and then mounted with Permount (Fisher, Waltham, MA).

2. Neutrophil accumulation

Chloroacetate esterase (CAE) staining for neutrophils was performed using the naphthol AS-D chloroacetate esterase kit (Sigma, St. Louis, MO) on paraffin embedded sections (5 µm). For blinded quantitation, samples were de-identified and 10 fields were photographed at 400x magnification and saved. For liver

tissue, the extent of CAE staining was quantified by counting the number of CAE-positive neutrophils per 1000 hepatocytes (69). For lung tissue, total CAE-positive cells were counted per 10 fields. During quantification, neutrophils were characterized by light pink (i.e. CAE-positive) staining and bi-lobed nuclear morphology.

3. Immunohistochemistry

Immunohistochemical staining for the marker F4/80 was performed as previously described (98). Formalin-fixed, paraffin embedded sections (5 µm) were deparaffinized and rehydrated with graded ethanol solutions. Endogenous peroxidase activity was quenched by incubating slides in a 3% H_2O_2 solution for 5 minutes at room temperature. Because the liver has high concentrations of biotin, an avidin-biotin blocking reagent (Dako, Carpinteria, CA) was used to reduce non-specific staining. Slides were then incubated in primary antibody for the F4/80 cell surface receptor (1:500 in 10% goat serum; Abcam, Cambridge MA) for two hours at room temperature. The Vectastain Elite ABC kit was used for detection (Vector Laboratories, Inc., Burlingame, CA). Briefly, tissue was incubated in a biotinylated anti-Rat IgG secondary antibody for 15 minutes at room temperature. Tissue was then washed and incubated in a solution containing avidin-bound horseradish peroxidase (HRP) for 30 minutes. The HRP substrate 3, 3'-diaminobenzidine (DAB) was added to tissue sections until positive (brown) staining was macroscopically visible. Slides were counterstained with Hematoxylin (Dako, Carpinteria, CA) for 1 minute, washed, dehydrated through graded ethanol and then mounted with Permount (Fisher,

Waltham, MA). Each slide contained a negative tissue section which does not receive primary antibody. The number of F4/80 macrophages in liver tissue was quantified by blinded counting of the number of F4/80 positive macrophages per 1000 hepatocytes in 200x photomicrographs.

   4. Immunofluorescence

Immunofluorescent detection of fibrin deposition was performed as described in (69). Briefly, frozen liver sections (6 μm thick) were fixed in 10% buffered formalin containing 2% acetic acid to solubilize all but cross-linked fibrin. Sections were blocked with PBS containing 10% horse serum before an overnight incubation at 4^0C with affinity purified rabbit anti-human fibrinogen IgG (Dako North America, Inc. Carpinteria, CA) diluted in blocking solution. Donkey anti-rabbit antibody conjugated to Alexa 488 (Invitrogen, Carlsbad, CA) was used as secondary antibody. Tissue was counterstained with Hoechst. Fluorescence for Alexa 488 and Hoechst was detected at excitation and emission wavelengths of 490 nm and 520 nm, and 350 nm and 460 nm, respectively. Immunofluorescence was visualized using a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) and Metamorph software

F. RNA isolation and real-time RT-PCR

The hepatic mRNA expression of select genes was detected by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR), which is routine for this group (29, 50, 98, 100).   PCR primers and probes for TNFα, PAI-1, IL-6, and β-actin were designed using Primer 3 (Whitehead Institute for Biomedical Research, Cambridge, MA). Primers and probes for KC, MIP-2, and IL-10 were

bought from Applied Biosystems as kits (Foster City, CA). All primers were designed to cross introns to ensure that only cDNA and not genomic DNA was amplified.   Total RNA was extracted from liver tissue by a guanidinium thiocyanate-based method (RNA STAT 60 Tel-Test, Ambion, Austin, TX). RNA concentrations were determined spectrophotometrically and 1 µg of total RNA was reverse transcribed using a kit (Quanta Biosciences, Gaithersburg, MD). PerfeCta qPCR Fast Mix (Quanta Biosciences, Gaithersburg, MD) was used to prepare the PCR reaction mixture.   This 2× mixture is optimized for TaqMan reactions and contains $MgCl_2$, dNTPs, and AccuFast Taq DNA Polymerase. Amplification reactions were carried out using the ABI StepOne Plus machine and software (Life Technologies, Grand Island, NY) with initial holding stage (95^0C for 30 seconds) and 50 cycles of a 2-step PCR (95^0C for 30 seconds, 60^0C for 20 seconds).   Fluorescent intensity of each sample was measured at each cycle to monitor amplification of the target gene. The comparative CT method was used to determine fold changes in mRNA expression compared to an endogenous reference gene (β-actin). This method determines the amount of target gene, normalized to an endogenous reference and relative to a calibrator ($2^{-\Delta\Delta Ct}$).

G. Luminex

Cytokine levels in plasma and BALF samples were measured using the MILLIPLEX MAP Magnetic Bead Mouse Cytokine/Chemokine Panel (Millipore, Billerica, MA). The plate was read using a Luminex 100 plate reader and Exponent software. MILLIPLEX MAP assay was performed according to

manufacturer's recommendations. Plasma (4x dilution) or BALF samples (undiluted) were added to a clear bottomed black 96 well plate. Antibody-immobilized beads were added to samples and the plate was incubated overnight at 4ºC on a plate shaker at 600 rpm. Detection antibody was added to wells and incubated for 1 hour followed by the addition of strepavidin-phycoerythrin. Quantitative analysis of the assay was performed using the Luminex 100™ IS. Data capture and analysis were performed using the Luminex Xponent software.

H. Microbiota analysis

1. Bacterial DNA isolation and sequencing

DNA was extracted from frozen cecal pellet and plasma using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's directions. Briefly, cecal content (75-100 mg) was rapidly weighed, recorded, and placed into a 2 mL microcentrifuge tube on ice. Lysis buffer was added to each sample and the sample was vortexed until thoroughly homogenized. Homogenized sample was heated at 70ºC for 5 minutes and then vortexed at full speed for 1 minute to pellet particles. Supernatant was removed and added to a new tube with an InhibitEX tablet (supplied by kit) which forms a matrix to pellet DNA-degrading enzymes. Samples were centrifuged to pellet inhibitors bound to the matrix; remaining supernatant was added to a new tube and incubated with proteinase K. DNA was then precipitated with ethanol and collected using the QIAmp spin column as directed by manufacturer's instructions.

100 ng of cecal DNA was sent to Baylor University School of Medicine's Alkek Center for Metagenomics and Microbiome Research for 16S rRNA sequencing on the 454 FLX-titanium Platform. The V3–V5 16S rRNA gene variable regions were amplified by PCR using 454 adapter-linked and bar-coded primers 357F and 926R, as described previously (101). Negative controls (water) and positive controls (*Staphylococcus aureus* USA300 genomic DNA) were included in all steps to control for contamination. Amplicons were purified using a SPRI bead clean-up step, quantitated by picogreen assay, normalized, pooled, and then sequenced on a 454 instrument using the FLX Titanium chemistry.

2. Bacterial DNA analysis

The raw sequencing dataset, consisting of an average of 28,590 reads per sample with an average read length of 253 nucleotides, was deconvoluted via the PyNAST alignment tool (102) using the Greengenes reference database (August 2013 release) (103) and quality filtered with cutoff parameters of: less than 200 and greater than 1,000 bp, quality score below 30, number of mismatches in primer greater than 0, number of ambiguous bases greater than 6, and maximum homopolymer runs greater than 6. Following quality filtering, 514,622 sequences remained from the original 531,138 with an average read length of 253 nucleotides. Chimeric sequences were removed by reference and de novo chimeric filtering with UCHIME (104) utilizing the Greengenes reference database (August 2013 release) (103). Operational taxonomic unit tables based on sequences

within the filtered data set were then generated by taxonomic binning via the Quantitative Insights Into Microbial Ecology tool (Qiime) and its various components (104). Open reference OTU picking was performed via Usearch 6.1 software (105) using 97% similarity to the clustered Greengenes database from August 2013 (106) while removing singleton OTUs from the OTU table as further quality filtering to improve specificity. The taxonomic classifications of sequence reads were identified from the phylum to the species level by the uclust based consensus taxonomy classifier (Bokulich, Rideout et al. (submitted)) with phylogenetic tree creation by Fast Tree (107). For diversity measures, an even sequence sampling depth of 16,000 sequences was used to avoid artifacts due to differing numbers of sequences per sample. PCoA analyses were performed to compare microbial community structure between samples utilizing weighted UniFrac measures (108). Linear effect size (LefSe) analyses were performed using the freely distributed Galaxy LefSe analysis module hosted at huttenhower.sph.harvard.edu/lefse/ (109). Hypothesis testing using the Dirichlet-Multinomial Distribution was performed via the HMP package for R (110) within the R Statistical Computing Environment (111)

I. **Statistical Analysis**

Results are reported as means ± standard error mean (SEM; n = 4-7). ANOVA with Bonferroni's post-hoc test (for parametric data) or Mann-Whitney Rank Sum test (for nonparametric data) was used for the determination of

statistical significance among treatment groups, as appropriate. A *p* value less than 0.05 was selected before the study as the level of significance.

CHAPTER III

OLIGOFRUCTOSE PROTECTS AGAINST ENHANCED LIVER INJURY

CAUSED BY ARSENIC IN A MODEL OF HIGH FAT DIET INDUCED NON-

ALCOHOLIC STEATOHEPATITIS – ROLE OF THE ECM

A. Introduction

Exposure to high concentrations of arsenic (As) is associated with a myriad of health effects including skin lesions, hypertension, cardiovascular disease, respiratory disease, and malignancies of the skin and internal organs (112). The liver, a major site of arsenic metabolism, is a known target of arsenic toxicity (113). Indeed, chronic arsenic exposure causes hepatomegaly, non-cirrhotic portal fibrosis and portal hypertension (113-115). The concentrations of arsenic found in the US water supply are generally considered lower than those necessary to cause overt liver damage, however lower levels of arsenic can cause more subtle changes in the liver (46) and can sensitize the liver to injury by another insult (47). Therefore, while arsenic levels in the US may not be high enough to directly injure the liver, there is evidence that low levels of arsenic may interact with other risk-modifying factors to cause hepatic injury.

Obesity is another major health concern for the US and globally. More than one third of American adults are obese (116), and it is expected that the number

of obese individuals in the US will double by 2050 (117). Obesity is associated with a myriad of health effects including insulin resistance, diabetes, and non-alcoholic fatty liver disease (NAFLD). NAFLD is a spectrum of disease states including simple steatosis, non-alcoholic steatohepatitis, and fibrosis and cirrhosis. Studies have shown that about 20-30% the general population has NAFLD (118) however studies have shown that the prevalence of NAFLD can more than double in obese cohorts (119). Despite the high incidence of NALFD in obese populations, only a minority of obese individuals ever progress to more severe stages of liver disease, such as non-alcoholic steatohepatitis. Risk factors for primary NAFLD include obesity, type II diabetes and dyslipidemia (120). However it is likely that other unidentified risk factors contribute to progression to more severe stages of liver disease (i.e. NASH).

Interestingly, there is potential overlap in areas of risk for arsenic exposure and obesity. For example, rural communities, which have both artesian water supplies that are unregulated by Clean Water Act, often have high arsenic concentrations; these areas also generally have a high prevalence of obesity. In a study investigating the possible interaction between obesity and arsenic, this group demonstrated that low levels of arsenic synergistically enhance liver damage caused by high fat diet (98). These data suggest that underlying risk factors, such as obesity, may modify the risk of hepatic injury caused by subhepatotoxic levels of arsenic exposure. However, the mechanism(s) underlying enhanced liver injury remain unknown.

The role of the gut:liver axis in liver disease is well recognized. Indeed, liver injury is associated with dysbiosis, disruption of gut barrier integrity, and leakage of inflammatory bacterial-derived components (i.e., endotoxin) from the gut into the systemic circulation. Obesity can affect gut permeability by decreasing the barrier function of the epithelium (78). Additionally, both obesity and arsenic have been shown to alter gut flora (121). Interestingly, probiotics, live bacteria cultures which promote commensal bacteria growth, can preserve intestinal barrier integrity and protect against liver injury in animals (81, 122). Clinical studies have shown that probiotic supplementation improves patient outcome compared to standard therapy (123) via gut-mediated mechanisms. Prebiotics, dietary compounds that favor the growth and maintenance of commensal bacteria, may also confer protective effects in the setting of liver injury via gut-mediated mechanisms. For example, Cani et al showed that the prebiotic oligofructose (OFC) can protect against inflammation and endotoxemia by regulating the microbiota of animals fed high fat diet (78). Recently, arsenic exposure has been shown to also cause dysbiosis in mice (124), which could, in principle, exacerbate the effect of HFD. The purpose of the current study was to test this hypothesis and to determine if the prebiotic oligofructose can protect against enhanced liver injury caused by arsenic in a mouse model of NAFLD.

B. EXPERIMENTAL PROCEDURES

1. Animals and treatments

Animals were exposed to combinations of HFD, arsenic and the prebiotic oligofructose as described in Chapter II.

2. Clinical Chemistry

Plasma levels of ALT and AST were determined as described in Chapter II

3. Lipid determination

Lipids were extracted from snap-frozen liver as described in Chapter II. Triglycerides and non-esterified fatty acids were determined as described in Chapter II.

4. Immunostaining

Hematoxylin and eosin staining, immunohistochemical staining for F4/80 and immunofluorescent staining for fibrin were performed on liver tissue as described in Chapter II.

5. RNA isolation and real-time PCR

Details for RNA isolation and real time PCR are described in Chapter II.

6. Cecal DNA extraction

DNA was extracted from cecal content as described in Chapter II.

7. Microbiome analysis

Metagenomic analysis of the cecal microbiome was performed as described in Chapter II.

8. Statistical analysis

Statistical analysis was performed as described in Chapter II.

C. Results

1. Body and organ weight

All animals gained weight and there was no mortality or morbidity in any group during the course of the study. As observed previously (98), arsenic did not significantly affect food consumption or body mass gain in animals fed either LFD or HFD (Figure 3.1A and B). High fat diet significantly increased food consumption ~20% and body mass gain ~50% compared to low fat diet alone (Figure 3.1C). Whereas OFC supplementation did not affect food consumption, it did significantly attenuate the increase body mass gain caused by HFD; this effect of OFC was independent of the presence of arsenic in the diet.

2. OFC protects against liver Injury

HFD significantly increased the percent liver to body weight compared to low fat diet (Figure 3.2B). Liver weight to body weight percent was not significantly affected by concomitant arsenic exposure or by oligofructose feeding. As expected by the hepatomegaly observed, 10 weeks of HFD feeding dramatically increased lipid accumulation in the liver (Figure 3.2A); this pathologic change comprised both macrovesicular and microvesicular steatosis. Changes in quantity of hepatic triglycerides and non-esterifieid fatty acids (NEFA) (Figure 3.2B) were also determined. HFD alone increased hepatic triglyceride and NEFA content compared to LFD alone by more than 4 fold. Arsenic administration did

Figure 3.1: Model parameters

(A) Animals were exposed to arsenic (or tap) water for 11 weeks. One week after

the start of arsenic exposure experimental diet (e.g. LFD, HFD, or HFD +OFC)

was provided as described in Chapter II. (B) Body weight and food consumption

were recorded once per week for the duration of the experiment.   Data are

shown as +/- SEM ($n = 6\text{-}10$). [a], $p < 0.05$ compared to tap water control; [b], $p <$

0.05 compared to LFD; [c], $p < 0.05$ compared to no OFC.
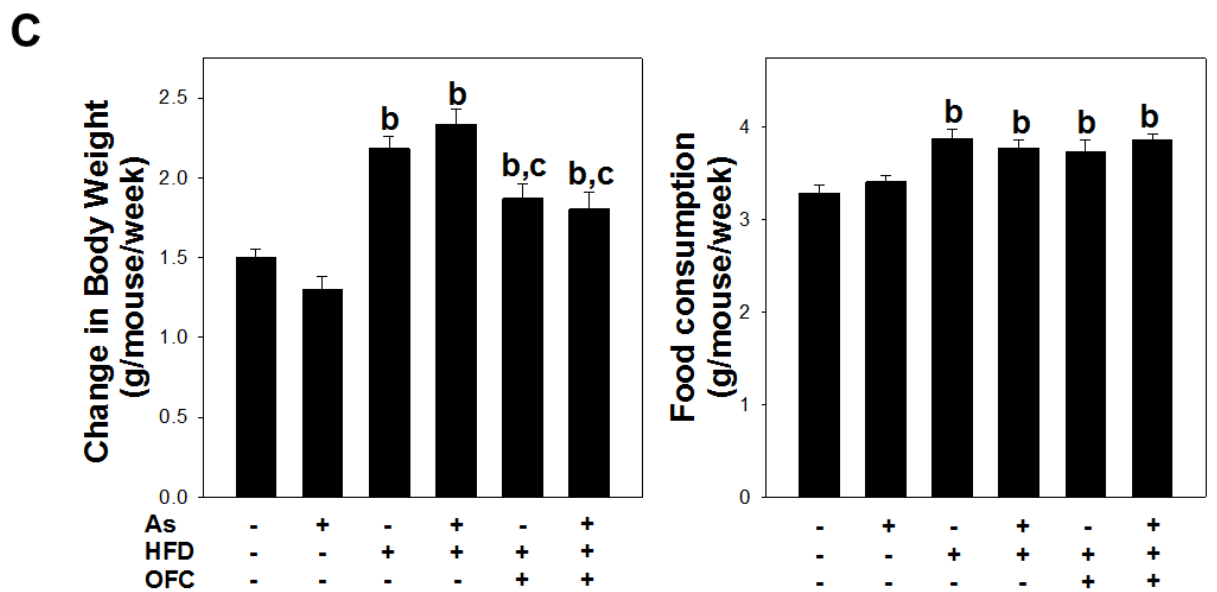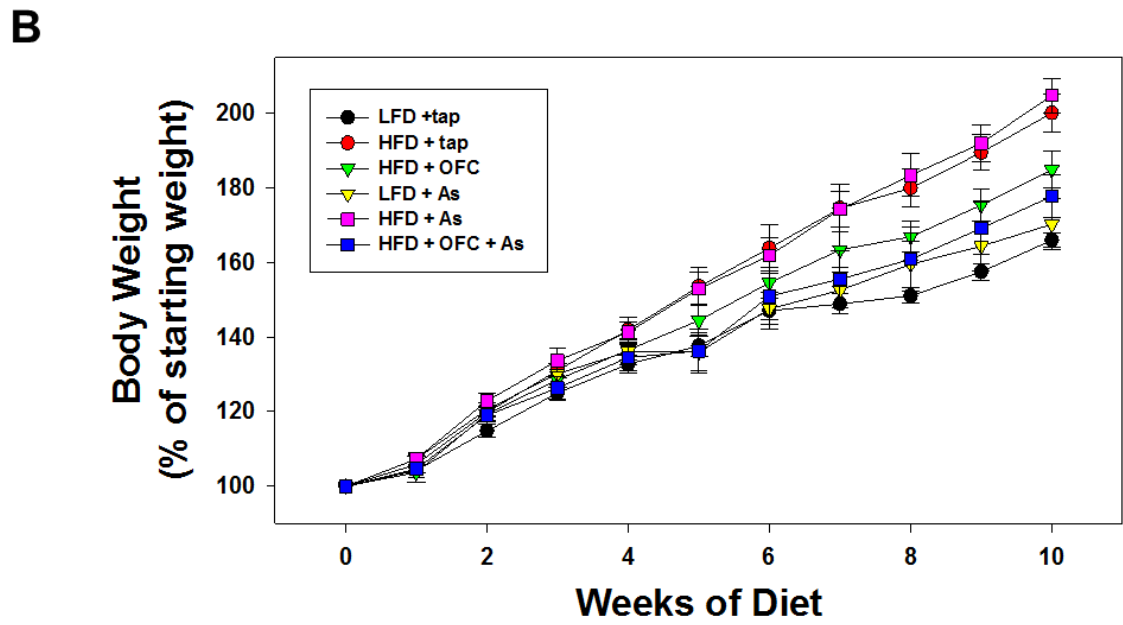
Figure 3.2: OFC protects against liver injury and steatosis.

(A) Representative photomicrographs depicting hematoxylin & eosin staining

(H&E, 200x; top left) are shown (B) Liver to body weight percent (LW/BW%) was

determined as described in Materials and Methods. Hepatic non-esterified fatty

acid (NEFA) and triglyceride (TG) content (top right) were determined using kits

as described in Materials and Methods. Plasma levels of alanine and aspartate

aminotransferase were determined as an index of liver injury. Quantitative data

are shown as +/- SEM (n =6-10). [a], p < 0.05 compared to tap water control; [b], p

< 0.05 compared to LFD; [c], p < 0.05 compared to no OFC.

A

	LFD	HFD	HFD + OFC
Tap			
As			

B
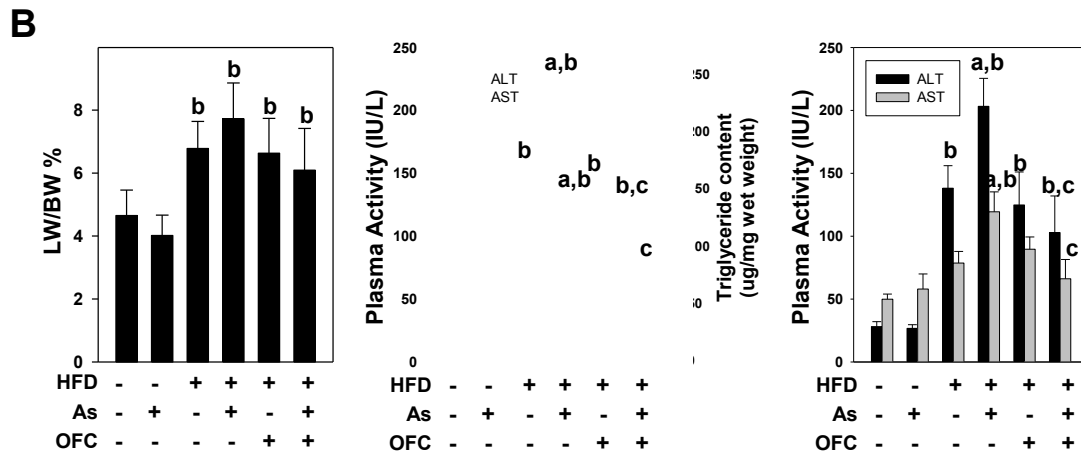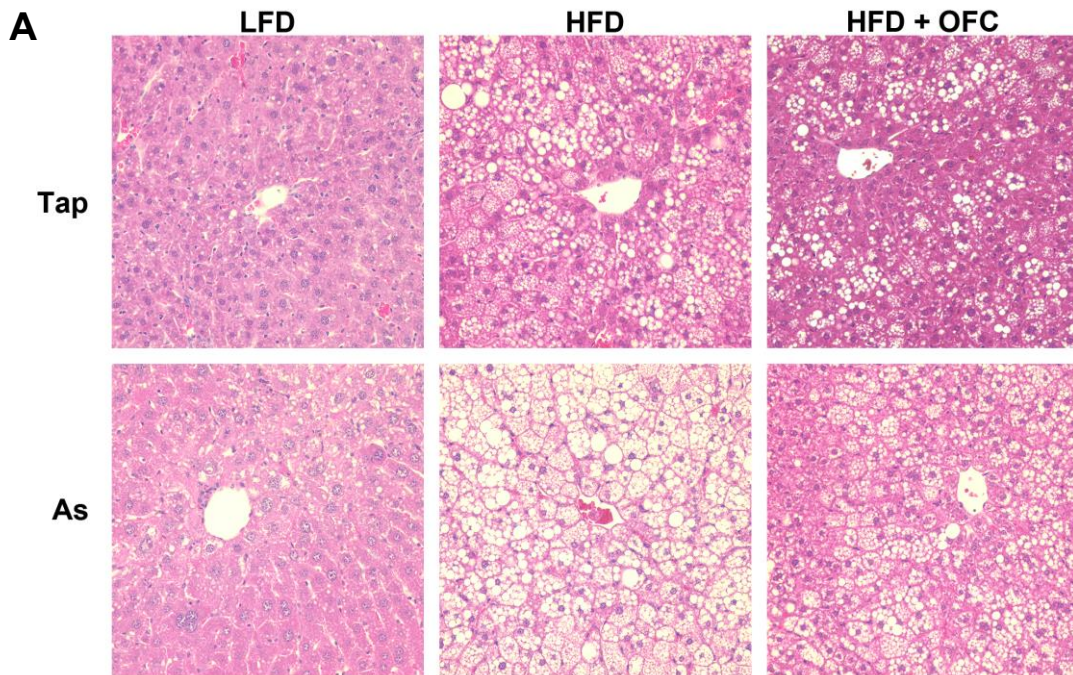
not affect steatosis or hepatic triglyceride or NEFA content when, in either LFD- or HFD-fed animals. Oligofructose administration blunted fat accumulation and significantly decreased triglyceride content compared to animals that received either HFD or arsenic + HFD without OFC. Interestingly, OFC had no significant effect on NEFA content of the liver.

