

Abstract

Tetyana Kobets

Modeling alcoholic hepatitis in mice: modified acute-on-chronic binge ethanol feeding and alcohol liquid diet models.

(Under the direction of Ivan Rusyn, M.D., Ph.D.)

Ethanol contributes to a wide spectrum of alcohol-induced liver injury in humans. The use of animal models enables understanding of the driving forces of liver damage. However, features and patterns of some liver pathology, such as alcoholic hepatitis, cannot be completely reproduced by existing animal models.

Two experimental designs were implemented in order to reproduce an acute-on-chronic pattern of liver injury. In the first experiment, six-week old male C57/BL6J mice were randomly assigned to the injections of CCl₄ (0.2ml/kg) or olive oil as vehicle for 6 weeks. Three days after cessation of CCl₄ administration, mice received daily gavages of ethanol (4-5g/kg) or saline vehicle for 8 days (binge ethanol feeding model). In the second study after pre-injections with CCl₄ mice were fed ad libitum with alcohol-containing modified Lieber-DeCarli liquid diet for 6 weeks (modified alcohol liquid diet model).

Serum alcohol concentration and biomarkers of liver injury, histopathological evaluation of liver tissue sections, and the expression of genes, markers of myofibroblast formation, were assessed. The results of the study demonstrate that administration of low

doses of CCl_4 with following consumption of alcohol liquid diet caused the development of liver lesions similar to those in human alcoholic hepatitis.

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List of Abbreviations:

°C - Degrees Celsius

$2^{\Delta\Delta Ct}$ - Delta Delta Ct

ADH - Alcohol Dehydrogenase

AH - Alcoholic Hepatitis

Alb - Albumin

ALDH - Aldehyde Dehydrogenase

ALKP - Alkaline Phosphatase

ALT - Alanine Transaminase

ANOVA - Analysis of Variance

AST - Aspartate Amino Transferase

CCl₄ - Carbon Tetrachloride

cDNA - Complementary DNA

CYP2E1 - Cytochrome P450 2E1

CYP450 - Cytochrome P450

DNA - Deoxyribonucleic Acid

EtOH - Ethyl Alcohol, Ethanol

HFD - High Fat Diet

NAD - Nicotinamide Adenine Dinucleotide

NHIS - the National Health Interview Survey

OO - Olive Oil

ROS - Reactive Oxygen Species

WHO - World Health Organization

Chapter 1.

Literature Review

1.1. Alcohol and its consumption.



Molecular Formula: C₂H₆O
Average mass: 46.068001 Da
Systematic name Ethanol [1]

Alcohol is an organic compound with a hydroxyl functional group bound to a carbon atom [2]. Ethanol (EtOH), a volatile, flammable, and colorless liquid with slight odor, belongs to the class of acrylic alcohols found in alcoholic beverages. It naturally occurs as a byproduct of the metabolic process of yeast (e.g., *Saccharomyces cerevisiae*).

In the human body, the presence of EtOH results not only from alcohol ingestion, but also from endogenous ethanol production. The formation of endogenous ethanol has been shown to result from the microbial fermentation of carbohydrates in the gastro-intestinal tract [3], with concentrations rarely exceeding 0.040 mg/dl [4]. Additional evidence has indicated the possibility of intracellular ethanol synthesis [5].

Alcoholic beverages are widely consumed worldwide due, in part, to easy accessibility. The world's highest alcohol consumption levels are found in developed

countries, including Western and Eastern Europe. In 2005, worldwide per capita consumption of alcoholic beverages was estimated at 6.13 liters of pure alcohol for persons aged 15 years or older [6]. Alcohol drinking patterns differ widely among age groups and gender, and range from low consumption levels to daily heavy drinking. The frequency distribution of alcohol drinking status in the U.S. in 2011 according to the National Health Interview Survey (NHIS) is as follows: 52% of adults aged 18 and over were current regular drinkers, 14% were current infrequent drinkers, 6% were former regular drinkers, 9% were former infrequent drinkers, and 20% were lifetime abstainers. Men were more likely to be current regular drinkers than women (60% compared to 44%, respectively) [7].

1.2. Why is alcohol a compound of concern?

The harmful use of alcohol is considered to be a global problem as it compromises both individual and social development [6]. According to the Global Status Report on Alcohol and Health in 2011, alcohol ranks eighth among global risk factors for death, and is the third leading global risk factor for disease and disability. The harmful use of alcohol results in approximately 2.5 million deaths each year, almost 4% of all deaths worldwide [6]. Excessive drinking is a major determinant for various long-term health effects, including neuropsychiatric disorders (e.g., epilepsy), cardiovascular diseases, liver diseases (e.g., hepatitis, cirrhosis), and various forms of cancer. Among European and American patients that undergo liver transplantation, alcoholic liver disease (ALD) is the second most common diagnosis [6].

Moreover, alcohol is associated with many serious social issues, including crime and violence, child neglect and abuse, and reduced productivity in the workplace [6]. Alcohol misuse and harmful drinking patterns are correlated with injuries such as traffic accidents.

Health outcomes from alcohol consumption depend on a series of factors, many of which are related to levels and patterns of alcohol consumption, drinking culture, alcohol regulation (or lack thereof), and alcoholic beverage quality [6].

Excessive alcohol consumption is a particularly grave threat to men. They have higher rates of total burden of disease attributed to alcohol: 7.4%, compared to that in women, 1.4%. Globally, among all male deaths, 6.2% are attributable to alcohol consumption; in women this percentage is lower (1.1%) [6,7].

1.3. Main pathways of alcohol metabolism.

Alcohol intake typically occurs through the oral route, with 5% absorbed in the oral cavity, almost 20% absorbed rapidly through the stomach and 75% through the small intestine [8]. The rate of absorption depends on volume, concentration, nature of the alcoholic beverage, presence and absence of food in the stomach, permeability of gastric and intestinal tissues, and genetic variation [9]. After absorption into the blood-stream, alcohol is distributed quickly throughout the body [10]. The distribution of alcohol into the tissues of the body depends on the water content and blood supply of the tissues.

Alcohol is highly diffusible through cell membranes and is metabolized by most tissues. After ethanol is ingested, first pass metabolism occurs in the oral cavity and gastric cells [11]. The liver is the major site of alcohol metabolism and one of the major targets for alcohol-induced organ damage [12].

Ethanol is metabolized through oxidative (major) and non-oxidative routes [13]. In the oxidative route, ethanol is oxidized to acetaldehyde, a highly reactive and toxic byproduct, by several pathways that require the presence of specific enzymes (Figure1).

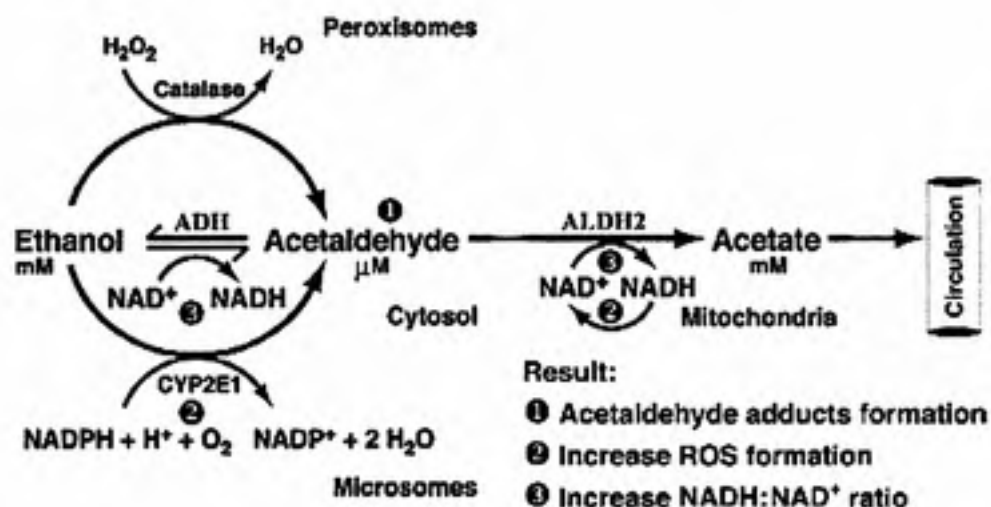
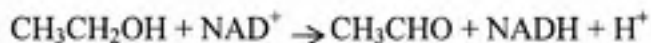


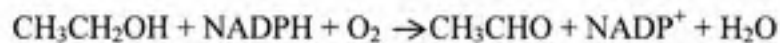
Figure 1. Oxidative pathways of alcohol metabolism. Adapted from [12]

Alcohol dehydrogenase (ADH), present in the cytosol, is primarily responsible for the major pathway of oxidative metabolism of ethanol in the liver [12]. This redox reaction requires an intermediate electron carrier and the coenzyme nicotinamide adenine dinucleotide (NAD⁺), which is reduced by two electrons to form NADH:



NADH is then oxidized by a series of chemical reactions in the mitochondria (e.g., the mitochondrial electron transport chain) [12].

Cytochrome P450 (CYP 450) is a major enzyme involved in the ethanol-inducible microsomal ethanol oxidizing system [14]. Isozymes of CYP 450, including CYP2E1, CYP1A2, and CYP3A4, are predominantly located in microsomes and contribute to ethanol oxidation in liver and other tissues where ADH activity is low (e.g., brain). The reaction catalyzed by the microsomal ethanol oxidizing system is:



Chronic alcohol consumption induces CYP2E1 expression through decreased proteolysis in the liver, which then assumes the important role in metabolizing ethanol at elevated alcohol concentrations [15].

Another enzyme, **catalase**, is located in peroxisomes and oxidizes ethanol in the presence of a hydrogen peroxide generating system such as the enzyme complex NADPH oxidase and the enzyme xanthine oxidase. This is considered a minor pathway of alcohol oxidation [12].

After formation, acetaldehyde is subsequently metabolized by mitochondrial aldehyde dehydrogenases (ALDHs) to acetic acid, or acetate, which occurs endogenously in the human body. Most of the acetate resulting from alcohol metabolism enters the circulation and is eventually metabolized to CO_2 in cardiac, skeletal muscle, and brain cells [12].

Non-oxidative alcohol metabolism is minimal, and predominantly occurs in organs where oxidative metabolism is rare or absent (e.g., pancreas and heart) (Figure 2) [16-18]. Different pathways of this metabolism result in the formation of fatty acid ethyl esters (FAEEs) and phosphatidylethanol [12].

The esterification of ethanol with endogenous fatty acids or fatty acyl coenzyme A to FAEEs occurs under the influence of FAEE synthase, the enzyme that catalyzes this reaction [19].

The formation of poorly metabolized phosphatidylethanol, a fat molecule containing phosphorus, requires the enzyme phospholipase D (PLD), which breaks down phospholipids to generate phosphatic acid [20]. This pathway usually occurs in the presence of high level of circulating alcohol [12].

Non-metabolized alcohol is eliminated in small quantities by the kidneys into urine (0.5–2.0%), lungs with breath (1.6–6.0%) and by the skin in the sweat (< 0.5) [16].

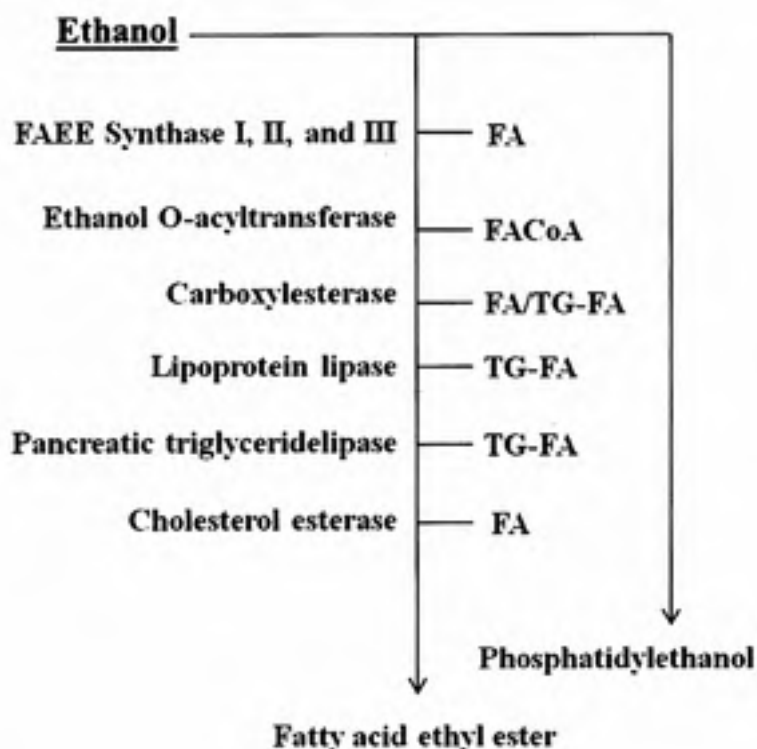


Figure 2. Non-oxidative pathways of ethanol metabolism. Adapted from [19]. FA- fatty acid; FACoA- fatty acyl coenzyme A; FAEE- fatty acid ethyl esters; TG, triglyceride.

1.4. Factors that influence alcohol metabolism.

In general, after the consumption of ethanol, blood alcohol concentration peaks within 30 to 45 minutes. Alcohol absorption is influenced by a number of factors, including the presence of food and the type of food in the gastrointestinal tract when alcohol is consumed [21]. The rate at which alcohol is absorbed depends on how quickly the stomach empties its contents into the intestine. The higher the dietary fat content, the more time this emptying will require and the longer the process of absorption will take [22].

Another factor that influences the alcohol absorption rate is gender. Thus, despite the fact that females tend to eliminate alcohol faster, they have been shown to develop a greater blood alcohol concentration compared to that in men after equal alcohol intake [23].

Alcohol is metabolized more slowly than it is absorbed. The liver can metabolize only a certain amount of alcohol per hour, regardless of the amount that has been consumed. The rate of alcohol metabolism depends, in part, on the functioning of metabolizing enzymes in the liver, which varies among individuals and is believed to have genetic determinants [24,25]. For example, polymorphism of *ADH* occurs at the *ADH1B* and *ADH1C* gene locations and is associated with varying levels of enzymatic activity [26]. The *ADH1B*1* form is found predominantly in Caucasian and Black populations, whereas *ADH1B*2* frequency is higher in Chinese and Japanese populations [27]. Individuals carrying the *ADH1B*2* and *ADH1B*3* alleles tend to metabolize and eliminate alcohol at a faster rate than those with the *ADH1B*1* form [28,29].

Genetic polymorphism of the *ALDH2* gene, which together with *ALDH1* is responsible for the metabolism of acetaldehyde, has the allelic variants *ALDH2*1* and

*ALDH2*2*. Because this genetic variation inhibits acetaldehyde metabolism and usually is virtually inactive, individuals with the *ALDH2*2* allele show an increased acetaldehyde level after alcohol consumption [30].

Several *CYP2E1* polymorphisms with evidence of functional importance have been identified. For instance, the polymorphism of the *CYP2E1*5A* allele (*RsaI/PstI* and *DraI* RFLP; c2/C genotype) has been shown to cause increased enzyme activity [31], whereas the *CYP2E1*5B* (*RsaI/PstI* RFLP; c2/D genotype) and *CYP2E1*6* alleles are responsible for the reduction of the enzyme activity [32].

1.5. Metabolic consequences of alcohol consumption.

Numerous detrimental consequences of the different pathways of ethanol metabolism contribute to tissue damage via several mechanisms (Figure 3). These consequences include disruption of carbohydrate and lipid metabolism, production of nitric oxide, and formation of reactive oxygen species (ROS) and other harmful metabolic intermediates such as adducts [12].

As was previously mentioned, one of the major pathways of oxidative alcohol metabolism, which involves ADH and ALDH, results in the generation of NADH by reduction of NAD in both the cytosol and mitochondria. The increased NADH/NAD ratio has profound effects on the metabolism of carbohydrates and lipids [14, 33]. For example, gluconeogenesis is impaired, substrate flow through the citric acid cycle is diminished and acetyl-coA is diverted towards ketogenesis and fatty acid synthesis [34]. Either directly or under the influence of adiponectin and tumor necrosis factor- α (TNF- α), ethanol may affect the activity of peroxisome proliferator-activated receptor alpha (*PPAR*- α), sterol regulatory

element-binding protein 1 (*SREBP-1*), and 5' adenosine monophosphate-activated protein kinase (*AMPK*). This also activate lipogenic pathways and inhibit fatty acid oxidation [34, 35]. In addition to the disruption of fatty acid synthesis and oxidation, ethanol alters lipid droplet (LD, the storage form of TG) metabolism in hepatocytes and very low-density lipoprotein secretion from the liver. This effect, together with the inhibition of mitochondrial fatty acid β -oxidation, contributes to the pathogenesis of fatty liver (steatosis), the initial stage of ALD [34].

Another major pathway of ethanol oxidation involves CYP2E1 in conjunction with free iron and is responsible for ROS formation and subsequent oxidative stress [14]. Ethanol-induced formation of ROS can also be attributable to overproduction of NADH, damage to mitochondria, activation of Kupffer cells, and reduction in the level of certain antioxidants (e.g., mitochondrial and cytosolic glutathione, vitamin E) [36]. ROS interact with and alters lipoprotein lipids, mitochondrial membranes, DNA, and proteins, ultimately causing necrosis or apoptosis. Also, ROS stimulate the release of TNF- α from Kupffer cells, which play an important role in the development of inflammatory reactions and are implicated in tissue damage and formation of fibrosis in the liver [12, 36,37].

Acetaldehyde formed as a result of ethanol oxidation and ROS interact with amino acids and other molecules, potentially resulting in the formation of stable and unstable adducts (e.g., malondialdehyde adduct). Formation of protein adducts in hepatocytes impairs protein secretion, which has been proposed to play a role in hepatomegaly [38]. Acetaldehyde is also capable of forming DNA adducts such as 1,*N*²-propanodeoxyguanosine, which are known to be carcinogenic [38]. Acetaldehyde also forms adducts with

neurotransmitters (dopamine) and forms salsolinol, which may contribute to alcohol dependence [12].

Acetate, another byproduct of ethanol metabolism, increases blood flow into the liver depresses the central nervous system, as well as affects various metabolic processes [39].

Ethanol metabolism intends to increase oxygen uptake from the blood by hepatocytes in the centrilobular area of the liver lobule, resulting in tissue hypoxia in perivenous regions [40, 41]. The perivenous hepatocytes are the first to show evidence of damage from chronic alcohol consumption [42]. Centrilobular hypoxia can be further enhanced by ethanol-induced changes in liver blood flow [43]. Alcohol causes vasoconstriction by an imbalance between nitric oxide/endothelin-1 interactions. Thus, at high-ethanol blood levels, hypoxia might ensue from the combination of reduced perfusion and increased oxygen demand. When blood ethanol levels subsequently decline, lobular perfusion is restored, potentially leading to reperfusion injury [44].

