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The Effect of Alcohol Consumption on Adipokine Secretion

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The Effect of Alcohol Consumption on Adipokine Secretion

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ABSTRACT

The Effect of Alcohol Consumption on Adipokine Secretion

by

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Alcoholic Fatty Liver Disease (AFLD) is caused by excessive alcohol consumption and is a leading cause of liver related mortalities, with currently no treatments available. The goal of this project was to establish the effect of alcohol consumption on adipose tissue-derived secreted factors, adiponectin and C1q TNF Related Proteins 1-3 (CTRP1-3). We propose that excessive alcohol consumption will reduce circulating levels of adiponectin and CTRPs 1-3. Mice were fed a Lieber-Decarli control or alcohol diet for 10-days with a gavage (NIAAA model) or 6-weeks with no gavage (chronic model). Serum and adipose tissue were collected and CTRPs 1-3 and adiponectin levels were examined by immunoblot analysis. Our results indicate that long-term alcohol consumption effects adipokine secretion in a sex specific manner. Further research will be needed to explore the physiological relevance of these findings, to determine if these changes are beneficial to combat the negative effects of excessive alcohol consumption.

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CHAPTER 1

LITERATURE REVIEW

Alcoholic Fatty Liver Disease (AFLD) is one of the leading causes of mortality in the United States and around the world (Bataller and Gao 2011; Parry 2011; Kema et al. 2015). Fatty liver is defined as the accumulation of excess lipids in the liver. According to the NIAAA (National Institute on Alcohol Abuse and Alcoholism), a standard drink contains about 14 grams of pure alcohol. The NIAAA defines binge drinking as having a blood alcohol concentration (BAC) level of 0.08 g/dL, which typically occurs after 4 drinks for women and 5 drinks for men at a time. Heavy drinking is defined by the NIAAA as binge drinking for 5 or more days within a month. Chronic alcohol consumption disrupts lipid synthesis and can lead to hepatic steatosis, hepatitis, and cirrhosis of the liver (Nagy et al. 2016). Hepatic steatosis is primarily asymptomatic, but the accumulation of lipids can serve as the beginning of more serious forms of fatty liver disease such as: hepatitis (fatty liver with inflammation), cirrhosis (hepatitis with fibrosis), and liver failure. Not only does this disease cause damage to the liver but it also affects the body as a whole. And a specific area of interest is the adipose tissue.

Adipose tissue has been found to be more than just an organ for storing fat, as it also plays a role in whole-body metabolism and is responsible for synthesis and secretion of many hormones (Ahima and Flier 2000; Coelho et al. 2013). The balance between lipogenesis (fat synthesis) and lipolysis (breakdown of fat) determines fat accumulation. Lipogenesis occurs in the adipose tissue as well as the liver. It is stimulated by an increase in calories and inhibited by fasting. Lipolysis occurs in adipose tissue and breaks down fat into fatty acids for energy production. Once broken down fatty acids are transported from the adipose tissue through the bloodstream to the liver, muscle, and other tissues for oxidation (Coelho et al. 2013). Alcohol consumption affects adipose tissue mass, adipokine secretion, adipose tissue hydrolysis, and results in the release of excess fatty acids that are transported to the liver and deposited as triglycerides (Kema et al. 2015). Changes to the adipose tissue have an opportunity to affect the levels of adipokines, cell-signaling proteins secreted from the adipose tissue. Adipokines have an effect on insulin sensitivity, glucose and fatty acid metabolism, and the inflammatory process (Peterson et al. 2013). There are many different adipokines secreted from adipose tissue but this project aims to look at a select few because of their roles in lipid metabolism and fatty acid oxidation. C1q/TNF-related proteins (CTRPs) are highly conserved paralogs of adiponectin consisting of a signal peptide, a short variable region, a collagen domain, and a globular C1q (Complement Component 1q) domain (Wong et al. 2008). Because the adiponectin globular domain closely resembles TNFα, proteins with the C1q domain are classified as the C1q/TNF protein family (Wong et al. 2008).

Table 1.1 Adipokines and Cytokines

Molecule	Function/effect	Molecular Weight Observed
CTRP1	Metabolic and cardiovascular functions, lowers blood glucose levels and protects from diet-induced obesity and insulin resistance. Promotes glucose uptake and fatty acid oxidation in skeletal muscle.	35 kDa
CTRP2	Promotes lipid uptake from the blood	38kDa
CTRP3	Stimulates liver lipid metabolism and attenuates diet-induced fatty liver disease.	25kDa
Adiponectin	Increases fatty acid oxidation in the liver and stimulates glucose uptake in skeletal muscle.	34kDa

Table 1.1 (continued)	
Leptin	Signals to the brain about body fat stores. Regulation of appetite and energy expenditure. Stimulates fatty acid oxidation in the liver and skeletal muscle. Stimulates glucose uptake in skeletal muscle. Prevents lipotoxicity Pro-fibrogenic
PAI-1	Inhibitor of the fibrinolytic system by inhibition of activation of plasminogen
IL-6	Pro-inflammatory, lipid and glucose metabolism, regulation of body weight Induces insulin and leptin resistance
TNF-α	Pro-inflammatory cytokine that induces PAI-1, increases fatty acid release from adipocytes, and increases lipogenesis in hepatocytes.

Adipokines

CTRP1

C1q/TNF-related protein 1 (CTRP1) is expressed at its highest levels in adipose tissue (Rodriguez et al. 2016). CTRP1 has been found to lower blood glucose and activate AMPK (AMP-activated protein kinase) to control fatty acid metabolism in skeletal muscle (Peterson et al. 2012; Rodriguez et al. 2016). And chronic over expression of CTRP1 has been found to enhance skeletal muscle fat oxidation and reduce insulin resistance caused by a high-fat diet (Rodriguez et al. 2016). CTRP1 has also reduces the formation of plaque and increases aldosterone production (Shabani et al. 2016). In a CTRP1 KO model, the loss of CTRP1 in mice fed a high fat diet resulted in decreased expression of multiple genes associated with lipid metabolism in the adipose tissue (Rodrgiuez et al. 2016).

CTRP2

C1q/TNF-related protein 2 (CTRP2) is a mouse paralog closely related to adiponectin, which promotes glycogen accumulation and activates the AMPK signaling pathway to increase fatty acid oxidation. Because CTRP2 induces glycogen accumulation it may also lower blood glucose (Wong et al. 2008). And because of the role of adiponectin in enhancing insulin resistance, CTRP2 could also play a role in improving insulin resistance.

CTRP3

CTRP3 (C1q/TNF-related protein 3) increases liver lipid metabolism and inhibits inflammation. Previous work has shown that overexpressing CTRP3 as well as daily injections of CTRP3 reduces high-fat diet induced fatty liver (Peterson et al. 2013). Over expressing CTRP3 in a high fat model showed a decrease in the synthesis of triglycerides and a decrease in circulating levels of TNF-α (Peterson et al. 2013). CTRP3 decreases blood glucose by suppressing gluconeogenic expression in the liver (Peterson et al. 2010). It has been found that human patients with non-alcoholic fatty liver disease (NAFLD) exhibited reduced levels of circulating CTRP3 (Zhang et al. 2017). Therefore, restoring CTRP3 levels has been shown as a possible treatment for NAFLD (Peterson et al. 2013). But it is still a question if it could be used to alleviate AFLD, because AFLD is caused by ethanol-induced lipogenesis and decreased lipid oxidation and NAFLD is primarily caused by an excessive accumulation of lipids in the liver (Breitkopf 2009; Coelho et al. 2013; Fujii 2014; Parker 2018). Identifying the affect of alcohol on levels of CTRP3 will provide further insight to this question.