HFD feeding also increased the appearance of necroinflammatory foci in the liver (Figure 3.2A). Circulating levels of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined as indices of liver damage (Figure 3.2B). HFD alone significantly increased ALT levels, but did not significantly affect AST levels. Whereas arsenic did not significantly affect plasma levels of either ALT or AST in the LFD group, it significantly enhanced the increase in these variables caused by HFD under these conditions by ~50%. The addition of OFC to animals receiving HFD did not affect the transaminase levels in the absence of arsenic. However, OFC administration completely blunted the increase in both ALT and AST caused by the combined exposure of arsenic and HFD.

3. OFC protects against inflammation

Immunohistochemistry for F4/80, a marker of activated macrophages, was performed as an index of inflammation (Figure 3.3A). Neither arsenic nor HFD alone altered the number of F4/80 positive macrophages; however the combination of arsenic + HFD significantly increased the number of hepatic

Figure 3.3: OFC protects against hepatic inflammation.

(A) Representative photomicrographs of immunohistochemical staining for F4/80 (200x) are shown. (B) Number of F4/80 positive cells were counted per 1000 hepatoctyes as described in Chapter II. C. Real time RT-PCR was performed for the pro-inflammatory geneTNFα as described in Chapter II. Data are shown as +/- SEM ($n = 6$-10). [a], p < 0.05 compared to tap water control; [b], p < 0.05 compared to LFD; [c], p < 0.05 compared to no OFC.

A

	LFD	HFD	HFD + OFC
Tap			
As			

B

F480 positive cells (/1000 hepatocytes)

b

c

HFD	-	-	+	+	+	+
As	-	+	-	+	-	+
OFC	-	-	-	-	+	+

TNFα

HFD	-	-	+	+	+	+
As	-	+	-	+	-	+
OFC	-	-	-	-	+	+
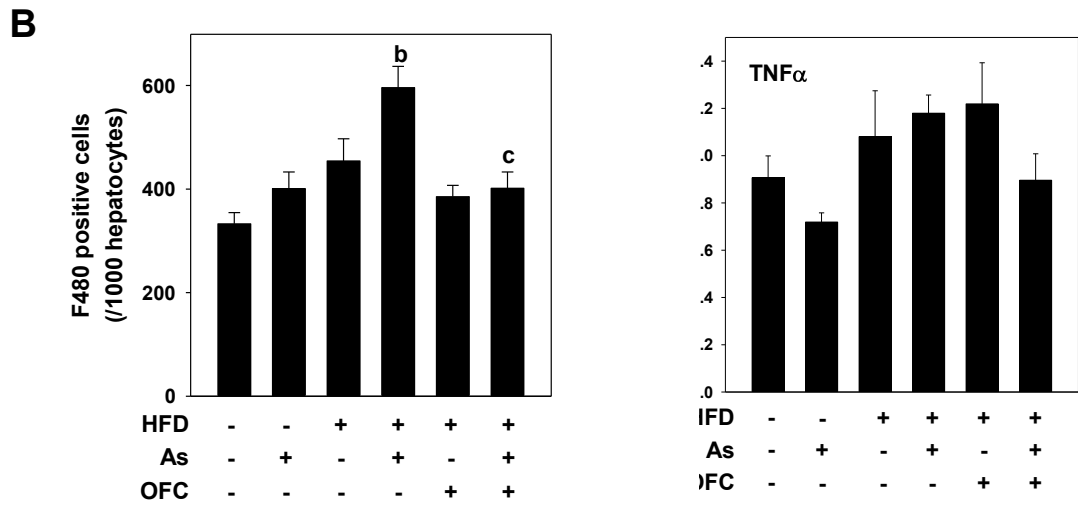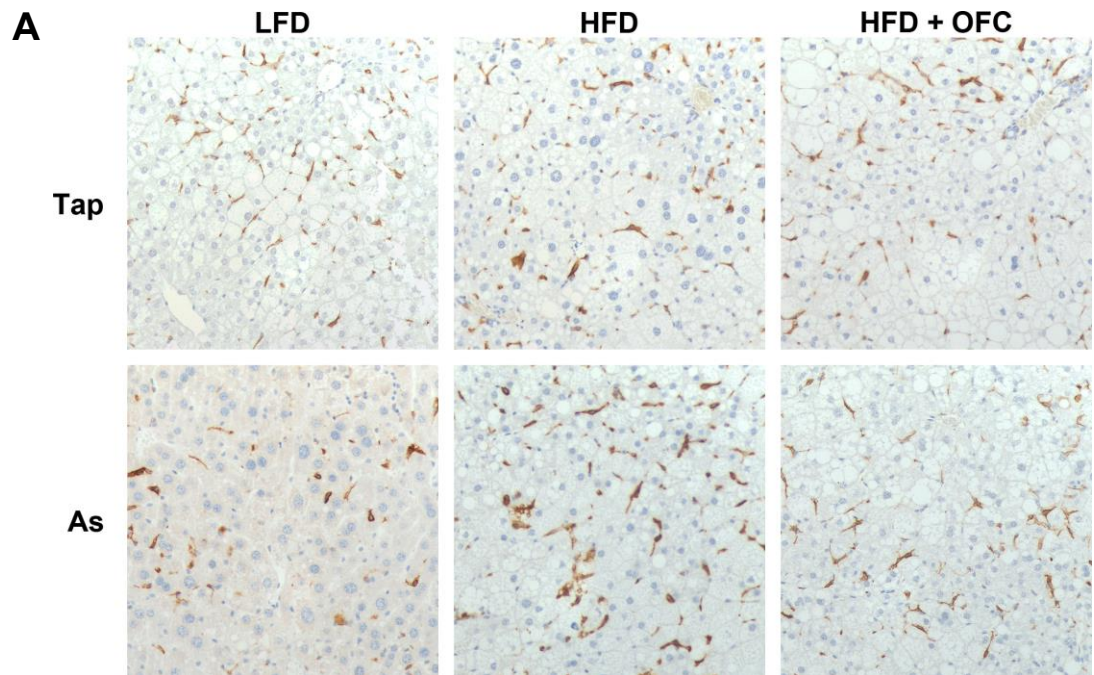
macrophages compared to arsenic or HFD alone, as was observed previously

(98). The administration of OFC significantly blunted the increase in number of

activated macrophages caused by the combination of arsenic and HFD and

values were similar to those of LFD controls.

4. OFC protects against fibrin deposition

Previously, the enhancement of HFD-induced liver injury caused by arsenic

correlated with a significant increase in hepatic expression of PAI-1 (98). PAI-1

is a major inhibitor of both tissue-type plasminogen activator (tPA) and

urokinase-type plasminogen activator (uPA), and is therefore a key inhibitor of

fibrin degradation (i.e., fibrinolysis) by plasmin. Fibrin deposition was determined

by immunofluorescent staining and quantitation of fluorescent signal. LFD-fed

animals given either tap or arsenic-containing water had minimal fibrin deposition

in the liver (Figure 3.4A). HFD alone increased deposition of fibrin by ~2-fold. As

observed previously (98), the combination of HFD + arsenic significantly

increased the expression of PAI-1 and fibrin deposition in liver (Figure 3.4B).

OFC administration almost completely prevented the changes caused by arsenic

under these conditions.

5. Effects on the microbiome

The effects of arsenic, HFD, and OFC on relative abundance and total

abundance of bacteria was determined at the phylum level (Figure 3.5A). As

expected, the most abundance bacteria phyla were Bacteroidetes and

Figure 3.4: OFC protects against sinusoidal fibrin accumulation.

A. Representative photomicrographs (400x) of immunofluorescent staining for fibrin are shown. B. Fibrin immunofluorescence (green color) was quantitated using Metamorph software. C. Hepatic PAI-1 mRNA expression was determined using real time RT-PCR. Data are shown as +/- SEM ($n = 6$-10). [a], p < 0.05 compared to tap water control; [b], p < 0.05 compared to LFD; [c], p < 0.05 compared to no OFC.

A

LFD | HFD | HFD + OFC

Tap

As

B

HFD	−	−	+	+	+	+
As	−	+	−	+	−	+
OFC	−	−	−	−	+	+

PAI-1

HFD	−	−	+	+	+	+
As	−	+	−	+	−	+
OFC	−	−	−	−	+	+

48
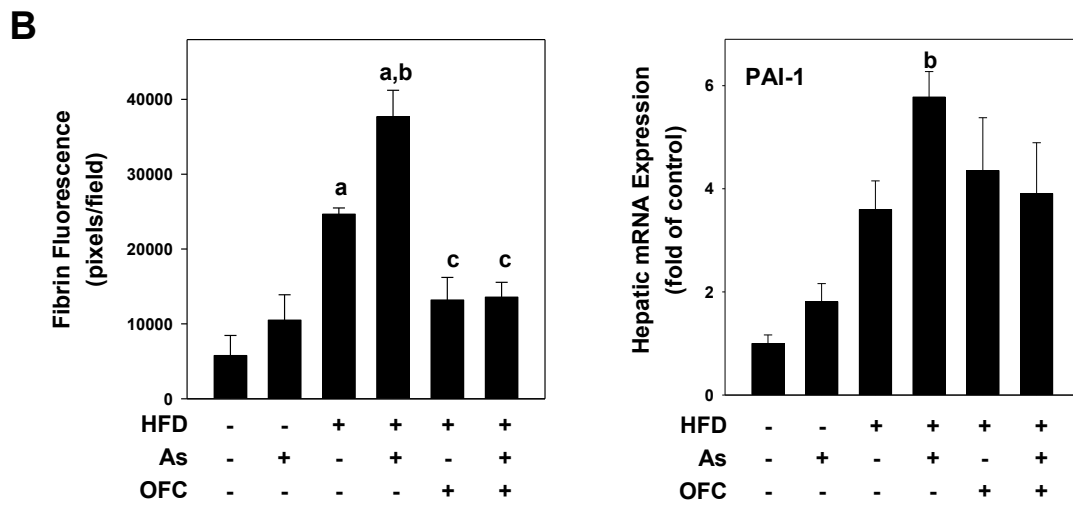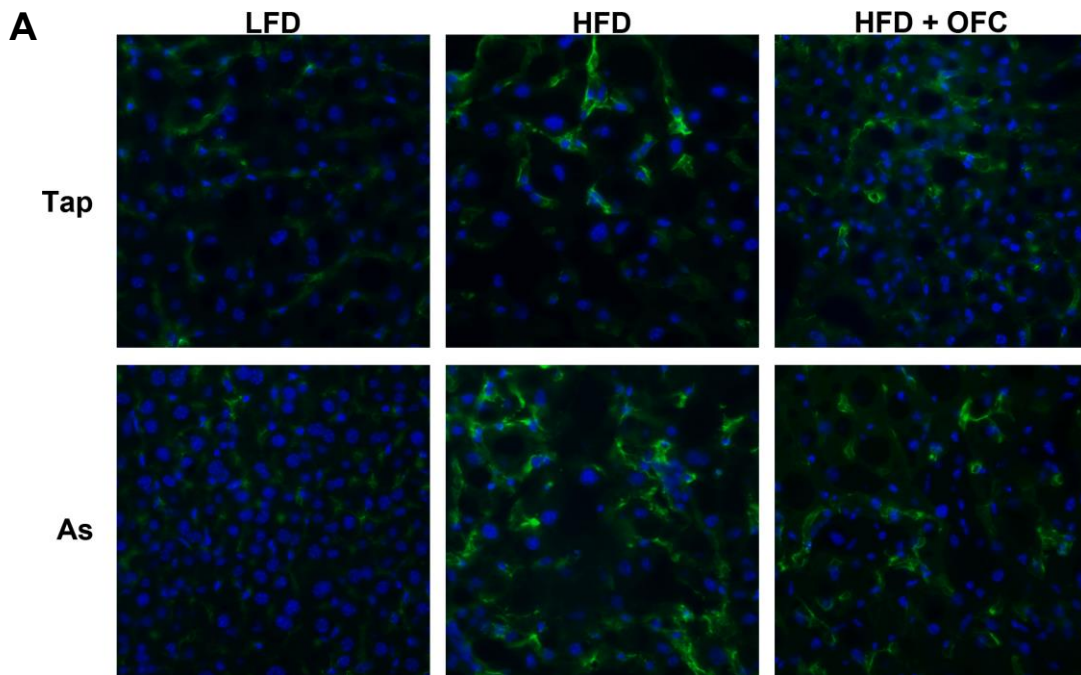
Firmicutes. Arsenic exposure alone caused a loss of the Verrucobiorobia in animals that were fed a LFD. Interestingly, this effect was lost in animals that were fed a HFD; however, the combination of arsenic and HFD did lead to an increase in the gram-negative phyla Bacteroidetes. Oligofructose feeding increased the relative abundance of Bacteroidetes with a concomitant decrease in Firmicutes compared to all other groups that did not receive the prebiotic. Additionally, OFC restored the growth of Tenericutes which was lost in animals that received a HFD. OFC also resulted in a robust increase in total bacteria compared to animals that did not receive OFC.

α diversity (Figure 3.5B) was used as a measure of diversity within a sample. Interestingly, the greatest α diversity was observed in animals exposed to a LFD. HFD feeding reduced α diversity and the combination of HFD and OFC resulted in the lowest α diversity of all six groups. β diversity was used as a measure of diversity between samples (Figure 3.5C). As expected, samples that represent a single treatment group clustered more closely together, suggested a higher degree of similarity among the samples.

Linear discriminant analysis effect size (LefSe) was used to identify the enrichment of bacteria within treatment groups. When all six treatment groups were compared, unique enrichment profiles were observed for each group (Figure 3.6A). In a LefSe comparison between the LFD and LFD + arsenic, enrichment was primarily seen in animals that received tap water, suggesting that arsenic exposure resulted in decreases in these bacteria including the

Figure 3.5: Effects of HFD, arsenic, and OFC on bacterial abundance and diversity.

A. The top stacked bar chart shows the relative abundance of bacteria phyla for each group. Absolute abundance (bottom) was determined by averaging the total number of OTUs found in each bacterial phyla per group. B. Alpha diversity, a measure of unique OTUs present in a given sample, is shown as an index of diversity within a sample. Alpha rarefaction curves were determined by counting the number of sequences found in a sampling of bacterial DNA. During the first sampling, all sequences identified were counted towards the number of observed OTUs; for each additional sampling, only unique OTUs are counted and added to the number of observed OTUs for that sample. This process is repeated until a total of 16,000 sequences are sampled. C. Beta diversity is shown as a measure of diversity between samples. This measure was determined using principle coordinate analysis of the phylogenetic diversity between samples and the differences in OTU abundance between samples. Samples that are closer in proximity have microbiomes that are more similar in term of phylogenetic diversity and OTU abundance.

A

Abundance

Count

HFD	-	-	+	+	+	+
As	-	+	-	+	-	+
OFC	-	-	-	-	+	+

Taxon
- Other
- Acidobacteria
- Actinobacteria
- Bacteroidetes
- Cyanobacteria
- Firmicutes
- Fusobacteria
- Proteobacteria
- Tenericutes
- Verrucomicrobia
- Thermi

B

α Diversity

Treatment
- HFD + As
- HFD + OFC
- HFD + OFC + As
- HFD + tap
- LFD + As
- LFD + tap

OTUs observed

of Sequences Sampled

C

β Diversity

PC2 (17%)

Treatment
- HFD + As
- HFD + OFC
- HFD + OFC + As
- HFD + tap
- LFD + As
- LFD + tap

PC1 (60%)

PC3 (6%)

51

Figure 3.6: HFD, arsenic, and OFC exposure caused unique changes to the microbiome.

LefSe analysis was used to determined enrichment of taxa between groups. Effect size is determined using linear discriminant analysis (LDA). A. LefSe analysis of all six treatment groups is plotted on a cladogram. Each treatment group is represented by a unique color; highlighted taxa indicate enrichment of that taxa within the treatment group. Effect size is represented by the size of the circular intersection points between the taxa. B. Bar plot of LefSe analysis comparing the gut microbiomes of animals fed either LFD or a LFD and arsenic (top). Each bar represents a bacterial taxa that was enriched by LFD. The length of the bar represents the effect size. C. Bar plot of LefSe analysis comparing the gut microbiomes of animals fed either HFD (blue), HFD and arsenic (red), or HFD, arsenic, and OFC (green). The length of the bar represents effect size.

A All Groups

Legend (left):
- HFD+As
- HFD+OFC
- HFD+OFC+As
- HFD+tap
- LFD+As
- LFD+tap

Legend (right):
- a: Bifidobacterium
- b: Bifidobacteriaceae
- c: USF_S24_7
- d: UGF_S24_7
- e: S24_7
- f: USF_Clostridiaceae
- g: UGF_Clostridiaceae
- h: Clostridiaceae
- i: USG_Coprococcus
- j: Coprococcus
- k: USF_Lachnospiraceae
- l: UGF_Lachnospiraceae
- m: Lachnospiraceae
- n: USF_Mogibacteriaceae
- o: UGF_Mogibacteriaceae
- p: Mogibacteriaceae
- q: USG_Ruminococcus
- r: Ruminococcus
- s: Ruminococcaceae
- t: USO_Clostridiales
- u: UGO_Clostridiales
- v: UFO_Clostridiales
- w: USG_Allobaculum
- x: Allobaculum
- y: USF_Erysipelotrichaceae
- z: UGF_Erysipelotrichaceae
- a0: muciniphila
- a1: Akkermansia
- a2: Verrucomicrobiaceae

B

LFD+tap

Taxon
Verrucomicrobia
muciniphila
Verrucomicrobiae
Akkermansia
Verrucomicrobiaceae
Verrucomicrobiales
Ruminococcus
gnavus
USG_Bifidobacterium
Bifidobacteriales
Actinobacteria
Bifidobacteriaceae
Bifidobacterium
OSG_Bifidobacterium

LDA SCORE (log 10)

C

HFD+As   HFD+OFC+As   HFD+tap

Taxon
Mogibacteriaceae
UGF_Mogibacteriaceae
USF_Mogibacteriaceae
USG_Turicibacter
Turicibacter
Turicibacteraceae
Turicibacterales
S24_7
UGF_S24_7
USF_S24_7
Bacteroidetes
Bacteroidales
Bacteroidia
Lactobacillales

LDA SCORE (log 10)

53

commensal Bifidobacterium (Figure 3.6B). Comparisons between animals fed

HFD with or without arsenic and OFC showed that arsenic exposure was

associated with a decrease in Mogibacteriacaceae and Turicibacter (Figure

3.6C).

D. DISCUSSION

The GI tract is home to approximately 100 trillion gut microbes (125). The

microbes residing in the gut regulate many important physiological functions

including metabolic processing, energy production, and immune system

development (125). Therefore, it is not surprising that dysbiosis of the gut flora is

associated with a myriad of disease states locally in the GI tract including

diarrhea/malabsorption (126, 127) and irritable bowel disease (128, 129).

Because of the critical importance of the microbiome in immune surveillance and

tolerance, changes in the gut microbiome are also linked to autoimmune and

diseases (130-132). The gut microbiome does not only contribute to diseases of

the GI tract. Indeed, several studies indicated that distal organ systems,

including the CNS, are negatively affected by gut dysbiosis (133).

A major mechanism by which gut dysbiosis may contribute to systemic

disease is via changing/impairing the GI tract barrier function. For example,

rodent models of obesity have been associated with altered gut microbiota that

favor gut leakiness (78, 79) and endotoxemia (78, 80). Furthermore, dysbiosis

associated with obesity contributes to the development of inflammation (78, 80)

metabolic syndrome (134, 135), and non-alcoholic fatty liver injury (79). The

understanding that the GI tract contributes to liver injury (i.e., the gut-liver axis) has been understood for over a century and was first shown experimentally more than 60 years ago by Rutenberg et al, when they demonstrated that nonabsorbable antibiotics protected against diet-induced liver injury (136). Since then, many studies using a myriad of models including antibiotics (137, 138), and germ-free mouse models (139) have provided additional evidence implicating the microbiome in the susceptibility and development of liver disease. Today, the gut-liver axis and enteric dysbiosis, including that caused by a HFD, is a generally accepted component in the development of liver disease.

Although the role of arsenic in altering the microbiome is less well studied, arsenic is known to alter the microbial components of soil (140). A recent study by Lu et al has shown that high levels of arsenic can cause dysbiosis in mice (124). In their study, metagenomic analysis of the gut flora of mice exposed to 50 ppm arsenic showed that arsenic significantly altered the abundance of some microbial families and also resulted in functional changes in the microbiome's metabolic profile (124). The changes in the gut flora caused by arsenic in the current study were more subtle than those seen by Lu and colleagues; this difference may be attributed, in part, to the >10-fold lower arsenic concentrations used in the current study. Nevertheless, arsenic exposure did change the mouse microbiome in the current study; importantly, arsenic further exacerbated the dysbiosis caused by HFD feeding in mice. Importantly, supplementation with oligofructose, a prebiotic that has been demonstrated to protect against dysbiosis and favor commensal bacteria, almost completely prevented the enhancement of

HFD-induced liver injury caused by arsenic. These results therefore support the hypothesis that arsenic mediates liver injury under these conditions via mechanisms that involve the gut-liver axis.

There are several potential mechanisms by which the microbiome may mediate arsenic-induced liver injury. First, the changes in the microbiome caused by diet (HFD and OFC) may affect arsenic metabolism and absorption. Indeed, interactions between gut microbes and arsenic are bidirectional. For instance, Pinyayev et al showed that bacteria from the mouse cecum can metabolize arsenic (141) and others have shown that the human gut flora can metabolize arsenic into toxic metabolites (142, 143). Whereas this is distinctly possible, it should be noted that concentrations that are 10-fold higher than used here were not directly hepatotoxic (47). Alternatively, the effects of arsenic on the microbiome may mediate the observed hepatotoxicity. Indeed, arsenic exposure expanded the population of Gram negative bacteria that could produce endotoxins, which are well known for contributing to fatty liver injury. However, whether the effects are driven by microbiome changes on arsenic levels, or by arsenic changes on the microbiome, these changes may potentiate arsenic toxicity locally on the gut epithelium (144) and contribute to enhanced arsenic toxicity distally in the liver.

In addition to direct effects on the microbiome, the protective effect of OFC feeding under these conditions could also be 'upstream' of arsenic. Specifically, OFC feeding significantly attenuated the increase in hepatic lipids caused by HFD, both in the presence and absence of arsenic. The multi-hit hypothesis of

fatty liver disease involves not only an increase in gut-derived products that damage the liver, but also an increased sensitivity to the damage caused by these gut-derived products. Indeed, fatty livers are more sensitive to a second toxic 'hit,' including gut-derived LPS (145, 146). Therefore, the favorable effects of OFC on hepatic steatosis may indirectly protect against the injury caused by HFD and/or arsenic by blunting this sensitization. In support of this hypothesis, enhanced liver injury caused by arsenic + HFD co-exposure was not due to an increase in steatosis per se, but rather due to an enhanced hepatic inflammatory response. Therefore, OFC may also be protecting against damage in this model by preventing hepatic sensitization caused by HFD-induced endotoxemia. Such a mechanism is supported by previous studies that arsenic-exposed livers are more sensitive to exogenous LPS (47) and that liver injury here in OFC-fed mice was insensitive to arsenic administration.

In conclusion, protection against enhanced liver injury conferred by OFC is likely due to both direct effects on the microbiome, as well indirect effects on hepatic sensitization. These findings are in-line with previous work, in which OFC supplementation was shown to protect against direct effects of HFD on the gut microbiome and barrier function (80). Prebiotics favor an environment in which commensal bacteria can thrive, in lieu of direct delivery of commensal bacteria (i.e., probiotics). As such, this approach may yield more predictable and stable results. Nevertheless, these results serve proof-of-principle that protecting against microbiome changes caused by arsenic can prevent the enhancement of inflammatory liver injury caused by this environmental contaminant.