Chronic alcohol consumption also leads to a reduction of ATP synthesis in the liver, consequently causing impairment of metabolism of hepatocytes and potentially leading to tissue damage [45]. Ethanol depresses the activity of mitochondrial complexes, which causes abnormalities in the mitochondrial respiratory chain. Such changes include impaired electron transport and proton translocation, decreased cytochrome b content, and reduced function of the ATP synthase complex [46]. In addition, alcohol intake increases the intestinal permeability to a variety of substances such as bacterial endotoxins and lipopolysaccharide [43]. Endotoxins released by gram-negative bacteria are taken up by Kupffer cells, causing the release of mediators which are cytotoxic to hepatocytes and chemoattractive to neutrophils. This leads to inflammation in liver tissue [47].

Chronic ethanol consumption may also influence various other metabolic pathways, thus contributing to metabolic disorders such as fatty liver and excessive levels of lipids in the blood (i.e., hyperlipidemia), accumulation of lactic acid in the body fluids (i.e., lactic acidosis), excessive production of chemical compounds such as ketones in the body (i.e., ketosis), and elevated levels of uric acid in the blood (i.e., hyperuricemia) [48,49].

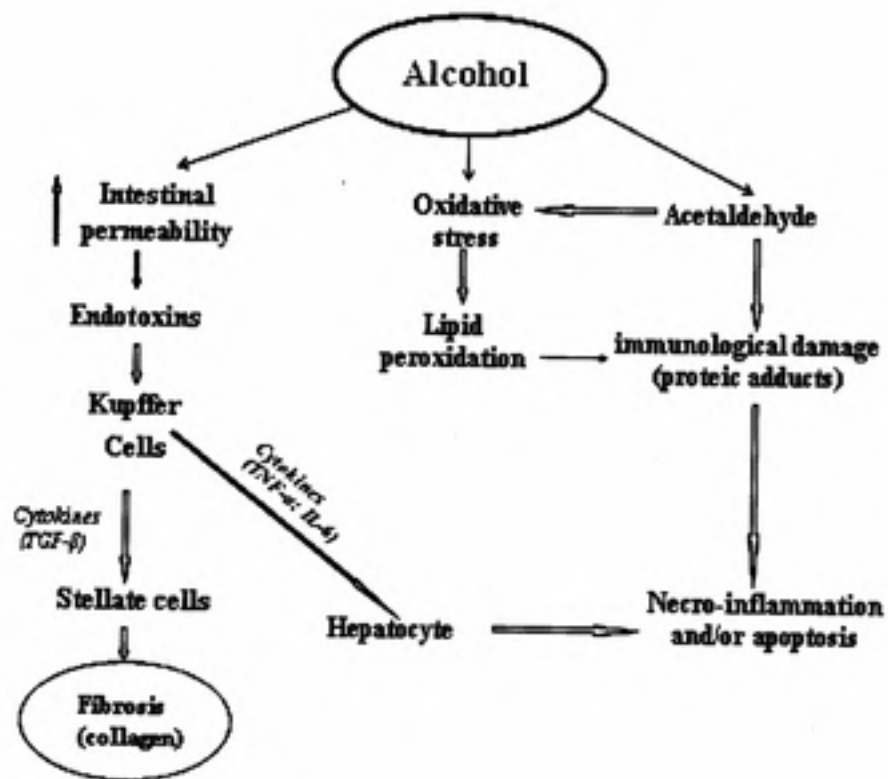


Figure 3. Mechanism of alcohol-induced hepatic damage. Adopted from [12].

1.6. Alcohol-induced liver disease.

Alcohol as a direct hepatotoxin, particularly in large concentrations, causes a wide spectrum of alcohol-related liver pathology in humans. Additionally, alcohol also acts as a “permissive agent” by causing liver injury in association with a wide variety of other factors [50,51]

ALD is a common condition with a high mortality rate. However, the reason why ALD progresses to advanced disease in only a relatively small proportion of heavy drinkers remains elusive [43]. The pathogenesis of ALD is a complex and multifactorial process which includes several overlapping events that initiate and perpetuate alcoholic liver injury. A primary factor in ALD pathogenesis is genetic background and its complex interrelationship with direct ethanol hepatotoxicity, as well as with and alcohol-induced metabolic and immunological changes. Secondary factors, such as nutritional and hepatotoxic co-morbid conditions, can critically contribute to the development of liver disease [33, 34].

ALD encompasses a spectrum of liver injury ranging from steatosis and fibrosis to cirrhosis and hepatocellular carcinoma [52]. All of these stages can be present simultaneously rather than appearing as evolutionary stages of the disease [37]. Histologically, they can be grouped into three stages of ALD: steatosis (fatty liver), steatohepatitis, and chronic hepatitis with hepatic fibrosis or cirrhosis [53].

Steatosis, the excessive accumulation of fat in hepatocytes, is the earliest stage of ALD and the most common alcohol-induced liver disorder [37]. This stage is typically reversible if alcohol consumption is stopped [54].

Alcohol steatohepatitis (ASH) is defined by the presence of nonspecific steatosis (mainly macrovesicular), inflammatory infiltrates, and hepatocellular damage [55]. Steatosis causes increased susceptibility to additional liver injuries caused by other agents such as drugs, toxins, and endotoxins [56].

Individuals with ASH may develop progressive fibrosis. Fibrotic tissue is typically located in pericentral and perisinusoidal areas in livers affected by ALD. Collagen deposits and bridging fibrosis are evident, events that both precede the development of regenerative nodules and liver cirrhosis [57].

Further progression of the disease causes the development of cirrhosis, the irreversible replacement of normal functional hepatic parenchyma with collagen (scar tissue) and regenerative nodules, which may result in liver failure [34]. Cirrhosis is usually micronodular, but may occasionally be mixed micronodular and macronodular [58]. Micronodular cirrhosis is associated with an increased risk of hepatocellular carcinoma [59].

Alcoholic hepatitis (AH) represents a spectrum of alcohol-induced liver disease ranging from mild to severe, life-threatening injury, and often presents acutely against a background of chronic liver disease [42]. The true prevalence is unknown, but histologic studies of patients with ALD suggest that AH may be present in as many as 10%-35% of hospitalized alcoholic patients [60]. Even individuals with only a relatively mild presentation of AH, however, are at high risk of progressive liver injury, with cirrhosis developing in up to 50% of cases [61]. Although abstinence from alcohol does not guarantee complete recovery, the likelihood that AH will progress to permanent damage is increased among those who continue to abuse alcohol [53].

1.7. Animal models for ALD.

Several animal models for ALD, described in Table 1, are currently widely used. They enable the study of the driving forces of liver damage, and how associations of alcohol with other factors can influence the severity of liver injury [62]. However, existing models do not completely reproduce all features and complex patterns of some liver diseases, such as AH, without the addition of a secondary insult (e.g. iron, high fat diet, vitamin supplementation,) [63-66].

Because a comprehensive model does not currently exist, ALD studies using animals are designed to answer specific questions about different aspects of the disease [62]. Among animals commonly involved in alcohol liver research (primates, dogs, rabbits, and rodents), rats and mice are used most frequently due in part to their relatively well-defined genetic background and the availability of diverse genetic traits [67]. Existing models differ in the amount of alcohol administered to animals and patterns of its consumption [64].

Lieber-DeCarli diets were designed to study alcohol liver injury as a result of nutritional deficiency. This method has several advantages, one of them being that the content of the diet can be easily manipulated. For example, it is easy to gradually increase alcohol concentration in the diet [66]. It has been previously shown that the development of liver steatosis occurred in mice that were administered this diet [62, 64]. However, this model does not mimic human drinking patterns because animals are forced to consume alcohol in the diet, which is their only source of food and water. In rodents, the intake of Lieber-DeCarli diet alone did not cause inflammation, fibrosis or cirrhosis [68].

Administration of alcohol in drinking-water also results in the development of hepatic steatosis, and has the advantage of being the simplest mode of alcohol administration to

laboratory animals. Moreover, it mimics human behavior patterns of intermittent alcohol consumption and changes in dietary intake [63]. The model allows the gradual increase of alcohol concentration during the feeding period. Furthermore, this model benefits over others when long-term effects of ethanol consumption are the focus of the study [68]. However, it may be difficult to maximize and control alcohol intake using this protocol, because animals can develop an aversion to alcohol that will affect their fluid consumption. Steatosis and inflammation achieved by this model do not progress to hepatic fibrosis and cirrhosis [64], and thus the model does not effectively mimic advanced stages of human alcoholic diseases.

To date, only the use of the Tsukamoto–French intragastric feeding model has resulted in the development of initial stages of liver fibrogenesis [69]. In this model, alcohol is delivered directly into the stomach via an implanted catheter and using an infusion pump. There are several advantages of this model, including the simulation of binge drinking by continuous delivery of alcohol 24 hours a per day, a characteristic cycling in urine alcohol concentrations due to diurnal variation in basal metabolism and hormones [66]. Additionally, high blood alcohol concentrations can be achieved and sustained because alcohol intake is controlled. However, this model does not imitate the pattern of alcohol consumption seen in humans. It does not follow normal rodent nocturnal feeding patterns, and the diet is not nutritionally adequate for induction of cytochrome P450 [70]. Furthermore, the implementation of this protocol is expensive and requires trained specialists to perform surgery and constant monitoring of animal health status [67].

An alternative to intragastric feeding is direct gastric gavage in which alcohol is administered as a bolus dose [71]. This model is particularly well suited for acute (binge) alcohol administration and allows for the control of the amount of alcohol administered. Oral

gavage is suitable to mimic acute alcohol intoxication. Nevertheless, this model can be stressful for animals, which will affect their eating habits [67].

To increase the amount of ingested alcohol to levels consistent with the human pathology of chronic alcohol abuse, alcohol-preferring rodents have been selectively bred to exhibit a relatively high preference for alcohol, such as Wistar rats, and have been used in alcohol studies [72]. The use of alcohol-preferring rats to study mechanisms of ALD has the advantage of oral "self-administration" that mimics human drinking patterns. However, despite the fact that alcohol-preferring rats consumed alcohol over a prolonged period and expressed markers of ALD, animals did not develop hepatic fibrosis-cirrhosis [73].

Table 1. Comparison of rodent models of alcohol feeding. Adapted from [64].

Model (name, species, BAC, pathology)	Advantages/disadvantages
<p><i>Lieber-DeCarli oral liquid diet</i> Mice, Rats 100–160 mg/dL</p> <p>Animals develop steatosis, minor inflammatory infiltrates</p>	<ul style="list-style-type: none"> -Oral delivery, strict nutritional equality with controls. -Activation of Kupffer cells by increased LPS -Can be combined with oral gavage to model chronic-binge patterns of alcohol consumption -Pathological changes do not progress beyond steatosis, metabolic oxidative stress in the absence of a secondary stress
<p><i>Ad libitum oral alcohol in drinking water</i> Mice, Rats 50–100 mg/dL</p> <p>Animals develop steatosis, minor inflammatory infiltrates</p>	<ul style="list-style-type: none"> -Mimics human consumption and delivery to the gastrointestinal tract. -Activation of Kupffer cells by increased LPS -Pathological changes do not progress beyond steatosis, metabolic, and oxidative stress in the absence of a secondary stress
<p><i>Tsukamoto-French intragastric cannulation, enteral feeding model</i> Mice, Rats As high as 500–600 mg/dL, depending on amount of alcohol.</p> <p>Animals develop steatosis, inflammatory cell infiltration, necrosis and fibrosis</p>	<ul style="list-style-type: none"> -Progressive pathological changes including fibrosis with activation of Kupffer cells and inflammatory networks -Larger dosage of alcohol than oral feeding methods -Enteral delivery, maintains nutritional equality with controls -Requires surgical expertise for insertion of cannula, which remains in place through duration of treatment. -Bypasses effects of alcohol on oral-pharyngeal mucosa and upper GI tract. -Contributes to dysbiosis and bacterial overgrowth in the GI tract -BAC must be closely monitored to avoid alcohol toxicity
<p><i>Oral gavage</i> Mice, Rats</p> <p>Can be in excess of 500 mg/dL</p> <p>Animals develop steatosis and mild inflammatory cell infiltrates</p>	<ul style="list-style-type: none"> -Allows for administration of increased dosage of alcohol -Models binge drinking, more difficult for chronic consumption -Pathological effects when combined with chronic oral ingestion mimics human pathology -Bypasses oral mucosa and upper GI -Stressful for animals, with risk of upper GI trauma -BAC must be closely monitored to avoid alcohol toxicity
<p><i>Alcohol preferring rodents</i> (alcohol-preferring (P), inbred alcohol-preferring (iP), and alcohol-nonpreferring (NP) rats) 40–130 mg/dL</p> <p>Animals do not develop cirrhosis</p>	<ul style="list-style-type: none"> -Natural oral feeding model, mimics human behavior, animals progressively develop HCC in the absence of cirrhosis (rats) -Pathological progression from steatosis, oxidative stress, inflammation, necrosis and fibrosis are uncharacterized

Chapter 2.

Introduction

Alcohol-induced liver damage is a major cause of acute and chronic liver disease worldwide [6]. Although the etiology of ALD is known, additional studies are required to clarify pathways of pathogenesis and subsequently provide sufficient information to develop strategies for prevention and treatment of the toxic effects of alcohol.

AH is a syndrome of progressive, inflammatory alcohol-induced liver injury. Typically, AH is described as an acute-on chronic type of liver damage, in which acute events overwhelm the ability of the liver to respond to alcohol-induced damage [74].

The variety of animal models has contributed to a greater understanding of pathological changes caused by alcohol and its metabolism, as well as by factors other than alcohol that can influence the severity of liver injury [62]. However, the majority of animal models of ALD do not develop classical patterns of alcoholic liver injury, such as AH, seen in humans [63]. This reflects the challenging task to develop a well-controlled mechanistic study model that replicates human liver disease.

For the current study, two experimental designs with specific emphasis on the acute-on-chronic nature of AH were developed. In order to mimic this pattern, administration of low doses of CCl_4 , a commonly used chemical to initiate liver fibrosis in rodent models [75], were followed by ethanol treatment (a trigger). The sequential administration of CCl_4 and ethanol not only reproduces an acute-on-chronic pattern, but also helps to avoid bias that adjuvant agents combined with ethanol can themselves induce liver damage.

Table 1. Comparison of rodent models of alcohol feeding. Adapted from [64].

Model (name, species, BAC, pathology)	Advantages/disadvantages
<p>Lieber-DeCarli oral liquid diet Mice, Rats 100–160 mg/dL</p> <p>Animals develop steatosis, minor inflammatory infiltrates</p>	<ul style="list-style-type: none"> -Oral delivery, strict nutritional equality with controls. -Activation of Kupffer cells by increased LPS -Can be combined with oral gavage to model chronic-binge patterns of alcohol consumption -Pathological changes do not progress beyond steatosis, metabolic oxidative stress in the absence of a secondary stress
<p>Ad libitum oral alcohol in drinking water Mice, Rats 50–100 mg/dL</p> <p>Animals develop steatosis, minor inflammatory infiltrates</p>	<ul style="list-style-type: none"> -Mimics human consumption and delivery to the gastrointestinal tract. -Activation of Kupffer cells by increased LPS -Pathological changes do not progress beyond steatosis, metabolic, and oxidative stress in the absence of a secondary stress
<p>Tsukamoto-French intragastric cannulation, enteral feeding model Mice, Rats As high as 500–600 mg/dL, depending on amount of alcohol.</p> <p>Animals develop steatosis, inflammatory cell infiltration, necrosis and fibrosis</p>	<ul style="list-style-type: none"> -Progressive pathological changes including fibrosis with activation of Kupffer cells and inflammatory networks -Larger dosage of alcohol than oral feeding methods -Enteral delivery, maintains nutritional equality with controls -Requires surgical expertise for insertion of cannula, which remains in place through duration of treatment. -Bypasses effects of alcohol on oral-pharyngeal mucosa and upper GI tract. -Contributes to dysbiosis and bacterial overgrowth in the GI tract -BAC must be closely monitored to avoid alcohol toxicity
<p>Oral gavage Mice, Rats</p> <p>Can be in excess of 500 mg/dL</p> <p>Animals develop steatosis and mild inflammatory cell infiltrates</p>	<ul style="list-style-type: none"> -Allows for administration of increased dosage of alcohol -Models binge drinking, more difficult for chronic consumption -Pathological effects when combined with chronic oral ingestion mimics human pathology -Bypasses oral mucosa and upper GI -Stressful for animals, with risk of upper GI trauma -BAC must be closely monitored to avoid alcohol toxicity
<p>Alcohol preferring rodents (alcohol-preferring (P), inbred alcohol-preferring (iP), and alcohol-nonpreferring (NP) rats) 40–130 mg/dL</p> <p>Animals do not develop cirrhosis</p>	<ul style="list-style-type: none"> -Natural oral feeding model, mimics human behavior, animals progressively develop HCC in the absence of cirrhosis (rats) -Pathological progression from steatosis, oxidative stress, inflammation, necrosis and fibrosis are uncharacterized

The results of the first experiment demonstrate that binge ethanol feeding via oral gavage caused minimal effect in liver tissues of mice indicating this model was not successful for the development of alcohol-induced hepatitis clinically relevant to that seen in humans. In contrast, the results of the second study with the administration of low doses of CCl₄ with following consumption of alcohol liquid diet resulted in the development of steatosis, necrosis, bridging fibrosis, and inflammatory lesions, which were exacerbated over the time period. These histopathological findings allowed us to conclude that this model represents liver pathological changes that mimic features of alcoholic hepatitis in humans.

Chapter 3.

Materials and Methods.

3.1. Animals and experimental design of modified acute-on-chronic binge ethanol feeding model.

Male, six-week old, C57BL/6J mice (Jackson Laboratory Bar Harbor, ME) (n = 70) were housed in regular cages in a temperature-controlled (24°C) room, with a 12/12-hr light/dark cycle, and given ad libitum access to purified water and food. The experimental design is described in Figure 4, panel A.