Adiponectin

Adiponectin was one of the first adipokines to be discovered and is well studied. Adiponectin is an adipokine involved in regulating glucose levels as well as the breakdown of fatty acids (Karbowska and Kochan 2006). Adiponectin is exclusively secreted by adipose tissue and abundantly present in the blood stream. Adiponectin stimulates insulin secretion, fatty acid oxidation in the liver, glucose uptake in skeletal muscle, and suppresses TNF-α and IL-6 expression, all factors that are disrupted with ETOH feeding. Chronic ETOH consumption causes a significant decrease in circulating levels of adiponectin, and correlated with the development of liver injury (Xu et al. 2003; Song et al. 2008; Tan et al. 2012). Adiponectin is believed to also play a protective role against alcoholic liver disease in mice as levels increase significantly with consumption of a high fat diet with ethanol (You et al. 2005). Adiponectin is an anti-inflammatory adipokine known to promote appropriate lipid storage, preventing ectopic fat storage in places such as the liver (Lang and Steiner 2017). Circulating levels of adiponectin are shown to be affected by alcohol consumption, although, there is some variability as adiponectin levels have been shown to be unaffected (Tan et al. 2012), suppressed (Chen et al. 2007; Yu et al. 2010), or increased (Sierksma et al. 2004; Pravdova et al. 2009; Mandrekar and Fulham 2016) with the consumption of alcohol. It is suspected that oxidative stress induced by acute alcohol exposure reduces the secretion of adiponectin (Tang et al. 2003), indicating that time since last dose of ethanol can affect results.

Leptin

Leptin is one of the most studied adipokine (Hiney et al. 1999; Roth et al. 2003; Strbák et al. 2003; Lang and Steiner 2017). It plays a role in food intake, energy expenditure, lipolysis, fatty acid oxidation, and lipogenesis (Lang and Steiner 2017).

Leptin also prevents lipotoxicity, which causes cell damage due to the accumulation of lipids in areas other than adipose tissue, such as the liver (Bertolani and Morra 2008). Leptin has been shown to be pro-fibrogenic, as an absence of leptin resulted in reduced liver fibrosis (Leclercq et al. 2002). The effects of alcohol on leptin levels vary among studies; it has been shown to increase (Kiefer et al. 2002; Obradovic 2002; He et al. 2015), decrease (Hiney et al. 1999), and be unchanged (Strbák et al. 2003) using a range of chronic alcoholic models (Lang and Steiner 2017). Circulating levels of leptin have been shown to be affected by alcohol consumption, although, similarly to adiponectin, there is variability. Some models have shown leptin levels to increase (He et al. 2003; Roth et al. 2003; Sierksma at al. 2004; Pravdova et al. 2009) with alcohol consumption while others have shown leptin levels to decrease (Hiney et al. 1999; Tan et al. 2012) with alcohol consumption.

Cytokines

PAI-1

Plasminogen Activator Inhibitor-1 (PAI-1) inhibits plasminogen activation that breaks down fibrin and is regulated by levels of TNF-α (Hou et al. 2004). As alcoholic fatty liver disease progresses, there is an accumulation of extracellular matrices that leads to fibrosis. Plasma activator inhibitor-1 (PAI-1) regulates fibrinolysis. (Arteel 2008).

IL-6

Interleukin 6 (IL-6) acts as a pro inflammatory cytokine. Studies have shown IL-6 to play an important part in protection of the liver through liver repair and preventing apoptosis (Hong et al. 2002).

TNF-α

TNF-α is a pro-inflammatory cytokine and increased levels have been documented in animal models of AFLD (McClain et al. 1998). TNF-α contributes to hepatic steatosis by inducing the expression PAI-1. It increases fatty acid release from adipocytes increasing lipogenesis in hepatocytes, and inhibits the β-oxidation of fatty acids (Arteel 2008).

Because of adipokines' positive affects on lipid oxidation, alcohol consumption will result in decreased circulating levels of adiponectin, CTRP1, CTRP2, and CTRP3.

CHAPTER 2

THE EFFECT OF ALCOHOL CONSUMPTION ON THE CIRCULATING LEVELS OF THE NOVEL ADIPOKINES: CTRP1, CTRP2, AND CTRP3

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ABSTRACT:

The goal of this project was to establish the effect of alcohol consumption on a group of novel adipose tissue-derived secreted factors: C1q TNF Related Proteins 1-3 (CTRP1, CTRP2, and CTRP3). Adipose tissue secretes several circulating proteins, called adipokines, which exert a multitude of biological effects important for human health. However, adipose tissue is extremely sensitive to alcohol consumption, leading not only to disrupted fat storage, but also to disruptions in adipokine production. Changes to adipokines could have widespread biological effects and potentially contribute to alcohol-induced ailments. To test the effects of alcohol consumption on adipokines, male and female mice were randomized to a Lieber-DeCarli control diet or Lieber-DeCarli 5% (v/v) ethanol diet for either: 1) 10-days followed by a single gavage of 5 g/kg ethanol on the 11th day (the NIAAA model); or 2) 6-weeks with no binge added (chronic model). In response to the NIAAA model, female mice fed ethanol had an ~200% increase in circulating levels of adiponectin, an ~25% increase in CTRP1, a 25% decrease in CTRP2 and 75% reduction in CTRP3. Whereas, in the male mice ethanol decreased circulating CTRP2 by ~25%, with no changes observed in adiponectin, CTRP1, or CTRP3. The effects of ethanol on CTRP2 levels disappeared after 6-weeks of ethanol feeding (chronic model), as CTRP2 levels were not different between control and ethanol fed mice of either sex. Whereas, in the chronic model, ethanol more than doubled circulating adiponectin levels in both male and female mice. Surprisingly, chronic ethanol feeding resulted in a dimorphic effect on circulating CTRP1. Briefly, CTRP1 levels were increased by ~125% in ethanol fed female mice but were 50% lower in ethanol fed male mice. Lastly, circulating CTRP3 levels were decreased in female mice, with no change in male mice. Combined, this is the first study to document the effects of alcohol on the circulating levels of CTRP1, CTRP2, and CTRP3. Understanding the impact of excessive alcohol consumption on adipokine production and secretion could identify novel alcohol-induced mechanisms of human disease and identify novel potential pharmaceutical targets for treatment development. Lastly, these results confirm earlier findings that alcohol consumption has sex-specific effects.