CHAPTER IV

CHARACTERIZATION OF THE LINK BETWEEN LIVER AND LUNG INJURY

IN THE SETTING OF ALCOHOL EXPOSURE – THE ROLE OF TNFα

A. <u>Introduction</u>

Alcohol use is widespread in the United States with more than half of

Americans reporting alcohol consumption on a weekly basis (147). It has long

been known that chronic ethanol consumption can lead to development of a

myriad of health effects spanning a variety of organs including the brain, gut, liver

and lungs. Indeed, the effects associated with chronic alcohol consumption have

made alcohol the third leading risk factor globally for disease and disability (148)

at a cost of more than $166 billion annually to treat the medical consequences of

alcohol abuse (33). Despite the known health risks, alcohol consumption remains

pervasive in today's culture. *In toto*, alcohol consumption is responsible for ~6%

of all disability-adjusted life years (DALY) lost in the United States (149), most of

which are attributable to alcohol-induced toxicity.

The liver is well recognized as a major target of alcohol-induced organ injury.

This is due in part to the high concentrations of alcohol reached in the portal

blood as well as the detrimental effects of ethanol metabolism (e.g., oxidative

stress). Alcoholic liver disease (ALD) is actually a spectrum of disease states

that include simple steatosis (fat accumulation), steatohepatitis, and in more

severe cases fibrosis and cirrhosis. A major focus in ALD therapy is to treat the

decompensation associated with end-stage ALD (i.e., cirrhosis). Indeed, the

sequelae of a failing liver (e.g., ascites, portal hypertension, and hepatorenal

syndrome) are generally what causes death in end-stage ALD. Although the

successful treatment of these secondary effects prolongs the life of ALD patients,

this therapy is only palliative; there are currently no therapies to halt or prevent

the progression of ALD.

Alcohol consumption can also contribute to lung damage. In contrast to the

liver, alcohol consumption does not appear to directly cause any overt pulmonary

pathology; however, alcohol is known to exacerbate the severity of lung damage

from other causes. It has been estimated that up to 190,000 people suffer from

acute lung injury (ALI) annually in the United States (52). The risk of developing

more severe forms of ALI such as acute respiratory distress syndrome (ARDS) is

significantly higher in people who meet the diagnostic criteria for alcohol use

disorders (53). Whereas alcohol consumption does not cause overt pathology in

the lung, studies have shown that experimental alcohol exposure can lead to

subtle changes to this organ, which has been coined the 'alcoholic lung

phenotype.' This effect of alcohol is characterized by dysregulated inflammatory

response (55, 56), oxidative stress (58, 150), and tissue remodeling (59, 60).

The dysregulated inflammatory response caused by alcohol in the lung is

dependent on both the amount and duration of alcohol consumption and is

suspected to be a key player in alcohol-related lung dysfunction (61). The exact

mechanisms that mediate the development of the alcoholic lung phenotype and

increased risk of ALI/ARDS remain unclear. Better understanding of this complex process could identify potential therapeutic targets to treat or prevent alcohol-related lung dysfunction, such as ALI/ARDS.

It is now clear that many human diseases are a multi-stage, multi-hit process; it is therefore not surprising that multiple cells within a target organ contribute to disease pathology. Indeed, system level analyses of disease, and organ-organ interactions, are again gaining the attention of the research community. Several studies indicate interdependence between liver and lung in organ pathology. For example, mortality in ALI in the setting of severe hepatic disease is almost 100% (151). In experimental models of liver injury, vascular permeability and leukocyte activation in lungs are increased (152). Studies investigating the multi-organ effects of endotoxemia indicate that interaction between the liver and lung are not only likely, but may occur via alcohol-induced mediators (75) including TNFα (76), an inflammatory cytokine markedly increased in ALD (77).

The purpose of this study was to characterize a model of concomitant liver and lung injury in the setting of chronic alcohol exposure. It was hypothesized that chronic alcohol exposure acts as a first 'hit' that sensitizes these organs to a second inflammatory stimulus (e.g., bacterial lipopolysaccharide; LPS). The parallel mechanisms of liver and lung injury in this '2-hit' model were explored. Furthermore, the potential interdependence of liver and lung injury via systemic release of TNFα was also determined.

B. Experimental procedures

1. Animals and treatments

Animals were exposed to ethanol and/or LPS as described in Chapter II. Some animals also received the TNFα blocker etanercept as described in Chapter II.

2. Histology

Liver and lung tissue was stained with hematoxylin & eosin and chloroacetate esterase as described in Chapter II.

3. Clinical Chemistry

Serum levels of alanine aminotransferase (ALT) and aspartate aminostransferase (AST) were determined using standard kits as described in Chapter II.

4. RNA isolation and real-time RT-PCR

RNA was isolated from liver and lung tissue as described in Chapter II. Real time RT-PCR was performed for the genes TNFα, IL-6, IL-10, KC, MIP-2, and the endogenous control β-actin as described in Chapter II.

5. Luminex

Luminex technology was used to determine the levels of TNFα, IL-10, MIP-2, and KC in the plasma and BAL as described in Chapter II

6. Statistical analysis

Data were analyzed as described in Chapter II.

C. Results

Throughout the duration of dietary exposure, all mice gained ~0.6 g/week, with no differences between dietary and treatment groups. In saline-treated mice fed control diet, liver weights (as percent of body weight) were 4.7± 0.1 g, whereas ethanol-fed mice had liver weights of 4.8 ± 0.2 g. Liver weights in both groups were unchanged by LPS exposure.

1. Ethanol-induced steatosis enhanced LPS-induced liver injury

As expected, 6 wks exposure to EtOH-containing diet caused mild liver damage, characterized predominantly by microvesicular fat accumulation in midzonal regions of the liver (Figure 4.1A; H&E staining). LPS administration to pair-fed animals increased the accumulation of inflammatory foci in liver. Feeding EtOH-containing diet increased the severity and frequency of necroinflammatory foci caused by LPS administration. Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined as indices of liver injury (Figure 4.1B). Chronic ethanol exposure significantly increased ALT and AST levels in the plasma. LPS alone did no significantly affected circulating levels of ALT or AST. The combination of ethanol exposure and LPS significantly increased plasma ALT and AST 4 hours after LPS administration; 24 hours after LPS administration, AST values remained significantly elevated compared to controls

Figure 4.1: Effects of chronic ethanol pre-exposure on LPS induced liver injury and inflammation.

(A) Representative photomicrographs of liver tissue after hematoxylin & eosin staining (200x; H&E) and chloroacetate-esterase staining (400x; CAE). (B) Plasma levels of alanine-aminotransferase (ALT; black bars) and aspartate-aminotransferase (AST; grey bars) were used as an index of liver injury. (C) Quantitation of hepatic neutrophil content. Data are shown as +/- SEM [a], p < 0.05 compared to pair fed controls, [b] p < 0.05 compared to LPS alone.
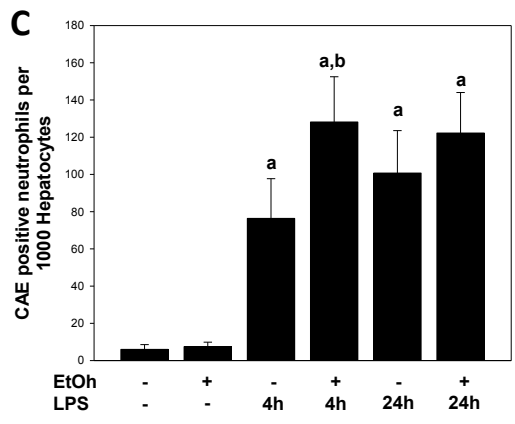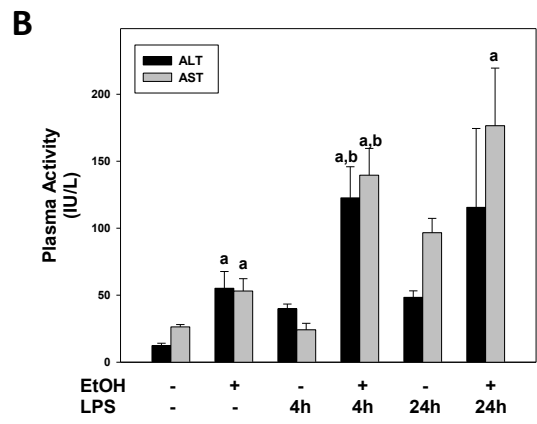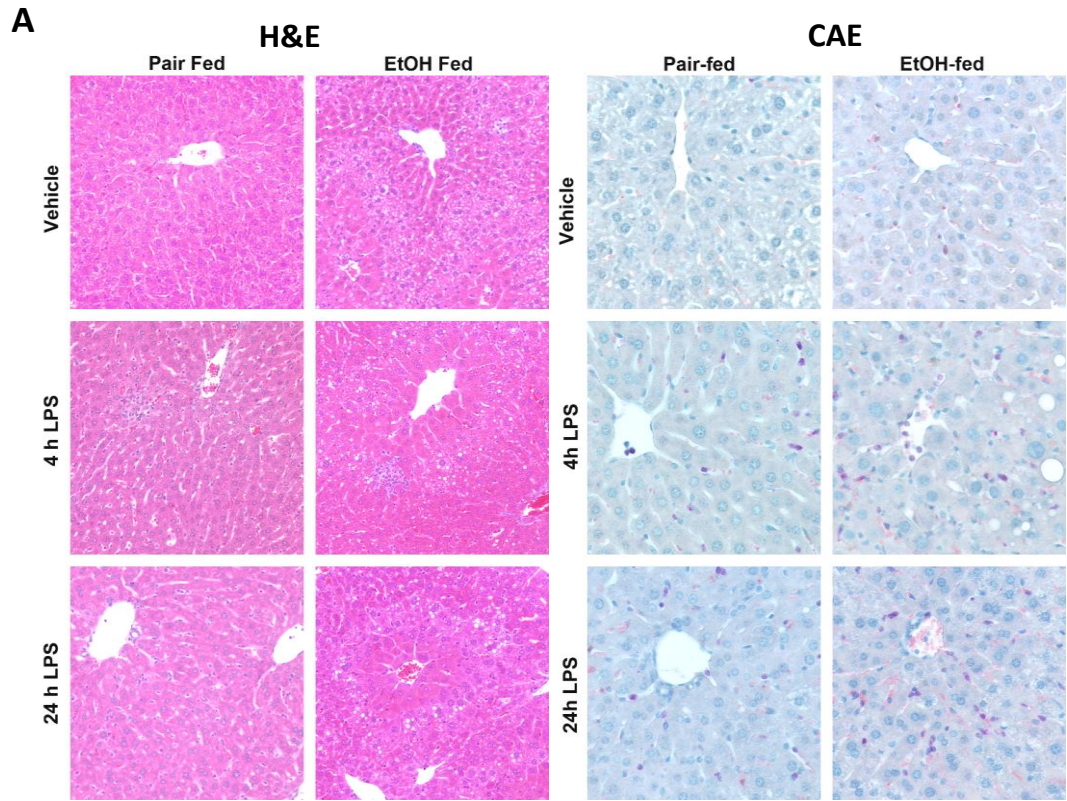
In addition to hematoxylin and eosin staining, Figure 4.1 also shows staining for neutrophils using chloroacetate esterase staining (CAE staining). Chronic ethanol exposure did not significantly affect neutrophil infiltration compared to their pair-fed counterparts (Figure 4.1A, right panels). As was expected, LPS increased the number of neutrophils in the liver compared to vehicle controls. Ethanol pre-exposure dramatically enhanced neutrophil infiltration caused by LPS.

2. Ethanol enhanced LPS-induced lung injury and inflammation

In liver, LPS injection caused noticeable pathologic changes as early as 4 h after administration (Figure 4.1A). Pathology in the lung tissue was also changed as early as 4 h as evidenced by inflammatory damage, however alveolar hemorrhage was not observed until the 24 hour timepoint (Figure 4.2). At this timepoint, LPS increased areas of alveolar hemorrhage in the lung, which can be observed in H+E staining, as indicated by an increase in the number of red blood cells in the pulmonary interstitium (Figure 4.2A, left panels). Hemorrhage was macro-heterogeneous, with some areas exhibiting a higher degree of hemorrhaging than others. Analogous to findings in liver, EtOH enhanced LPS-induced lung damage, as characterized by an increase in the frequency and severity of alveolar hemorrhage caused by LPS. The increase in alveolar wall thickness caused by LPS also appeared to be more pronounced after ethanol exposure. CAE staining of the lung tissue showed a significant increase in neutrophils in the lung tissue 4 h after LPS administration. Chronic ethanol

Figure 4.2: Effects of chronic ethanol pre-exposure on LPS induced lung injury and inflammation.

(A) Representative photomicrographs of lung tissue after hematoxylin & eosin staining (400x; H&E) and chloroacetate-esterase staining (400x; CAE). (B) Cell number in the bronchioalveolar lavage fluid (BALF). (C) Quantitation of pulmonary neutrophil content. Data are shown as +/- SEM [a], $p < 0.05$ compared to pair fed controls, [b] $p < 0.05$ compared to LPS alone.
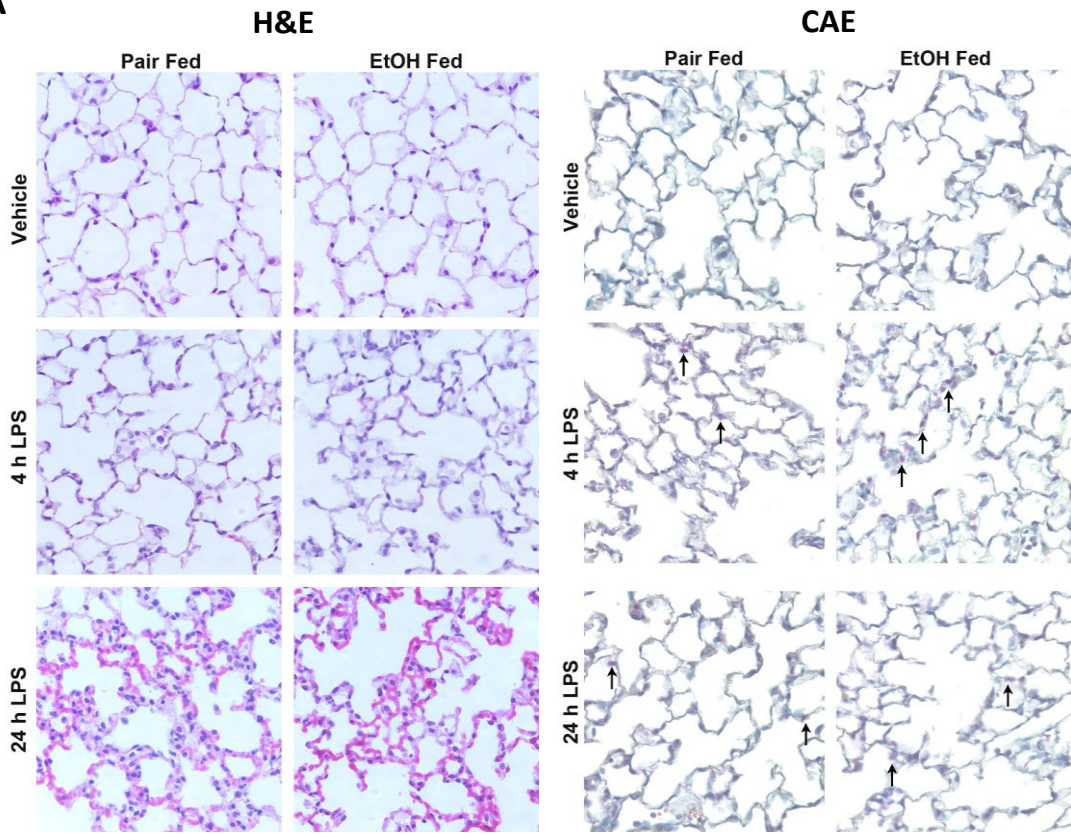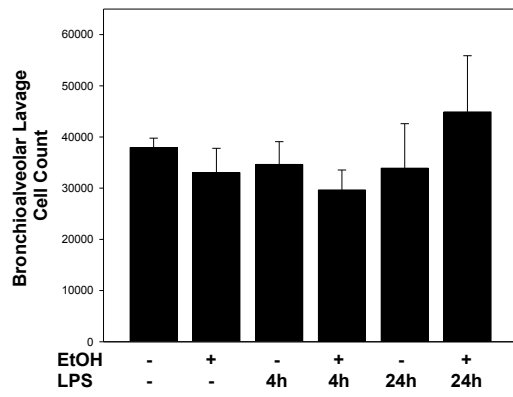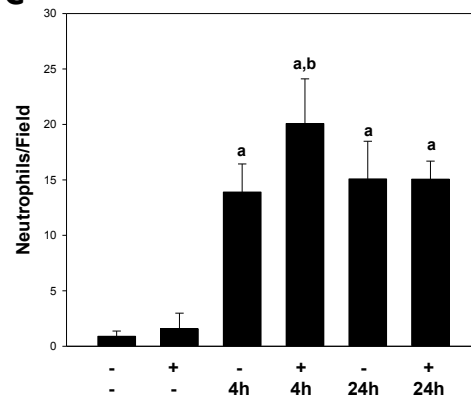
exposure enhanced the effect of LPS at the 4 hour timepoint. Incidentally, 24 hours after LPS administration, neutrophil infiltration persisted, but was no longer significantly different between ethanol and pair-fed groups.

3. **Ethanol differentially alters cytokine/chemokine expression in the liver and lung**

The pathologic assessment of liver (Figure 4.1) and lung (Figure 4.2) indicates that chronic ethanol exposure enhanced the inflammation and damage caused by LPS in both organs caused by LPS. To further characterize the mechanisms by which ethanol causes these effects, the mRNA expression of key inflammatory mediators (TNFα, IL-6, IL-10, PAI-1, MIP-2, and KC) were determined via real time RT-PCR (Figure 4.3A). Chronic alcohol exposure alone caused no significant changes in mRNA expression of these select cytokines in either the liver or lung. However, LPS administration alone significantly increased hepatic mRNA expression of TNFα, IL-6, IL-10, PAI-1, and MIP-2, with PAI-1 and MIP-2 being the most robustly induced; LPS did not significantly affect the hepatic mRNA expression of KC. As expected, chronic ethanol exposure enhanced the increase in TNFα and IL-6 mRNA expression levels seen with LPS alone in the liver.

Similar to the results seen in the liver, LPS administration alone increased the pulmonary mRNA expression of all mediators; however, the increase in TNFα was much less pronounced compared to liver and MIP-2 was the inflammatory mediator that was most robustly altered. In contrast to the liver, chronic ethanol

Figure 4.3: Effect of chronic ethanol exposure on LPS-induced cytokine expression.

(A) Cytokine mRNA expression in the liver and lung at the four-hour LPS time point. (B) Inflammatory cytokine levels in the plasma and bronchoalveolar fluid (BALF) at the four-hour time point. Data are shown as +/- SEM [a], p < 0.05 compared to pair fed controls, [b] p < 0.05 compared to LPS alone.

A

Liver

Legend: TNFa, I-L6, IL-10, PAI-1, MIP-2, KC

mRNA Expression
(fold of control)

Lung

B

Plasma

Legend: TNFa, IL-10, MIP-2, KC

Protein level
(pg/mL)

BAL

| LPS | - | - | + | + |
| EtOH | - | + | - | + |
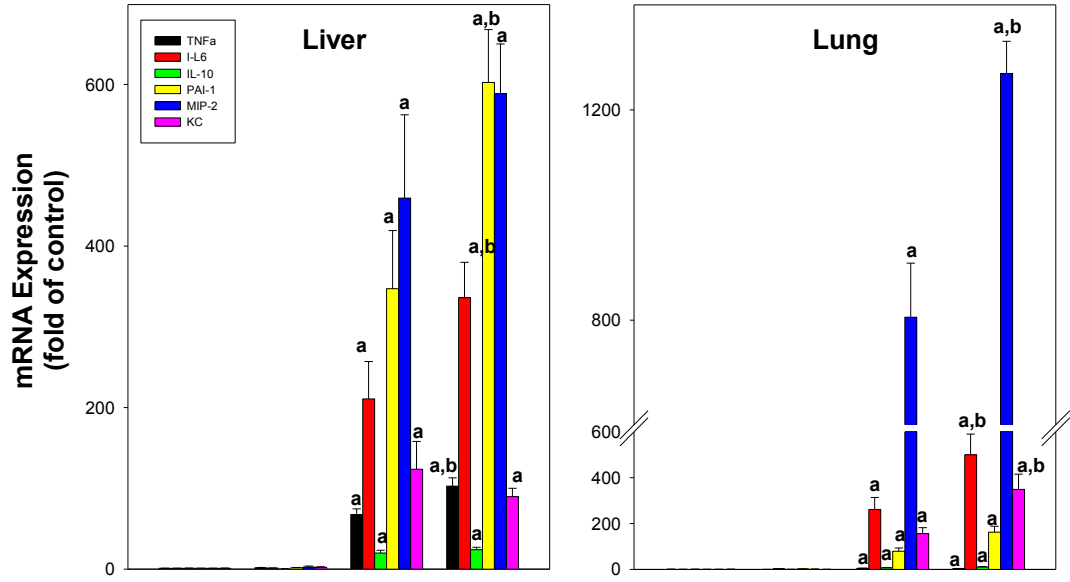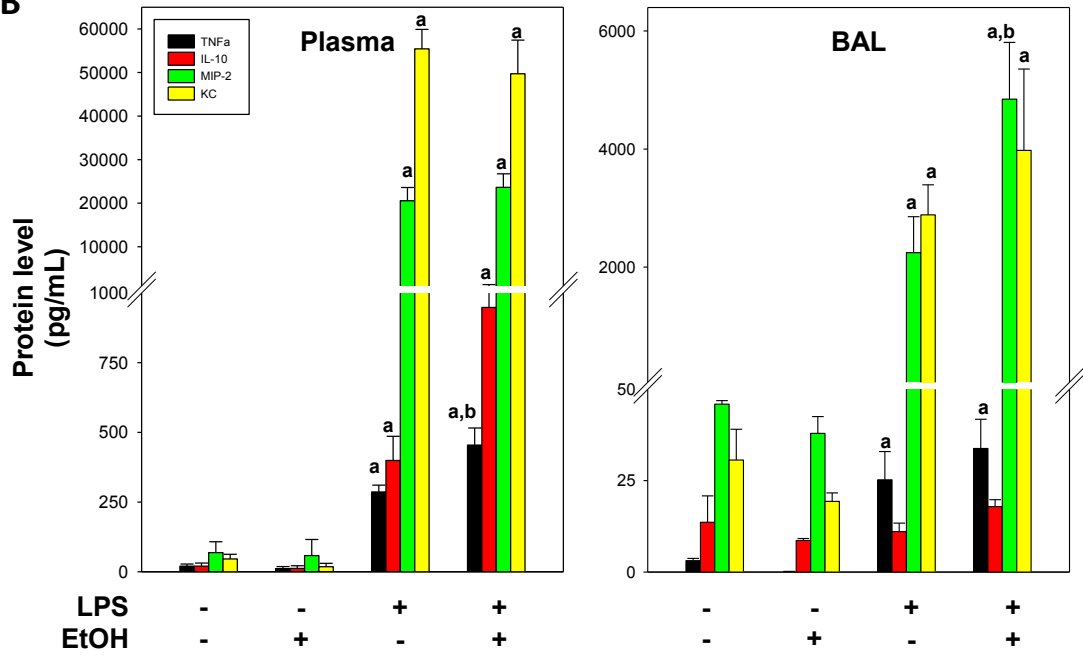
exposure did not significantly enhance the increase in TNFα expression seen with LPS alone. However, ethanol did enhance the increases in IL-6, PAI-1, and MIP-2 mRNA caused by LPS in the lung, but the pattern of that enhancement differed from liver tissue. Indeed the increase in MIP-2 caused by EtOH + LPS in the lung was >2-fold higher than observed in the liver. Additionally, ethanol exposure significantly enhanced the increase in expression of KC in the lung, an effect not observed in liver tissue.

The liver is the major contributor to the increase in inflammatory mediators in plasma in response to i.p. LPS administration (153). Likewise, BALF contains inflammatory mediators produced by lung tissue. The presence of the select mediators (TNFα, IL-10, MIP-2 and KC) in the plasma and BALF were therefore determined via Luminex assay (Figure 4.3B). With the exception of IL-10 in the BALF, LPS treatment increased the levels of all four inflammatory mediators in the plasma and BALF. In plasma, chronic ethanol exposure enhanced the increase in systemic IL-10 and TNFα caused by LPS, but did not significantly affect MIP-2 and KC. In contrast, TNFα and IL-10 levels in the BALF were both insensitive to ethanol preexposure, but the increase in MIP-2 and KC caused by LPS administration was dramatically enhanced.