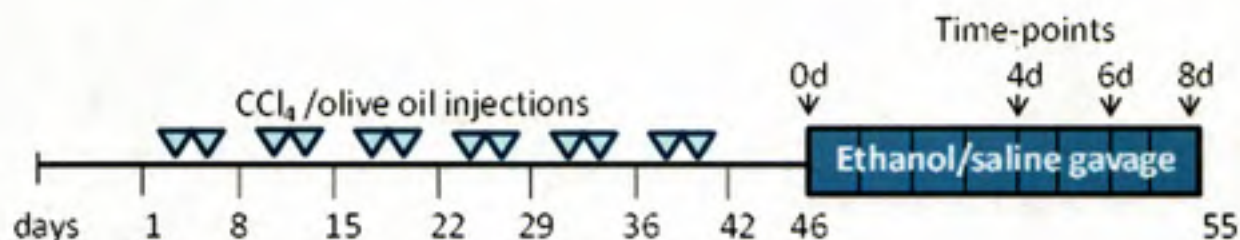


Figure 4.A. Experimental design of modified binge ethanol feeding model

After an acclimation period (4 days), the mice were randomly allocated into four groups. Two groups were injected intraperitoneally with olive oil vehicle while the other two groups received injections of CCl_4 (0.2 ml/kg, diluted in olive oil) twice a week for 6 weeks. EtOH treatment was initiated in the second stage of the study, 3 days after the final injection of CCl_4 or olive oil. EtOH (4-5 g/kg) or saline were administered through daily gavages for 8

days. Alcohol treatment was initiated with a dose 4 g/kg. On the next two days of the gavage feeding period the dose was increased to 5 g/kg. The increase in alcohol concentration caused the survival rate of mice to decrease. Because of that, we decreased alcohol concentration to 4 g/kg and maintained this EtOH concentration throughout the rest of the feeding period. Mice were weighed daily. Mice were sacrificed at 0 days, 4 days, 6 days, and 8 days of alcohol treatment. Before sacrifice, mice were anesthetized with nembutal (100 mg/kg intraperitoneal injection). The livers, kidneys, and stomachs were excised and stomach contents, blood, and urine were collected. A slice of the median lobe of the liver and a slice of the kidney from each animal was fixed in 10% neutral buffered formalin for 48 hours. Histopathological examination was conducted using hematoxylin and eosin-stained and Masson's trichrome-stained sections. The remaining tissue was frozen immediately in liquid nitrogen and stored at -80°C for subsequent analysis. All animals were treated humanely and with regard for alleviation of suffering. The experiments were approved by the Institution Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

3.2. Animals and experimental design of modified alcohol liquid diet model.

Male, six-week old, C57BL/6J mice (Jackson Laboratory) (n = 64) were housed in regular cages in a temperature-controlled (24°C) room, with a 12/12-hr light/dark cycle, and given ad libitum access to purified water and food. Figure 4, Panel B shows the experimental design of the study.

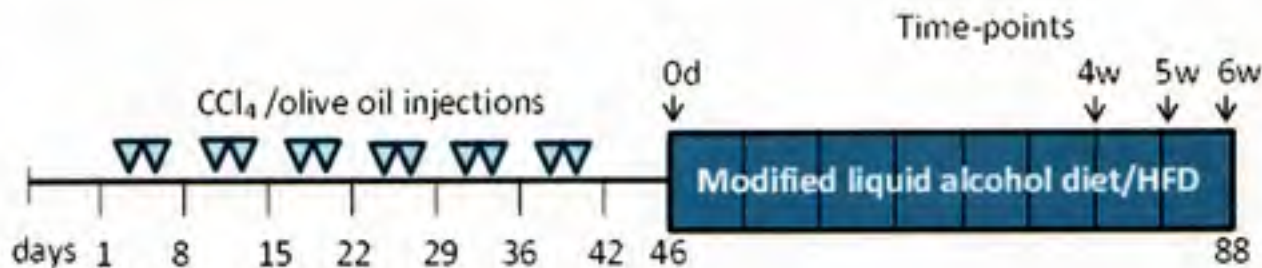


Figure 4.B. Experimental design of alcohol liquid diet model.

After an acclimatization period (4 days), the mice were randomly allocated to four groups. Two groups received intraperitoneal injections of olive oil vehicle, while the other two groups received injections of CCl₄ (0.2 ml/kg, diluted in olive oil) twice a week for 6 weeks. Alcohol treatment was initiated 3 days after the last injection of CCl₄ or olive oil. At the second stage of the study, the mice were fed ad libitum a modified Lieber-DeCarli high fat liquid diet (HFD; Cat # 710270; Dyets, Inc, Bethlehem, PA) for 6 weeks. The diet contained 35% of calories from fat (corn oil), 12% from carbohydrate, 18% from protein, 35% from maltose dextrin, and an additional 35% from ethyl alcohol (experimental groups). Alcohol treatment was initiated with a dose 5 g/kg/day and gradually increased to 17 g/kg/day by day 19 of ethanol treatment. An attempt to increase the dose of EtOH to 21 g/kg/day did not succeed; mice started to drastically lose weight. Because of that, we decreased alcohol concentration in the diet to 17 g/kg/day and maintained this EtOH concentration throughout the rest of the feeding period. During the study, the mice had free access to non-caloric rodent chow pellets. Each group of mice was treated in an identical manner. Animals were euthanized at 0 day, 4, 5, and 6 weeks of alcohol consumption. Before sacrifice mice were anesthetized with nembutal (100 mg/kg intraperitoneal injection). The livers, kidneys, and stomachs were excised and stomach contents, blood, and urine were

collected. A slice of the median lobe of the liver, as well as a slice of the kidney from each animal was fixed in 10% neutral buffered formalin for 48 hours. Histopathological examination was conducted on sections stained with hematoxylin and eosin, and Masson's trichrome-stain. The remaining tissue was frozen immediately in liquid nitrogen and stored at -80°C for subsequent analysis. All animals were treated humanely and with regard for alleviation of suffering. The experiments were approved by the Institution Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

Serum samples were analyzed for common biomarkers of liver injury alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALKP) and levels of serum albumin by using an automated blood chemical analyzer, VT350 Ortho-Clinical Diagnostics Company (Rochester, NY).

Liver sections were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at a 5µm thickness. Afterwards these sections were stained with hematoxylin and eosin and Masson's trichrome. Hematoxylin and eosin stained sections were evaluated for several histological indices. Scoring was performed by two veterinary pathologists. For steatosis, the extent of lesions was scored based on the affected area of the liver cells as follows: 1 < 10%, 2 < 25%, 3 < 50% and 4 > 50%. Other lesions, such as hypertrophy, anisonucleosis, vacuolization, and cellular infiltration and pigment-laden macrophages/inflammation, were graded in the standard manner: 1 - minimal, 2 - slight, 3 - moderate and 4 - severe.

Fibrosis score was based on the count of relative proportion of collagen accumulation to the normal liver tissue, and calculated for Masson's trichrome-stained liver

tissue sections using image analysis software (Image-Pro Plus.5.1, Media Cybernetics, Inc., Rockville, MD).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted from mouse liver tissues using Qiagen miRNeasy kits (Qiagen Sciences, Inc; Germantown, MD). Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosciences, Carlsbad, CA). Gene expression was determined for the next genes: actin, alpha 2, smooth muscle, aorta (*Acta2*) and tissue inhibitor of metalloproteinase (*Timp1*). All primers were purchased from Applied Biosystems (Carlsbad, CA) and their gene expression assay numbers are Mm00725412_s1 and Mm00441818_m1, respectively. The relative level of mRNA for each gene was determined using the $2^{-\Delta\Delta Ct}$ method [76] as compared to the housekeeping gene glucuronidase, beta (*Gusb*) (gene expression assay number Mm00446953_m1). The results are presented as a fold change for each mRNA in the liver of mice treated with EtOH and pre-injected with CCl₄ to those from mice who were treated with vehicle and previously received injections of CCl₄.

Statistical analysis. Results are presented as mean \pm SD. Statistical analyses were performed using one-way ANOVA with pair-wise comparison by Tukey's test between the groups within each time-point, with the exception of 0 day time-point. An unpaired two-tailed Student's t-test was used to determine statistical significance between groups from the 0 day time-point. Statistical analysis was performed using Graphpad Prism software (GraphPad Software Inc, La Jolla, CA); p-values < 0.05 were considered significant.

Chapter 4

Results.

4.1. Results of modified acute-on-chronic binge ethanol feeding.

4.1.1. Body and liver weight.

Body weight was measured to monitor the overall health of mice throughout the experiment. Figure 5 shows body weight change during the study. Noticeable 6% decrease in body weight was observed after administration of treatment and vehicle through oral gavage (day 46). This may be explained by stress and change in eating habits due to esophagus irritation caused by the procedure. After mice adjusted to the procedure, on day 48 their body weight increased.

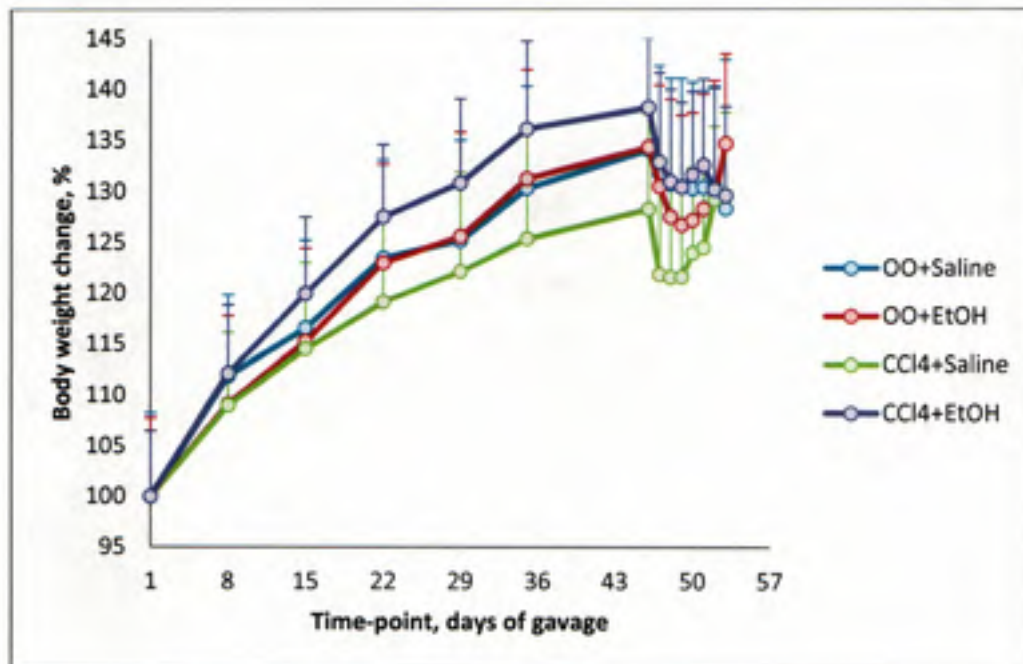


Figure 5. Body weight change during the study. OO+Saline - represents the control group

that received vehicle only; OO+EtOH - represents the group pretreated with vehicle and treated with EtOH in the second stage of the study; CCl₄+Saline - represents the group that was pretreated with CCl₄ and received vehicle in the second stage of the study; CCl₄+EtOH - represents the group that was pretreated with CCl₄, and treated with ethanol in the second stage. OO - olive oil; Saline - NaCl 0.9%; EtOH - ethanol; CCl₄ - carbon tetrachloride.

During sacrifice, liver weight was determined and adjusted to body weight. Figure 6 shows there were no significant differences between groups pretreated with vehicle and groups pretreated with CCl₄, as well as between groups that were treated with vehicle and groups treated with ethanol.

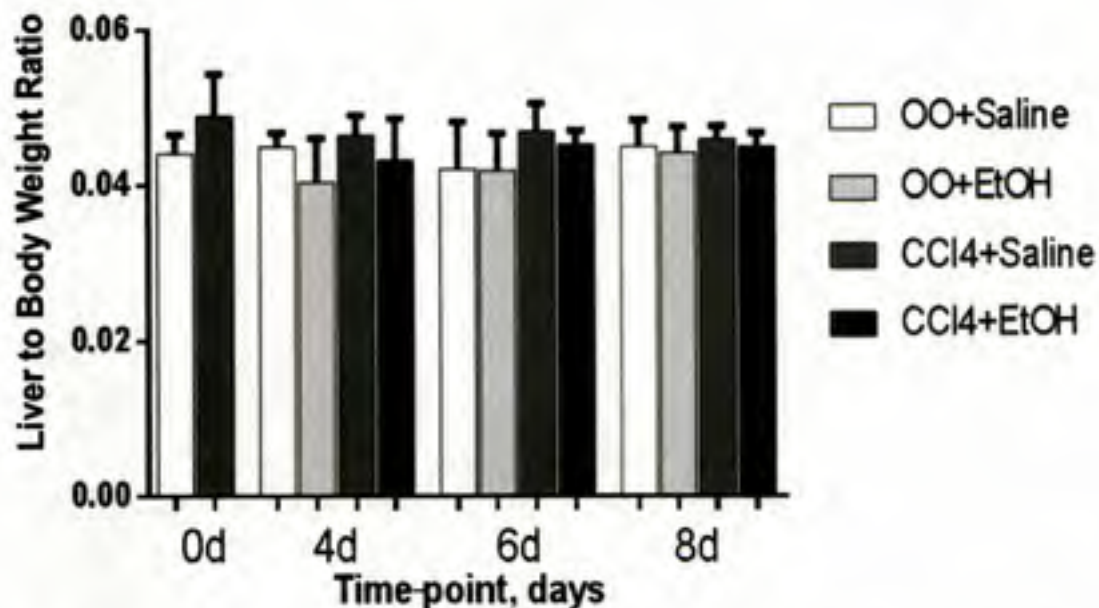
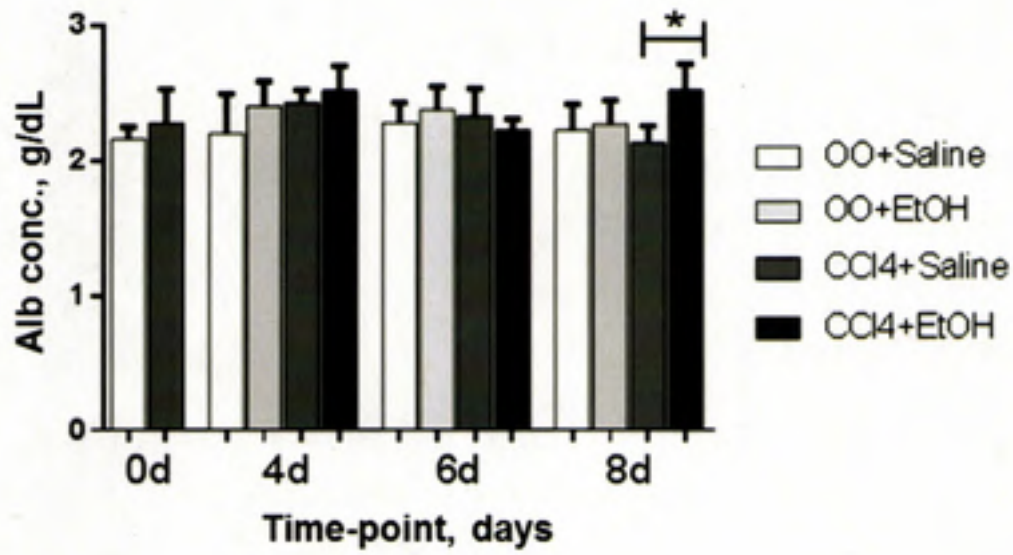


Figure 6. Liver to body weight ratio. OO+Saline – represents the control group that received vehicle only; OO+EtOH - represents the group pretreated with vehicle and treated with EtOH in the second stage of the study; CCl₄+Saline - represents the group that was pretreated with CCl₄ and received vehicle in the second stage of the study; CCl₄+EtOH - represents the group that was pretreated with CCl₄, and treated with ethanol in the second stage. OO - olive oil; Saline - NaCl 0.9%; EtOH - ethanol; CCl₄ - carbon tetrachloride. Statistical analyses were performed using one-way ANOVA with pair wise comparison Tukey's test between the groups within 4 day, 6 day, and 8 day time-points. Unpaired two-tailed Student's t-test was used to determine statistical significance between groups from the 0 day time-point.

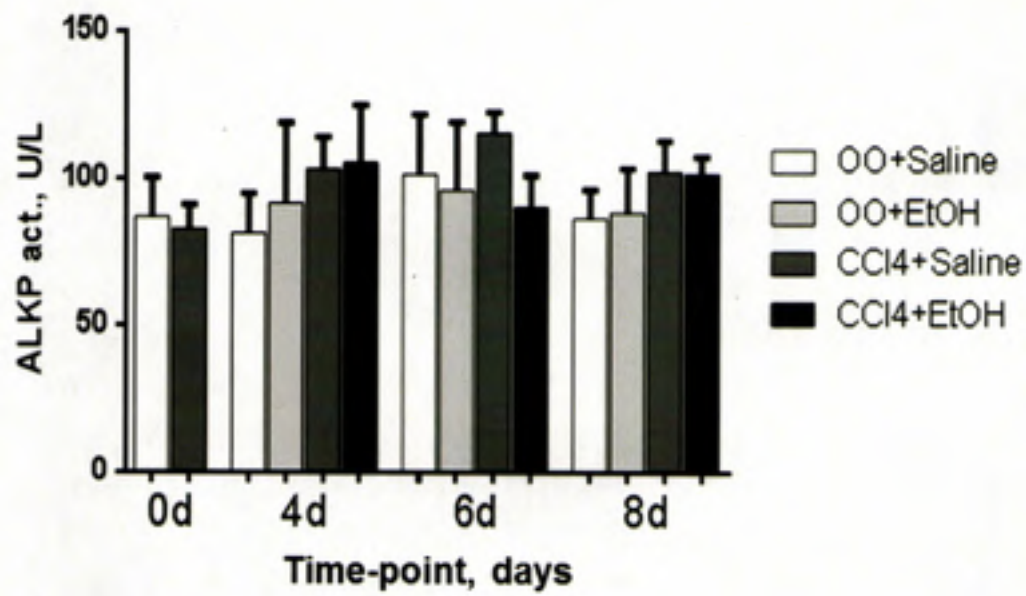
4.1.2. Analysis of serum markers of liver injury.

Transaminase enzymes, ALT and AST, together with ALKP are known biomarkers of cell damage caused by a spectrum of pathological injury that occurs in liver disease including steatosis, inflammation, apoptosis, and fibrosis. Hypoalbuminemia can also be an indicator of liver pathology. Serum samples were analyzed for these biomarkers (Figure 7, Panels A-D). As can be noticed from the figure, biomarkers did not show response to alcohol treatment as the levels of their activity were in range of normal clinical chemistry values. Albumin concentration level remained the same across all groups, although in the group treated with EtOH, at the 8 day time-point concentration of albumin was significantly higher than in the group treated with CCl₄ only (2.5 g/dL compared to 2.1g/dL respectively). ALKP activity was slightly (~20 units) elevated in groups pretreated with CCl₄, although statistical analysis showed that this change was not significant. Levels of AST activity inconsistently varied across the groups and time-points. At the 0 day time-point there was a significant difference in AST activity between the group treated with oil as a vehicle and the group treated with CCl₄ alone (52.6 U/L compared to 65.2 U/L respectively). There was no significant difference in the levels of ALT activity in all groups, and the difference between groups that were sacrificed on 0 day time-point (~30units) was found to be insignificant.