INTRODUCTION

The detrimental effects of chronic alcohol abuse have been well-documented with established long-term health conditions such as cardiovascular disease [1], respiratory distress [2], gastrointestinal dysfunction, alcoholic liver disease [3-6], cancer [7], and metabolic dysfunction [8] . Excessive alcohol consumption not only causes initial injury via direct toxic effects (i.e. oxidative stress) to the individual tissues, but also results in secondary indirect injury through elevations in inflammatory cytokines and ectopic fat deposition [9, 10]. Although little can be done to prevent the acute toxic effects of alcohol consumption, understanding and reducing secondary alcohol-induced injury is a key component of treating the long-term health conditions associated with chronic alcohol consumption.

Adipose tissue is not only the primary location for the storage of excess lipids, but is also a major contributor to the production of circulating inflammatory cytokines [11]. Furthermore, chronic alcohol consumption results in high levels of adipose-tissue oxidative stress, leading to elevations in inflammation and hyperlipolysis [11]. Therefore, alcohol-induced disruptions to adipose tissue function contribute to the wide-spread development of secondary alcohol-related health conditions. Females have a higher amount of adipose tissue (higher percent body fat), and this may account for the increased susceptibility of females to the chronic effects of alcohol [5, 7, 12-14].

In addition to the direct effects of alcohol on adipose tissue, alcohol consumption can also lead to disruptions in adipokine secretion. Adipokines are bioactive proteins secreted by adipose tissue which can have significant endocrine effects on regulating human health and disease. Circulating levels of the two most widely studied adipokines, leptin and adiponectin, are significantly affected by alcohol consumption [10, 15-29]. Leptin is primarily a satiety signal; therefore, its role in ethanol-related health effects is unclear. However, adiponectin stimulates fatty acid oxidation and inhibits both the activity and production of inflammatory cytokines [10, 30, 31], thus supporting our hypothesis that alcohol-induced disruptions to adipokine production can contribute to the development alcoholic related disease.

In 2004 Wong et al identified a novel family of adipokines, referred to as Complement C1q Tumor Necrosis Factor-Related Proteins (CTRPs) [32, 33]. Reflecting profound biological potency, the initial characterization of these adipose tissue-derived CTRP factors demonstrate wide-ranging effects upon metabolism, inflammation, and survival-signaling [32-52]. As alcohol alters the expression of both leptin and adiponectin, we hypothesized that chronic alcohol abuse would also affect other adipokines. To test this hypothesis, we chose to examine three novel adipokines that have not been examined in relation to alcohol consumption: CTRP1, CTRP2, and CTRP3. All three of these proteins have been demonstrated to improve lipid handling in unique ways in response to high fat diet. Briefly, elevated circulating CTRP1 levels attenuate body weight gain in response to a high fat feeding through increased lipid oxidation in skeletal muscle [36], CTRP2 improves serum lipid clearance in obese mice [37, 46], and CTRP3 attenuates high fat diet-induced hepatic steatosis [38, 53]. In summary, all three of these adipokines have documented, unique methods to improving lipid handling in ways that could diminish the secondary damage caused by chronic alcohol abuse. Therefore, the purpose of this project was to establish the effects of alcohol on the circulating levels of these proteins. The results of this study could produce new understanding to the mechanism by which chronic alcohol consumption leads to ectopic fat deposition and other health issues and identify novel potential pharmaceutical targets for treatment of alcohol-induced disease.

METHODS

Animal model

Forty female mice (C57BL/6) and thirty-seven male mice (C57BL/6) were used for this study. Mice were housed in polycarbonate cages on a 12-h light-dark photocycle with *ad libitum* access to water and food, except as specified. At the time points indicated, animals were anesthetized with isoflurane and euthanized via cardiac puncture. Serum samples were prepared according to manufacture's instructions (Sarstedt, Cat#41.1500.005). The gonadal fat pads were excised, snap frozen in liquid nitrogen, and stored at −80 °C until further analysis. All animal procedures were conducted in accordance with institutional guidelines, and ethical approval was obtained from the University Committee on Animal Care (protocol #P151201; East Tennessee State University, Animal Welfare Assurance number is A3203-01). Animals were checked/weighed daily and euthanized (counted as dead), via CO_2 inhalation, based on the presence of any of the following criteria for humane endpoints: unconsciousness, intractable seizures, labored breathing or respiratory distress, inability to ambulate or maintain upright position, diarrhea or constipation, or the inability to eat or drink.

Ethanol Feeding

Two independent ethanol feeding models were employed: The first model was the 11-day chronic plus binge model, also known as the NIAAA model [54]. This model reportedly mimics hepatic steatosis and liver injury, which occurs in many alcoholic hepatitis patients (26). Briefly, 12-week old mice were acclimatized to a control liquid diet (Bio-serv; cat# F1259SP) for 4 days followed by 10 days on the Lieber-DeCarli ethanol diet (5% v/v ethanol; Bio-serv; cat# F1258SP) *ad libitum.* On the morning of the 11th day (1 hour into light cycle) food was removed and replaced with water and the mice were given a single gavage of ethanol (5 g kg^{-1}). After gavage, cages were placed on heating pads to prevent hypothermia, as described [54]. Nine hours post gavage, mice were anesthetized with isoflurane, until the absence of reflex was observed, and the euthanized by exsanguination, and tissue/serum samples were collected and processed for analysis.
In the second model (chronic model), 8-week old male and 12-week old female mice were acclimatized to a liquid diet *ad libitum*, without the addition of alcohol for 1-

week and then gradually transitioned from 1-5% Lieber-DeCarli ethanol diet (v/v ethanol) over the course of the next 2 weeks, then maintained on 5% ethanol (v/v ethanol) for the remaining 4 weeks. This feeding protocol is believed to reflect chronic ethanol abuse, beginning with low volumes and increasing over time [55]. On the morning of the final day, food was removed, and mice were fasted 9 hours, anesthetized with isoflurane until the absence of reflex was observed, and the euthanized by exsanguination, and tissue/serum samples were collected and processed for analysis. The 9-hour time point was selected to be consistent with the NIAAA model protocol [54].

Control Fed mice were placed on an ethanol free isocaloric control diet (Bio-serv; cat# F1259SP) supplemented with maltose dextrin (to match the calories of ethanol), for use as experimental controls. Food intake in ethanol fed mice was measured daily and mice on the control diet had their food intake limited to match the daily intake for the previous day of the corresponding ETOH-fed mice. Overview of animals in each experimental group are listed in table 1.