4. Effect of etanercept on indices of injury and inflammation.

Previous studies have established that TNFα production is critical for subsequent pulmonary chemokine (e.g., MIP-2 and KC) production and injury in experimental models of ARDS (154). It is unclear, however, whether the source

of TNFα needs to be pulmonary or systemic in order to see this effect in the lungs (155). Therefore, etanercept was used to deplete systemic TNFα, then measured the effects of etanercept on indices of damage and inflammation in the liver and lung. As seen in Figure 4, co-administration of etanercept during the course of the study partially protected against liver injury, as determined by AST levels (Figure 4.4B), but did not affect steatosis or inflammation caused by ethanol or LPS (Figure 4.4A), respectively. Etanercept administration decreased levels of freely soluble TNFα in response to the combination of LPS in the plasma and BALF, by 33% and 60%, respectively. Interestingly, etanercept administration caused a 1.3-fold decrease in plasma MIP-2 levels which correlated with a decrease in MIP-2 mRNA expression in the liver. Etanercept also caused decreased MIP-2 in the BALF as well as a 3.2-fold decrease in KC levels in the BALF. The decrease in BALF KC levels correlated with a 3.5-fold decrease in pulmonary KC mRNA expression.

D. <u>Discussion</u>

It is now generally accepted that human disease can be a multi-stage, multi-hit process in which various cells within a target organ contribute to disease pathology. In addition to cell-cell interactions within a single organ, recent studies have begun to highlight the importance of more complex interactions, including those between multiple organs. One example of such inter-organ interaction in the setting of alcohol exposure is the critical link between the

Figure 4.4: Etanercept protects against enhanced cytokine levels in the BALF without concomitant protection against liver injury.

(A) Representative photomicrographs of liver tissue (200x; top) and lung tissue (400x; bottom) after hematoxylin & eosin staining. (B) Fold change in indices of liver injury (ALT, AST), plasma and pulmonary cytokine levels, and mRNA expression in the liver and lung. Data are shown as +/- SEM [a], $p < 0.05$ compared to no etanercept.

A

H&E

Liver

EtOH + LPS   EtOH + LPS + Etan

Lung

B

EtOH + LPS vs. EtOH + LPS + Etan
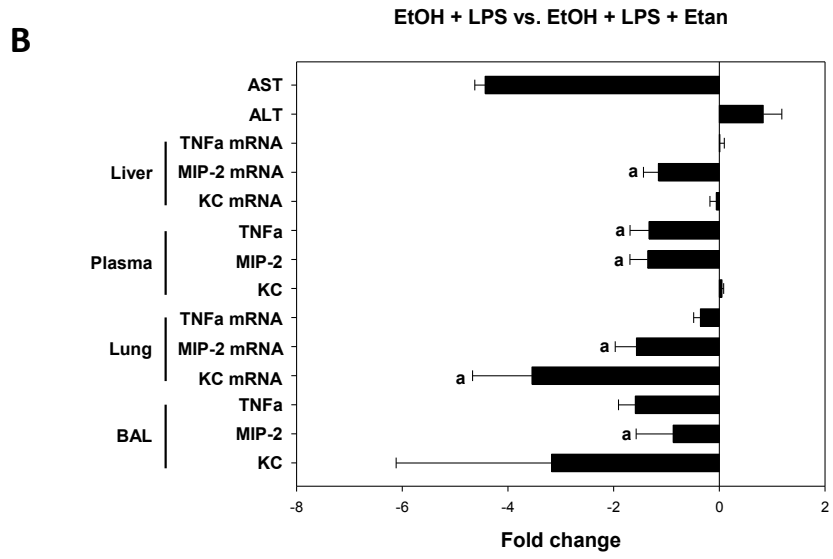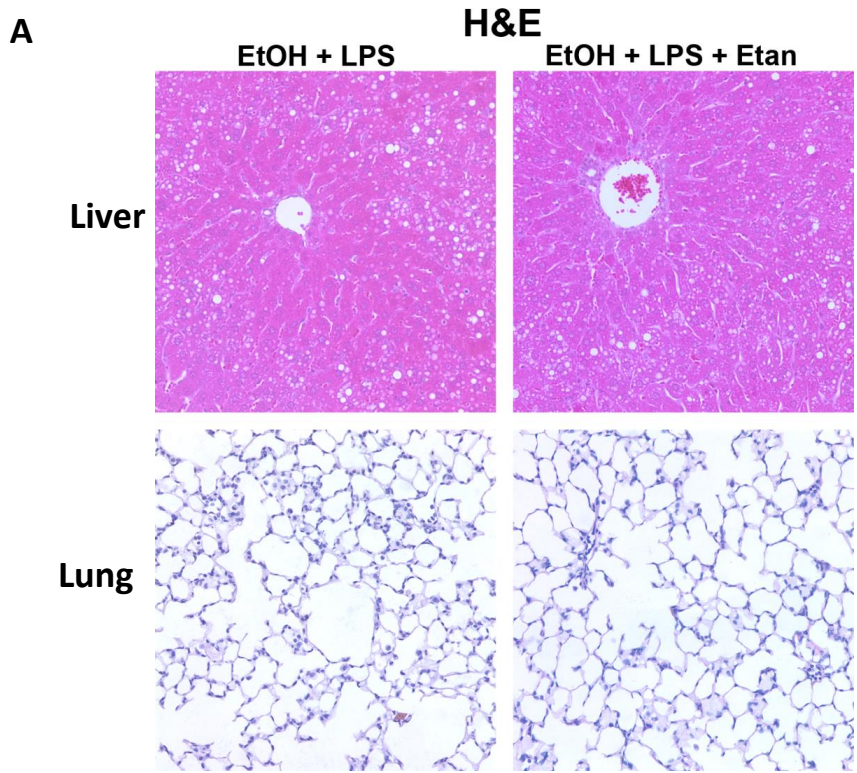
intestine and the liver, or the gut-liver axis (156). Indeed, a myriad of

experimental and clinical studies have demonstrated that alcohol-induced gut

dysbiosis and subsequent impairment of the gut barrier results in the release of

gut-derived toxins (e.g. LPS) and endotoxemia (157-159).   Studies using

antibiotics (137), germ-free mouse models (160), and probiotics (161) have

confirmed that endotoxemia caused by alcohol consumption drives injury in

experimental models of ALD. Today, the gut-liver axis and enteric dysbiosis is a

widely-accepted component in the development of ALD as well as other liver

disease (156, 162).

This group is particularly interested in potential interactions between the liver

and lung, i.e. the liver-lung axis, in the context of alcohol exposure. As

mentioned in the introduction, liver disease is associated with increased

incidence of acute lung injury and enhanced mortality. These and other findings

have suggested a potential interaction between the liver and lung that may

represent an important aspect of the pathophysiology of acute lung injury,

particularly in the setting of sepsis. Bringham and collaborators tested the

possible interaction between the liver and lung in a study performed in an *in situ*

perfused piglet preparation (152). Their model revealed that hypoxemia and

pulmonary edema were only observed when the liver and lung circulations were

connected. Their results indicate that while endotoxemia could directly cause

pulmonary vasoconstriction and leukocyte sequestration, interaction between the

liver and lung was required for severe inflammatory response and oxidative injury

to the lung. Additional studies have also shown that pulmonary injury induced by systemic endotoxin can be altered by mediators released from the liver (e.g. TNFα) (84, 152). Unfortunately, few studies have investigated the potential interactions between the liver and lung in the setting of chronic alcohol exposure. Therefore the goal of this study was to characterize a model of concomitant liver and lung injury caused by LPS after chronic ethanol administration. The Lieber-Decarli model of chronic alcohol administration was chosen for this study because this model has been used previously to study effects of alcohol exposure on both the liver (163-167) and lung (62, 168, 169), albeit separately. Systemic LPS administration served as an inflammatory stimulus to induce hepatic inflammation and an ARDS-like phenotype in the lung.

The effects of the Lieber-DeCarli rodent model of ethanol exposure on the liver is well-characterized and is predominated by hepatic steatosis (163). Six weeks of exposure to the Lieber-Decarli diet does not cause overt liver injury or hepatic inflammation (163), but sensitizes the liver to other toxic stimuli. In this study, LPS served as an inflammatory insult that induces hepatic inflammation and injury. In this 2-hit model, alcohol pre-exposure enhanced liver injury and inflammation, which corroborates previous findings in this model. Ethanol exposure has been shown to prime immune cells, including Kupffer cells, to release cytotoxic mediators including TNFα after stimulus (77, 170, 171). In this study enhanced liver injury correlated with enhanced mRNA expression and plasma levels of TNFα in response to LPS, which previous studies have shown to be predominantly derived from Kupffer cells (172).

Acute lung injury is associated with increased release of pro-inflammatory cytokines (173) in the lung and neutrophil migration into the interstitium (174). In this study, ethanol pre-exposure enhanced alveolar hemorrhage and neutrophil infiltration caused by LPS. Studies have shown that neutrophil migration into the lung is at least partially mediated by the chemokines MIP-2 and KC (175-177). Indeed, both MIP-2 and KC have been linked to acute lung injury caused by systemic LPS (178, 179) by mediating neutrophil recruitment via activation of CXCR2 (180). The work presented here shows that LPS-induced MIP-2 and KC expression in the lung was enhanced by ethanol exposure. Furthermore, increases in these chemokines correlated with increased neutrophil recruitment. Interestingly, increased expression of MIP-2 and KC in the lung and BAL have been shown to be downstream of TNFα signaling (88), which was not enhanced by ethanol pre-exposure in the lung in our model. In contrast, hepatic TNFα mRNA expression and systemic levels of TNFα were enhanced by the combination of ethanol + LPS. As mentioned previously, the role of Kupffer cell-derived TNFα in liver injury is well recognized (181-183), however hepatic-derived TNFα has also been shown to contribute to pulmonary injury. A study by Kono et al demonstrated that depletion of hepatic Kupffer cells protected against pulmonary inflammation and injury and correlated with decreased TNFα expression (183). These findings suggest that systemic inflammation mediated by increased release of TNFα by hepatic macrophages (i.e. Kupffer cells), may be partially responsible for driving pulmonary MIP-2 and KC expression, and subsequent pulmonary injury, as presented here.

In order to determine the role of systemic/hepatic TNFα in enhanced pulmonary injury, the effect of depleting systemic TNFα with etanercept was determined. Etanercept is a fusion protein of the human p75 TNF receptor and the Fc portion of human IgG1. This TNFα binding antibody has been approved by the FDA for treatment of rheumatoid arthritis and psoriasis. In the work presented here, etanercept administration resulted in a 1.7 and 1.9 fold decrease in systemic and pulmonary TNFα levels, respectively, compared to animals that were exposed to ethanol + LPS in the absence of etanercept. This incomplete blocking of systemic and pulmonary TNFα was likely due to the robust increase in TNFα caused by systemic LPS administration, particularly in the plasma; in fact, LPS administration caused more than a 10 fold increase in systemic TNFα levels compared to their vehicle-treated, ethanol-fed counterparts. Despite the lack of complete TNFα knockdown, etanercept decreased TNFα levels in the plasma by >100 pg/mL.

Etanercept under these conditions provided partial protection against inflammatory injury in the lung caused by the combination of ethanol and LPS. Etanercept administration blunted enhanced MIP-2 and KC levels in the lung, resulting in a 1.6 and 3.5 fold decrease, respectively. These results support previous findings that TNFα signaling was upstream of enhanced MIP-2 expression in experimental ARDS (184). The authors of that study showed that TNFR1$^{-/-}$ mice were partially protected against increased MIP-2 expression in a model of acute lung injury (184). Whereas the protective effect of TNFα depletion was significant, it was not complete. Indeed, TNFα is likely one of

several factors that may contribute to the 'alcoholic lung phenotype. For example, PAI-1 has been linked to liver and lung injury (29, 50, 185) and was differentially affected by ethanol pre-exposure in this model. Additionally, oxidative stress and transitional tissue remodeling are known to play important roles in liver injury and inflammation. Therefore, complete protection against injury in either organ will likely require multiple interventions or an intervention that protects against multiple mechanisms of injury (i.e., Kupffer cell blockade). Nevertheless, these results establish a strong link between systemic TNFα and lung damage in this model of alcohol-enhanced ARDS.

As mentioned above, the priming effect of ethanol on the hepatic inflammatory response is relatively well established. In contrast, the effect of ethanol exposure on lung has predominantly focused on impaired bacterial clearance and subsequent increased susceptibility to pulmonary infections (186-188). Indeed, others have shown that ethanol preexposure impairs alveolar macrophage function and inflammatory cytokine release (168, 189, 190). However, there are two important differences between the current study and those that have reported decreased immune response after alcohol. Specifically, the timing of LPS administration has been shown to play an important role in alcohol-induced liver injury. Previous studies have shown that if LPS is administered during acute alcohol intoxication (or w/in 1 day of a bolus dose of alcohol), the hepatic inflammatory response is blunted. In contrast, if LPS is given distal to alcohol intoxication, the inflammatory response is synergized (170). Indeed, most studies that have documented a decrease in pulmonary

immune response in the context of alcohol exposure used acute models of intoxication in which LPS was administered within hours of alcohol exposure (186, 191-194). Indeed, others have shown that chronic alcohol exposure can enhance pulmonary inflammation post-intoxication. For example, a recent study by Liu et al showed that chronic ethanol exposure enhances the pulmonary inflammatory response caused by insult (195). Additionally, Boe et al showed that chronic ethanol exposure enhances LPS-induced neutrophil migration, as was observed in the current study (196). Therefore it is likely that the timing of a stimulus – whether during intoxication or distal to intoxication – plays a critical role in pulmonary response after alcohol exposure. This timing effect could create a 'perfect storm' in the lung that clearance of infection is impaired during alcohol intoxication, but the inflammatory response and normal tissue damage caused by that infection is enhanced later. Such a paradigm would explain the fact that ethanol exposure increases both the incidence of lung infections and the likelihood of developing ARDS in response to a lung infection in the human population.

In conclusion, the current study characterizes a model of alcohol-induced liver and lung sensitization which enhances injury caused by systemic LPS. These data suggest that the pulmonary response to ethanol is dependent on systemic release of TNFα, most likely from the liver (Figure 4.5). Additionally, although ethanol pre-exposure enhanced both liver and lung inflammation, both organs experienced a unique pro-inflammatory cytokine profile indicating that similar, yet

Figure 4.5 Proposed mechanism of interdependent injury.

This work supports the hypothesis that systemic TNFα plays an important role in enhanced pulmonary injury caused by the combination of chronic ethanol exposure and LPS. The combination of ethanol and LPS caused significant increases in hepatic TNFα. Interestingly, the combination of ethanol and LPS caused a significant increase in MIP-2 mRNA expression and protein levels in the lung tissue and BAL, respectively. Previous studies have shown that MIP-2 expression in the lung is driven, at least in part, by TNFα signaling. The work here demonstrated that depleting systemic TNFα with etanercept partially protects against increased MIP-2 expression the lung. This protection against MIP-2 correlated with protection against pulmonary injury.
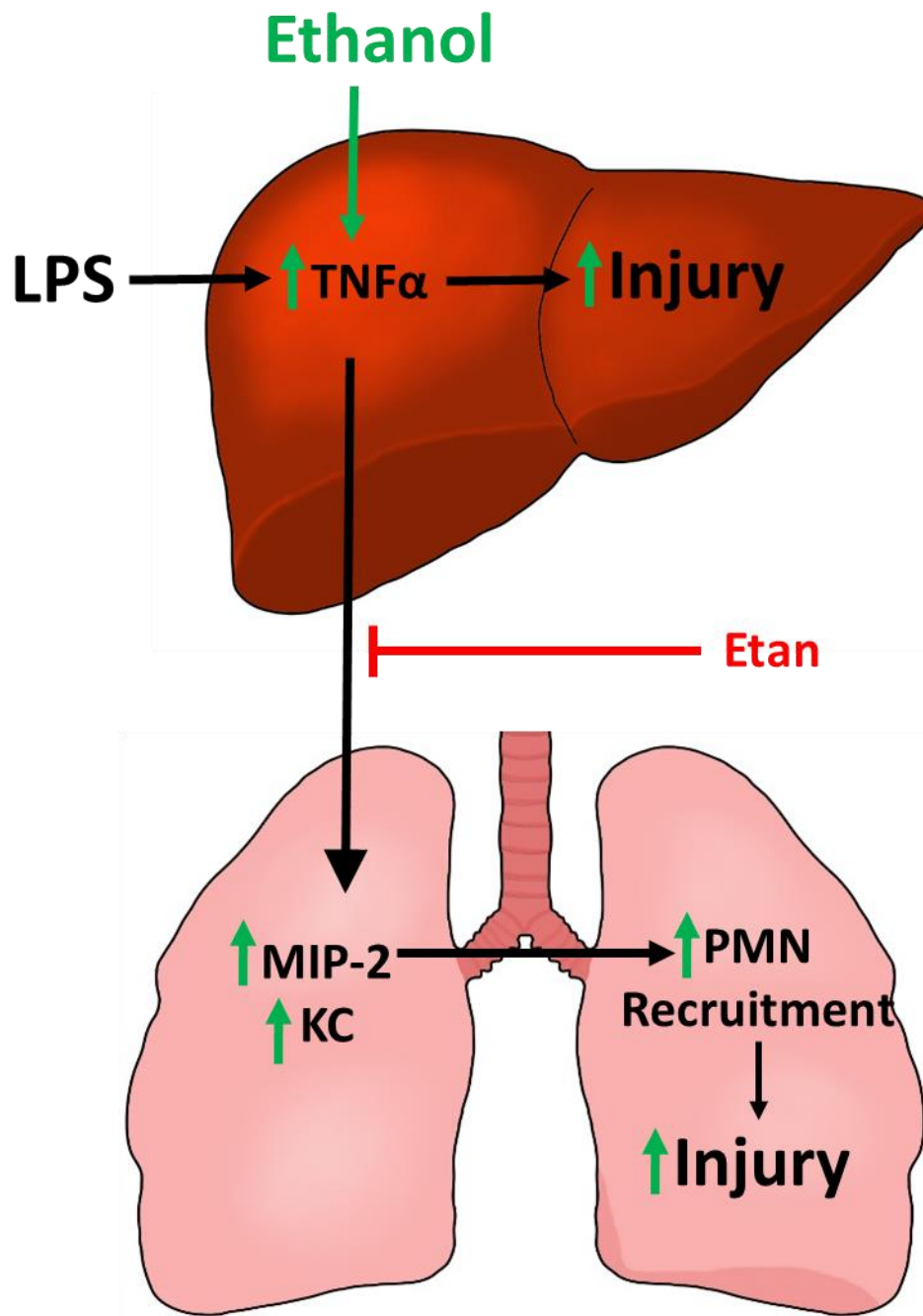
distinct, mechanisms may be contributing to injury in these two organs. Other studies have suggested that injury to the liver and lung may be linked, potentially via systemic mediators such as TNFα. Enhanced MIP-2 and KC expression in the lung, without a concomitant increase in pulmonary TNFα further support a potential role for hepatic-derived TNFα as a mediator of pulmonary injury in this model. Since etanercept is already approved for human use for treatment of rheumatoid arthritis and psoriasis, the translational value of these findings is high.

CHAPTER V

ROLE OF THE EXTRACELLULAR MATRIX IN THE LIVER IN A MODEL OF

CHRONIC ALCOHOL EXPOSURE

A. **Introduction**

The extracellular matrix (ECM) consists of a diverse group of proteins and

sugars including collagens, glycoproteins and proteoglycans. Together, these

fibrous macromolecules create a scaffold that provides structure to tissues and a

substratum for cells. In addition to their structural role, these ECM components

serve as signaling molecules via interactions with cell-surface receptors and

impact cell-to-cell communication by binding and retaining soluble mediators

including cytokines, chemokines, and growth factors (3). Proteases and

protease inhibitors present in the ECM serve as regulators of matrix degradation

important for the maintenance of the ECM homeostasis as well as remodeling of

the matrix composition in disease (2). Together, the diverse range of the ECM

components work bi-directionally with surrounding cells to create a dynamic and

responsive microenvironment that regulates cell signaling, recruitment and tissue

function.

The role of the ECM is well established in the setting of chronic liver injury,

such as alcoholic liver disease (ALD). Indeed, robust changes in the hepatic

ECM is the hallmark of later stages of liver disease (fibrosis and cirrhosis) which

are characterized by dramatic increases in collagen deposition (197). Given the dominance of collagenous changes in the hepatic ECM during fibrosis/cirrhosis, many studies have focused on the mechanisms underlying changes in collagen deposition that lead to hepatic fibrosis. However, the alterations of the hepatic ECM during fibrosis are much more diverse than simply an increase in collagen, and include changes in the deposition and distribution of a myriad of other ECM proteins (e.g., laminin and vitronectin), that may also contribute to fibrogenesis (48).

Hepatic ECM changes are not only more diverse than simply changes to collagen, but also not only relegated solely to fibrogenesis per se. Indeed, it is becoming increasingly understood that the hepatic ECM responds dynamically to stress. For example, changes in the expression of ECM proteins such as fibronectin (28, 90), and fibrin (29, 49, 50) have been observed in models of hepatic inflammation. Importantly, blocking these ECM changes blunts, at least in part, hepatic injury in these models. Therefore, transitional changes to the ECM may contribute to early disease initiation and/or progression long before fibrosis phases of liver disease.

Currently there exists a great need to characterize qualitative and quantitative changes in the ECM protein composition in earlier stages of disease that may be targeted for therapeutic benefit (198). Although previous studies have shown that subtle changes in the ECM may contribute to the development of inflammatory liver injury, the research in this area has thus far been restricted to the hypothesis-driven study of single ECM components. A more 'omic

approach has been previously hampered by the difficulties associated with the low abundance and insolubility of many ECM proteins. Therefore, a goal of this study was to develop a multi-step liver extraction that would allow for the enrichment and solubilization of ECM proteins. A second goal of this work was to use proteomic analysis to determine global changes in the extracellular matrix in a model of inflammatory liver damage.

B. Experimental procedures

1. Animals and treatment

Animals were exposed to ethanol and/or LPS as described in Chapter II

2. ECM extraction

Liver tissue was submitted to a 3-step extraction process that allows sequential isolation of loosely-bound extracellular proteins, cellular proteins, tightly bound extracellular proteins, and highly crosslinked, insoluble proteins to be differentially separated as described in Chapter II.

3. LC-MS analysis.

Liver tissue extracts underwent liquid chromatography and mass spec analysis as described in Chapter II

4. Analysis

LC-MS data was analyzed as described in Chapter II

5. Statistical analysis

Statistical analysis was performed as described in Chapter II.

C. <u>Results</u>

For this study, a previously described 3-step ECM fractionation method was adapted for use with mouse liver tissue (95-97). The ECM isolation method was performed on hepatic tissue of from and a model of ASH which does not cause significant fibrosis. Three sequential extraction steps were used in order to reduce cellular protein contamination and allow for greater resolution of low expressing ECM proteins (Figure 5.1).

The first step of the extraction involved treatment with 0.5 M NaCl. This step removes loosely-bound proteins by displacing polyionic interactions between proteins. The ECM proteins extracted during this step likely include newly formed and/or degraded ECM components and ECM-binding proteins that are loosely associated with the matrix (e.g., proteins that bind to ECM glycosaminoglycans; (199). Following NaCl extraction, the remaining liver tissue was decellularized using 1% SDS. This step solubilizes cytoplasmic and nuclear membranes, allowing for the release of cellular proteins. Decellularization was followed by a denaturing step with 4 M guanidine HCl (GnHCl) buffer (200). Following the denaturing extraction, an insoluble pellet enriched in highly insoluble, heavily cross-linked matrix proteins (i.e. type I collagens) remains (96). All three extracts as well as the insoluble pellet were subjected to enzymatic deglycosylation with chondroitinase ABC, endo-beta-galactosidase, and heparitinase II to remove the side chains chondroitin, keratin, and heparin sulfate of glycoproteins, respectively. This step increases the chance of proper

Figure 5.1: Scheme of extraction methodology.