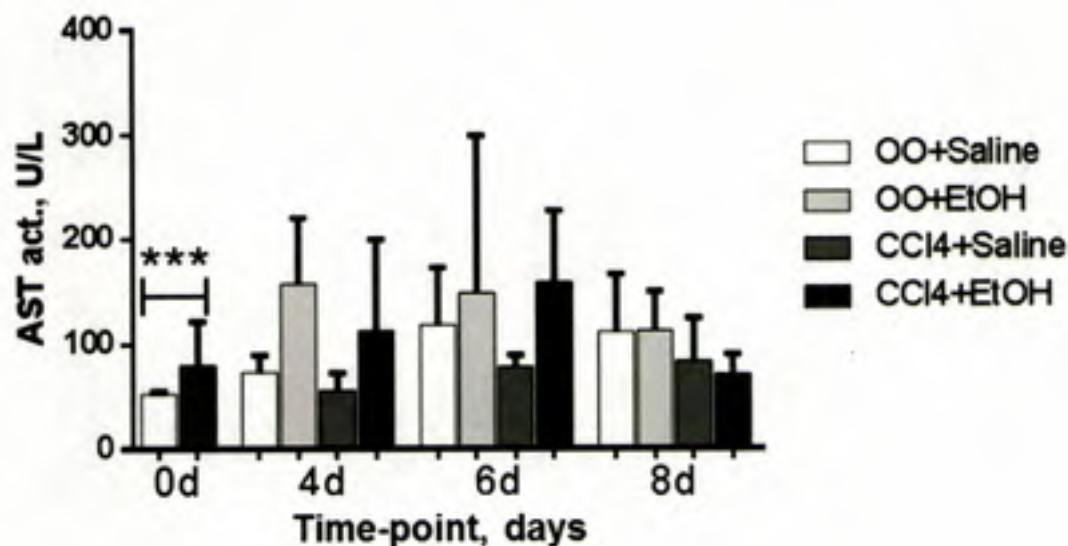
A.



B.



C.



D.

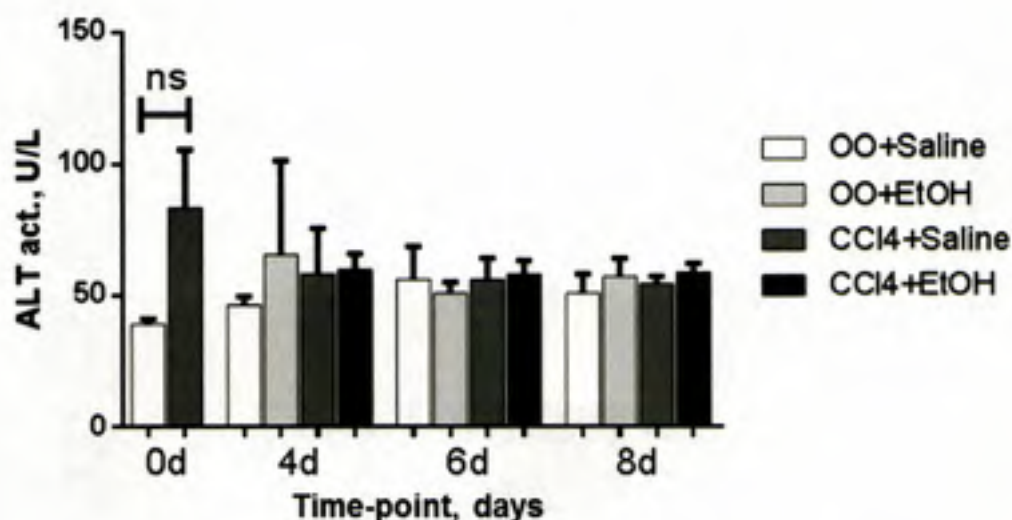


Figure 7. Activity of biomarkers of liver injury. A - Albumin concentration; B - Alkaline phosphatase activity; C - Aspartate amino transferase activity; D - Alanine amino transferase activity. Data are expressed in absolute units. Normal reference clinical chemistry values for mice are: Alb=2.5-3 g/dl; ALT=17-77U/L; AST=54-298U/L; ALKP=35-96U/L
 OO+Saline - represents the control group that received vehicle only; OO+EtOH - represents the group pretreated with vehicle and treated with EtOH in the second stage of the study;
 CCl₄+Saline - represents the group that was pretreated with CCl₄ and received vehicle in the second stage of the study; CCl₄+EtOH - represents the group that was pretreated with CCl₄,

and treated with ethanol in the second stage. OO - olive oil; Saline - NaCl 0.9%; EtOH - ethanol; CCl₄ - carbon tetrachloride. Statistical analyses were performed using one-way ANOVA with pair wise comparison Tukey's test between the groups within 4 day, 6 day, and 8 day time-points. Unpaired two-tailed Student's t-test was used to determine statistical significance between groups from the 0 day time-point. Asterisks denote significant differences from control mice of the corresponding time-point. * - $p \leq 0.05$, *** - $p \leq 0.001$, ns - not significant, $p > 0.05$.

4.1.3. Histopathological evaluation of alcohol-induced liver injury.

To evaluate potential degrees of liver injury, sections of liver stained with hematoxylin and eosin were evaluated for several histological indices. Based on the results of histopathological evaluation presented in the Figure 8, the assumption was made that there were minimal effects caused by ethanol treatment. Additionally, binge feeding with ethanol for 8 days did not cause significant phenotypic changes such as cholestasis or neutrophil infiltration in mice from both groups (pretreated with vehicle and pretreated with CCl₄).

Mice treated with CCl₄, regardless of EtOH administration, developed fibrotic lesions. In groups with liver injury caused by CCl₄, liver was restored after CCl₄ administration was stopped and only minimal hepatocellular injuries, like centrilobular necrosis, ballooning degeneration, and fatty deposition, were observed. However, bridging fibrosis was still observed in the liver sections of animals from groups pretreated with CCl₄.

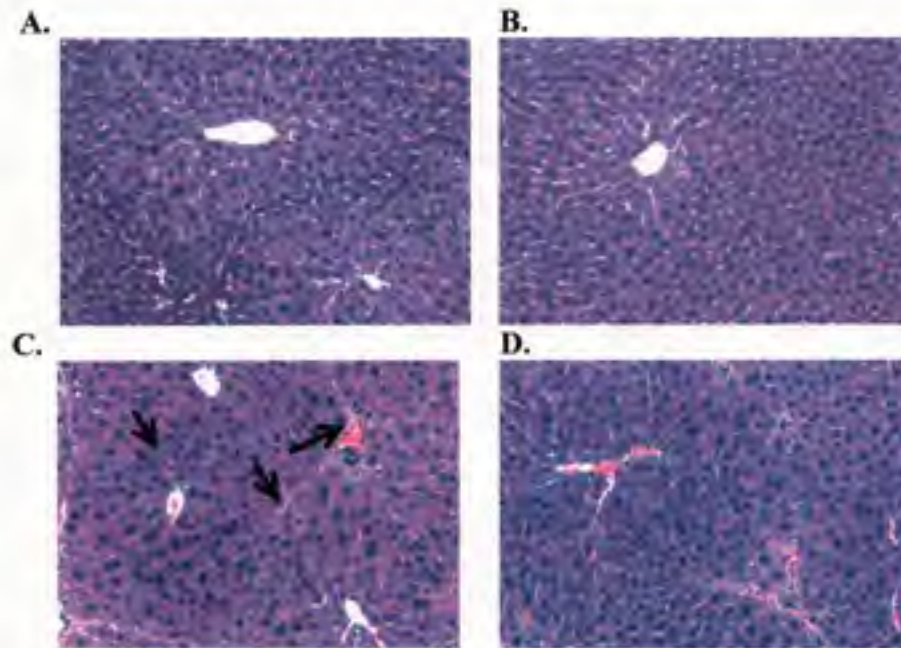


Figure 8. Liver sections stained with hematoxylin and eosin. **A** – representative liver section of an animal from the control group that received vehicle only; **B** - representative liver section of an animal from the group pretreated with vehicle and treated with ethanol in the second stage of the study; **C** - representative liver section of an animal from the group that was pretreated with carbon tetrachloride and received vehicle in the second stage of the study; **D** - representative liver section of an animal from the group pretreated with carbon tetrachloride and treated with ethanol in the second stage. Magnifications: 200X. Arrows show slight bridging fibrosis with mixed cellular infiltration (mainly lympho/monocytes) and presence of enlarged nuclei (anisonucleosis) (possibly due to the repeated dosing of CCl_4).

To estimate the level of fibrosis present, Masson's trichrome staining on liver sections was performed. Liver tissues harvested at the 0 day time-point demonstrated features of fibrin accumulation and inflammatory lesions. Figure 9, panel A shows thick fibrous septa in perivascular region. However, over the time period after CCl_4 administration was stopped fibrotic changes regressed (Figure 9, Panels B and C).

Figure 10 shows that the fibrosis ratio (estimated as the ratio of connective tissue to normal liver tissue) decreased after CCl_4 administration was stopped across the time-points (decrease up to 40% compared to the group from the 0 day time-point); however there was no significant effect of ethanol treatment.

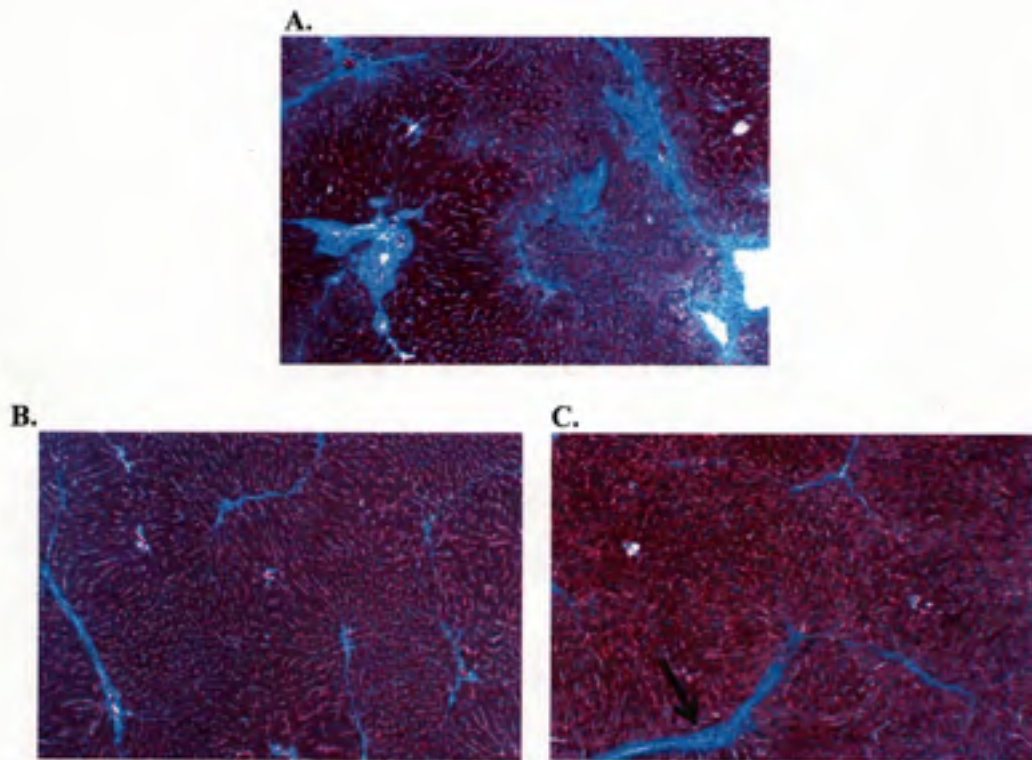


Figure 9. Liver sections stained with Masson's trichrome. A and B - representative liver section of animals from the group that was pretreated with carbon tetrachloride and received vehicle in the second stage of the study, the 0 day time-point (**A**) and 8 day time-point (**B**); **C** - representative liver section of an animal from the group pretreated with carbon tetrachloride and treated with ethanol in the second stage, 8 day time-point. Arrow shows bridging fibrosis. Magnification 40x.

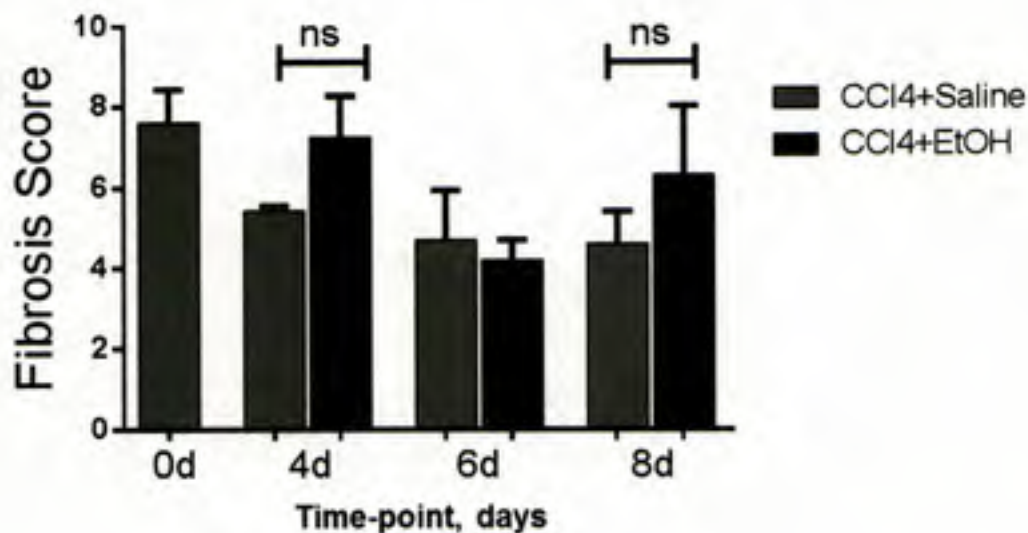


Figure 10. Fibrosis ratio in groups pretreated with CCl₄. Data are presented in percent change over control. CCl₄+Saline - represents the group that was pretreated with CCl₄ and received vehicle in the second stage of the study; CCl₄+EtOH - represents the group that was pretreated with CCl₄, and treated with ethanol in the second stage. Saline - NaCl 0.9%; EtOH - ethanol; CCl₄ - carbon tetrachloride. Statistical analyses were performed using one-way ANOVA with pair wise comparison Tukey's test between the groups within 4 day, 6 day, and 8 day time-points. Unpaired two-tailed Student's t-test was used to determine statistical significance between groups from the 0 day time-point. ns - not significant, $p > 0.05$

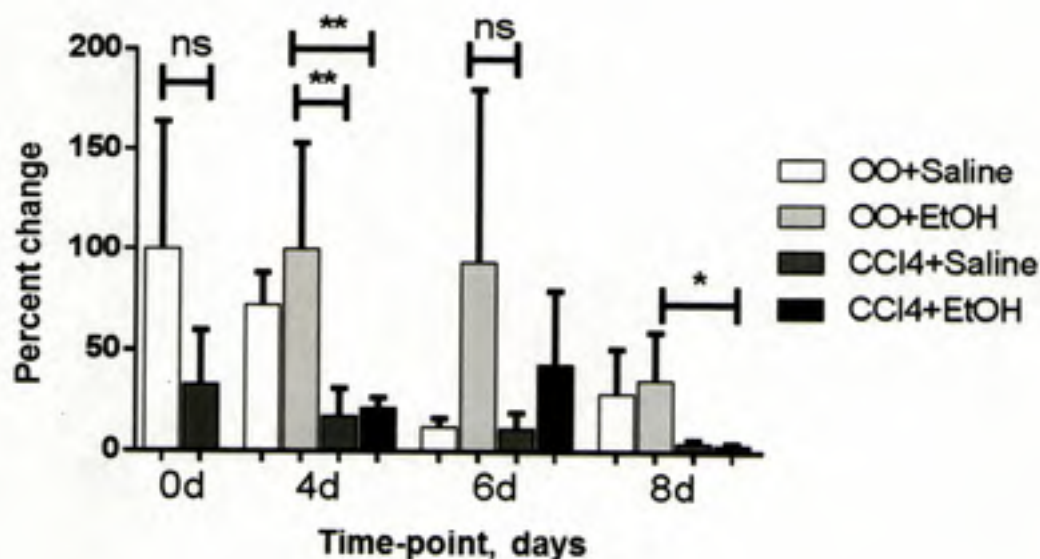
4.1.4. Hepatic expression of *Acta1* and *Timp1* genes.

Acta2 and *Timp1* genes are commonly used as a marker of myofibroblast formation. Therefore, the expression of these genes was determined to evaluate if differences in their expression may account for the different fibrosis ratio. Figure 11 panel A shows the gene expression of *Acta2*. The decrease in gene expression in groups treated with CCl₄ at the 0 day and 6 day time-points is statistically insignificant. In liver tissue harvested at the 4 day time-point in both groups pretreated with CCl₄, *Acta2* expression is significantly decreased (82% and 78% decrease) compared to the group in which mice were pretreated with olive oil and fed with alcohol. At the 8 day time-point, there was a significant 31% difference in gene

expression between the group that was treated with olive oil and ethanol and the group that was pretreated with CCl₄ and fed with alcohol.

The expression of *Timp1* better correlates with the fibrosis ratio (Figure 11, Panel B). In the group that was sacrificed at the 0 day time-point, there is a 156% increase in *Timp1* gene expression in tissues of mice from the group treated with CCl₄ only as compared to those from the group treated with olive oil only. However, this difference was found to be statistically insignificant. At the 4 day time-point gene expression in the group treated with CCl₄ and alcohol was significantly higher than that in groups treated with olive oil only and olive oil followed by ethanol treatment (59%, and 73% respectively). In groups sacrificed after 8 days of ethanol gavage feeding, *Timp1* expression in the group treated with olive oil and ethanol was 24% higher than in the group treated with CCl₄ and ethanol.

A.



B.