Table 2.1 Number of animals in each experimental group

	Male		Female	
Model	Control	ETOH	Control	ETOH
NIAAA model	10	8	6	6
Chronic Model	6	13	8	20

ETOH, ethanol fed; Control, fed matched control diet without ethanol

Immunoblot Analysis

Serum samples were diluted, and adipose tissues (gonadal fat pad) were homogenized in assay buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.1% Triton x-100, 0.5% sodium deoxycholate, 0.1% SDS), plus the addition of protease and phosphatase inhibitors (Bimake Cat#B14001 & B15001). Protein concentrations were measured by commercial assay (Pierce™; Cat#PI23236). Afterward, equal proportions of each sample were denatured at 95°C in SDS loading buffer (final concentration: 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.004% bromophenal blue, 0.125 M Tris HCl, pH 6.8). 30 μg per sample (adipose) or 1 μl serum was separated by gel electrophoresis (BioRad; cat#456-1046) and transferred to a nitrocellulose membrane (BioRad; Cat#162-0115), according the manufacturer's instructions. To confirm appropriate protein migration a protein standard was loaded with each blot (BioRad cat#1610374 or Thermo Scientific cat#26616). Membranes were blocked in 2% non-fat milk and probed with primary antibodies: CTRP1 (GW Wong Lab; Johns Hopkins University, Cat# anti-gCTRP1, RRID:AB_2716247), CTRP2 (Abnova Corporation Cat# H00114898-M01, RRID:AB_426121), CTRP3 (R and D Systems Cat# AF2436, RRID:AB_2067713), and Adiponectin (R and D Systems Cat# MAB1119, RRID:AB_2305045). After incubation with primary antibodies membranes were washed and probed with appropriate HRP-labeled secondary antibodies: Goat anti-rabbit (Thermo Fisher Scientific Cat#

31460 RRID:AB_228341), rabbit anti-goat (Thermo Fisher Scientific Cat# 31402, RRID:AB_228395), rabbit anti-rat (Thermo Fisher Scientific Cat# PA1-28786, RRID:AB_10983740), or goat anti-mouse (Cell Signaling Technology Cat# 7076, RRID:AB_330924). Chemiluminescence signals were visualized with Millipore (Cat# 17010A2). Quantification of signal intensity was performed using Alphaview Software (Alpha Innotech).

RNA Isolation

RNA was isolated according to commercial assay following manufacturer's instructions (Direct-zol Cat# R2070). Isolated RNA was eluted in 50 µl RNase-free water; purity (RIN ≥ 7.0) and concentrations were confirmed by microfluidic capillary electrophoresis (Agilent RNA 6000 Nano kit, #5067-1511, Agilent Technologies). 1 µg RNA was reverse transcribed according to manufacturer's instructions (Promega, Cat#A5001).

Quantitative real-time PCR

A 10-fold dilution series of DNA amplicons generated from a prepared sample was employed as a standard curve for each gene of interest, and the qPCR efficiency was determined for each gene (Bio-Rad Cfx thermocycler). All qRT-PCR primers displayed a coefficient of correlation greater than 0.95 and efficiencies between 90% and 110%. Primer sequences are listed in table 2. Briefly, 25 ng of cDNA was incubated in SYBR Green qPCR Master mix (Bimake.com, Cat# B21203) for an initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles each consisting of 95 °C for 15 s, and 60 °C for 1 min. After the last cycle specificity of amplification products were confirmed by analyzing melting curve profiles for primers and products. Data is reported as copy number normalized to the geometric mean of the reference genes Beta-actin (Actb) and Hypoxanthine-guanine phosphoribosyltransferase (Hprt1).

Table 2.2 PCR primer sequences

Gene Name	Forward	Reverse
Actb	CCTCCCTGGAGAAGAGCTATG	TTACGGATGTCAACGTCACAC
Hprt1	CAAACTTTGCTTTCCCTGGT	TCTGGCCTGTATCCAACACTTC
Adipoq	CCTGGCCACTTTCTCCTCATT	ATCCTGAGCCCTTTTGGTGT
CTRP1	TCCGAGCTCTGTTGACATGC	AAAGATTGACCAGCCCCTGG
CTRP2	TCCTGGGTACTCTTGGCCTG	AAGCATTGGGTCAGCAGCA
CTRP3	CATCTGGTGGCACCTGCTG	TGACACAGGCAAAATGGGAG

Abbreviations: Actb, Beta-actin; Hprt1, Hypoxanthine-guanine phosphoribosyltransferase; Adipoq, Adiponectin; CTRP, C1q and tumor necrosis factor related protein

Multiplex Serum Analysis

Circulating concentrations of Interleukin-6 (IL-6), Tumor necrosis factor alpha (TNF), Plasminogen activator inhibitor-1 (PAI-1) and leptin were determined using commercially available assays (Bio-Plex® Multiplex Immunoassay System, Bio-rad Cat# 171F7001M, 171G5023M, 171I50001, 171G5007M).

Statistical Analysis

Descriptive statistics (mean and standard deviations) were calculated for all measured variables. As the feeding models were not performed concurrently, each model was analyzed independently. Body weight and food intake data were analyzed by two-way repeated measures ANOVA followed by Tukey's multiple comparisons test. Survival curve was determined by log-rank (Mantel-Cox) test. An unpaired t test was used to compare immunoblot and gene expression data between control and ethanol fed groups. All statistical analysis was performed by Graphpad Prism 6.

RESULTS

Animal Characteristics

As expected, with pair feeding no differences in total food intake occurred between control and ethanol fed groups. Although during weeks 2 and 3 the body weights of the ethanol fed female mice were significantly higher than control fed, this difference normalized by week 4 and there was no further difference in body weights between the control and ethanol fed groups of either sex (Fig 1A-B). Further, no differences were observed in ethanol consumption between male and female mice when normalized to body mass (Fig 1C-D). All animals on the NIAAA feeding protocol survived through the end of the experiment. Unexpectedly, with 6-weeks of ethanol feeding the female mice had an approximate 50% mortality rate compared with a non-significant difference in mortality rate in identically treated male mice (Fig 1E-F).