Liver tissue from pair-fed (control) animals and animals exposed to ethanol, LPS, or the combination of ethanol + LPS underwent increasingly vigorous extraction with a NaCl buffer, SDS buffer, and GnHCl buffer as described in Materials and Methods.

Tissue

↓

NaCl Buffer

Pellet   Supernatant
               (NaCl Extract)
               **Loosely bound/new
               matrix**

↓

SDS Buffer

Pellet   Supernatant
               (SDS Extract)
               Cellular components

↓

GnHCl Buffer

Pellet   Supernatant
               (GnHCl Extract)
               Tightly bound matrices

↓

Final pellet
Insoluble fraction
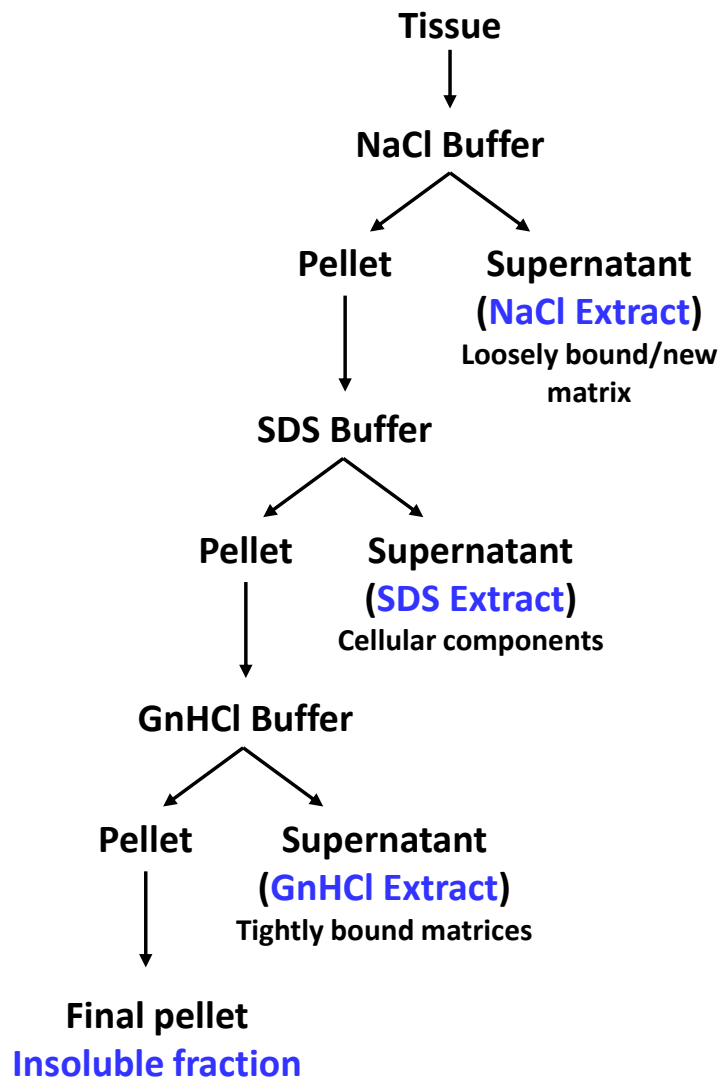
identification of proteoglycans by mass spectroscopy, which are infamous for being heavily post-translationally modified.

1. Analysis of LC-MS data

After LC-MS analysis (see Materials and Methods), Scaffold proteome software was used for visualization of the MS data. Across the four experimental groups, the NaCl extract contained 588 proteins, the SDS extract contained 511, the GnHCl extract contained 585, and the pellet contained 322 proteins. The Scaffold software allows classification of total proteins by gene ontology (GO) annotation. When sorted by GO annotation, the NaCl contained 89 extracellular proteins, the SDS extract contained 63, GnHCl contained 84, and pellet contained 46 extracellular proteins. Additional analysis was only performed on those proteins that were classified as extracellular proteins by GO annotation. The extracellular proteins identified by GO annotation were further categorized into four classes based on their role in the extracellular space as determined by a comprehensive literature search. These four groups include class 1) glycoproteins and proteoglycans, class 2) other ECM-associated proteins class 3) proteases and protease inhibitors and class 4) collagens. If no record could be found supporting the hypothesis that a protein was associated with the ECM, the protein was placed into a fifth group, class 5) other proteins. Table 5.1 shows a list of all the proteins from this study that were identified by GO annotation as extracellular, the classification of the extracellular proteins, and references supporting their association with the ECM.

Table 5.1: Extracellular proteins

Protein name	Accession Number	MW (kDa)	ECM function	Ref		Con	EtOH	LPS	EtOH +LPS
Proteoglycans and glycoproteins									
Alpha-1-acid glycoprotein 1	A1AG1_MOUSE	24	Acute phase serum protein	(201)	NaCl		⬛	⬛	⬛
					SDS				
					GnHCl				
					Pellet				
Alpha-2-HS-glycoprotein	FETUA_MOUSE	37	Inhibitor of ECM mineralization	(202)	NaCl	⬛	⬛	⬛	⬛
					SDS				
					GnHCl	⬛	⬛	⬛	⬛
					Pellet				
Arylsulfatase A	ARSA_MOUSE	54	ECM structure	(203)	NaCl		⬛		
					SDS				
					GnHCl				
					Pellet				
Beta-2-glycoprotein	APOH_MOUSE	39	Coagulation, phospholipid binding	(204)	NaCl				
					SDS				
					GnHCl	⬛	⬛	⬛	⬛
					Pellet				
Biglycan	PGS1_MOUSE	42	Regulation of collagen fibril formation, ECM-cell interactions	(205)	NaCl				
					SDS				
					GnHCl	⬛	⬛	⬛	⬛
					Pellet				
Corneodesmosin	CDSN_MOUSE	54	Adhesive protein	(206)	NaCl				
					SDS				
					GnHCl				
					Pellet	⬛		⬛	
Cysteine-rich with EGF-like domain protein 2	CREL2_MOUSE	38	Extracellular protein heavily glycosylated. ECM function unknown	(207)	NaCl	⬛	⬛	⬛	⬛
					SDS				
					GnHCl	⬛	⬛	⬛	⬛
					Pellet				

Protein	Accession		Function	Ref	Fraction	1	2	3	4
Decorin	PGS2_MOUSE	40	ECM-cell interactions, ECM assembly	(208)	NaCl				
					SDS				
					GnHCl	■	■	■	■
					Pellet				
Dermatopontin	DERM_MOUSE	24	ECM-cell interactions, ECM assembly	(209)	NaCl				
					SDS				
					GnHCl	■	■	■	■
					Pellet				
Dystroglycan	DAG1_MOUSE	97	Matrix organization, basement membrane assembly	(210)	NaCl	■	■	■	■
					SDS				
					GnHCl	■	■	■	
					Pellet				
Fibrinogen alpha chain	E9PV24_MOUSE	87	Hemostasis, cell signaling (fibrin(ogen) alpha chain)	(211)	NaCl			■	■
					SDS			■	■
					GnHCl		■	■	■
					Pellet				■
Fibrinogen beta chain	FIBB_MOUSE	55	Hemostasis, cell signaling	(211)	NaCl		■	■	
					SDS				
					GnHCl				
					Pellet				
Fibrinogen gamma chain	FIBG_MOUSE	49	Hemostasis, fibrinogen polymerization	(212)	NaCl		■	■	■
					SDS			■	■
					GnHCl	■	■	■	■
					Pellet	■			■
Fibronectin	FINC_MOUSE	273	Scaffolding, ECM organization, regulation of ECM-cell interactions	(213)	NaCl				
					SDS				
					GnHCl	■	■	■	■
					Pellet				
Galectin-1	LEG1_MOUSE	15	Matrix crosslinking, matrix organization	(214)	NaCl	■	■	■	
					SDS				
					GnHCl	■	■	■	■
					Pellet				
Galectin-3-binding protein	LG3BP_MOUSE	64	Cell-matrix adhesion	(215)	NaCl	■	■	■	■
					SDS		■	■	■
					GnHCl	■		■	■
					Pellet				

Protein	Accession		Function	Ref	Fraction	S1	S2	S3	S4
Granulins	GRN_MOUSE	63	Cell-matrix signaling	(216)	NaCl	■	■	■	■
					SDS	■	■	■	■
					GnHCl	■	■	■	■
					Pellet				
Haptoglobin	HPT_MOUSE	39	Inhibition of collagen degradation, cell migration	(217)	NaCl	■	■	■	■
					SDS	■	■	■	■
					GnHCl	■		■	■
					Pellet		■	■	■
Insulin-like growth factor-binding protein 2	IBP2_MOUSE	33	Binding/retainment of ILGF in the extracellular space	(218)	NaCl				
					SDS				
					GnHCl	■	■		
					Pellet				
Insulin-like growth factor-binding protein 4	IBP4_MOUSE	28	Binding/retainment of ILGF in the extracellular space	(218)	NaCl				
					SDS				
					GnHCl	■	■		
					Pellet				
Keratin, type I cytoskeletal 13	K1C13_MOUSE	48	Cell-matrix adhesion, cell migration	(219)	NaCl		■		■
					SDS				
					GnHCl				
					Pellet			■	
Leucine-rich HEV glycoprotein	Q91XL1_MOUSE	37	Cell-matrix adhesion	(220)	NaCl			■	■
					SDS				
					GnHCl				
					Pellet				
Lumican	LUM_MOUSE	38	Regulation of collagen fibril formation	(221)	NaCl				
					SDS				
					GnHCl	■	■	■	■
					Pellet	■	■		■
Microfibrillar-associated protein 5	MFAP5_MOUSE	18	Elastic fiber assembly, binding of fibrillin-1 and fibrillin-2	(222)	NaCl				
					SDS				
					GnHCl	■			■
					Pellet				
Fibrinogen alpha chain	E9PV24_MOUSE	87	Hemostasis, cell signaling (fibrin(ogen) alpha chain)	(211)	NaCl			■	■
					SDS			■	■
					GnHCl		■	■	■
					Pellet				■

Protein	Accession	kDa	Function	Ref	Fraction	Con	EtOH	LPS	EtOH +LPS
Ribonuclease inhibitor	RINI_MOUSE	50	Extracellular inhibitor of angiogenin which can degrade BM	(223)	NaCl				■
					SDS				
					GnHCl				
					Pellet				
Sulfated glycoprotein 1	SAP_MOUSE	61	Cell survival, cell signaling	(224, 225)	NaCl				
					SDS	■	■	■	■
					GnHCl	■	■	■	■
					Pellet	■	■	■	■
Syndecan-4	SDC4_MOUSE	21	ECM organization, signal transduction, cell adhesion	(226)	NaCl		■	■	■
					SDS	■			
					GnHCl	■	■	■	■
					Pellet				
Tubulointerstitial nephritis antigen-like	TINAL_MOUSE	52	Protein binding - fibronectins, collagens	(227)	NaCl	■	■	■	■
					SDS				
					GnHCl	■	■		■
					Pellet				
Vitronectin	VTNC_MOUSE	55	ECM-cell signal transduction, cell adhesion	(228)	NaCl	■	■	■	■
					SDS				
					GnHCl		■	■	■
					Pellet				

Other ECM-associated proteins

Protein	Accession	kDa	Function	Ref	Fraction	Con	EtOH	LPS	EtOH +LPS
14-3-3 protein epsilon	1433E_MOUSE	29	Protein binding	(229)	NaCl	■	■	■	■
					SDS				
					GnHCl				
					Pellet			■	
14-3-3 protein gamma	1433G_MOUSE	28	Protein binding	(229)	NaCl	■	■	■	■
					SDS				
					GnHCl		■	■	■
					Pellet				
14-3-3 protein sigma	1433S_MOUSE	28	Protein binding	(229)	NaCl				
					SDS				
					GnHCl				
					Pellet	■		■	

Protein	Accession	MW	Function	Ref	Fraction	Lane 1	Lane 2	Lane 3	Lane 4
14-3-3 protein zeta/delta	1433Z_MOUSE	28	Protein binding	(229)	NaCl	■	■	■	■
					SDS		■	■	■
					GnHCl				
					Pellet			■	
Actin, cytoplasmic 1	ACTB_MOUSE	42	Cortical cytoskeleton	(230, 231)	NaCl	■	■	■	■
					SDS	■	■	■	■
					GnHCl				
					Pellet	■	■	■	■
Actin, cytoplasmic 2	ACTG_MOUSE	42	Cortical cytoskeleton	(230, 231)	NaCl				
					SDS				
					GnHCl	■	■	■	■
					Pellet				
Alpha-2-macroglobulin receptor-associated protein	AMRP_MOUSE	42	Protein binding, soluble mediator internalization	(232)	NaCl	■	■	■	
					SDS		■		■
					GnHCl	■	■	■	■
					Pellet				
Alpha-actinin-4	ACTN4_MOUSE	105	Focal adhesion, cell adhesion	(233, 234)	NaCl	■	■	■	■
					SDS				
					GnHCl				
					Pellet			■	
Annexin A1	ANXA1_MOUSE	39	ECM binding, fibrinolysis	(235)	NaCl				■
					SDS				
					GnHCl	■			
					Pellet				
Annexin A2	ANXA2_MOUSE	39	ECM binding, fibrinolysis	(236)	NaCl	■	■	■	■
					SDS	■	■		
					GnHCl				
					Pellet	■	■	■	■
Annexin A5	ANXA5_MOUSE	36	ECM binding (collagen)	(237)	NaCl	■	■	■	■
					SDS				
					GnHCl				
					Pellet				
Apolipoprotein A-I	APOA1_MOUSE	31	ECM binding (collagen, fibronectin)	(238)	NaCl	■	■	■	■
					SDS	■	■	■	■
					GnHCl	■	■	■	■
					Pellet	■	■	■	■

Protein	Accession	MW	Function	Ref	Fraction	S1	S2	S3	S4
Apolipoprotein A-IV	APOA4_MOUSE	45	Lipid metabolism	(239)	NaCl	■	■	■	■
					SDS			■	■
					GnHCl	■		■	■
					Pellet	■	■	■	■
Apolipoprotein E	APOE_MOUSE	36	Lipid metabolism, cell signaling	(240)	NaCl	■	■	■	■
					SDS				
					GnHCl	■	■		■
					Pellet				
Apolipoprotein O-like	APOOL_MOUSE	29			NaCl	■	■	■	■
					SDS		■	■	■
					GnHCl				
					Pellet				
Beta-2-microglobulin	B2MG_MOUSE	14	Protein binding, cell-ECM interaction	(241)	NaCl				
					SDS	■	■	■	■
					GnHCl	■	■	■	■
					Pellet				
Calreticulin	CALR_MOUSE	48	Protein binding (collagens, laminin)	(242)	NaCl	■	■	■	■
					SDS	■	■	■	■
					GnHCl	■	■	■	■
					Pellet				
Calumenin	CALU_MOUSE	37	Pprotein binding, stabilization of fibulin-1	(243)	NaCl	■	■	■	■
					SDS				
					GnHCl				
					Pellet	■	■		■
Carbonic anhydrase 2	CAH2_MOUSE	29	pH regulation of ECM (other isozymes)	(244)	NaCl	■	■	■	
					SDS				
					GnHCl				
					Pellet				
Ceruloplasmin	CERU_MOUSE	121	Iron, copper binding	(245)	NaCl		■	■	■
					SDS				
					GnHCl				
					Pellet				
Chitinase-like protein 3	CHIL3_MOUSE	44	ECM turnover	(246)	NaCl			■	■
					SDS				
					GnHCl				
					Pellet				

Protein	Accession	kDa	Function	Ref.	Fraction	1	2	3	4
Clusterin	CLUS_MOUSE	52	Extracellular protein chaperone	(247)	NaCl				
					SDS			■	■
					GnHCl		■	■	■
					Pellet				
Cofilin-1	COF1_MOUSE	19	Cortical cytoskeleton, cell motility	(248)	NaCl				
					SDS				
					GnHCl	■	■	■	■
					Pellet				
C-reactive protein	CRP_MOUSE	25	Protein binding (fibronectin)	(249)	NaCl	■	■	■	■
					SDS		■	■	
					GnHCl				
					Pellet				
C-X-C motif chemokine 9	CXCL9_MOUSE	14	Soluble mediator (angiostatic cytokine)	(250, 251)	NaCl	■	■	■	■
					SDS				
					GnHCl			■	
					Pellet				
Endoplasmin	ENPL_MOUSE	92	Soluble mediator (TLR signaling)	(252)	NaCl	■	■	■	■
					SDS	■	■	■	■
					GnHCl				
					Pellet	■	■	■	■
Estradiol 17-beta-dehydrogenase 12	DHB12_MOUSE	35	Protein binding (heparin, fibronectin, collagen)	(253)	NaCl				
					SDS	■	■		
					GnHCl				
					Pellet				
Ferritin heavy chain	FRIH_MOUSE	21	Cell migration	(254)	NaCl	■	■	■	■
					SDS	■		■	■
					GnHCl		■	■	■
					Pellet				
Ferritin light chain 1	FRIL1_MOUSE	21	Cell migration	(254)	NaCl	■	■	■	■
					SDS	■	■	■	■
					GnHCl	■	■	■	■
					Pellet				
Glucose-6-phosphate isomerase	G6PI_MOUSE	63	Cell motility	(255)	NaCl	■	■	■	■
					SDS				
					GnHCl				
					Pellet				

Protein	Accession	#	Function	Ref	Extraction	C1	C2	C3	C4
Hepatoma-derived growth factor	HDGF_MOUSE	26	Decreted heparin-binding growth factor	(256)	NaCl	■	■	■	■
					SDS				
					GnHCl	■	■	■	■
					Pellet				
High mobility group protein B1	HMGB1_MOUSE	25	Heparin binding, cytokine	(257, 258)	NaCl	■	■	■	■
					SDS				
					GnHCl	■	■	■	■
					Pellet				
High mobility group protein B2	HMGB2_MOUSE	24	Heparin binding, cytokine	(257, 258)	NaCl		■	■	■
					SDS				
					GnHCl				
					Pellet				
Lumican	LUM_MOUSE	38	Regulation of collagen fibril formation	(221)	NaCl			■	■
					SDS				
					GnHCl				
					Pellet				
Moesin	MOES_MOUSE	68	Cell cortex organization	(259)	NaCl	■	■	■	■
					SDS				
					GnHCl				
					Pellet				
Myosin-9	MYH9_MOUSE	226	Cell migration, cortical actin organization	(260)	NaCl			■	■
					SDS	■	■	■	■
					GnHCl	■	■	■	■
					Pellet				
Neutrophil gelatinase-associated lipocalin	NGAL_MOUSE	23	ECM remodeling (modulation of MMP9 activity)	(261)	NaCl			■	■
					SDS				
					GnHCl			■	■
					Pellet				
Nucleobindin-2	NUCB2_MOUSE	50	Matrix maturation	(262)	NaCl				
					SDS				
					GnHCl				■
					Pellet				
Peptidyl-prolyl cis-trans isomerase A	PPIA_MOUSE	18	Matrix assembly of hensin	(263)	NaCl	■	■	■	■
					SDS	■	■	■	■
					GnHCl	■	■	■	■
					Pellet	■		■	

Protein	Accession	#	Function	Ref	Fraction	1	2	3	4
Pro-cathepsin H	CATH_MOUSE	37	Matrix degradation	(264)	NaCl	■	■	■	■
					SDS				
					GnHCl				
					Pellet				
S100-A13	S10AD_MOUSE	11	Calcium binding	(265)	NaCl	■	■	■	■
					SDS				
					GnHCl				
					Pellet				
S100-A9					NaCl		■	■	■
					SDS	■	■	■	■
					GnHCl		■	■	■
					Pellet				
Serum amyloid A-1	SAA1_MOUSE	14	Cell adhesion	(266)	NaCl			■	■
					SDS				
					GnHCl		■	■	■
					Pellet			■	■
Serum amyloid A-2	SAA2_MOUSE	14	Cell adhesion	(266)	NaCl				■
					SDS				
					GnHCl		■	■	■
					Pellet				■
Serum amyloid A-3	SAA3_MOUSE	14	Cell adhesion	(266)	NaCl				
					SDS				
					GnHCl			■	■
					Pellet				
Serum amyloid P-component	SAMP_MOUSE	26	Protein binding (type IV collagen, laminin)	(267, 268)	NaCl				■
					SDS				
					GnHCl				
					Pellet				
Superoxide dismutase [Cu-Zn]	SODC_MOUSE	16	Protein binding (heparin/heparan sulfate, type 1 collage)	(269)	NaCl	■	■	■	■
					SDS	■	■	■	■
					GnHCl	■	■	■	■
					Pellet	■	■	■	■
Thioredoxin	THIO_MOUSE	12	Soluble mediator (immunomodulatory cytokine)	(258)	NaCl	■	■	■	■
					SDS				
					GnHCl	■	■	■	■
					Pellet	■	■	■	■

Protein	Accession	kDa	Function	Ref	Fraction	Con	EtOH	LPS	EtOH +LPS
Tubulin beta-5 chain	TBB5_MOUSE	50	Microtubule formation, modified via ECM interactions	(231)	NaCl				
					SDS				
					GnHCl	■	■	■	■
					Pellet				
UPF0556 protein C19orf10 homolog	CS010_MOUSE	18	Soluble mediator (cytokine)	(270)	NaCl	■	■	■	■
					SDS				
					GnHCl				
					Pellet				
Vimentin	VIME_MOUSE	54	Microtubule formation	(271)	NaCl	■	■	■	■
					SDS				
					GnHCl	■	■	■	■
					Pellet	■	■		■
Vitamin D-binding protein	VTDB_MOUSE	54	Vitamin D binding	(272)	NaCl	■	■	■	■
					SDS	■	■	■	■
					GnHCl	■	■	■	■
					Pellet				

Proteases and protease inhibitors

Protein	Accession	kDa	Function	Ref	Fraction	Con	EtOH	LPS	EtOH +LPS
Alpha-1-antitrypsin 1-1	A1AT1_MOUSE	46	Inhibition of ECM proteases; inhibition of trypsin	(273)	NaCl	■	■	■	
					SDS				
					GnHCl		■	■	■
					Pellet	■	■		■
Alpha-1-antitrypsin 1-4	A1AT4_MOUSE	46	Inhibition of ECM proteases; inhibition of trypsin	(273)	NaCl	■	■	■	■
					SDS	■	■	■	■
					GnHCl				
					Pellet				
AMBP	AMBP_MOUSE	39	Precursor of protein HC (protein-ECM interactions) & bikunin (protease inhibitor),	(274-276)	NaCl			■	■
					SDS				
					GnHCl	■	■	■	■
					Pellet				
Alpha-2-macroglobulin	A2M_MOUSE	166	Inhibition of ECM proteases, inhibition of ADAMTS-7 and ADAMTS-12	(277)	NaCl	■	■		■
					SDS				
					GnHCl				
					Pellet				

Protein	Accession	#	Function	Ref	Fraction	1	2	3	4
Antithrombin-III	ANT3_MOUSE	52	Inhibition of ECM proteases, inhibition of thrombin	(278)	NaCl	■	■	■	■
					SDS	■	■	■	
					GnHCl		■		■
					Pellet				
Cathelin-related antimicrobial peptide	CRAMP_MOUSE	20	Inhibition of ECM proteases, inhibition of cathepsin L-cysteine protease activity	(279)	NaCl		■	■	
					SDS			■	■
					GnHCl		■	■	■
					Pellet				
Cathepsin B	CATB_MOUSE	37	ECM degradation	(264)	NaCl	■	■	■	
					SDS				■
					GnHCl				
					Pellet				
Cathepsin D	CATD_MOUSE	45	ECM degradation, activation of cathepsin B	(280)	NaCl	■	■	■	■
					SDS				
					GnHCl				■
					Pellet				
Cathepsin Z	CATZ_MOUSE	34	ECM degradation, integrin signaling	(281, 282)	NaCl	■	■	■	■
					SDS		■	■	■
					GnHCl			■	■
					Pellet				
Complement C3	CO3_MOUSE	186	Activation of complement system	(283)	NaCl			■	■
					SDS	■	■	■	■
					GnHCl				
					Pellet				
Dipeptidyl peptidase 4	DPP4_MOUSE	87	Protein cleavage (cytokines, chemokines), protein binding (collagen)	(284)	NaCl				
					SDS			■	■
					GnHCl		■		
					Pellet				
Ectonucleoside triphosphate diphosphohydrolase 5	ENTP5_MOUSE	47	Hydrolysis of extracellular ATP	(285)	NaCl		■		
					SDS				
					GnHCl				
					Pellet				
Fetuin-B	FETUB_MOUSE	43	Cysteine protease inhibitor, metalloprotease inhibitor, matrix mineralization	(286, 287)	NaCl	■	■	■	■
					SDS				
					GnHCl				
					Pellet				