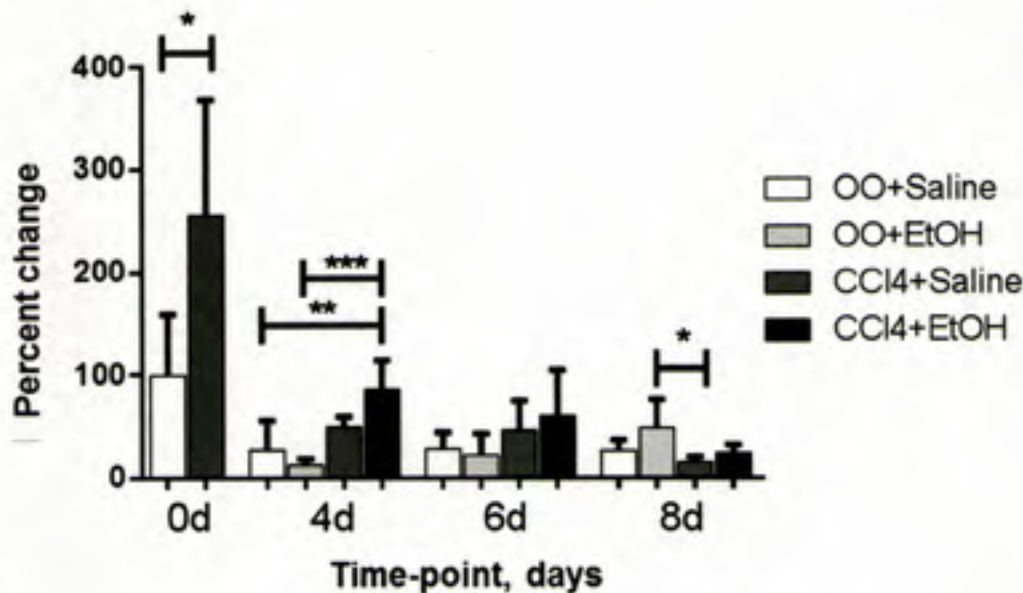


Figure 11. *Acta2* and *Timp1* gene expression. **A** - gene expression of *Acta2*, **B** - gene expression of *Timp1*. Expression was assessed using qRT-PCR, with *Gusb* as the housekeeping gene. Data are expressed as percent change over control of 2-dCt value. Olive Oil+Saline - represents the control group that received vehicle only; Olive Oil+EtOH - represents the group pretreated with vehicle and treated with EtOH in the second stage of the study; CCl₄+Saline - represents the group that was pretreated with CCl₄ and received vehicle in the second stage of the study; CCl₄+Saline+EtOH - represents the group that was pretreated with CCl₄, and treated with ethanol in the second stage. OO - olive oil; Saline - NaCl 0.9%; EtOH - ethanol; CCl₄ - carbon tetrachloride. Statistical analyses were performed using one-way ANOVA with pair wise comparison Tukey's test between the groups within 4 day, 6 day, and 8 day time-points. Unpaired two-tailed Student's t-test was used to determine statistical significance between groups from the 0 day time-point. Asterisks denote significant differences from control mice of the corresponding time-point. * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$, ns - not significant, $p > 0.05$.

4.2. Results of alcohol liquid diet feeding.

4.2.1. Body and liver weight.

Similarly to the previous experiment, body weight of mice and their liver weight was measured (Figure 12 and 13).

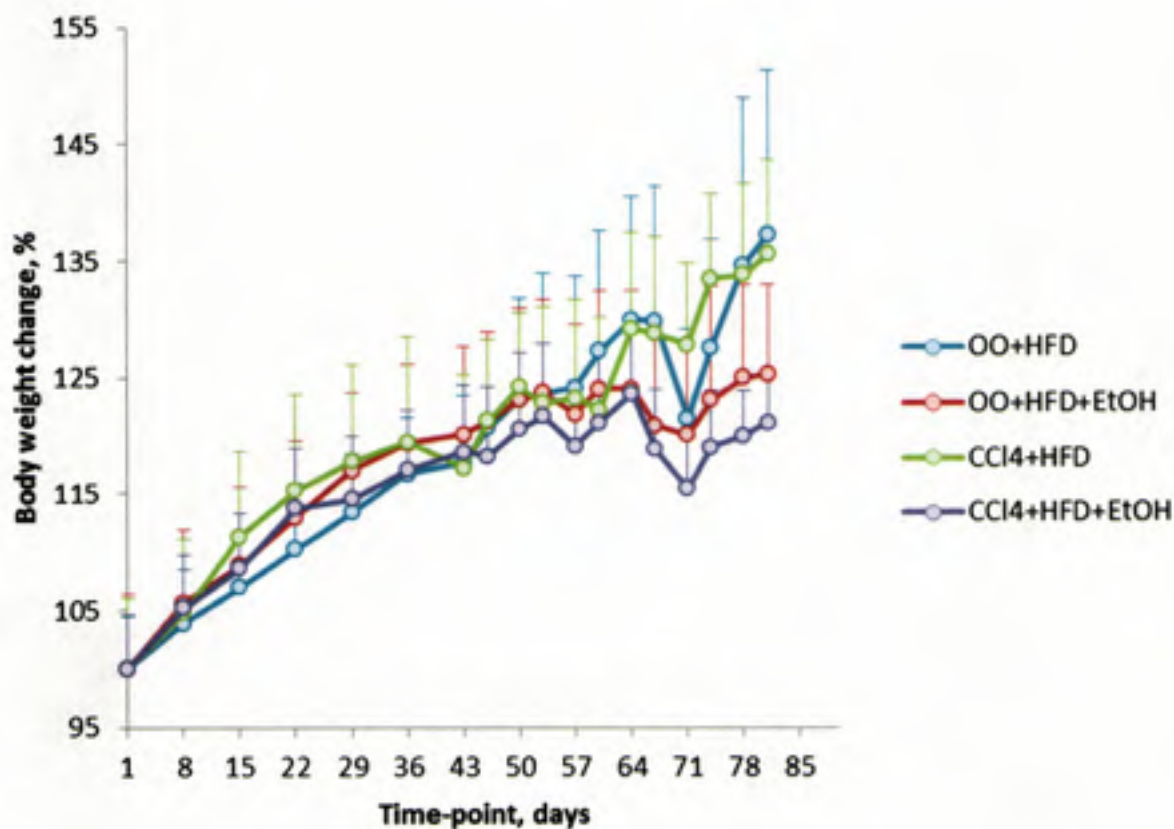


Figure 12. Body weight change during the study. OO+HFD - represents the control group that received vehicle and high fat diet; OO+HFD+EtOH - represents the group pretreated with vehicle and fed with alcohol liquid diet in the second stage of the study; CCl₄+HFD - represents the group that was pretreated with CCl₄ and fed with high fat diet in the second stage of the study; CCl₄+HFD+EtOH- represents the group that was pretreated with CCl₄, fed with alcohol liquid diet in the second stage of the study.

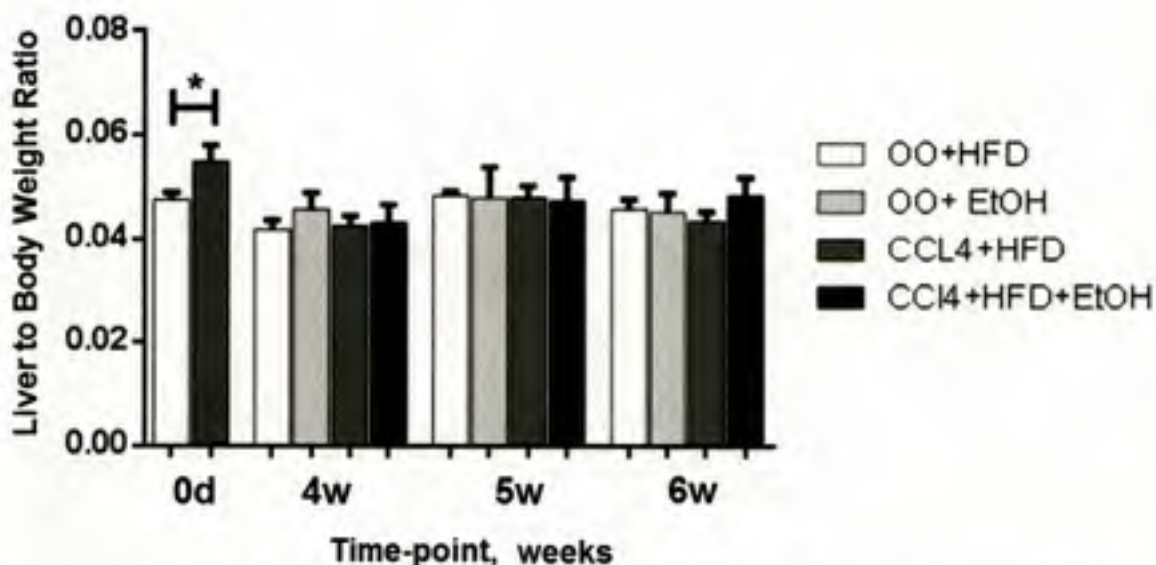


Figure 13. Liver to body weight ratio. OO+HFD - represents the control group that received vehicle and high fat diet; OO+HFD+EtOH - represents the group pretreated with vehicle and fed with alcohol liquid diet in the second stage of the study; CCl₄+HFD - represents the group that was pretreated with CCl₄ and fed with high fat diet in the second stage of the study; CCl₄+HFD+EtOH - represents the group that was pretreated with CCl₄, fed with alcohol liquid diet in the second stage of the study. Statistical analyses were performed using one-way ANOVA with pair wise comparison Tukey's test between the groups within 4 week, 5 week, and 6 week time-points. Unpaired two-tailed Student's t-test was used to determine statistical significance between groups from the 0 day time-point. Asterisks denote significant differences from control mice of the corresponding time-point. * - $p \leq 0.05$.

Groups that received the modified alcohol liquid diet had a slower rate of weight gain compared to that in groups given the high fat diet. The dramatic decrease in body weight on day 71 resulted from the attempt to increase the alcohol consumption to 21 g/kg (Figure 12). After alcohol consumption was reduced to 17 g/kg, mice began to gain weight back.

Figure 13 shows that the liver to body weight ratio of the group pretreated with CCl₄ at the 0 day time-point was significantly higher compared to the control group of the corresponding time-point. There was no significant difference in the liver to body weight ratio across the remaining groups.

4.2.2. Ethanol consumption and serum alcohol concentration.

In order to adjust alcohol consumption of mice, their diet uptake per day was measured. In general, each mouse consumed approximately 10 ml of diet per day. Based on these measurements we were able to calculate their alcohol consumption. Figure 14 confirms that mice from groups that were fed alcohol liquid diet had approximately the same rate of alcohol consumption.

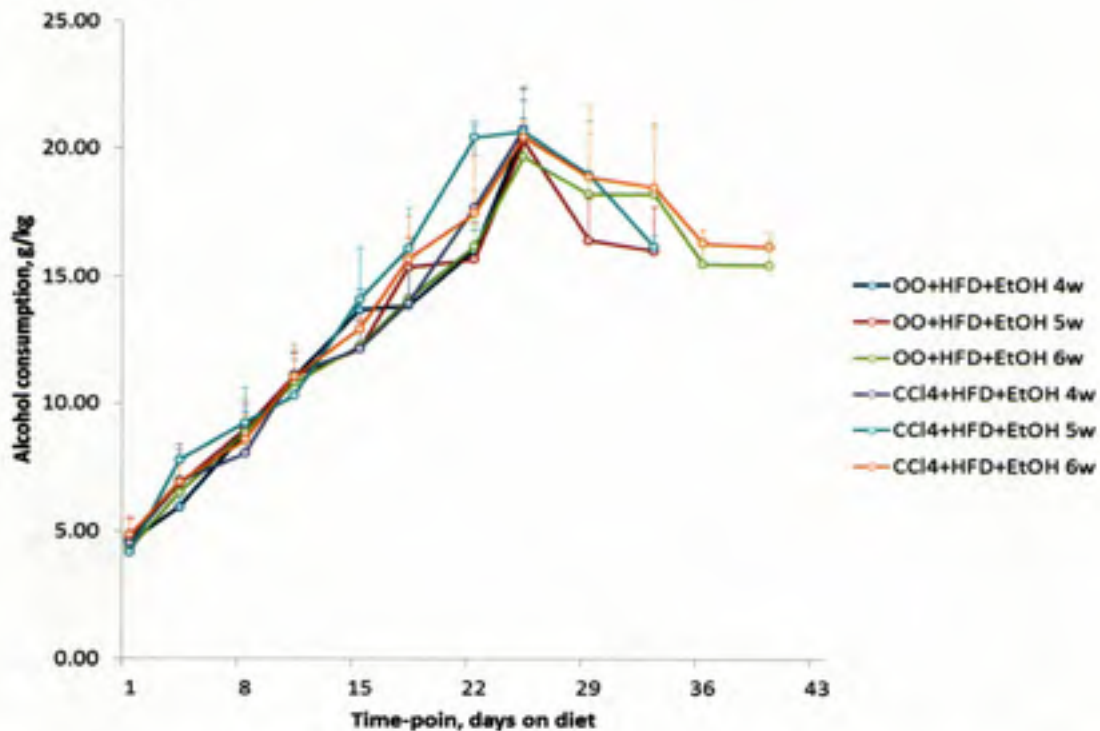


Figure 14. Alcohol consumption in groups that received alcohol liquid diet. OO+HFD - represents the control group that received vehicle and high fat diet; OO+HFD+EtOH - represents the group pretreated with vehicle and fed with alcohol liquid diet in the second stage of the study; CCl₄+HFD - represents the group that was pretreated with CCl₄ and fed with high fat diet in the second stage of the study; CCl₄+HFD+EtOH - represents the group that was pretreated with CCl₄, fed with alcohol liquid diet in the second stage of the study.

During the diet preparation process, maltose dextrin was added to the alcohol diet by mistake. Thus, 1ml of control diet had 1 Kcal, whereas in the alcohol diet caloric status was 1.36 Kcal/ml. High levels of dextrose in the diet were shown to prevent alcohol from stimulating the CYP2E1 enzyme's activity. Experiments with alcohol-fed rats confirm that in animals with a low percentage of dietary calories derived from dextrose, blood alcohol levels remain high and liver injury increases as compared with alcohol-fed rats whose diets contain more dextrose [77]. In order to analyze whether or not an unequal caloric status caused any bias, we determined adjusted caloric uptake by mice from all group. Figure 15 shows that the difference in caloric uptake between groups that received high fat diet and groups fed with modified alcohol liquid diet was minimal.

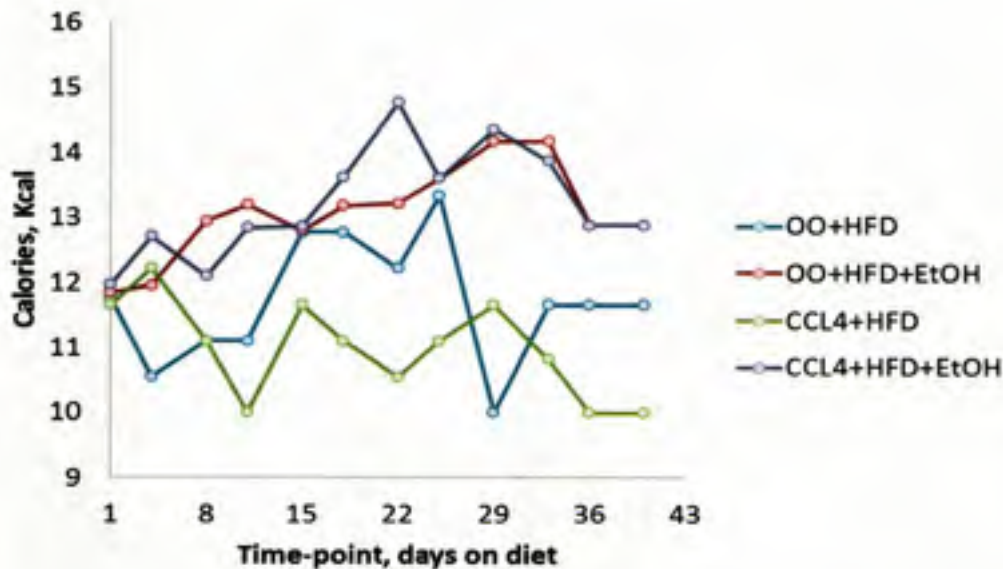


Figure 15. Caloric uptake from the high fat diet. OO+HFD - represents the control group that received vehicle and high fat diet; OO+HFD+EtOH - represents the group pretreated with vehicle and fed with alcohol liquid diet in the second stage of the study; CCL₄+HFD - represents the group that was pretreated with CCL₄ and fed with high fat diet in the second stage of the study; CCL₄+HFD+EtOH - represents the group that was pretreated with CCL₄, fed with alcohol liquid diet in the second stage of the study.

Serum alcohol concentration (SAC) was measured in mice from groups that received modified alcohol liquid diet, to confirm that they were consuming alcohol (Figure 16). Groups that were fed with the modified diet showed increased levels of serum alcohol concentration. Serum alcohol concentration was significantly increased in mice from the group that was pretreated with CCl₄ and later fed with alcohol liquid diet, compared to the remaining groups from the corresponding time-point. Lower serum alcohol concentration in groups pretreated with olive oil from the 6 week time-point may be explained by the fact that levels of serum alcohol depends on dark-light cycles; high levels are maintained throughout the dark period, and decrease at the beginning of the light period [78].

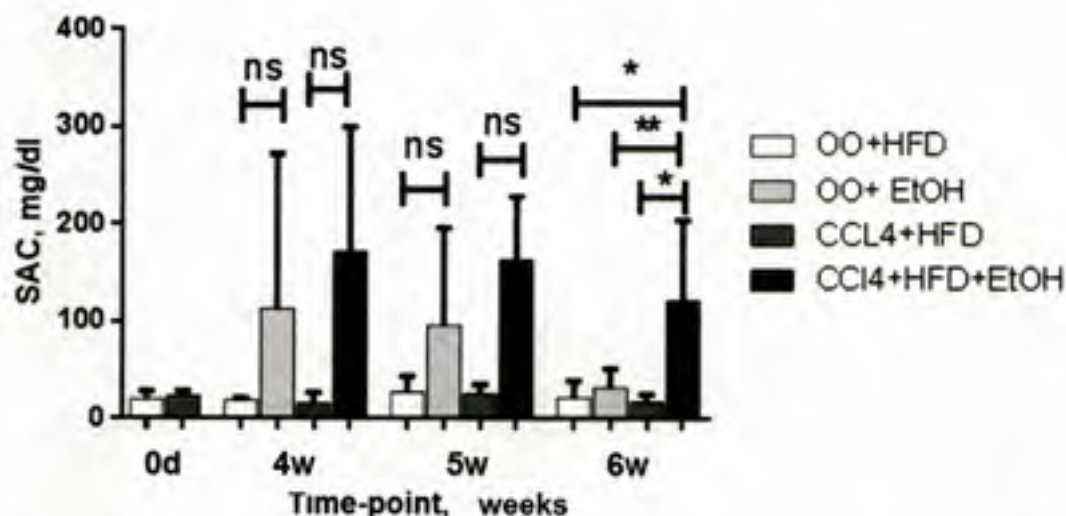


Figure 15. Serum alcohol concentration. OO+HFD - represents the control group that received vehicle and high fat diet; OO+HFD+EtOH - represents the group pretreated with vehicle and fed with alcohol liquid diet in the second stage of the study; CCl₄+HFD - represents the group that was pretreated with CCl₄ and fed with high fat diet in the second stage of the study; CCl₄+HFD+EtOH - represents the group that was pretreated with CCl₄, fed with alcohol liquid diet in the second stage of the study. Statistical analyses were performed using one-way ANOVA with pair wise comparison Tukey's test between the groups within 4 week, 5 week, and 6 week time-points. Unpaired two-tailed Student's t-test was used to determine statistical significance between groups from the 0 day time-point. Asterisks denote significant differences from control mice of the corresponding time-point.