Figure 2.1

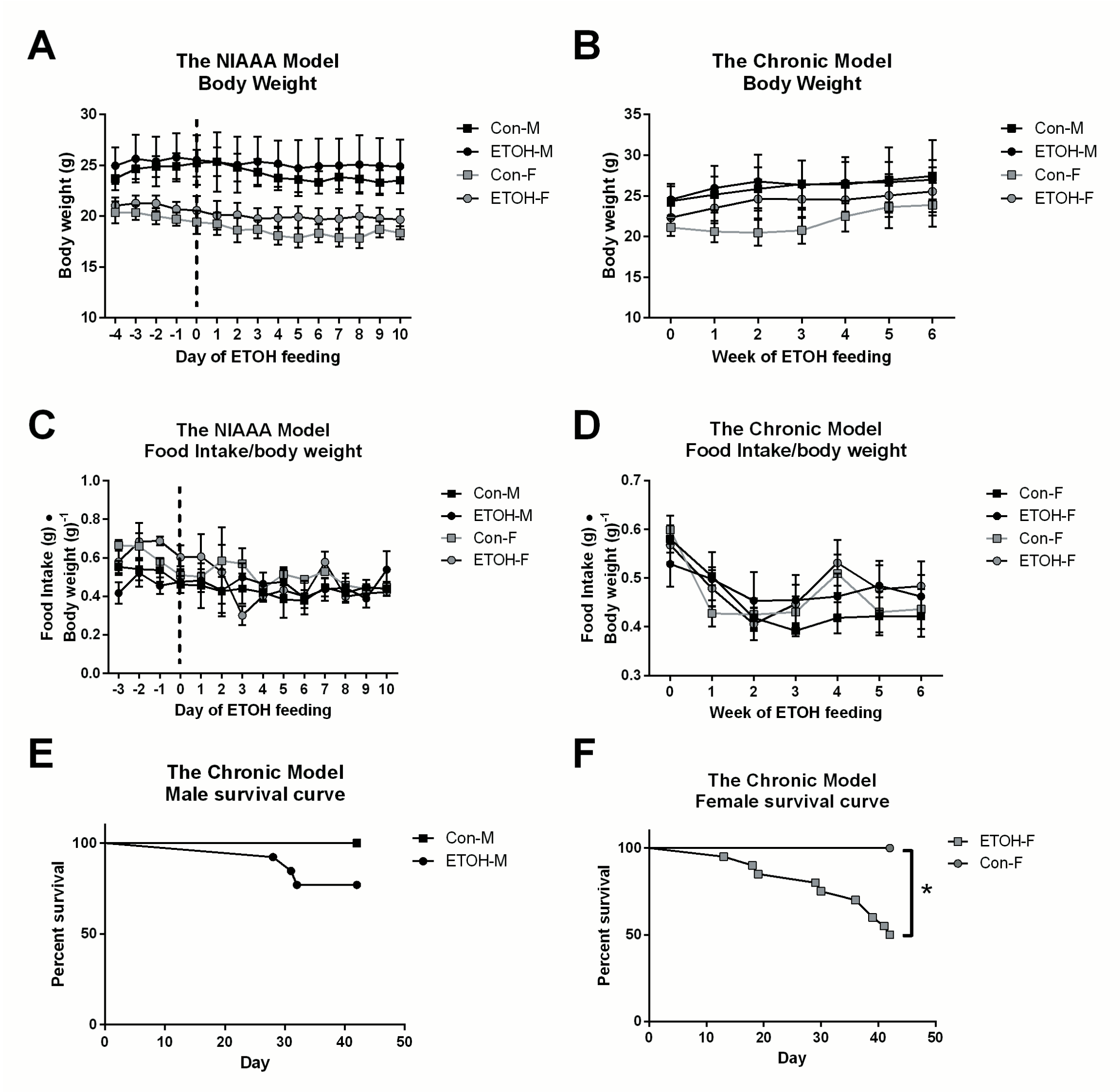


Figure 2.1 Animal Characteristics.

Separate cohorts of male and female mice were exposed to two separate ethanol feeding protocols: The NIAAA model (10 days, plus a binge on day 11) or the chronic model (6 weeks). No differences in body weights (A & C) or food intake normalized to body weight (B & D) were observed. There was no difference in the survival curve in ethanol compared with control fed male mice (E), however there was a significant difference in the female survival curve (F). Data reported as mean ± SD (A-D), data reported as absolute numbers (E & F), *=$p < 0.05$ ETOH vs Con. Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male; NIAAA, National Institute on Alcohol Abuse and Alcoholism.

Multiplex serum analysis

Data from multiplex analysis is reported in Table 2. Briefly, in both models ethanol increased circulating IL-6 levels in female mice, and 6 weeks of ethanol feeding increased TNF levels in female mice. On the other hand, PAI-1 levels were increased with ethanol feeding regardless of sex or feeding model. Lastly, leptin levels were significantly elevated with ethanol feeding in both sexes with the NIAAA model, with no differences noted after 6 weeks of feeding (Chronic model).

Table 2.3 Serum Multiplex analysis

	The NIAAA Model			
	Con-M	ETOH-M	Con-F	ETOH-F
IL-6 (pg/mL)	186 ± 61	292 ± 315	82 ± 112	3627 ± 1875*
TNF (pg/mL)	215 ± 57	443 ± 274	529 ± 410	602 ± 318
PAI-1 (ng/mL)	4.0 ± 1.2	10.9 ± 4.5*	3.8 ± 1.3	12.4 ± 7.2*
Leptin (ng/mL)	6.0 ± 3.4	16.3 ± 9.5*	2.4 ± 1.8	41.6 ± 26.7*
	Chronic model			
	Con-M	ETOH-M	Con-F	ETOH-F
IL-6 (pg/ml)	57 ± 54	45 ± 23	36 ± 10	81 ± 55*
TNF (pg/ml)	427 ± 117	514 ± 532	338 ± 79	590 ± 263*
PAI-1 (ng/ml)	2.87 ± 0.32	3.9 ± 0.90*	3.66 ± 0.47	6.96 ± 3.2*
Leptin (ng/mL)	12.8 ± 11.6	3.4 ± 2.2	10.4 ± 4.7	7.7 ± 5.0

All data is reported as mean ± standard deviations. *=$p< 0.05$ Con compared with ETOH. Abbreviations: ETOH, ethanol fed; Con, control fed, M, Male; F, Female; IL-6, Interleukin-6; TNF, Tumor necrosis factor alpha; PAI-1, Plasminogen activator inhibitor-1.

Circulating Adipokines

In response to the NIAAA model, female mice fed ethanol had an increase in circulating levels of both adiponectin and CTRP1 and a decrease in both CTRP2 and CTRP3. Whereas, in the male mice ethanol decreased circulating CTRP2 with no changes observed in adiponectin, CTRP1, or CTRP3 (Fig 2).
In the chronic model, circulating CTRP2 levels were not different between control and ethanol fed mice regardless of sex. Whereas, 6 weeks of ethanol feeding more than doubled circulating adiponectin levels in both male and female mice. Unexpectedly, chronic ethanol feeding had a dimorphic effect on CTRP1 levels: CTRP1 levels were increased in female mice but lowered by ~50% in male mice. Lastly, circulating CTRP3 levels were decreased in female mice, with no change in male mice.