Protein	Accession	#	Function	Ref	Fraction	1	2	3	4
Gelsolin	GELS_MOUSE	86	Actin filament capping	(288)	NaCl	■	■	■	■
					SDS				
					GnHCl			■	■
					Pellet				
Ectonucleoside triphosphate diphosphohydrolase 5	ENTP5_MOUSE	47	Hydrolysis of extracellular ATP/ADP (signaling)	(286)	NaCl		■		■
					SDS				
					GnHCl				
					Pellet				
Hemopexin	HEMO_MOUSE	51	Heme binding	(289)	NaCl	■	■	■	■
					SDS				
					GnHCl	■	■	■	■
					Pellet				
Insulin-degrading enzyme	IDE_MOUSE	118	Metalloendopeptidase	(290)	NaCl				
					SDS				
					GnHCl				
					Pellet	■			
Inter alpha-trypsin inhibitor, heavy chain 4	ITIH4_MOUSE	100	Chain of ITI (protease inhibitor)	(291)	NaCl				
					SDS				
					GnHCl				
					Pellet				
Lysozyme C-1	LYZ1_MOUSE	17	Antibacterial, hydrolysis of β-1, 4-glycosidic linkages	(292)	NaCl				
					SDS				
					GnHCl				
					Pellet	■	■	■	■
Lysozyme C-2	LYZ2_MOUSE	17	Antibacterial, hydrolysis of β-1, 4-glycosidic linkages	(292)	NaCl		■	■	■
					SDS				
					GnHCl				
					Pellet				
Murinoglobulin-1	MUG1_MOUSE	165	Protease inhibitor	(293, 294)	NaCl	■	■	■	■
					SDS				
					GnHCl	■	■	■	■
					Pellet				
Myeloid bactenecin (F1)	O08692_MOUSE	19	Cysteine protease inhibitor	(295)	NaCl		■	■	■
					SDS		■	■	■
					GnHCl		■	■	■
					Pellet				

Protein	Accession	No.	Function	Ref.	Fraction	1	2	3	4
Phosphatidylethanolamine-binding protein 1	PEBP1_MOUSE	21	Serine protease inhibitor	(296)	NaCl	■	■	■	■
					SDS		■	■	■
					GnHCl	■	■	■	■
					Pellet				
Plasminogen	PLMN_MOUSE	91	Precursor of plasmin (serine protease)	(297)	NaCl				
					SDS				
					GnHCl		■	■	■
					Pellet				
Probable carboxypeptidase PM20D1	P20D1_MOUSE	56			NaCl	■	■	■	■
					SDS		■	■	■
					GnHCl				
					Pellet				
Serpinb9	O08797_MOUSE	42	Serine protease inhibitor, granzyme inhibitor)	(298)	NaCl				■
					SDS				
					GnHCl				
					Pellet				
Serine protease inhibitor A3K	SPA3K_MOUSE	47	Serine protease inhibitor, chymotrypsin inhibitor	(299)	NaCl	■	■	■	■
					SDS			■	■
					GnHCl				
					Pellet				
Serine protease inhibitor A3N	SPA3N_MOUSE	47	Serine protease inhibitor, chymotrypsin inhibitor	(299)	NaCl			■	■
					SDS				
					GnHCl				
					Pellet				
Serpin B5	SPB5_MOUSE	42	Serine protease inhibitor,	(299)	NaCl				
					SDS				
					GnHCl				
					Pellet			■	
Thioredoxin	THIO_MOUSE	12	Activation of transglutaminase	(300)	NaCl				
					SDS	■	■	■	■
					GnHCl				
					Pellet				
Transthyretin	TTHY_MOUSE	16	Hormone binding protein, cryptic protease, peptidase	(301) (302)	NaCl				
					SDS	■	■	■	■
					GnHCl	■	■	■	■
					Pellet				

Collagens						Con	EtOH	LPS	EtOH +LPS
Adiponectin	ADIPO_MOUSE	27	Inhibition of vascular matrix degradation (contains collagen-like domain)	(303, 304)	NaCl	■	■	■	■
					SDS				
					GnHCl				
					Pellet				
Collagen alpha-1(I) chain	CO1A1_MOUSE	138	Component of collagen fibrins	(303)	NaCl				
					SDS	■	■	■	■
					GnHCl	■	■	■	■
					Pellet	■	■	■	■
Collagen alpha-1(III) chain	CO3A1_MOUSE	139	Component of collagen fibrils	(303)	NaCl				
					SDS				
					GnHCl	■	■	■	■
					Pellet	■	■	■	■
Collagen alpha-1(IV) chain	CO4A1_MOUSE	161	Component of collagen network	(303)	NaCl				
					SDS				
					GnHCl	■	■		
					Pellet	■	■	■	■
Collagen alpha-1(V) chain	CO5A1_MOUSE	184	Fibrillogenesis, crosslinking	(303)	NaCl				
					SDS				
					GnHCl				
					Pellet		■		
Collagen alpha-2(I) chain	CO1A2_MOUSE	130	Component of collagen fibrils (contains collage-like domain)	(303)	NaCl				
					SDS	■			■
					GnHCl	■	■	■	■
					Pellet	■	■	■	■
Collagen alpha-2(IV) chain	CO4A2_MOUSE	167	Component of collagen network	(303)	NaCl				
					SDS				
					GnHCl				
					Pellet		■		■
Collagen alpha-2(V) chain	CO5A2_MOUSE	145	Fibrillogenesis, crosslinking	(303)	NaCl				
					SDS				
					GnHCl		■		
					Pellet	■	■		■
Mannose binding protein	MBL2_MOUSE	26	soluble pattern recognition receptor	(305)	NaCl				
					SDS				
					GnHCl	■	■	■	■
					Pellet				

Extracellular - ECM interactions unknown

60 heat shock protein, mitochondrial	CH60_MOUSE	61
78 glucose-regulated protein	GRP78_MOUSE	72
Aminoacyl tRNA synthase complex-interacting multifunctional protein 1	AIMP1_MOUSE	34
Arginase-1	ARGI1_MOUSE	35
BolA-like protein 1	BOLA1_MOUSE	14
BolA-like protein 3	BOLA3_MOUSE	12
Carboxylesterase 1C	EST1C_MOUSE	61
Clathrin heavy chain 1	CLH1_MOUSE	192
Epididymal secretory protein E1	NPC2_MOUSE	16
Group XIIB secretory phospholipase A2-like protein	Q8VC81_MOUSE	22
Heat shock cognate 71 protein	HSP7C_MOUSE	71
Hepcidin	HEPC_MOUSE	9
Hypoxia up-regulated protein 1	HYOU1_MOUSE	111

Interferon-inducible GTPase 1	IIGP1_MOUSE	48
Lactotransferrin	TRFL_MOUSE	78
Macrophage migration inhibitory factor	MIF_MOUSE	13
Major urinary protein 12	A2CEK7_MOUSE	21
Major urinary protein 17	MUP17_MOUSE	21
Major urinary protein 20	MUP20_MOUSE	21
Major urinary protein 3	MUP3_MOUSE	21
Major vault protein	MVP_MOUSE	96
Monocyte differentiation antigen CD14	CD14_MOUSE	39
Multiple coagulation factor deficiency protein 2 homolog	MCFD2_MOUSE	16
Myeloperoxidase	PERM_MOUSE	81
Nuclease-sensitive element-binding protein 1	YBOX1_MOUSE	36
Polymeric immunoglobulin receptor	PIGR_MOUSE	85
Profilin-1	PROF1_MOUSE	15

|		
Protein CREG1	CREG1_MOUSE	24
Protein Gm20425	E9Q035_MOUSE	108
Pyruvate kinase PKM	KPYM_MOUSE	58
RNA binding motif protein, X-linked-like-1	RMXL1_MOUSE	42
Serotransferrin	TRFE_MOUSE	77
Serum albumin	ALBU_MOUSE	69
Serum paraoxonase/arylesterase 1	PON1_MOUSE	40
Translationally-controlled tumor protein	TCTP_MOUSE	19
UPF0369 protein C6orf57 homolog	CF057_MOUSE	12
Xanthine dehydrogenase/oxidase	XDH_MOUSE	147

2. 3-step serial extraction creates 4 extracts with distinct protein profiles

For initial analysis, the ECM proteins present in each of the four extracts from control animals was compared (Figure 5.2, top) without regard to protein classification. Each extract had a unique extracellular protein profile consisting of not only ECM proteins that were shared among all four extracts (7), but also proteins unique to particular extracts. Indeed, there were 16, 3, 15, and 7 proteins unique to the NaCl, SDS, GnHCl, and pellet fractions, respectively in livers from control animals. These results indicate that the extraction approach was effective at separating the proteins.

When ECM proteins were compared between treatment groups, interesting patterns of protein presence in the extracts were revealed (Figure 5.3, lower). For example, some proteins (e.g., plasminogen), if present, located consistently to the same extraction fraction across the treatment groups. The effect of treatments on other proteins was more complex than the simple presence and absence. For example, whereas annexin A1 was found in the insoluble pellet in samples from control animals, it was absent in the liver from animals exposed to ethanol or LPS alone; however, the combination of ethanol and LPS caused this protein to accumulate in the NaCl fraction. Likewise, although fibrin(ogen) gamma chain peptides were found in all treatment groups, the location of these signals was unique to each treatment condition. These

Figure 5.2: Liver extracts have unique protein profiles based on fraction type and experimental group.

The number of proteins unique to or shared by all four extractions of pair-fed animals is shown (A). Bubble graphs are used to show presence of plasminogen, annexin A1 and fibrin(ogen) gamma chain across the four extracts (x-axis) and the four experimental groups (y-axis).
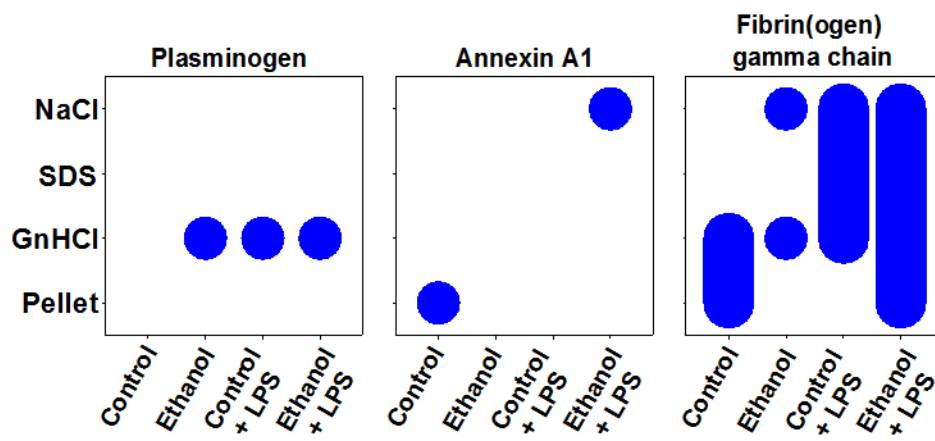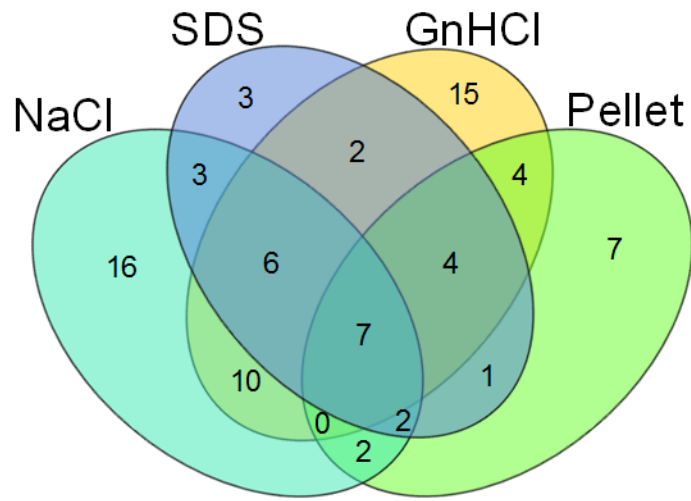
changes likely represent differences in the synthesis, degradation and/or maturity of the ECM proteins.

3. Qualitative changes to the ECM proteome in response to stress

The highest abundance of ECM was found in the GnHCl fraction of the control liver, which was closely followed by the NaCl fraction. Of the proteins in the NaCl and SDS extracts the majority were ECM-associated proteins (class 2; Figure 5.3); this effect was expected as these are generally loosely associated with the extracellular matrix and are easily solubilized. Additionally, the low abundance of collagens (class 3) in the NaCl and SDS fractions was not surprising as collagens are often tightly cross-linked and require denaturing conditions for solubilization. As expected, the denaturing conditions created by GnHCl more than doubled the number of proteoglycans and glycoproteins in the GnHCl extract compared to the NaCl and SDS fractions. The number of collagens in the GnHCl extract were also dramatically increased compared to the NaCl and SDS fractions. The pellet fraction contained the fewest proteins of all four extracts; this fraction has the greatest number of collagens (8) which are known to be highly cross-linked to the point of insolubility (306). These results indicate that the 3 step extraction process was successful in separating and enriching the ECM proteins.

Exposure to ethanol and LPS, alone or combined, did not change the general pattern of proteins found in the various extracts (Figure 5.3), but both tended to increase the number of ECM proteins. For example, ethanol exposure

Figure 5.3: Ethanol and LPS cause dynamic changes in the matrisome.

Each row represents a separate extract and each column represents a separate

experimental group comparison. In the left column, the number of proteins

unique to the control (blue) or ethanol (yellow) groups and the number of proteins

shared between the two experiment groups (green) *in toto* (Venn diagram) or

separated by protein class (stacked bar graph) are shown. Similarly, in the right

column, Venn diagrams show the total number of proteins unique to control

(blue) or LPS (yellow) group and shared between these two groups (green) and

stacked bar graphs show changes in protein number by protein class. The size

of the Venn diagrams are proportional to the number of proteins represented.
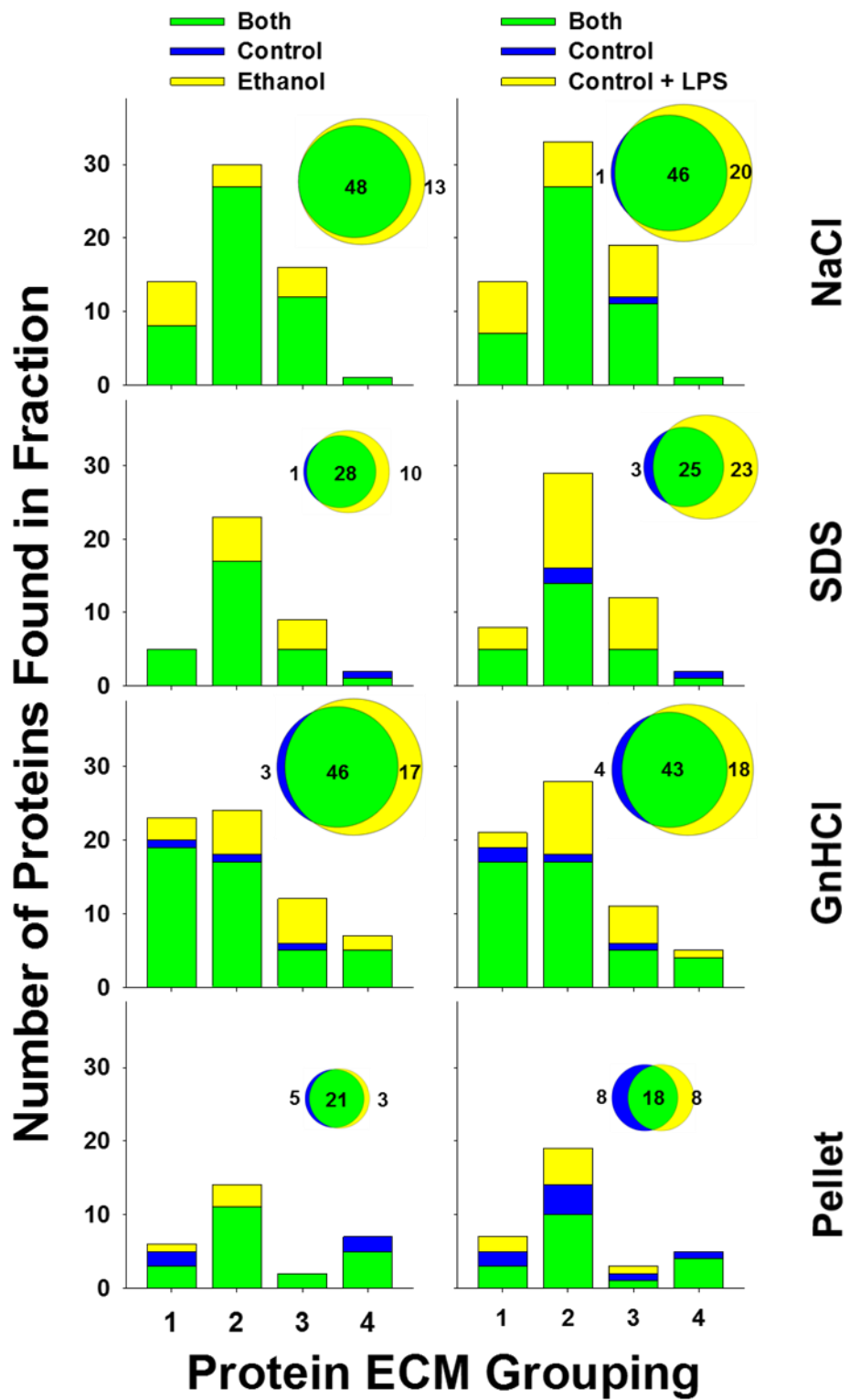
caused a net increase in the number of ECM proteins by ~22%. These changes were spread across the first 3 protein classes [(1) proteoglycans and glycoproteins, (2) ECM associated proteins, and (3) protease and protease inhibitors] with the NaCl, SDS and GnHCl extracts all increasing to a similar extent. The least responsive protein class were the collagens (class 4), which did not show a net increase with ethanol or LPS exposure. Likewise, the pellet fraction responded the least dynamically to ethanol or LPS exposure, and actually showed a net loss in total proteins. Figure 5.4 (left panels), shows the distribution of the proteins in the various extracts between all 4 treatment groups.

In addition to proteins that changed in their extraction pattern in response to ethanol (e.g., see Figure 5.2, lower panel), there were 22 proteins whose presence was unique to ethanol exposure compared to control; these include fibrin(ogen) α and β chains, cytokeratin 13, vitronectin, plasminogen, high mobility group protein B2, and collagens IVα2 and Vα2. Likewise, LPS exposure caused the appearance of several proteins that were unique compared to control. Eighteen of these proteins were shared with ethanol exposure (e.g., fibrin(ogen) α and β chains, cytokeratin 13, vitronectin, plasminogen, high mobility group protein B2, and collagen IVα2), but also 14 that were unique to LPS compared to ethanol, including serpin B5 (maspin), serine protease inhibitor A3N (Serpin A3N), and CXC motif chemokine 9 (CXCL9).

Previous work has shown that ethanol pre-exposure sensitizes the liver to inflammatory injury caused by a second insult (i.e. LPS) (170, 307). Furthermore, previous studies have suggested that changes in the ECM

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composition can contribute to the sensitizing effect of ethanol pre-exposure (29, 50, 308). In this study, the combination of chronic ethanol exposure and a second hit of LPS caused unique changes in the ECM protein profile of the liver. Indeed, the combination of EtOH + LPS resulted in the appearance of 4 unique proteins that were not present in livers from animals exposed to either ethanol or LPS alone, including serum amyloid P and serpine B9.

4. **Quantitative changes to the extracellular proteome caused by stress.**

The composition of extracellular environment is tightly regulated and drastic changes, such as the presence or absence of ECM proteins, can produce functional changes in the surrounding tissue. Therefore, it was not surprising that the presence of the majority (73 proteins) of the extracellular matrix proteins was not altered by any exposure regardless of fraction type. However, several proteins that did not change qualitatively changed in abundance. In order to understand better the more subtle changes on the expression of extracellular proteins in the four fractions, quantitative changes in the expression that were not changed qualitative was determined (Figure 5.4, right panels).

Several proteins change quantitatively in response to ethanol and/or LPS exposure relative to control animals in the livers. Notable (>4-fold increase) changes caused by LPS included ferritin heavy chain (15-fold increase) in the NaCl fraction, haptoglobin and myosin-9 (11- and 9-fold, respectively) in the SDS fraction, and fibrinogen γ chain and haptoglobin (14- and 7-fold, respectively) in the GnHCl fraction. Notable changes caused by ethanol include myosin-9 (6-

Figure 5.4: Liver extracts show quantitative changes in the matrisome.

Venn diagrams (left column) show all proteins within an extract and indicate the number that are shared between or that are unique to the four experimental groups. Bubble plots (right column) show quantitative changes in protein expression of proteins that were shared by all four experimental groups. The bubble plots show fold change in protein expression caused by LPS (y-axis), ethanol (x-axis), and the combination of ethanol + LPS (bubble size). Each bubble represents a single extracellular protein; bubble color indicates the protein's class.
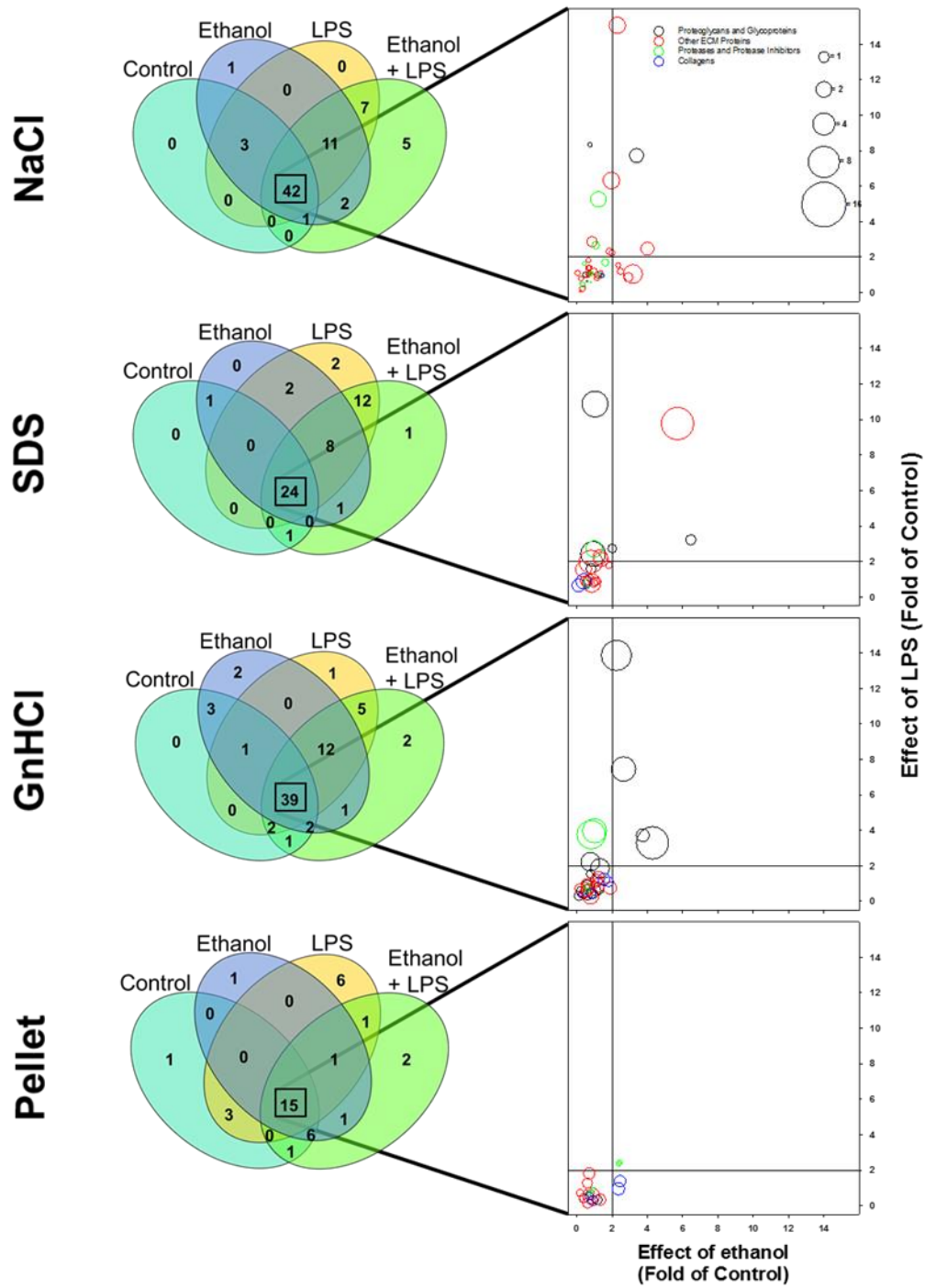
fold) and cysteine-rich with EGF-like domain protein 2 (CREDL2; 6-fold) in the SDS fraction. As was observed for qualitative analysis (Figure 5.3), the pellet fraction was the most stable of the fractions with very few changes in the expression level of ECM proteins.