* - $p \leq 0.05$, ** - $p \leq 0.01$, ns - not significant, $p > 0.05$.

4.2.3. Analysis of serum markers of liver injury.

Activity of ALT, AST, and ALKP and the concentration of albumin in serum was measured in order to determine if indicators of liver injury were increased in response to the treatment. Figure 17 shows that levels of albumin concentration remained the same across 0 day and 5 week time-points. At the 4 week time-point, albumin concentration was significantly higher in groups pretreated with olive oil only (2.8 g/dL) compared to the group treated with CCl_4 only (2.3 g/dL). At the 6 week time-point animals pretreated with olive oil only had significantly higher albumin concentration compared to that in both groups pretreated with CCl_4 (2.77 g/dL compared to 2.27 g/dL and 2.4 g/dL) (Panel A).

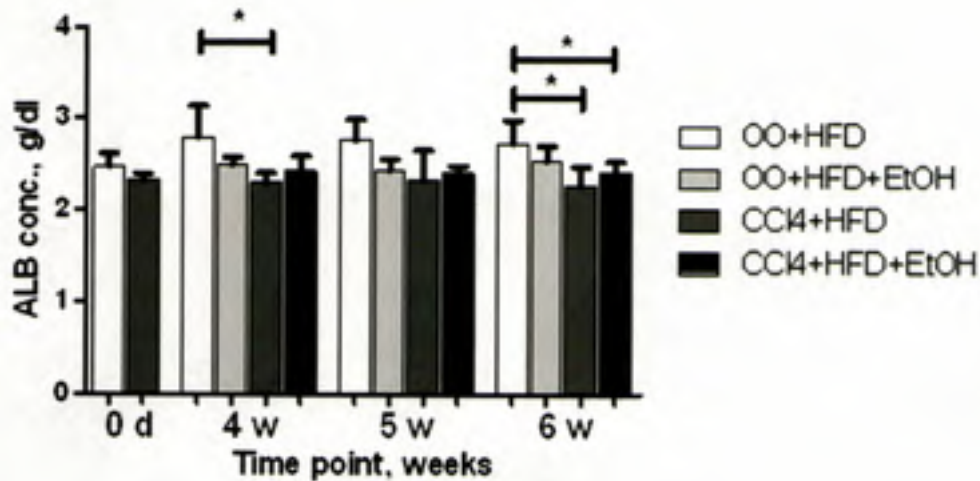
Groups pretreated with CCl_4 also showed greater ALKP activity as compared to that in group treated with olive oil only at the 4 week time-point (81.7 U/L and 90.5 U/L compared to 49 U/L respectively) (Figure 17, Panel B). However, after 4 weeks there were no significant differences in the enzyme activities across all groups.

AST activity at the 4 week time-point was significantly higher in mice from the group treated with olive oil only as compared to mice from the group pretreated with olive oil and later fed with alcohol diet (169.3 U/L compared to 52 U/L respectively). At the 6 week time-point the activity of the enzyme in the group treated with CCl_4 was significantly higher compared to the remaining groups (150 U/L compared to 53.3 U/L in group treated with olive oil, 2.8 U/L in group treated with Olive oil and fed with alcohol liquid diet, and 77.8 U/L in group pretreated with CCl_4 and fed with alcohol diet). At the 0 day and 5 week time-points, AST activity levels did not show significant differences across all groups (Figure 17, Panel C).

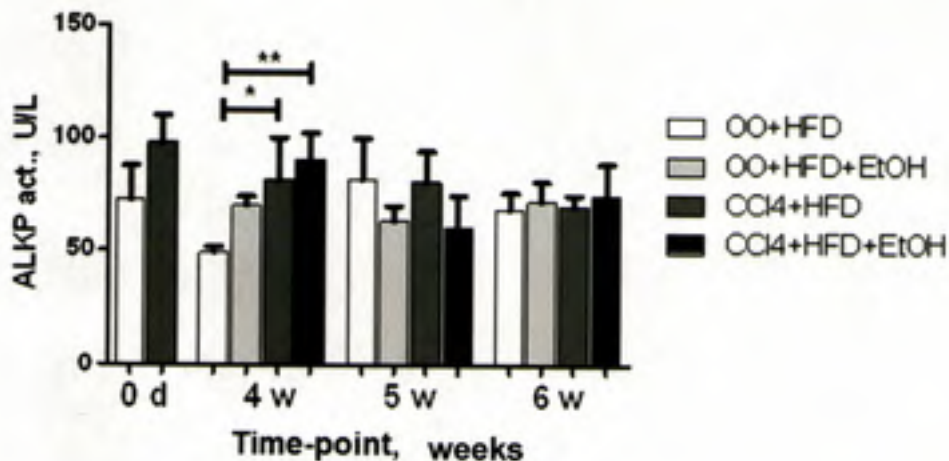
Activity of ALT did not show response to the alcohol treatment; only at the 4 week time-point was it significantly higher in the group treated with olive oil compared to the group pretreated with olive oil and later fed with alcohol diet (22 U/L compared to 12 U/L respectively) (Figure 17, Panel D).

Hence, these data demonstrate that clinical biomarkers were not good predictors of liver injury. This may be explained by the fact that cellular release of enzymes decreased due to a reduction in the number of functional hepatocytes.

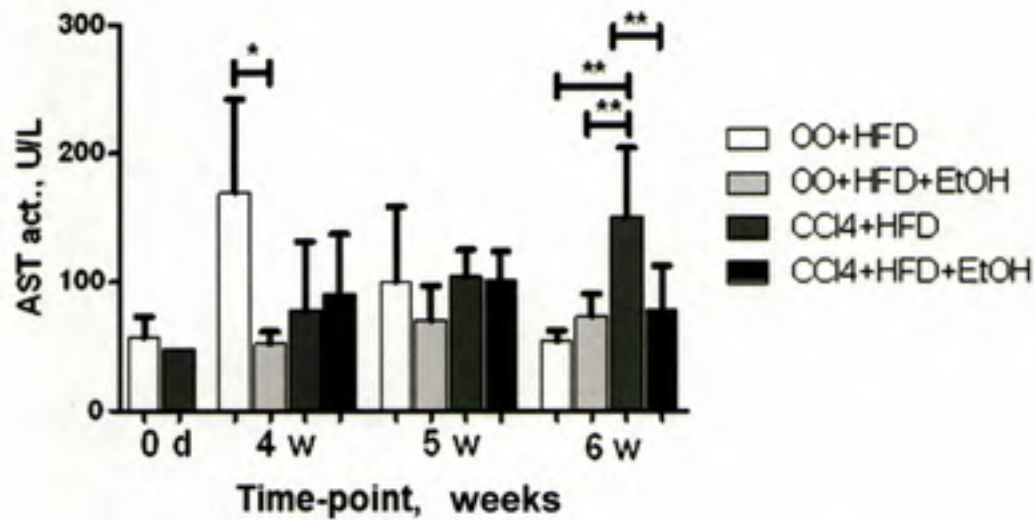
A.



B.



C.



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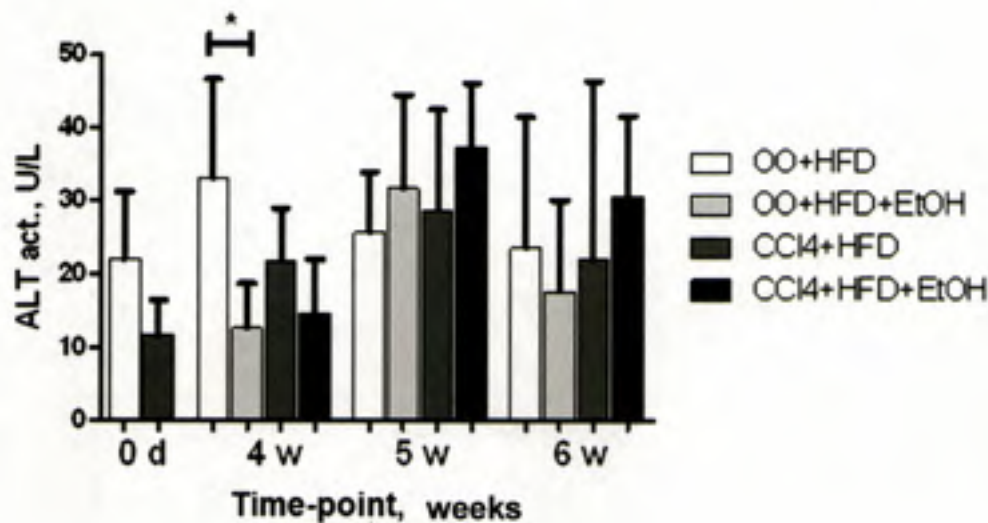


Figure 16. Serum biomarkers of liver injury. A - Albumin concentration; B - Alkaline phosphatase activity; C - Aspartate amino transferase activity; D - Alanine amino transferase activity. Normal reference clinical chemistry values for mice are: Alb=2.5-3 g/dl; ALT=17-77U/L; AST=54-298U/L; ALKP=35-96U/L. OO+HFD - represents the control group that received vehicle and high fat diet; OO+HFD+EtOH - represents the group pretreated with vehicle and fed with alcohol liquid diet in the second stage of the study; CCl4+HFD -

represents the group that was pretreated with CCl₄ and fed with high fat diet in the second stage of the study; CCl₄+HFD+EtOH - represents the group that was pretreated with CCl₄, fed with alcohol liquid diet in the second stage of the study. Data are expressed as absolute values. Statistical analyses were performed using one-way ANOVA with pair wise comparison Tukey's test between the groups within 4 week, 5 week, and 6 week time-points. Unpaired two-tailed Student's t-test was used to determine statistical significance between groups from the 0 day time-point. Asterisks denote significant differences from control mice of the corresponding time-point. * - $p \leq 0.05$, ** - $p \leq 0.01$, ns - not significant, $p > 0.05$.

4.2.4. Histopathological evaluation of alcohol-induced liver injury.

The hematoxylin and eosin-stained liver sections were evaluated for several histological indices. There were no abnormal findings in the livers of mice treated with olive oil alone (Figure 18). Liver sections from group that received HFD alone showed that diffuse vacuolar changes consisted of deposition of small lipid vacuoles and glycogen-like materials in the hepatocytes (Figure 18, Panel B). In contrast, chronic intake of EtOH with HFD either pretreated with olive oil or CCl₄ resulted in remarkable steatosis with small and mid-to large lipid vacuoles in the midlobular area (Figure 18, Panels C and F). In the EtOH with HFD-treated group, centrilobular hepatocytes exhibited slight to moderate hypertrophy with anisonucleosis, while there was not clear hepatocellular necrosis.

Characteristic liver lesions induced by CCl₄ treatment consisted of hepatocellular anisonucleosis and mixed cellular inflammation with pigment-laden macrophages in the centrilobular area (Figure 18, Panels D, E, and F). These nuclear and inflammatory changes were observed in livers of almost all mice from CCl₄-treated groups and there were no clear differences in the extent of these lesions in each time-point, with the exception of weak fibrotic changes. In addition, weak fibrosis was observed in livers of mice from CCl₄-treated

groups although apparent bridging fibrosis was hardly seen in the current experimental condition.

Figure 19 shows individual and total pathology scores based on the histopathology analysis. Steatosis developed after 4 weeks of high fat diet consumption. In group treated with CCl_4 , the steatosis score was significantly lower compared to that in the remaining groups at 4 and 6 week time-points, although at the 5 week time-point this decrease was found to be insignificant (Figure 19, Panel A).

Mice that received alcohol liquid diet also developed liver necrosis. Figure 19, panel B represents statistically significant difference in necrosis score between groups treated with alcohol liquid diet and modified high fat diet. Furthermore, in groups pretreated with CCl_4 and fed with alcohol diet, necrosis was exacerbated over the course of treatment.

Administration of CCl_4 caused inflammatory lesions in liver tissue. Figure 19, panel C shows the significant differences between groups pretreated with olive oil and groups pretreated with CCl_4 across all time-points. Moreover, groups treated with CCl_4 showed the regression of fibrotic changes over the course of time after administration of the chemical was stopped. In groups that were pretreated with CCl_4 and fed alcohol liquid diet, fibrosis score increased during the diet feeding period.

Pretreatment with CCl_4 followed by consumption of modified alcohol liquid diet caused more severe liver damage compared to that in the rest of the groups. Moreover, over the time course of treatment this damage was exacerbated (Figure 19, Panel D).

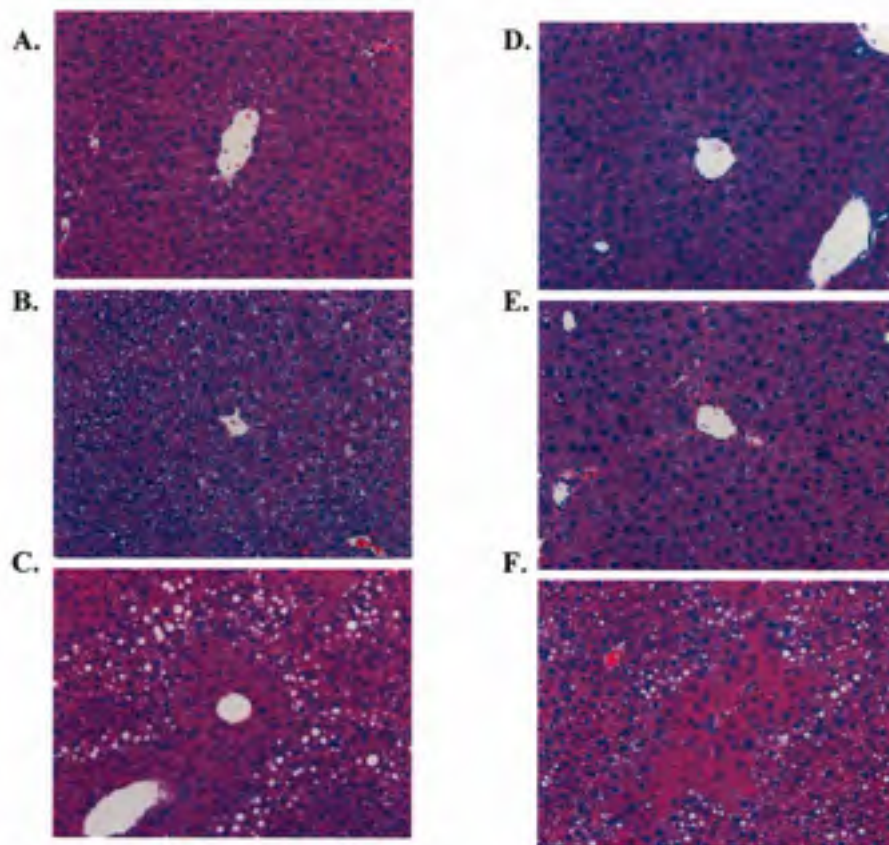
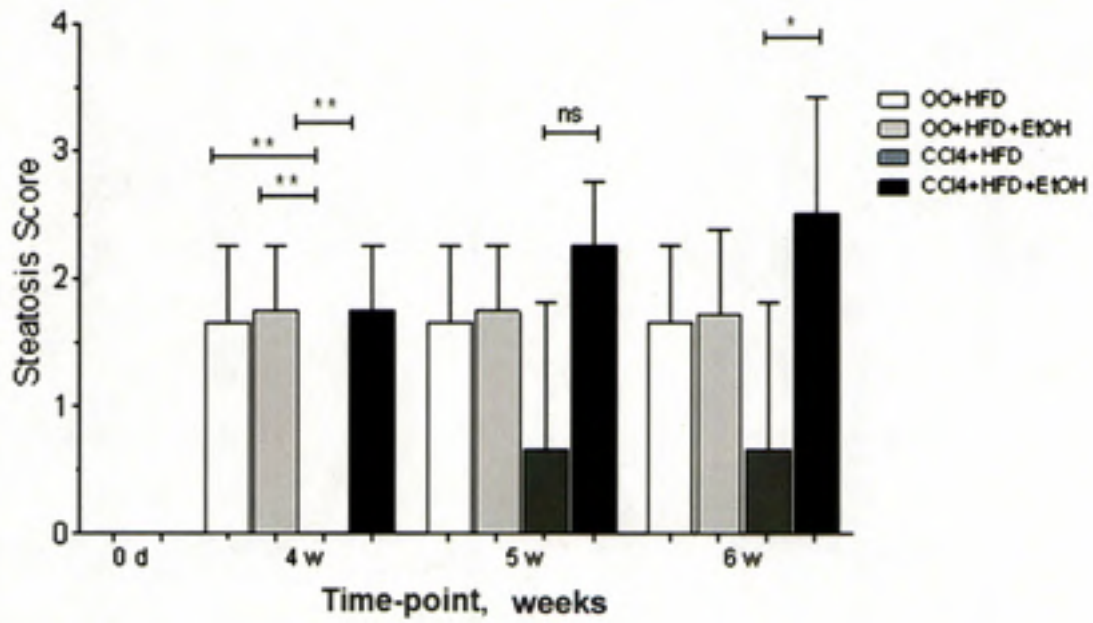
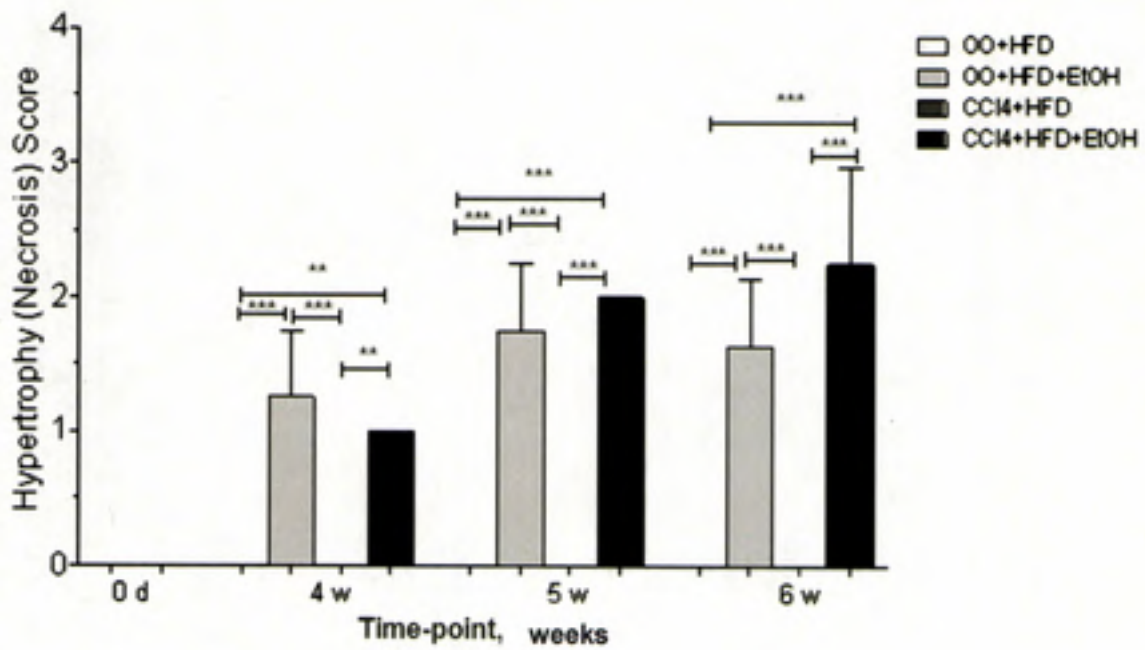