Figure 2.2

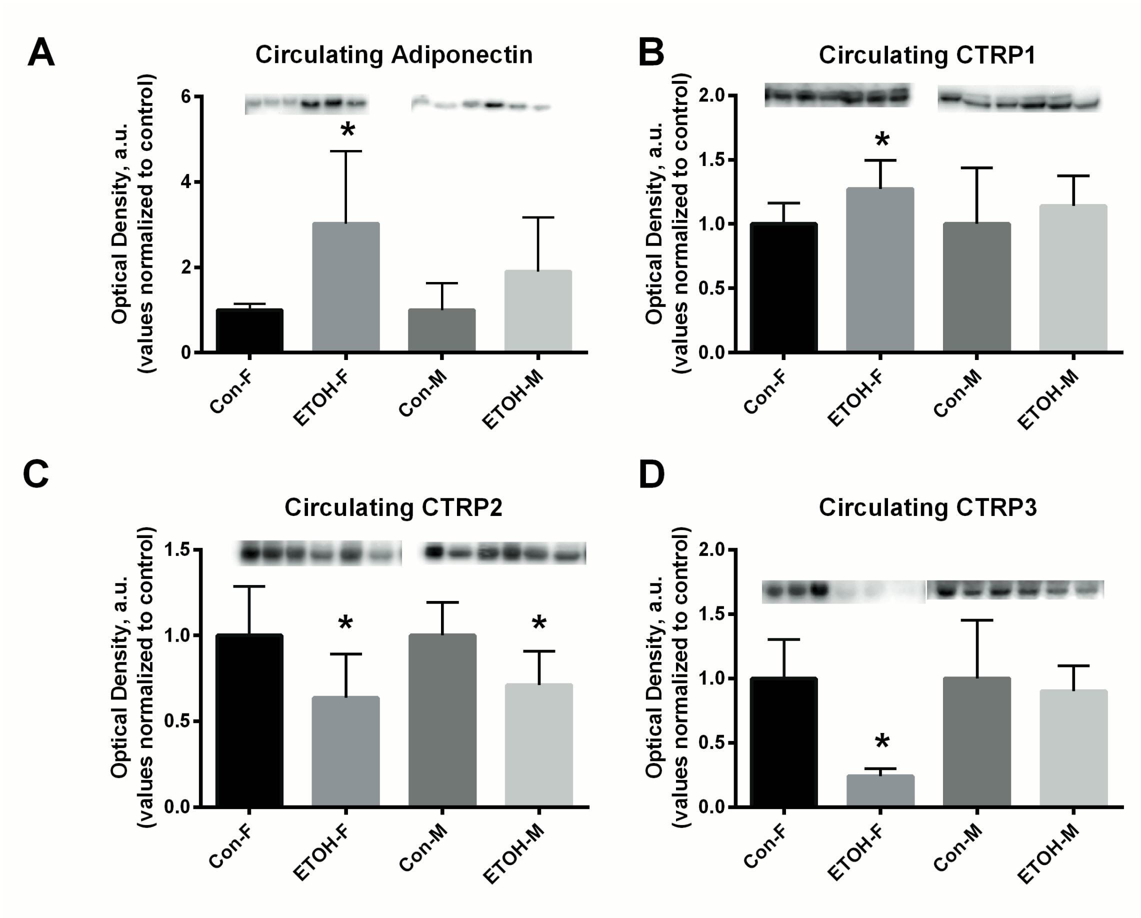
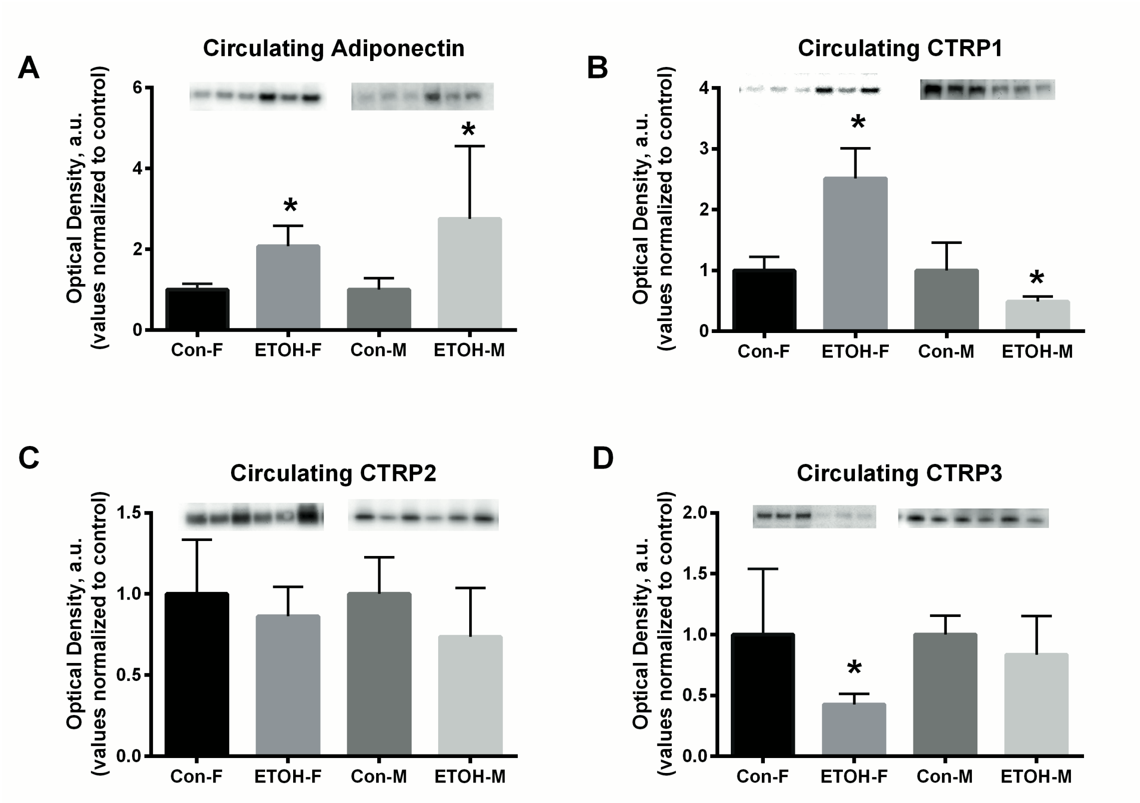


Figure 2.2 Circulating Adipokines in the NIAAA model of ethanol feeding.

Circulating adiponectin, CTRP1, CTRP2, and CTRP3 levels were determined by immunoblot in serum collected from mice on the 10-day plus binge model of ethanol feeding (the NIAAA model). Data reported as mean ± SD. Male and female blots were performed and analyzed independently, and values were normalized to control fed within each sex. n=6, and *=p < 0.05 ETOH vs Con. Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male; NIAAA, National Institute on Alcohol Abuse and Alcoholism. Raw data files for data presented in Figure 2 are attached as supplemental data (S1).

Figure 2.3

Figure 2.3 Circulating Adipokines in the Chronic model of ethanol feeding.



Serum was collected from the mice after 6 weeks of ethanol feeding and circulating levels of adiponectin, CTRP1, CTRP2, and CTRP3 were determined by immunoblot analysis. Data reported as mean ± SD. Male and female blots were performed and analyzed independently, and values were normalized to control fed within each sex, n=6, and *=$p < 0.05$ ETOH vs Con. Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male. Raw data files for data presented in Figure 3 are attached as supplemental data (S2).

Adipokine Gene Expression and Tissue Content

Gonadal adipose adipokine gene expression and protein content levels were analyzed to determine if differences in serum could be contributed to changes in tissue expression. However, no differences were observed in adipokine gene expression (Fig 4), indicating that changes to gene expression, at least in the gonadal fat pads, were not responsible for the changes in circulating adipokine levels. The adipose tissue protein content (Fig 5) was also analyzed and overall there was no difference in protein levels in the adipose tissue in response to either alcohol-feeding model. These data indicate that the alcohol feeding models used in this study did not alter tissue level expression of these adipokines, at least in the gonadal fat pads.