Some proteins responded to the combination of ethanol and LPS exposure differentially than a simple additive effect. For example, although LPS alone did not change endoplasmin levels and ethanol only increased it 3-fold, the combination of ethanol and LPS increased this protein 11-fold in the NaCl fraction. A similar effect was seen on granulin expression in the SDS fraction, which was increased by 5-fold by LPS + ethanol despite no effect by ethanol alone and only a 2-fold increase by LPS. Additionally, although both LPS and ethanol alone increased CRELD2 the combination of ethanol + LPS actually decreased the levels in the SDS fraction. In the GnHCl fraction, proteins that were differentially regulated by the combination of ethanol and LPS include hemopexin and alpha-1-microglobulin/bikunin precursor (AMPB).

D. <u>Discussion</u>

The aim of this work was to develop and characterize a method of hepatic ECM protein extraction and analysis that would provide both the sensitivity to identify low expression proteins and the power to observe global changes in the ECM using proteomics. As mentioned in the Introduction, the study of the hepatic ECM proteome (i.e., matrisome) has largely been 'collagenocentric' and 'fibrisocentric – that is, centered on the dramatic increase in collagen deposition during fibrosis, a quasi-permanent scarring of the organ. However, the

matrisome of the healthy and diseased liver is significantly more diverse than collagen ECM. Indeed, studies have shown that in addition to collagen, laminin (306) and vitronectin (309, 310) are also increased during fibrogenesis. Furthermore, proteomic-based studies in other organs haves demonstrated that the matrisome responsds dynamically in composition after insult (95-97). Previously, this group showed that fibrin ECM accumulation correlates with inflammatory liver injury in several models, and may well play a causal role (29). Additionally, Gillis et al. have shown a similar role for fibronectin ECM in experimental ALD (28). However, little is known about global changes in the hepatic ECM during inflammatory liver injury.

Global changes in the ECM may mediate tissue function via mechanisms that fall generally into three categories, including physical, biochemical, and signaling. Physical properties of the ECM include the matrix topography, organization, and crosslinking (2). These physical properties support the structural role of the ECM, but can also allow regulation of cell migration both by serving as a physical barriers or enhancer to that function (26). For example, fibrin matrices are permissive to chemotaxis and activation of monocytes and leukocytes (311, 312). Physical changes in the ECM can cause tissue rigidity resulting in reduced organ function. Although such physical changes to the ECM can of course indirectly influence the biochemistry of the liver (e.g., hemostasis-induced hypoxia), changes to the mastrisome can also directly cause biochemical changes that are independent of structural changes. For example, ECM components can facilitate interactions between ligands and receptors (313),

bind and retain chemokines (3), and regulate activation of growth factors (i.e.

TGFβ, (3). ECM molecules also can serve directly as signaling molecules via

interactions with cell surface receptors, including integrins (314). Because of the

multi-faceted roles of many ECM molecules, any single change in the

extracellular matrix can trigger a cascade of dependent changes that influence

the composition and properties of the ECM. For example, biglycan serves as a

structural component which regulates collagen fiber assembly but upon release

from the matrix by can serve as a signaling molecule binding to TLR4 receptors

(315).

In the current study, the individual and combined effects of two

experimental exposures (ethanol and LPS) on the hepatic matrisome were

determined. The rationale for selecting LPS is that it induces a robust

inflammatory response in the liver. Exposure of the liver to low levels of LPS is

common and occurs through multiple means, including both acute and chronic

alcohol consumption (316). Whereas inflammatory responses triggered by small

doses of LPS are typically non-injurious, other stresses can synergistically

enhance the hepatotoxic response to LPS. Indeed, in addition to increasing

circulating LPS, ethanol also enhances inflammation and liver damage caused by

acute LPS exposure (71, 170). This '2-hit' paradigm is a common factor in fatty

liver diseases (34). The results here demonstrate that the hepatic matrisome

responded dynamically to both stresses, not only increasing the net amount of

protein associated with the matrisome, but also differentially changed the

amounts (Figure 5.5) and likely the composition (Figure 5.3) of the proteins found.

This study was able to validate changes in the hepatic matrisome that have been reported previously after LPS and/or ethanol exposure. Indeed, this work validated that the fibrin(ogen) ECM is dramatically altered by LPS and enhanced by the combination of LPS + ethanol. In the current study, LPS exposure caused a nearly 14-fold increase in the fibrinogen gamma chain in the GnHCl extract. Additionally, both LPS and ethanol caused fibrinogen chains to appear in the NaCl and SDS extracts, suggesting an increased rate of turn-over of this ECM component. The fibrin(ogen) gamma chain is a major component of fibrin clots which form upon cleavage of the fibrinogen gamma chain, causing its polymerization into insoluble fibrin fibers (317). Therefore, the localization of this robust increase in fibrinogen gamma chain in the GnHCl fractions suggests that there was an increase in fibrin(ogen) gamma chain polymerization into a less-soluble and more highly cross-linked form. Furthermore, the combination of LPS + ethanol resulted in presence of the fibrin(ogen) gamma chain in the insoluble pellet, which suggests additional modifications that increased insolubility (e.g., crosslinking). LPS also caused the appearance of serum amyloid A-1 and A-2 (SAA1 and SAA2) proteins, which are acute phase proteins whose production and secretion by hepatocytes is increased by LPS (318, 319). In fact, LPS administration resulted in the presence of or increased the levels of several other acute phase proteins including haptoglobin, complement C, and ceruloplasmin, all of which are known to be increased in liver by LPS administration (320-322).

Proteomic analysis of liver tissue from animals exposed to chronic ethanol exposure also validated changes that others have shown to be caused by ethanol. Previous studies have shown that ethanol exposure enhances fibronectin expression and deposition prior to the onset of fibrosis (28). In this model of alcoholic steatohepatitis, we found that fibronectin expression was increased by nearly 2-fold in the GnHCl extract compared to control livers. Additionally, ethanol exposure increased the level of collagen 1α1, which has previously been shown to be increased in patients (323) and in animal models of alcoholic steatohepatitis (324). Recent studies have also implicated the high-mobility group protein B1 as a contributor to ALD and have shown that expression is increased in both the liver and serum after alcohol exposure (325). In our study, HMGB1 was increased in the NaCl fraction by 2-fold.

Expression of several other matrix proteins were altered by chronic ethanol exposure. For example, vitronectin levels were increased in the GnHCl extracts of ethanol-treated liver. The extracellular glycoprotein vitronectin has previously been linked to hepatic fibrosis. Indeed, end-stage hepatic fibrosis is characterized by decreased circulating levels of vitronectin due to decrease in hepatic function and an increase in vitronectin deposition locally in the liver (326, 327). The findings of this study suggest that more subtle changes in vitronectin expression occur prior to the onset of fibrosis and hepatic decompensation. Ethanol also resulted in the appearance of galectin-1. Galectin-1 is glycosaminoglycan-binding lectin (328), which has been associated with cell proliferation and adhesion through modulation of glycoprotein crosslinking.

Galectin-1 may also play a role in hepatic inflammation and fibrinogenesis (329). For example, galectin-1 has been shown to be protective against experimental inflammatory liver injury (330). This role may be in contrast to later stages of liver disease that correlate increased galectin-1 secretion from activated HSCs in hepatic fibrosis (331, 332). Although additional studies will be required to validate functional changes in these proteins after alcohol exposure, these data suggest that ethanol is likely contributing to a myriad of changes in the extracellular matrix composition, many of which have not yet been fully investigated.

Changes in expression of protease and protease inhibitors can also contribute to inflammatory liver injury and fibrogenesis. In the current study, a number of matrix-associated proteases were present in the GnHCl extract of ethanol-exposed liver including plasmin(ogen), antithrombin III, dipeptidyl peptidase, and alpha-1-antitrypsin. Plasmin(ogen) is a well-known serine protease which degrades fibrin into fibrin degradation products and is tightly regulated by a series of activators (urokinase and tissue-type plasminogen activators) and inhibitors (i.e. plasminogen-activator inhibitor 1; PAI-1). In this study, both ethanol and LPS resulted in the presence of plasminogen in the GnHCl extract, suggesting that plasmin(ogen) was tightly associated with the ECM after either exposure. LPS also resulted in the presence of protease inhibitors, such as serpine B5 (maspin) and PAI-1. Several other proteases and protease inhibitors that may be critical for ECM homeostasis [e.g., transthyretin, phosphatidylethanolamin-binding protein-1 (PEBP1), and serine protease

inhibitor A2K] increased in response to ethanol and/or LPS. These data support the notion that transitional remodeling of the hepatic matrisome is likely bidirectional and driven by both increased secretion of matrix proteins as well as changes in ECM degradation.

As mentioned above, ethanol is well-known to synergize liver damage caused by LPS exposure. In the current study, we found that the combination of ethanol and LPS resulted in unique changes to the hepatic matrisome compared to either ethanol or LPS alone. Indeed, fibronectin and biglycan expression were all differentially increase by co-exposure. Fibronectin accumulation caused by ethanol may contribute to hepatic inflammation via stimulation of KCs (308). Biglycan expression was also dramatically increased by the combination of ethanol + LPS. Research suggests that biglycan may play a role in fibrotic liver disease (315, 333). This small proteoglycan was first recognized as a structural component and signaling molecule in the ECM due to its association with collagen fibrils and its role in collagen fiber organization (334, 335). In addition to its structural role, biglycan has been implicated in inflammation by retaining pro-inflammatory cytokines including TNFα and TGFβ (336) and activating TLR4 signaling (337); interestingly, in order to contribute to the inflammatory response, biglycan must be released from the collagens of the ECM by one of several MMPs (338). Areas with increased bigylcan expression have also been associated with increased inflammatory cell migration (339). These data suggest that biglycan expression may be increased in pre-fibrotic stages of liver disease. In contrast, the combination of ethanol and LPS resulted in a synergistic

decrease in the serine protease inhibitor phosphatidylethanolamine-binding protein-1 (PEBP1). This enzyme has been shown to inhibit trypsin-like serine proteases including thrombin, but not trypsin or tissue-type plasminogen activator (296). Multiple studies have identified PEBP1 as a critical player in metastasis (340), and have defined it as a metastasis suppressor gene (MSG) (341). Additional studies which implicate a role for PEBP1 in inhibition of NFκB (342), a well-recognized player in hepatic inflammation (343) further support a potential role for PEBP1 in inflammatory liver injury. It is likely that other changes in the ECM previously undetermined may also contribute to hepatic inflammation and injury.

In summary, an extraction method to enrich the hepatic ECM was developed and characterized. The results demonstrate that the hepatic matrisome responds dynamically to both acute (LPS) and chronic (ethanol) stresses, long before more dramatic fibrotic changes to the liver. It is likely that these transitional changes to the hepatic ECM contribute, at least in part, to the pathologic responses to these stresses. It is also interesting that several ECM proteins responded similarly to both stresses, suggesting a common mechanism in both models. Nevertheless, there were responses that were unique to the individual and combined effects. These results therefore also serve as a foundation for future analyses in hepatic models of liver disease.

CHAPTER VI

DISCUSSION AND CONCLUSIONS

A. <u>Restatement of goals and questions</u>

The overall goal of the work described in this dissertation was to further elucidate the role of ECM remodeling in liver injury and the potential role of such changes on the pathology of other organs, such as the lung. The experiments in Chapter III were performed to identify the potential role of gut dysbiosis in driving fibrin accumulation and liver injury in a model of enhanced fatty liver disease caused by the combination of HFD and arsenic. Chapter IV aimed to characterize a model of concomitant liver and lung injury in the context of chronic alcohol exposure that will allow further investigation of parallel and interdependent mechanisms of injury in these two organs, including transitional tissue remodeling. Finally, Chapter V of this dissertation describes a new method of protein extraction which can be used for analysis of the extracellular matrix proteome. Together, these studies shed new insight into the complex mechanisms that mediate transitional tissue remodeling and injury in the liver and lung.

B. Major findings of this dissertation

1. Dysbiosis contributes to hepatic sensitization and transitional tissue remodeling during NAFLD

Previous studies from this group have shown that coexposure to low concentrations of arsenic and high-fat diet (HFD) enhanced liver injury and inflammation (98); importantly, these effects of arsenic correlated with an increase in proinflammatory fibrin ECM deposition. However, whether enhanced hepatic injury in this model was due to interactions between arsenic and HFD locally in the liver or distally in the gut was unclear. Therefore, the study described in Chapter III was designed to identify changes in the gut microbiome after concomitant HFD and arsenic exposure and to test the therapeutic potential of the prebiotic oligofructose (OFC) in this model; a major endpoint was whether or not this intervention would blunt the increase in fibrin ECM deposition caused by the combination of arsenic and HFD.

Changes in the gut microbiome caused by HFD, as described in Chapter III support the link between HFD and dysbiosis previously demonstrated by others (78, 134). Additionally, this study found that arsenic alone impacted the gut microbiome, leading to a net loss of commensal bacteria and expansion of the population of Gram negative bacteria. Increases in Gram negative bacteria, which harbor a lipopolysaccharide wall, can contribute to endotoxemia and have been linked to liver injury (344, 345). Therefore, the increase in Gram negative bacteria caused by arsenic may contribute to liver injury by enhancing HFD-induced endotoxemia. Another potential mechanism of enhanced injury may be

'upstream' of arsenic exposure. For instance, HFD-induced steatosis and endotoxemia may sensitize the liver to otherwise innocuous levels of arsenic. HFD has previously been shown to sensitize the liver to injury by other factors, including LPS (145, 146).

The work presented in Chapter III demonstrated a protective effect of OFC. Protection conferred by OFC was evidenced by a reduction of liver steatosis, fibrin ECM deposition and inflammation that is normally seen with HFD-feeding and a prevention of expansion of Gram negative bacteria caused by arsenic exposure, coupled with an expansion of potentially protective bacterial populations. Protection against enhanced liver injury that was conferred by OFC is likely mediated by both direct effects on the microbiome as well as potentially indirect effects on hepatic sensitization. For example, protection against increases in Gram negative bacteria, which can contribute to endotoxemia, may confer protection against liver injury. Indeed, Adachi and colleagues demonstrated that depletion of Gram negative bacteria by enteral antibiotic administration protected against endotoxemia and liver injury caused by alcohol exposure (344). Additionally, OFC has been shown to protect against HFD-induced endotoxemia (80). In agreement with this previous work, we found that OFC protects against steatosis caused by HFD-feeding to animals. Steatosis and endotoxemia are two factors known to sensitize the liver to injury (34, 35). Therefore, protection against these changes by OFC feeding may diminish HFD-induced hepatic sensitization and, ultimately, protect against increased sensitivity to arsenic toxicity.

It is hypothesized that the enhanced HFD-induced liver injury caused by arsenic exposure is mediated at least in part via deposition of fibrin ECM. This hypothesis is supported by previously established proinflammatory effects of fibrin ECM in liver (29), as well as mechanisms hypothesized to be involved in the atherogenic effects of arsenic in the vasculature (346). Here, this enhanced fibrin accumulation caused by arsenic was almost completely blunted by OFC administration. These data support a role for transitional matrix remodeling in the setting of arsenic hepatotoxicity. Fibrin ECM accumulation in the liver can be mediated via an increase in deposition (via activation of the coagulation cascade), and/or via a decrease in degradation (i.e., fibrinolysis) caused by PAI-1 induction (29). In the work presented here, PAI-1 expression was significantly increased by the combination of arsenic and HFD. The implications of potential crosstalk between transitional tissue remodeling in the setting of fatty liver disease is a focus of future work in this laboratory. The results of the current study lend weight-of-evidence that transitional changes in the hepatic ECM play a unifying role in the initiation and/or progression of fatty liver disease, be it from alcohol or from diet (HFD).

2. Chronic ethanol exposure causes unique pro-inflammatory changes in the liver and lung after systemic LPS administration

Chapter IV of this dissertation aimed to characterize a model of concomitant liver and lung injury in the context of chronic ethanol exposure and to establish a potential link between injury of these two organs. As mentioned in the Introduction, work by this group and that of the Roman laboratory has been

pursuing in parallel the role of transitional matrix changes in inflammatory liver and lung injury. Whereas the detrimental effects of chronic alcohol exposure on the liver and lung are well established separately, potential links between the two are less well characterized. However, clinical studies have suggested a potential interaction between the liver and lung; for example, hepatic cirrhosis increases mortality due to acute lung injury to nearly 100% (152). Additionally, an experimental model of endotoxic shock has shown that LPS-induced lung damage required interaction with the liver (84). Therefore, the damage to liver and lung caused by transitional ECM changes may not only be parallel pathologic responses, but interdependent mechanisms. Elucidating these mechanisms therefore offers the possibility to develop novel therapeutic approaches. Prior to these critical experiments being performed, the model needed to be developed.

In the work presented here, ethanol preexposure enhanced LPS-induced injury and inflammation in both the liver and the lung. Interestingly, ethanol preexposure caused changes to the mRNA expression of inflammatory cytokines that was unique to each organ. For example, ethanol preexposure caused a significant increase in TNFα mRNA expression in the liver which correlated with a significant increase in plasma levels of TNFα in animals exposed to ethanol and LPS. In contrast, ethanol preexposure did not alter TNFα expression in LPS-exposed lungs. Ethanol preexposure did, however, enhance mRNA expression of the TNFα sensitive genes MIP-2 and KC in the lung. Both MIP-2 and KC have been shown to contribute to pulmonary injury caused by LPS (154, 178) and

blocking TNFα expression has been shown to protect against increases MIP-2 and KC in the lung (88). To address the potential role of TNFα in driving pulmonary MIP-2 levels, we used a TNFα binding antibody, etanercept, in an attempt to block enhanced pulmonary injury caused by ethanol exposure. As expected, etanercept blunted plasma TNFα levels. Etanercept also protected against enhanced pulmonary mRNA expression of MIP-2 and KC in lung and also caused a significant decrease in MIP-2 levels in the BALF. Attenuation of the increase in MIP-2 correlated with protection against pulmonary injury, as indicated by pulmonary pathology. These findings suggest that enhanced systemic TNFα caused by ethanol preexposure may contribute to pulmonary injury. Additionally, Kono and colleagues have suggested that KC-derived inflammatory mediators, including TNFα, contribute to pulmonary injury after systemic LPS exposure (347). Therefore future studies by our lab should aim to elucidate the potential contribution of hepatic-derived TNFα using models that specifically block the release of TNFα from the liver. This work also lays the foundation to investigate the key role of ECM changes in both organs and their independence in pathology.

3. **The hepatic matrisome undergoes dynamic changes after ethanol and LPS exposure.**

The first 2 studies build on our understanding of the role of transitional ECM changes in liver damage (Chapter III) and develop a new model to leverage these questions in the interplay between liver and lung damage (Chapter IV); however, a limitation of previous studies is that they generally investigated

changes in one ECM protein at a time (e.g., fibrin; Chapter III) and ignore

structural changes that may occur with altered ECM protein turnover. Although

the role of the ECM is well established in the setting of chronic liver injury and

alcoholic liver disease (ALD), the study of the hepatic ECM changes has been

focused primarily on dramatic changes in the ECM during fibrogenesis and

specifically the contribution of collagen ECM in driving this process. However,

recent studies have shown that multiple ECM proteins contribute to fibrogenesis

(48), and that the hepatic ECM is altered prior to fibrogenesis (28). Therefore the

goal of Chapter V was to adapt a sequential protein extraction method developed

for cardiovascular research (95-97) to facilitate LC-MS analysis of global

changes in the hepatic matrisome after chronic alcohol exposure. Such an

approach could discover new proteins that are changed by insults. Furthermore,

the sequential extraction methods yield new insight into potential changes in

turnover.

The 3-step protein extraction described in Chapter V allowed efficient

extraction of the hepatic ECM proteome into four distinct extracts. These

consisted of loosely-associated ECM proteins (NaCl extract), intracellular and

membrane-associated proteins (SDS extract), tightly bound ECM proteins

(GnHCl extract), and highly insoluble ECM components (the remaining pellet). A

total of 147 extracellular proteins were identified in this study (Table 5.1).

Qualitative analysis of the ECM proteins in each fraction demonstrated that both

ethanol and LPS caused dynamic changes in the ECM. While neither ethanol

nor LPS changed the general pattern of proteins found in the various extracts,

both tended to increase the number of ECM proteins. Despite no changes in the general pattern of proteins, there were 22 proteins and 14 proteins whose presence was unique to ethanol and LPS exposure, respectively. These unique subsets of proteins may play critical roles in the hepatic response to stress. There were also quantitative changes in protein abundance caused by ethanol or LPS that may impact function. Interestingly, the combination of ethanol and LPS caused both qualitative and quantitative changes in the hepatic ECM. Analysis of these global changes in the hepatic matrisome may identify new key players in the development of liver injury, the roles of which can be validated in future studies. Furthermore, analysis of changes specific to chronic ethanol exposure may reveal new insights into the mechanisms of liver sensitization caused by alcohol, thus leading to potential therapeutic targets. Most importantly, this work emphasizes dynamism in the hepatic ECM proteome responses to stress that opens a new critical area of future research. This work also lays the foundation for future studies, such as investigating the interactive changes between the hepatic and pulmonary ECM proteome, using the model established in Chapter IV.

C. Significance of new findings

The findings of Chapter III have identified dysbiosis as a potential mechanisms by which HFD feeding may predispose individuals to increased toxicity from environmental toxicant, such as arsenic. While the levels of arsenic exposure to most Americans is typically considered sub-toxic, data presented

here suggest that other factors which alter the gut microbiome, like HFD, may sensitize the liver to injury by otherwise innocuous exposures. Such interactions are becoming more relevant as the incidence of NAFLD continues to increase both nationally as well as globally. Indeed, the estimated prevalence of NAFLD in the US may be as high as 19% (39), however only a minority of these individuals will progress to later stages of disease (40-42). A better understanding of the interactions that occur between diet and environmental exposures will offer new insights into potential risk factors for the development of NAFLD in the human population and allow better identification of at-risk populations.

The role of inter-organ crosstalk during disease states such as multi-organ failure and sepsis is well established but mechanisms underlying these interactions can be very complex and largely remain incompletely understood. Chapter IV characterized a model of concomitant liver and lung injury in the setting of chronic alcohol exposure. Characterization of this model will bring attention to the parallel mechanisms of injury in these two organs as well as the potential for extensive interdependence between them. Better understanding of the contribution of hepatic-derived mediators in the increased susceptibility to lung injury after alcohol exposure may provide new insights into the mechanisms underlying dysregulated immune response and tissue remodeling in the lung.

Chapter V describes a new method of hepatic matrisome extraction and analysis. Implementation of this new method will allow identification of new players in fatty liver disease paradigm. Furthermore, adaptation of this method

will allow analysis of global changes in the extracellular matrix in other models of liver injury (i.e., NAFLD) and in other organs (i.e., the lung). Identifying new targets in disease progression could potentially be beneficial for individuals with early stages of liver disease by offering a means to prevent progression to later stages of disease.

D. **Strengths and Weaknesses**

1. **Strengths**

There are several strengths of this dissertation. One such strength is that it identifies gut dysbiosis as a new potential mechanism mediating interaction between diet-induced liver disease and environmental toxicants (i.e., arsenic exposure). The findings of this study provide additional support for the use of prebiotics as a potential therapeutic for early stages of liver disease, and potentially as a prophylactic therapy to protect against environmental liver disease. This work also describes an initial characterization of a new model of concomitant liver and lung injury after chronic alcohol exposure. Characterization of this new model will allow a better understanding of how alcohol affects the entire person (or mouse) rather than an individual organ. Use of this model may also provide new insights into why some lung-specific approaches to the study and treatment of pulmonary injury after alcohol exposure are not completely effective and that systemic therapies (e.g., etanercept) may confer new avenues of treatment.