Figure 17. Liver sections stained with hematoxylin and eosin. A and B - representative liver sections from the control group that received vehicle, 0 day and 6 week time-points, respectively; C-representative liver section from the group pretreated with vehicle and fed with alcohol liquid diet in the second stage of the study, 6 week time-point; D and E - representative liver sections from the group that was pretreated with CCl_4 and fed with high fat diet in the second stage of the study, the 0 day and 6 week time-points, respectively; F - representative liver section of the group that was pretreated with carbon tetrachloride, fed with alcohol liquid diet in the second stage of the study, the 6 week time-point. Magnification 200X

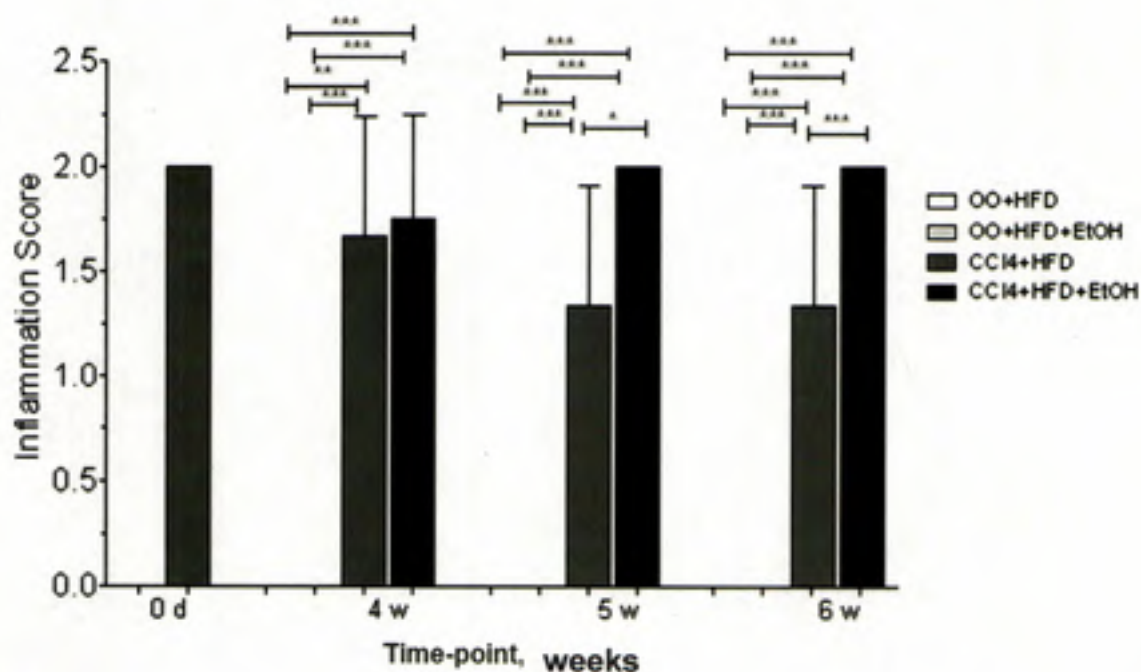
A.



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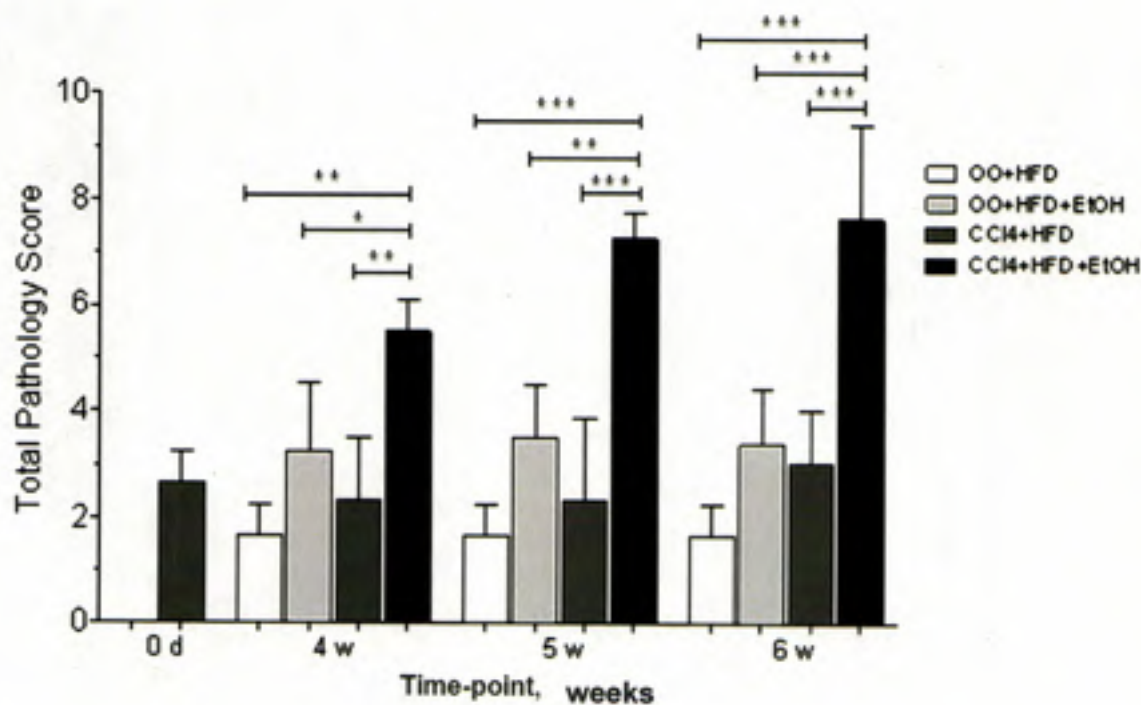


Figure 18. Histopathology scores, individual and total. A - Steatosis score; B - Hypertrophy score; C - Inflammation score; D - Total pathology score. For steatosis the extent

of lesions were scored based on the affected area of the liver cells as follows: 1 < 10%, 2 < 25 %, 3 < 50%, and 4 > 50 %. Other lesions, such as hypertrophy, anisonucleosis, vacuolization, and cellular infiltration and pigment-laden macrophages/inflammation, were graded in the standard manner: 1 - minimal, 2 - slight, 3 - moderate and 4 - severe. There are characteristic histopathological changes induced by the cotreatment of high fat diet with EtOH (fatty changes and hypertrophy of hepatocytes) and CCl₄ (inflammatory changes and nuclear changes). Statistical analyses were performed using one-way ANOVA with pair wise comparison Tukey's test between the groups within 4 week, 5 week, and 6 week time-points. Unpaired two-tailed Student's t-test was used to determine statistical significance between groups from 0 day time-point. Asterisks denote significant ($p < 0.05$) differences from control mice of the corresponding time-point. * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$, ns - not significant, $p > 0.05$.

In order to evaluate fibrosis, Masson's trichrome staining for liver sections was provided (Figure 20). Mice pretreated with CCl₄ developed more severe degree of bridging fibrosis and inflammatory lesions compared to groups treated with olive oil.

Fibrosis score was estimated based on the ratio of fibrotic tissue to normal liver tissue. Figure 21 supports the results of visual analysis. There was a significant increase in collagen accumulation in liver tissues of mice pretreated with CCl₄ compared to groups treated with olive oil across all time-points of the study. A slight decrease of fibrosis score was noted over the time period in the group that received a high fat diet. Mice that received alcohol liquid diet showed a decrease in fibrosis score at the 5 week time-point; however, at the 6 week time-point, the ratio of fibrosis increased again.

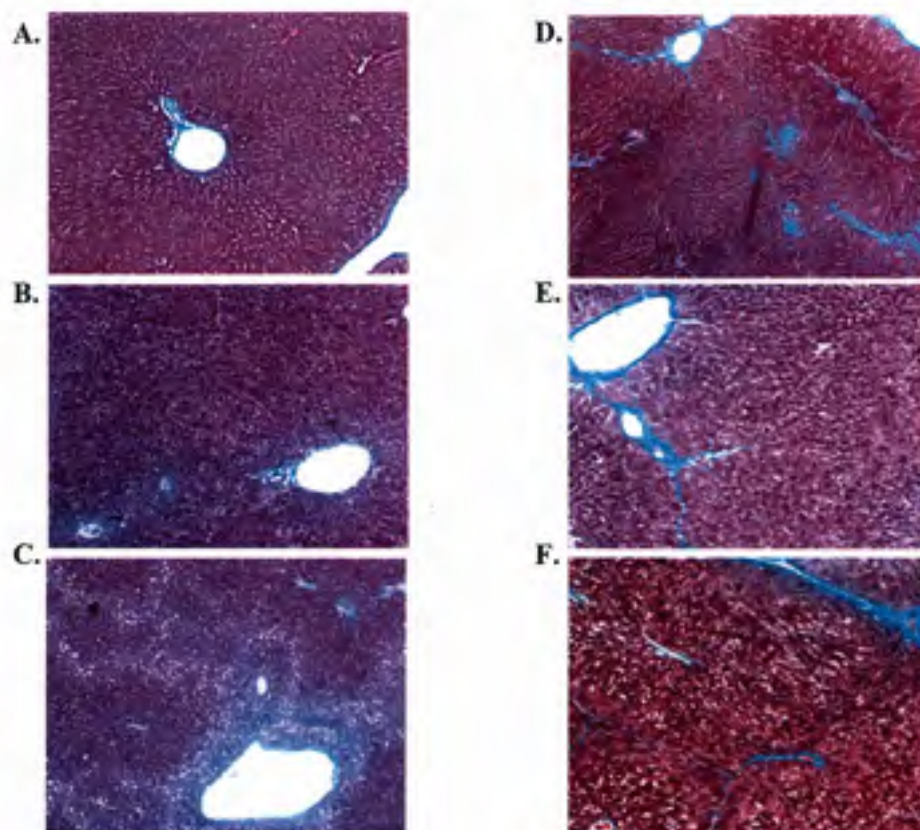


Figure 20. Liver sections stained with Masson's trichrome. A and B - representative liver sections from the control group that received vehicle, 0 day and 6 week time-points, respectively; C - representative liver section from the group pretreated with vehicle and fed with alcohol liquid diet in the second stage of the study, 6 week time-point; D and E - representative liver sections from the group that was pretreated with CCl_4 and fed with high fat diet in the second stage of the study, 0 day and 6 week time-points, respectively; F - representative liver section of the group that was pretreated with carbon tetrachloride, fed with alcohol liquid diet in the second stage of the study, 6 week time-point. Magnification 40X

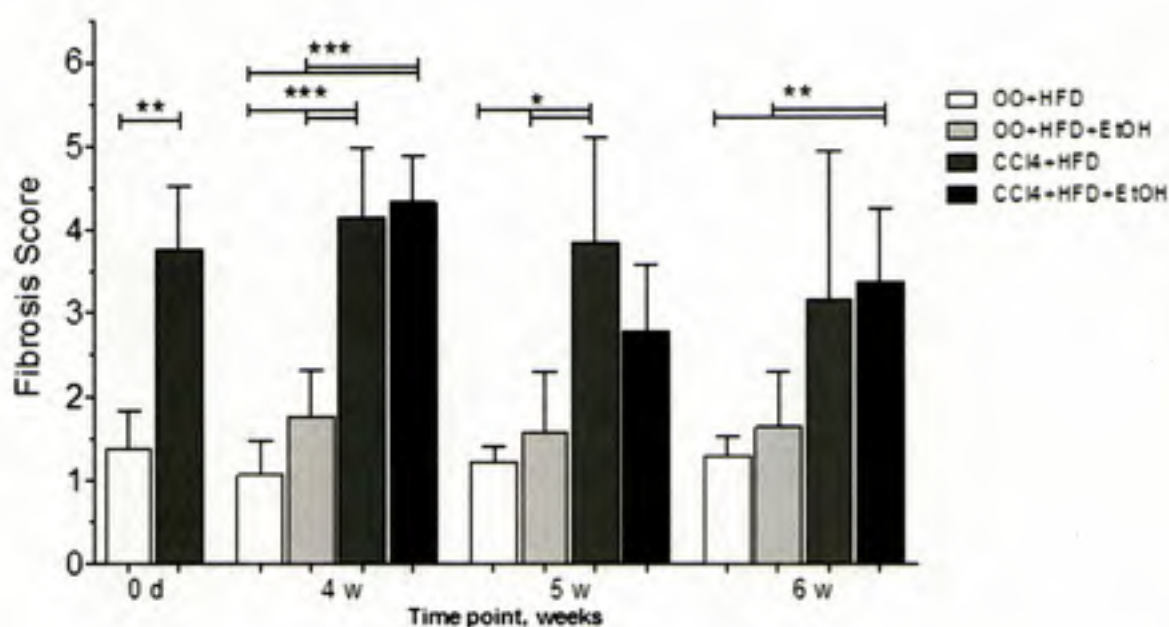


Figure 19. Fibrosis score. OO+HFD – represents the control group that received vehicle and high fat diet; OO+HFD+EtOH - represents the group pretreated with vehicle and fed with alcohol liquid diet in the second stage of the study; CCl₄+HFD - represents the group that was pretreated with CCl₄ and fed with high fat diet in the second stage of the study; CCl₄+HFD+EtOH - represents the group that was pretreated with CCl₄, fed with alcohol liquid diet in the second stage of the study. Statistical analyses were performed using one-way ANOVA with pair wise comparison Tukey's test between the groups within 4 week, 5 week, and 6 week time-points. Unpaired two-tailed Student's t-test was used to determine statistical significance between groups from 0 day time-point. Asterisks denote significant differences from control mice of the corresponding time-point. * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$.

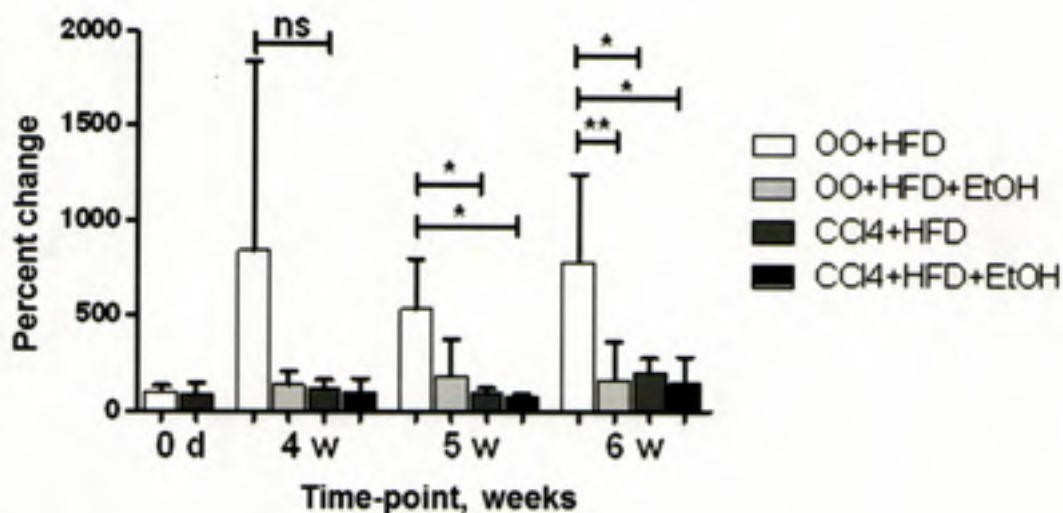
4.2.5. Hepatic expression of *Acta1* and *Timp1* genes.

The expression of *Acta2* and *Timp1* was assessed using qRT-PCR, with *Gusb* as the housekeeping control gene (Figure 22).

The expression of *Acta2* gene was significantly increased at 5 and 6 week time-points compared the remaining groups. At the 4 week time-point, this increase was not significant. The expression of *Acta2* did not show correlation with fibrosis score.

The *Timp1* gene expression decreased during the time course of high fat diet feeding and increased in groups that received alcohol liquid diet were not confirmed by statistical analysis. At the 5 week time-point, however, *Timp1* gene expression in the group treated with CCl₄ and alcohol liquid diet was significantly higher compared to the group that was pretreated with olive oil and received high fat diet (50% increase).

A.



B.