Figure 2.4

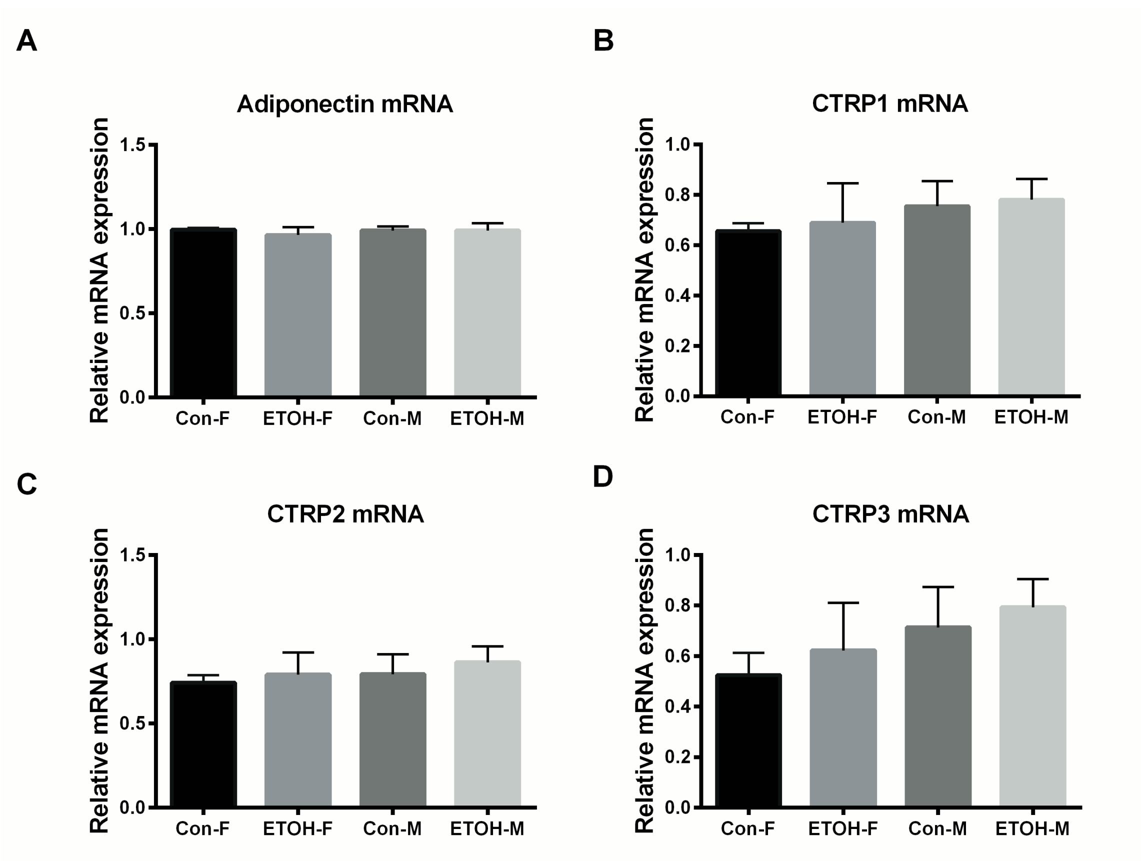


Figure 2.4 Tissue Adipokine Gene Expression.

After 6 weeks gonadal adipose tissue samples were collected from the mice. There were no significant differences found in the gene expression of adiponectin, CTRP1, CTRP2, or CTRP3 (A-D). Data reported as mean ± SD and normalized to geometric mean Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male.

Figure 2.5

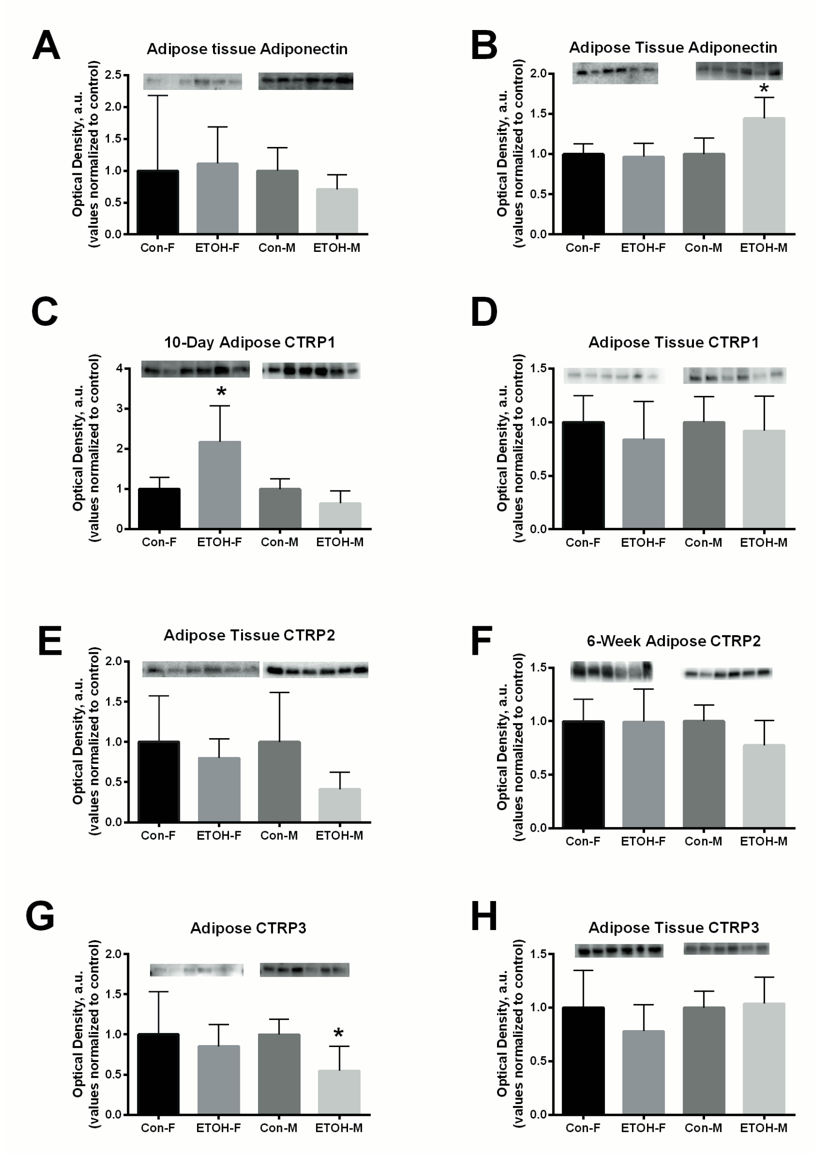


Figure 2.5 Tissue Adipokine Protein Content.

Gonadal adipose tissue samples were collected from the mice after ethanol feeding for 6 weeks or 10 days (NIAAA) and adiponectin, CTRP1, CTRP2, and CTRP3 and levels were examined by immunoblot analysis. Data reported as mean ± SD Male and female blots were performed and analyzed independently, and values were normalized to control fed within each sex, n=6, and *=$p < 0.05$ ETOH vs Con. Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male.

DISCUSSION

The major finding of this study is that excessive ethanol consumption effects adipokine levels in a sex specific manner. The expanding family of adipokines, with their multiple biologically relevant functions, provide abundant research targets for the development of novel therapies for the treatment/prevention of human disease. The purpose of this project was to specifically examine the effects of ethanol abuse on three novel adipokines (CTRP1, CTRP2, and CTRP3). These adipokines have documented lipid regulating functions and their disruption could contribute to the development of ectopic fat deposition. Ectopic fat deposition is a major contributor to the secondary injury caused to tissues due to excessive alcohol consumption [9, 10].