Additionally, a new method of protein extraction has been validated for use with liver tissue, effectively allowing the analysis of low-expressing and highly insoluble hepatic ECM proteins. LC-MS analysis of these extracts has opened the door for a discovery-based research approach that can help to expand the foundation of new hypothesis-driven studies and possibly increase the identification of new therapeutic targets. Importantly, this work also demonstrates that the hepatic ECM proteome responds dynamically to stress. Whereas such dynamism for the ECM is well understood in other organs and systems, most work in the liver has focused predominantly on hepatic fibrosis.

Another strength of this work is the use of whole animal models. Most of the experiments described here were performed *in vivo*. As mentioned previously, liver disease and the effects of alcohol use are complex phenomena which are not limited to a single organ or cell type. The use of *in vivo* models in the experiments described here allowed the study of interactions between multiple organs, including the gut, liver, and lung. Indeed, all of the questions that were addressed here likely could not be sufficiently recapitulated in a simpler model system (e.g., cultured cells).

2. Weaknesses

Although the use of animal models of disease is a strength, it is also a potential source of complexity. There is almost always an inverse relationship between the validity of the model and the degree to which the model can be controlled and therefore how certain our conclusions may be. Therefore,

although *in vivo* is a more valid approach than simpler models, the 'black box' is also much larger. Furthermore, although *in vivo* research is preferred for the study of inter-organ phenomena, no animal model can completely recapitulate the human condition. Indeed, there is currently no rodent model that exactly represents the progression of fatty liver disease, increased susceptibility to lung injury, or the typical human microbiome. It is therefore possible that the observed changes in the microbiome or mastrisome are unique to the species tested and have little direct relevance to human beings.

The work presented here identified the microbiome as a site for interaction between a HFD and the environmental contaminant arsenic. This study focused on changes in the microbiome caused by arsenic, HFD and OFC administration rather than the potential effects that the microbiome might be having on these parameters. Lu et al have shown that arsenic-induced dysbiosis does cause functional changes in the gut metabolome (2). Future studies will be necessary to pare out additional, bidirectional changes in the microbiome and arsenic metabolism.

One weakness of this project is the use of systemic administration of etanercept to block TNFα investigate the potential interaction between liver and lung injury. Whereas the liver is known to contribute to enhanced systemic levels of pro-inflammatory cytokines, including TNFα, systemic etanercept binds TNFα, regardless of source. Studies that aim to identify the role of hepatic-derived mediators in the development of lung injury will require an experimental model which allows specific blockage of liver-derived TNFα.

The experiments described in Chapter V identified changes in the hepatic matrisome after alcohol and LPS exposure. Unfortunately, due to the cost of LC-MS analysis, study samples were pooled prior to LC-MS. This is a significant weakness of this study as qualitative changes in protein expression could have been driven by a single sample. Future studies would be strengthened by analysis of individual samples as separate runs, or via 'multiplexing' the samples with protein tagging. Additionally, the experiments described herein did not determine the functional significance of the changes in the ECM. Future studies will be required to validate that changes in the matrisome caused by LPS and/or ethanol cause functional change *in vivo* and *in vitro*.

E. <u>Future Directions</u>

While the experiments described in this dissertation answered specific gaps in our knowledge of ECM remodeling, it has also created new questions that will need to be addressed in future studies. Some of these are discussed below.

1. Does HFD-induced dysbiosis change arsenic metabolism and/or absorption?

Because the experiments described in Chapter III were largely concerned with the effect of arsenic, HFD, and OFC on the gut microbiome, the bidirectional effects of dysbiosis on arsenic metabolism and absorption were not determined. Lu and colleagues have previously shown that higher levels of arsenic can cause changes in the gut metabolome (124) and that dysbiosis caused by bacterial infection altered arsenic metabolism (348). *In vitro* studies have also shown that

the bacteria of both the mouse and human gut can metabolize arsenic in vitro (141-143) . Such findings suggest that dysbiosis caused by HFD could cause dramatic changes in arsenic metabolism. An important endpoint of future studies would include hepatic levels of arsenic and arsenic metabolites. Indeed, increased levels of arsenic in the liver of HFD-fed mice may contribute to enhanced liver injury in the model presented here. On a related note, it would be interesting to determine if arsenic has global effects on the hepatic matrisome; this question is especially valid, since previous work by others has shown that very low concentrations of arsenic cause hepatic endothelial remodeling (46) Work by others in our group are pursuing these questions using the ECM proteomic approach developed in Chapter V.

2. Does liver-derived TNFα contribute to the alcoholic lung phenotype?

In the experiments described in Chapter IV, etanercept was used to identify the potential role of extra-pulmonary TNFα in driving lung inflammation. While studies have shown that hepatic Kupffer cells are responsible for ~90% of the increase in plasma TNFα caused by LPS (172) the role of liver-derived TNFα needs to be determined using a model in which one can specifically block TNFα release from the liver. One approach to this problem would be to use Kupffer cell chimeric mice to impair production of tumor necrosis factor α converting enzyme (TACE) in Kupffer cells. TACE is an enzyme required for cleavage and systemic release of TNFα into the blood. The use of such chimeras would target TACE deficiency to the Kupffer cells and thereby block hepatic TNFα signaling. These

studies would identify the contribution of hepatic TNFα in the lung alterations caused by chronic alcohol exposure. The protocol for generating the chimeras to perform these studies are currently being optimized.

3. What are the functional effects of changes in the hepatic matrisome of LPS and ethanol-exposed livers?

The method described in Chapter V identified dynamic changes in the hepatic matrisome after ethanol and/or LPS exposure. However, the functional effects of these changes has yet to be determined. Others have shown that the extracellular matrix from decellularized tissues can be solubilized and used to coat cell culture plates where it serves as a substratum for cells *in vitro* (349). By using plates coated with the ECM of animals exposed to ethanol or LPS, the functional effects of changes in the ECM composition on cell migration activation could be determined.   *In vivo* experiments using inhibitors that block the interactions between the ECM and cell surface receptors may also provide insight into the functional effects of changes in the hepatic matrisome after alcohol and LPS exposure. Indeed, previous studies from our group have shown that the integrin inhibitor CycloRGDfV can protect against enhanced liver injury and inflammation caused by acute ethanol exposure and LPS (350).

4. What changes does alcohol exposure cause to the lung matrisome?

Experimental alcohol exposure has been shown to contribute to the alcoholic lung phenotype which is characterized by oxidative stress (58), dysregulated

immune response (186, 189), and tissue remodeling of the lung (59, 351, 352).

Indeed, studies have shown that alcohol exposure increases the expression of

fibronectin by pulmonary fibroblasts (68). Additional studies have shown that

ethanol also causes increased expression and activity of matrix

metalloproteinases (MMPs) (62).   Furthermore, the fibronectin-rich matrix

produced by alcohol-exposed alveolar macrophages appears to contribute to

functional changes in the lung after alcohol exposure, including monocyte

activation (353). A discovery-based method, such as that described in Chapter

V, would allow for analysis of global changes to the pulmonary matrisome after

alcohol exposure.   Furthermore, identification of new players in transitional

tissue remodeling in the lung would expand the foundation for new hypothesis-

driven studies investigating the role of ECM proteins in alcohol-related

susceptibility to acute lung injury.

Summary and Conclusions

The overall goal of the work described in this dissertation was to build upon the

unifying hypothesis that changes in the ECM contributes to hepatic pathology as

well as pathology in other organs, such as the lung. The experiments described

in Chapter III further support the critical role of fibrin deposition in the

development of steatohepatitis and the potential contribution of arsenic exposure

on hepatic tissue remodeling. These findings suggest that future studies by our

group should include investigation of changes in the hepatic extracellular matrix

in models of non-alcoholic liver disease.   Chapter IV characterized a new model

of concomitant liver and lung injury in the context of chronic alcohol exposure that will lay the foundation for testing the hypothesis that liver injury is critical to transitional ECM remodeling in the lung.   Finally, Chapter V described a new proteomic strategy for characterizing the extracellular proteome which will allow identification of new players in transitional tissue remodeling in the liver and potentially the lung in future experiments. Taken together, this work has shed new insight into the complex interplay between tissue injury and matrix dyshomeostasis.

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ABBREVIATIONS

ADAMTs   disintegrin and metalloproteinase with thrombospondin motif

ADH   Alcohol dehydrogenase

ALD   Alcoholic liver disease

ALI   Acute lung injury

AMPB   Alpha-1-microglobulin/bikunin precursor

ALT   Alanine aminotransferase

ARDS   Acute respiratory distress syndrome

As   Arsenic

ASH   Alcoholic steatohepatitis

AST   Aspartate aminotransferase

BAL(F)   Bronchioalveolar lavage (fluid)

BSA   Bovine serum albumin

BW   Body weight

CAE   Chloroacetate esterase

CID   Collision induced dissociation

CRELD2   Cysteine-rich with EGF-like domain protein 2

CYP2E1   Cytochrome P450 2E1

ECM   Extracellular matrix

Etan   Etanercept

ETD   Electron transfer dissociation

EtOH   Ethanol

FA   Formic acid

GAPDH   Glyceraldehyde 3-phosphate dehydrogenase

GI	Gastrointestinal
GnHCl	Guanidine Hydrochloride
GO	Gene ontology
H&E	Hematoxylin & eosin
HFD	High fat diet
HMGB1	High mobility group protein B1
HSCs	Hepatic stellate cells
i.p.	intraperitoneal
IL-10	Interlukin-10
IL-1b	Interleukin-1β
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
KC	Keratinocyte chemoattractant
LC	Liquid chromatography
LefSe	Linear effect size
LFD	Low fat diet
LPS	Lipopolysaccharide
LW	Liver weight
MD	Maltose-dextrin
MIP-2	Macrophage inflammatory protein
MMPs	Matrix metalloproteinases
MS	Mass spectroscopy
NaCl	Sodium chloride
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acid
OFC	Oligofructose
OTU	Operational taxonomic unit

PAI-1	Plasminogen activator inhibitor
PCoA	Principle coordinate analysis
PEBP1	Phosphatidylethanolamine-binding protein
RANTES	Regulated on activation, normal T cell expressed and secreted
SAA1	Serum amyloid A1
SAA2	Serum amyloid A2
SDS	Sodium dodecyl sulfate
TG	Triglycerides
TGFβ	Transforming growth factor beta
TIMP	Tissue inhibitor of matrix metalloproteinases
TNFα	Tumor necrosis factor-alpha

CURRICULUM VITAE

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EDUCATION

2010-2012   M. S. in Pharmacology & Toxicology, University of Louisville, Louisville, KY

2006-2010   B.S. in Biology, Xavier University, Cincinnati, OH

PROFESSIONAL EXPERIENCE

2012-2014   Graduate Research Fellow, University of Louisville, Department of Pharmacology & Toxicology

2010-2011   Graduate Research Assistant, University of Louisville, Department of Pharmacology & Toxicology

2009-2010   Lab assistant, Cincinnati Children's Hospital, Department of Developmental Biology

2008-2009   Undergraduate researcher, University of Louisville, Department of Pharmacology & Toxicology

PROFESSIONAL MEMBERSHIPS AND ACTIVITIES

2013-present Society of Toxicology, Student member

2013-present Ohio Valley Society of Toxicology

2010-present University of Louisville Alcohol Research Center

HONORS AND AWARDS

12/2014	The Dean's Citation
09/2014	1st Place Poster Award at the NIEHS Tamborro Symposium
09/2013	Battelle Minority/Women Travel Award for the 2013 Society of Toxicology Meeting
09/2013	Invitation for Platform Presentation, Ohio Valley Society of Toxicology Regional Meeting, University of Louisville, Louisville, KY
09/2013	School of Medicine Research Committee Travel Award
09/2013	Graduate Student Council Travel Award
2008-2010	Member of Alpha Epsilon Delta Honors Society, Xavier University
2006-2010	Xavier University Academic Scholarship
2006-2010	Dean's List, Xavier University

SERVICE

Service to Department

01-04/2014   Pharmacology and Toxicology Admissions Committee
This committee serves as the committee who reviews interviews applicants to the departments master's and doctoral programs. I served as a student representative and took part in review of applications and interview with applicants. As the student representative, I was also responsible for of hosting students during their stay in Louisville.

2013-2014   Pharmacology and Toxicology Planning Committee
This meets as needed to plan department events including the annual summer picnic and the annual holiday party. I served as the student representative.

2012-2014   Pharmacology and Toxicology Student Affairs Committee
This committee met as needed to discuss department curriculum, requirements, and student concerns. As one of the student

representative, I served as a liaison and an advocate for the student body.

Service to the Institution

08/2014   Professional Development (PLAN) workshop panelist
                 This workshop, titled Managing life as a Graduate student, included a panel of 4 graduate students who engaged in a discussion with new graduate students regarding time management and professional development during graduate school. I served as a panelist, representing the School of Medicine's graduate program.

2013-2014   University of Louisville School of Medicine Website Advisory Committee
                 This student committee was organized to aid in the organization of the School of Medicine's updated website. I served as a graduate student representative to voice the needs and concerns of the graduate students and their programs during the website's design.

2013   Educational Enterprise Committee, Scholarship Subcommittee
                 This committee was established as part of the 21st Century University initiative. Over the course of several months, this committee met weekly or bi-weekly to produce a plan that clarified the School of Medicine's vision for educational excellence in the areas of scholarship, service, and curriculum. I served as a graduate student representative to the committee as well as a member of the Scholarship subcommittee.

2012-2013   Vice Dean of Research Search Committee
                 This committee was charged with the task of identifying a list of semifinal candidates for the position of Vice Dean of Research. I served as the graduate student representative to the committee. As such, I took an active part in the reviewing of applications as well as applicant interviews.

08/2013   Integrated Program in Biomedical Sciences Student Panelist

10/2011   Judging assistant, Research!Louisville

10/2010   Judging assistant, Research!Louisville

Service to the Community

03/2013	Head Judge, Cellular and Molecular Biology, Manual High School Science Fair
02/2013	Judge, St. Patrick's School Science Fair
03/2012	Judge, Cellular and Molecular Biology, Manual High School Science Fair

EDUCATION ACTIVITIES

Lectures/labs

3/14	Guest lecturer (Alcohol Pharmacology), Basics of Pharmacology, University of Louisville
9/13	Facilitator for Autonomic Pharmacology Simulation Lab, Pharmacology, University of Louisville School of Medicine
3/13	Teaching Assistant and lecturer (Alcohol Pharmacology), Basics of Pharmacology, University of Louisville

Professional Development

2013-2014	Graduate Teaching Assistant Academy

LEADERSHIP

2013-2014	Graduate Student Council Representative for the Department of Pharmacology &Toxicology
2013-1014	President, Pharmacology &Toxicology Graduate Student Organization
2012-2013	Vice President, Pharmacology & Toxicology Graduate Student Organization
2011-2012	Secretary, Pharmacology & Toxicology Graduate Student Organization, University of Louisville
2010-2014	Class Representative, Department of Pharmacology & Toxicology, University of Louisville

2009-2010   Secretary, Biology Club, Xavier University

FUNDING

2011-present   NIEHS T-32 Training Grant

2010-2011   Graduate Research Fellowship, Integrated Programs in
              Biomedical Sciences

ABSTRACTS AND PRESENTATIONS

ORAL PRESENTATIONS

Local/Regional

1. Research Seminar 03/14 The role of transitional ECM in the parallel and
   interdependent mechanisms of liver and lung injury. University of Louisville
   Alcohol Research Center, University of Louisville, Louisville, KY

2. Platform Presentation, 09/13, The prebiotic oligofructose protects against
   liver injury caused by arsenic in a model of NASH. Ohio Valley Society of
   Toxicology, University of Louisville, Louisville, KY

3. Research Seminar, 11/12, Parallel and interdependent mechanism of
   alcohol-induced organ injury in the liver and lung. University of Louisville
   Alcohol Research Center, Louisville, KY

4. Research Seminar, 8/11 OVSOT Summer Meeting. CycloRGDfV protects
   against LPS-induced liver injury caused by ethanol in mice.

5. Research Seminar, 03/11, Integrin inhibitor Cyclo-RGDfV blunts enhanced
   LPS-induced liver injury caused by ethanol in mice. University of Louisville,
   Seminar in Pharmacology and Toxicology, Louisville, KY.

POSTERS

National

1. Poster Presentation 09/14, The prebiotic oligofructose protects against
   enhanced liver injury caused by arsenic in a model of NASH, Tamburro
   Symposium on Environmental Chemicals and Liver Disease, Louisville, KY

2. Poster Presentation 3/14 The prebiotic oligofructose protects against enhanced liver injury caused by arsenic in a model of NASH, Society of Toxicology, Phoenix, AZ

3. Poster Presentation 11/13, The prebiotic oligofructose protects against enhanced liver injury caused by arsenic in a model of NASH, American Association for the Study of Liver Disease, Washington D.C.

4. Poster Presentation, 11/11, Integrin inhibitor Cyclo-RGDfV blunts enhanced LPS-induced liver injury caused by ethanol in mice. American Association for the Study of Liver Disease, San Francisco, CA.

Local/Regional

1. Poster Presentation 09/14, Characterization of the liver:lung axis in alcohol-induced lung damage, Research!Louisville, Louisville, KY

2. Poster Presentation 09/13, The prebiotic oligofructose protects against enhanced liver injury caused by arsenic in a model of NASH, Research!Louisville, Louisville, KY

3. Poster Presentation, 10/12, Integrin $\beta3^{-/-}$ mice are protected against enhanced alcohol-induced liver injury. Research!Louisville Louisville, KY

4. Poster Presentation, 10/11, Integrin $\alpha v\beta3$ mediates ethanol-enhanced liver damage caused by LPS. Research!Louisville, Louisville, KY.

5. Poster Presentation, 10/11, Ethanol sensitization to LPS-induced liver injury: protection by the integrin inhibitor, cycloRGDfV. Ohio Valley Society of Toxicology Regional Meeting, Dayton, OH.

ABSTRACTS

National

1. **Massey VL**, Poole LG, Torres E, Dolin C, Merchant ML, Ritzenthaler J, Roman J, Arteel GE. (2014) Characterization of the liver:lung axis in alcohol-induced lung damage. Hepatology 59 (S1) 616

2. Anders LC, Douglas AN, Mohammad MK, **Massey VL**, Kirpich IA, Falkner KC, Warner NL, Arteel GE, Cave M, McClain CJ and Beier JI (2014) Vinyl Chloride Predisposes The Liver To Injury: Implications For Human Exposure. Gastroenterology 146(5):S910. (Selected for oral presentation).

3. **Massey VL**, Schmidt RH, Tan M, Watson WH, Arteel GE. (2014) The prebiotic oligofructose protects against enhanced liver injury caused by

arsenic in a model of NASH *The Toxicologist. Supplement to Toxicological Sciences* 138(1) 1323

4. Anders L, Douglas A, Guardiola J, **Massey VL**, Kirpich I, Mohammad M, Falkner KC, Cave M, McClain C, Beier J. (2014) An environmental 2nd hit: effect of vinyl chloride exposure on liver injury in experimental hepatotoxicity and in humans. *The Toxicologist. Supplement to Toxicological Sciences.* 138(1) 1879

5. **Massey VL**, Schmidt RH, Tan M, Watson W, Arteel GE (2013) The prebiotic oligofructose protects against enhanced liver injury caused by arsenic in a model of NASH. Hepatology 58 (S1) 739A

6. Schmidt RH, **Massey VL**, Jokinen J, Falkner KC, Beier JI, Arteel GE (2013) Olanzapine-induced liver injury: direct metabolic effects on liver and exacerbation by high-fat diet. Hepatology 58 (S1) 704A

7. Schmidt RH, Tan M, Jokinen J, **Massey VL**, Zhong H, Arteel GE (2012) Arsenic enhances experimental liver disease via altering GI tract function: protection with prebiotics. Hepatology 56 (S1): 89A

8. **Massey VL**, Beier JI, Schmidt RH, Zhong H, Arteel GE (2011) Integrin inhibitor Cyclo-RGDfV blunts enhanced LPS-induced liver injury caused by ethanol in mice. Hepatology 54(S1):970A

9. Tan M, **Massey VL**, Ding X, Zhong H, States JC, and Arteel GE (2010) Arsenic pre-exposure delays cell cycle entry and progression in mouse liver after partial hepatectomy. Hepatology 52:462A

Local/Regional

1. **Massey VL**, Poole LG, Torres E, Dolin C, Merchant ML, Ritzenthaler J, Roman J, Arteel GE. (2014) Characterization of the liver:lung axis in alcohol-induced lung damage. Research!Louisville. Louisville, KY.

2. Christine DE, **Massey, VL**, Poole, LG, Siow, DL,Merchant, ML, Wilkey, DW, Arteel,GE. (2014) The hepatic 'matrisome' responds dynamically to stress: novel characterization of the ECM proteome. Research!Louisville. Louisville, KY.

3. Watson W, Burke T, **Massey VL**, Arteel GE, Merchant ML (2014) Activity of the zinc finger transcription factor HNF-4a is inhibited by arsenic in the livers of mice with diet induced non-alcoholic fatty liver disease. Research!Louisville. Louisville, KY.

4. Poole LG, **Massey VL**, Dolin C, Merchant M, Beier JI, Roman J, Arteel GE (2014) Ethanol-induced changes in the hepatic ECM directly enhance the pro-inflammatory response of macrophages. Research!Louisville. Louisville, KY.

5. Bushau, A, Anders L, Douglas A, Poole LG, **Massey VL**, Lang A, Falkner C, Cave M, McClain C, Beier J (2014) Mechanistic insights into vinyl chloride-induced liver injury. Research!Louisville. Louisville, KY.

6. **Massey VL**, Schmidt RH, Tan M, Watson WH, Arteel GE (2013) The prebiotic oligofructose protects against enhanced liver injury caused by arsenic in a model of NASH. Ohio Valley Society of Toxicology Annual Meeting, Louisville KY

7. **Massey VL**, Schmidt RH, Tan M, Watson WH, Arteel GE (2013) The prebiotic oligofructose protects against enhanced liver injury caused by arsenic in a model of NASH. Research!Louisville

8. Poole LG, Jokinen JD, **Massey VL**, Beier JI, and Arteel GE (2013) Sinusoidal endothelial cell-derived extracellular matrix regulates basal and stimulated macrophage activation. Research!Louisville.

9. **Massey VL**, Poole LG, Schmidt RH, Arteel GE (2012) Integrin β3$^{-/-}$ mice are protected against enhanced alcohol-induced liver injury. Research!Louisville Louisville, KY

10. **Massey VL**, Beier JI, Schmidt RH, Zhong H, Arteel GE (2011) Ethanol sensitization to LPS-induced liver injury: protection by the integrin inhibitor, cycloRGDfV. Ohio Valley Society of Toxicology Annual Meeting, Dayton, Ohio

11. **Massey VL**, Beier JI, Schmidt RH, Zhong H, Arteel GE (2011) Integrin αvβ3 mediates ethanol-enhanced liver damage caused by LPS. Research!Louisville Louisville, KY

PUBLICATIONS

Papers

1. Massey VL, Dolin C, Poole LG, Wilkey D, Merchant ML, Torres E, Roman J, Arteel GE. The hepatic 'matrisome' responds dynamically to stress: novel characterization of the ECM proteome (in preparation)

2. Massey VL, Torres E, Poole LG, Schmidt RH, Ritzenthaler J, Roman J, Arteel GE Characterization of a model of concomitant liver and lung injury in a 2-hit model of chronic alcohol exposure (in preparation)

3. Massey VL, Schmidt RH, Tan M, Stocke K, Neal RE, Barve S, Arteel GE. The prebiotic oligofructose protects against arsenic-enhanced liver injury in a model of NAFLD via modulation of the gut microbiome (in preparation)

4. Schmidt RH, Jokinen JD, Massey VL, Falkner KC, Shi X, Yin X, Zhang X, Beier JI, Arteel GE (2013) Olanzapine activates hepatic mammalian target of rapamycin (mTOR): New mechanistic insight into metabolic dysregulation with atypical antipsychotic drugs. J Pharmacol Exp Ther

5. Massey VL and Arteel GE (2012). Acute alcohol-induced liver injury. Frontiers in Physiology. 3 (193) 1-8

Book Chapters

1. Arteel GA, Massey VL, Ritzenthaler JD, Roman JR. The Gut/Liver/Lung axis and alcohol-induced-tissue pathology.