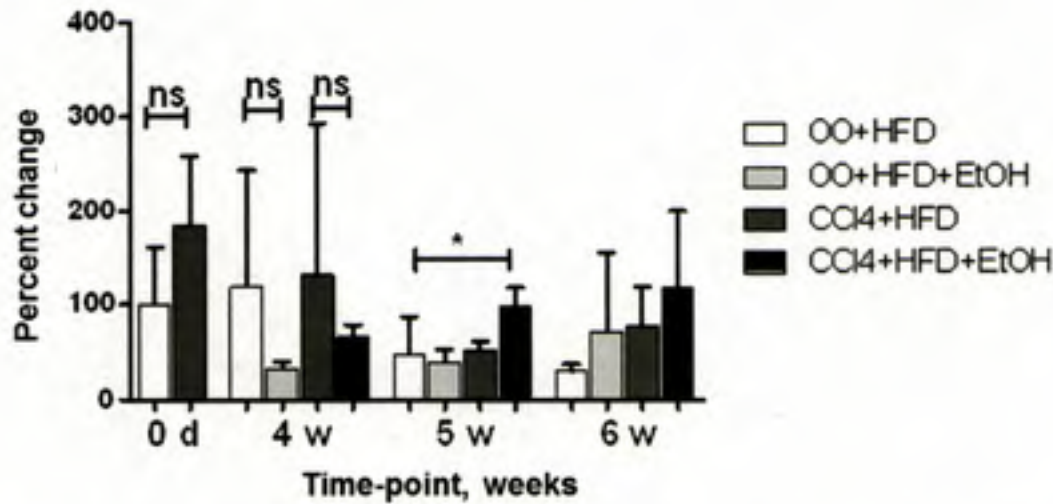


Figure 20. *Acta2* and *Timp1* gene expression. A - gene expression of *Acta2*, B - gene expression for *Timp1*. Expression was assessed using qRT-PCR, with *Gusb* as the housekeeping gene. Data are expressed as percent change over control of 2-dCt value. OO+HFD - represents control group that received vehicle and high fat diet; OO+HFD+EtOH - represents the group pretreated with vehicle and fed with alcohol liquid diet in the second stage of the study; CCl₄+HFD - represents the group that was pretreated with CCl₄ and fed with high fat diet in the second stage of the study; CCl₄+HFD+EtOH - represents the group that was pretreated with CCl₄, fed with alcohol liquid diet in the second stage of the study. Statistical analyses were performed using one-way ANOVA with pair wise comparison Tukey's test between the groups within 4 week, 5 week, and 6 week time-points. Unpaired two-tailed Student's t-test was used to determine statistical significance between groups from 0 day time-point. Asterisks denote significant differences from control mice of the corresponding time-point. *- $p \leq 0.05$, **- $p \leq 0.01$, ns - not significant, $p > 0.05$.

Chapter 5

Discussion, Limitations, Conclusions, and Future Directions.

Discussion.

The use of animals for modeling human functions, conditions, and pathological states is an integral part of medical and scientific research. However, there are a number of limitations to how these models can be used to understand the pathophysiology of human diseases.

The establishment of a compelling and human relevant model for alcohol-induced liver injury in rodents has faced many difficulties. The most rational way to provide a useful framework for future clinical trials in alcoholic hepatitis consists of the identification of relevant pathways and appropriate targets by matching them to the prevailing molecular profile of patients. However, features and patterns of some liver pathology, alcoholic hepatitis in particular, is not completely reproduced by existing models. The ability of animal models to reproduce the phenotypic hallmarks of alcohol-induced liver disease in humans, such as steatosis, inflammation, and necrosis, is typically a benchmark for model's success [66].

Alcoholic steatohepatitis in rodents with all characteristic features has only been achieved using the continuous, Tsukamoto-French survival, surgery-requiring intragastric feeding model [62]. However, this model requires surgical expertise for insertion of cannula and the implementation is time-consuming and expensive. Thus, the focus of our study was

to develop a mouse model of alcoholic hepatitis that does not require surgery or continuous force-feeding of alcohol.

In the first experiment, ethanol feeding using a gavage technique allowed us to model the binge drinking pattern observed in humans. Data obtained from a preliminary study showed that mice that received 14 intraperitoneal injections of CCl₄ (0.5 ml/kg, 3 times a week) followed by an EtOH gavage (5g/kg, for 5 days) develop significant increase in hepatic fibrosis, inflammatory changes, and moderate increase in hepatic steatosis at a significantly higher rate compared to groups treated with CCl₄ alone. Moreover, CCl₄-induced fibrotic changes regressed in the vehicle-only treated group but were maintained in ethanol-treated group. Under these experimental conditions, mice developed fibrotic lesions due to CCl₄ administration, but minimal effects were caused by ethanol treatment; likely due to the stress of daily gavage. Additionally, binge feeding with ethanol for 8 days did not cause significant phenotypic changes, such as cholestasis or neutrophil infiltration in the livers of mice from both groups (either pretreated with vehicle or pretreated with CCl₄). Mice treated with CCl₄ regardless of EtOH-administration developed fibrotic lesions. In groups in which liver injury was caused by CCl₄, liver damage was shown to be restored after administration of the chemical was stopped, resulting in minimal hepatocellular injuries such as centrilobular necrosis, ballooning degeneration, and fatty deposition. However, bridging fibrosis was still observed in the liver sections of animals from groups pretreated with CCl₄.

While in mice this model was not successful in achieving an alcohol hepatitis phenotype similar to that in humans, it could have a different outcome when used in different species (i.e., rats) due to interspecies differences.

The inability to induce alcoholic hepatitis in the first experiment motivated us to use another experimental protocol with longer alcohol induction of liver injury.

In rats and mice, the Lieber-DeCarli liquid diet treatment regimen induces the earliest form of damage to the liver and hepatocytes (i.e., fatty liver, characterized by fat deposits throughout the liver) but does not lead to the more serious forms of liver damage observed in humans (i.e., necrosis, inflammation and fibrosis) [62]. However, published studies suggest that the composition of the liquid diet, in which the alcohol is administered, can significantly influence the intensity of alcohol effects. For example, the amount and the type of fat in the diet will influence the intensity of alcohol-induced organ damage. Thus, it has been shown previously that using corn oil as a source of fat in a standard Lieber-DeCarli liquid diet model allowed to achieve development of steatohepatitis comparable to that in the Tsukamoto-French model [66].

The liver fat and the rate of ethanol elimination was found to be lower in rodents fed alcohol in high carbohydrate-low fat liquid diet compared to those fed identical amounts of alcohol in high fat-low carbohydrate diet [79]. In the current study we were able to achieve blood alcohol levels at sacrifice nearly as high as those observed previously in the ethanol feeding studies [78]. Additionally, we were able to induce fibrosis and inflammatory changes in the livers of treated mice by CCl₄ administration. It is important to mention that mice in negative control groups, which received vehicle and high fat diet only, exhibited no signs of liver damage, including steatosis. Mice from the positive control group treated with CCl₄ and high fat diet developed liver inflammation. In this group, liver injury regressed over time, whereas in group treated with CCl₄ and ethanol changes were maintained.

Clinical parameters of liver injury in both experiments did not show any treatment response. While in binge ethanol this feeding could be explained by insufficient liver damage, in the model where mice were fed alcohol liquid diet this can be explained by a depletion of enzymes or the reduction of their cellular release due to a decrease in functional hepatocyte number. Being unspecific for liver injury these parameters are not very reliable biomarkers, so it is important to find new more reliable clinical chemistry parameters of hepatotoxicity.

While further work will be necessary to identify additional biomarkers that correlate with liver injury, the administration of modified alcohol liquid diet model after CCl₄ treatment is likely to cause liver lesions with features similar to alcoholic hepatitis in humans.

Limitations.

During implementation of both protocols we faced a number of limitations. In particular, in the first study, binge ethanol feeding using oral gavage technique caused stress and changes in the eating habits of the mice. The mice survival rate decreased after the administered dose of alcohol was increased to 5g/kg, possibly due to intoxication from high alcohol concentration.

In the other experimental design, during diet preparation, maltose dextrin was added to the alcohol-liquid diet by mistake, which changed the caloric status of 1 ml of this diet to 1.36 Kcal compared to 1Kcal in the high fat diet. Specifically, it has been shown that high levels of dextrose in diet prevent alcohol from stimulating CYP2E1 enzyme activity, thus protecting liver from damage. In our study the survival rate of mice was indeed high, no mice

died during the course of diet treatment, but histopathological analysis confirmed that mice treated with ethanol developed liver injury. After we estimated caloric uptake of mice based on their diet consumption, the difference was not significant, probably due to the fact that mice fed with high fat diet alone on average consumed more diet than the treated group (Figure 14).

Under these experimental settings, it was difficult to monitor what volume of diet each mouse consumed. Although we tried to minimize this limitation by putting only two mice per cage, there was still a chance that one mouse consumed more than another. In order to prove the fact that mice received alcohol, we examined serum alcohol concentration levels (Figure 15).

Mice were housed in regular cages instead of metabolic in order to avoid hypothermia in mice that were fed with alcohol diet. This disabled urine collection for assessment of metabolomics profiles. Therefore, urine samples were collected only on the day of sacrifice.

Differences in hormonal status and alcohol metabolizing enzyme activity between different species can impact the susceptibility to alcohol-induced organ damage. In order to induce liver injury that mimics features of alcoholic hepatitis in humans, doses of alcohol were high (5 g/kg/day in binge feeding experiment, and 17 g/kg /day in modified liquid diet model). In humans, consumption of such a high doses leads to severe intoxication, coma and even death.

Mice from the C57BL/6 strain used in the current study present features of relatively high voluntary alcohol consumption. This strain has also been shown to be sensitive to alcohol-induced effects [80,81]; however, in human populations the individual susceptibility to alcoholic liver disease varies.

Another important concern is the fact that in humans, factors such as comorbidities (e.g., diabetes, renal diseases, and cardiovascular disorders), exposure to different environmental factors (cigarette smoke, drugs), and dietary status are largely involved in the pathogenesis of alcoholic liver disease. The experimental approach used in the current study does not account for multifactorial pathogenesis of AH [14].

Conclusions.

We have developed and evaluated two experimental mouse models in order to produce liver injury consistent with pathological changes seen in human patients.

Binge ethanol feeding via oral gavage caused minimal effects in liver tissues of mice. This indicates that this animal model was not successful for the development of alcohol-induced hepatitis clinically relevant to that seen in humans. While this experimental design in mice did not lead to severe liver damage, this approach may be effective in other species (e.g., rats).

Administration of low doses of CCl_4 followed by consumption of alcoholic liquid diet resulted in the development of steatosis, necrosis, bridging fibrosis, and inflammatory lesions, which were exacerbated over the treatment period. These histopathological findings allowed us to conclude that this model represents liver pathological changes that mimic features of alcoholic hepatitis in humans.

Elucidating molecular mechanisms and processes associated with the development of alcoholic liver disease in humans is critical for its treatment and prevention. However, investigating the molecular basis of alcoholic liver disease in humans is frequently impractical and inherently complex, whereas using relevant animal models may substantially

overcome the limitations of human-only studies. The results of our experiments show that we have successfully developed a novel mouse model of alcoholic hepatitis that resembles histopathological features of human disease. This model can be used to investigate the underlying mechanisms associated with the development of human hepatitis, as well as for disease treatment and prevention.

Future directions.

Alcoholic liver disease is a major health problem in the United States and developed countries. It is critical to uncover molecular mechanisms and processes associated with the development of alcoholic liver disease in humans to improve clinical management of the disease. In light of this, our model may be used for in-depth investigation of molecular mechanisms and processes of the pathogenesis of alcoholic liver disease. Specifically, future studies should be conducted to estimate other markers of liver injury in order to support our findings. For this purpose the level of hydroxyprolin can be assessed with the help of western blot technique, also gene expression of collagen alpha 1(*Col1*) can be analyzed using qRT-PCR.

Additionally, it is necessary to analyze how alcohol interacts with lipid metabolism. A hepatic free fatty acid profile should be evaluated.

Also, the histopathological evaluation of other tissues (kidney, stomach) would be helpful to estimate the influence of alcohol on other target organs. For this purpose, biological material collected in the study can be used in order to identify ethanol's molecular targets.

Furthermore, in humans, genetic factors greatly influence individual susceptibility to alcoholic liver disease. It remains very difficult to explain and predict individual susceptibility to alcoholic liver disease. Our mouse model may be very useful for studying the underlying mechanisms of this individual susceptibility.

Chapter 6

Practicum Report

I completed my public health practicum at the Division of Biochemical Toxicology in the U.S. FDA National Center for Toxicological Research (NCTR), in Jefferson, Arkansas, under the supervision of Dr. Igor Pogribny. I selected this public agency because NCTR is committed to conducting the scientific research necessary for the FDA to make far reaching decisions affecting public health. Through collaborative efforts with government, academic, and industry researchers, NCTR fosters national and international collaborations and helps develop, refine and apply technology to further improve safety evaluations of FDA-regulated products to protect public health and improve the quality of life for all Americans. NCTR researchers spread the principles of regulatory science globally. The Division of Biochemical Toxicology conducts fundamental and applied research specifically designed to define the biological mechanisms of action underlying the toxicity of products regulated by, or of interest to, the FDA. The risk-assessment research is firmly rooted in mechanistic studies focused on the understanding of toxicological endpoints, an approach that allows greater confidence in the subsequent risk assessments. These studies currently serve as the benchmark by which toxicological assessments are made by FDA and other federal agencies. In addition to providing basic information on toxicological endpoints, such as cancer, these experiments form the basis for mechanistic studies to ascertain if the response detected in the experimental model is pertinent to humans. Laboratory under the direction of Dr. Pogribny conducts a numerous studies to elucidate the epigenetic mechanisms of action of some

carcinogens and unravel new biomarkers that may be useful in the early detection or prevention of disease.

The goal of my practicum project was to answer the question what epigenetic mechanisms are involved in inter-strain variability of 1,3-butadiene (BD) genotoxicity. Multistrain approach used in the study can be beneficial for identifying groups that might be especially susceptible to butadiene toxicity. Once identified, such groups can be advised to take cautionary measures to protect themselves from BD exposure (i.e. limiting environmental tobacco smoke). This study can not only benefit in clarifying of pathogenesis pathways but also subsequently provide sufficient information to set strategies for preventing and curating the toxic effects of butadiene.

To answer this question I analyzed the status of histone modifications in kidney and liver tissues of mice (A/J, CAST/EiJ, and C57BL/6J, males and females) that were exposed to different doses of BD through inhalation (0, 13, or 425 ppm BD for 6 hr/day, 5 days/week, for 2 weeks). For this purpose I used western blot, an important technique in molecular biology which serves to help identify and quantify proteins and evaluate if they are up- or down-regulated in treated tissue compared to control tissues. It is known that inter-individual variability in humans plays a role in susceptibility to BD toxicity. This study attempted to model genetic variation and examine interstrain and gender differences in genotoxicity of BD.

It is now well understood that although the base sequence of DNA provides the "blueprint" for life, this blueprint can be modified through epigenetic changes such as methylation, phosphorylation, or acetylation of DNA or histones that are stably transmitted through cell divisions [82]. Histone modifications are proposed to affect chromosome

function through at least two distinct mechanisms. The first mechanism suggests modifications may alter the electrostatic charge of the histone resulting in a structural change in histones or their binding to DNA. The second mechanism proposes that these modifications are binding sites for protein recognition modules, such as the bromodomains or chromodomains that recognize acetylated lysines or methylated lysines, respectively. Currently several classes of post-translational histone modifications are known, among them methylation, phosphorylation, and acetylation [83]. Global histone modification patterns correlate with tumor phenotypes and prognostic factors in multiple tumor types [84].

For the practicum research, I focused on evaluation of the methylation and acetylation state of several lysine residues on histones H3 and H4 in the kidney, including H3K9me₃, H3K27me₃, H3K27ac, H4K20me₃, and H3K27ac in liver tissue. It is accepted that increased trimethylation these marks are important in maintaining genomic stability and chromatin structure [85]. High levels of histone acetylation were shown to be associated with gene activity with low levels being correlated with gene silencing [86].

For a western blot analysis, proteins were isolated from the tissue using lysis buffer and aliquots were prepared. Samples were denatured by heating in the presence of mercaptoethanol and proteins were resolved by polyacrylamide gel electrophoresis. Then proteins were transferred to PVDF membranes by electroblotting. After blocking membranes were incubated with primary antibodies that bind to the protein of interest, after the wash the membrane were exposed to secondary antibodies and visualized.

The analysis of data collected during the study showed the existence of inter-strain and inter-gender variability in the status of histone modifications. Specifically, H3K9me₃ in kidney of males from CAST/EiJ strain did not significantly change after the increase of

inhaled BD concentration, while in females this histone modification mark was decreased. It is important to mention that female mice have been shown to develop lung and liver tumors at lower exposure concentrations than males [85]. Therefore, trimethylation of histones can be involved in protective mechanisms. In male mice of A/J strain the level of H3K9me3 slightly increased in group that received high dose of BD compared to that in control and low dose groups, in females only group that received low dose of BD showed response to the exposure. Male mice of C57BL/6J strain showed dose-response relationship in trimethylation status, whereas in females it didn't change.

Trimethylation of H3K27 status in kidney was similar to that of H3K9. There was no significant inter-strain and inter-gender difference noticed in the H4K20 trimethylation, but in C57BL/6J, both males and females it was up-regulated in groups that were exposed with low and high dose of BD.

Acetylation of H3K27 in kidney and liver was also evaluated. In kidney of CAST/EiJ male mice who were exposed with low dose, it is up-regulated, but in high dose its expression is similar to that in control group. In females, at low dose it was downregulated, at high dose group H3K27ac increased, but still remained lower compared to control group. In C57BL/6J males it showed increased together with the administered dose, in females- H3K27 ac in low and high dose groups was decreased compared to that in control group. Hepatic H3K27ac was downregulated in CAST/EiJ males who received low and high doses compared to control, but didn't change in all groups of females. Males of A/J strain showed no exposure response, but in females it was increased in groups exposed to low and high doses of BD. Both genders of C57BL/6J strain showed similar increase in H3K27ac with the increase of the BD dose.

My practicum project is relevant to public health since BD is a ubiquitous environmental pollutant and a widely used industrial chemical. The multistrain approach mimics interindividual variability in the population.

Epigenetic changes are very relevant to public health. While genetic mutations are fixed in cells through cellular replication, epigenetic changes can potentially be reversed. Thus, if therapies are developed to influence epigenetics, the pathogenesis of disease could be altered.

During my time at NCTR, I achieved the following competencies laid out by the School of Public Health and Department Environmental Sciences and Engineering:

- ✓ Communication and Informatics
 - Engage in collective information sharing, discussion and problem solving
- ✓ Diversity and Cultural Competency
 - Show effective and productive skills in working with diverse individuals including co-workers, partners, stakeholders, and/or clients
- ✓ Leadership
 - Exercise productive organizational, time-management and administrative skills
- ✓ Professionalism and Ethics
 - Appreciate the need for lifelong learning in the field of public health
 - Consider the effect of public health decisions on social justice and equity
- ✓ Program Planning
 - Discuss social, behavioral, environmental, and biological factors that contribute to specific individual and community health outcomes
 - Identify needed resources for public health programs or research

✓ Systems thinking

- Interpret the results addressing the strengths and limitations of the inference(s)

✓ Environmental Sciences Competencies

- Describe the direct and indirect human, ecological and safety effects of major environmental and occupational agents
- Describe genetic, physiologic and psychosocial factors that affect susceptibility to adverse health outcomes following exposure to environmental hazards
- Describe federal and state regulatory programs, guidelines and authorities that control environmental health issues
- Specify approaches for assessing, preventing, and controlling environmental hazards that pose risks to human health and safety
- Explain the general mechanisms of toxicity in eliciting a toxic response to various environmental exposures
- Develop a testable model of environmental insult

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