The secondary finding of this study is that female mice are more sensitive to ethanol feeding than male mice. This observation has been repeatedly noted in the literature on a variety of experimental outcomes [5, 13, 56]. Specifically, in this study male and female mice consumed similar amounts of ethanol, normalized to body weight. However, female mice had significant inflammation (elevations in TNF and IL-6) and a significant mortality rate in ethanol-fed compared with control-fed mice. Conversely, there was no difference in TNF, IL-6, or mortality between control and ethanol fed male mice. It is important to note that ethanol consumption led to increased levels of PAI-1, indicating in both sexes alcohol consumption leads to damage tissues and disruptions to overall tissue homeostasis [57].

Leptin and Adiponectin

Leptin and adiponectin were the first adipokines discovered and are currently the most well studied. Circulating levels of both leptin and adiponectin have been documented to be affected by alcohol consumption, although to what extent alcohol alters these proteins is unclear. Briefly, leptin levels have been shown to increase with alcohol feeding in some models [10, 15-24], with no change in other feeding models [25], while decreasing in other alcohol feeding models [16, 23, 26]. Specifically, acute alcohol consumption decreases leptin levels in both human and animal models [21-23], however, acute reductions in leptin levels are gone approximately 9-hours post exposure [23]. On the other hand, leptin levels were significantly increased with withdrawl in alcohol patients [58, 59]. Combined, these data indicate that the time from last alcohol exposure could influence serum leptin results. Further variability in serum leptin levels with alcohol consumption could be associated to the feeding model. For example, ethanol increased leptin compared with pair-fed rats, but showed no difference compared with control ad libitum rats [25]. Our findings demonstrate that leptin levels can vary depending on the exposure model as leptin levels were increased in the NIAAA model (which includes a final ethanol bolus), but showed no difference in leptin levels after 6 weeks of chronic ethanol feeding.

Similarly, adiponectin levels have been shown to be suppressed [18, 24], not different [26], or elevated [12, 18, 19, 26-28] in response to alcohol consumption. It is suspected that oxidative stress induce by acute alcohol exposure reduces the secretion of adiponectin [29], indicating that time since last dose of ethanol can affect results. The cause of elevated levels of serum adiponectin seen in many alcohol feeding protocols has not been established. However, chronic alcohol abuse has demonstrated associations between circulating adiponectin levels and the severity of liver damage and elevated adiponectin levels in cases of cirrhosis [10, 60-62]. Our data supports the finding that circulating adiponectin levels increase with both NIAAA and chronic alcohol consumption. As circulating adiponectin levels increase with ethanol exposure, activating adiponectin and adiponectin mediated signaling pathways may not be a successful strategy for the prevention/treatment of ALD, as its levels are already increased with chronic ethanol exposure.

CTRP1

We hypothesized that the alcohol-induced loss of CTRP1 may exacerbate the effects of alcohol abuse due to decreased skeletal muscle lipid oxidation [36], resulting in excessive circulating lipid levels, thus promoting ectopic lipid accumulation. Both the NIAAA and chronic models showed CTRP1 levels were elevated in the female ethanol fed mice, compared with control fed mice, indicating that alcohol consumption does not inhibit CTRP1 levels in females. On the other hand, in the male mice, chronic ethanol feeding resulted in a significant reduction in CTRP1 levels. At this time, the mechanism responsible for the dimorphic effect of alcohol is unknown. This effect may be secondary to the observed increase in pro-inflammatory cytokines (TNF and IL-6). Regardless, alcohol-induced reduction in circulating CTRP1 levels requires further analysis to determine if similar results are shown in human alcoholics and whether restoration of CTRP1 would generate any protective effect in male mice.

CTRP2

As predicted CTRP2 levels decreased with ETOH feeding in both male and female mice during the 10-day plus binge (the NIAAA model) ethanol feeding protocol. However, there was no long-term alcohol-induced difference in CTRP2 in either sex. Therefore, the data does not support that alcohol-induced changes to circulating CTRP2 levels contribute to the adverse effects of chronic alcohol abuse.

CTRP3

Ethanol feeding reduced circulating CTRP3 levels in female, but not male, mice in response to both the NIAAA model and chronic feeding model. Alcoholic cirrhosis occurs at a higher rate in female alcoholic patients, at an earlier age, with a lower proportional amount of alcohol consumption [5, 12, 13]. As CTRP3 levels are selectively reduced in ethanol fed female mice, this provides a novel mechanism to explore the increase susceptibility of females to alcoholic cirrhosis. In support of this hypothesis, our previous work has shown that CTRP3 acts directly on liver tissue to stimulate lipid oxidation and attenuate diet-induced fatty liver disease [38]. In fact, in human subjects CTRP3 levels are reduced with diet-induced hepatic steatosis, or non-alcoholic fatty liver disease [63]. Further, we have also previously shown that CTRP3 suppresses lipid-induced elevations in pro-inflammatory cytokines, such as TNF. However, the effects of CTRP3 on reducing alcoholic fatty liver disease are yet to be explored. Chronic alcohol consumption disrupts lipid synthesis which leads the buildup of hepatic lipids, resulting alcoholic fatty liver and eventually alcoholic cirrhosis [5, 9, 13], the leading causes of liver failure and a leading cause of death in the Unites States [5, 13, 14, 56]. Thus, there has been renewed interest in developing effective therapeutic strategies to prevent alcoholic fatty liver disease [4, 6, 9, 13, 64, 65]. Our data, combined with the literature, identifies CTRP3 as an ideal candidate to develop novel treatments for alcoholic fatty liver disease.

Conclusion

Since the discovery of leptin and adiponectin, research into understanding the role of adipokines in human health has become a popular topic. The expanding family of adipokines, with their multiple functions, provides abundant research targets for the development of novel therapies. This study demonstrates that sex and mode of ethanol exposure can significantly influence the results indicating that the role of alcohol on adipokines should be studied via multiple models. Lastly, we have identified the sex specific alcohol-induced reduction in CTRP3 as a potential mechanism for the increased susceptibility of females to alcoholic cirrhosis. These findings warrant further study.

Figure Legends

Figure 1: Animal characteristics.

Separate cohorts of male and female mice were exposed to two separate ethanol feeding protocols: The NIAAA model (10 days, plus a binge on day 11) or the chronic model (6 weeks). No differences in body weights (A & C) or food intake normalized to body weight (B & D) were observed. There was no difference in the survival curve in ethanol compared with control fed male mice (E), however there was a significant difference in the female survival curve (F). Data reported as mean ± SD (A-D), data reported as absolute numbers (E & F), *=p < 0.05 ETOH vs Con. Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male; NIAAA, National Institute on Alcohol Abuse and Alcoholism.

Figure 2: Circulating Adipokines in the NIAAA model of ethanol feeding.

Circulating adiponectin, CTRP1, CTRP2, and CTRP3 levels were determined by immunoblot in serum collected from mice on the 10-day plus binge model of ethanol feeding (the NIAAA model). Data reported as mean ± SD. Male and female blots were performed and analyzed independently, and values were normalized to control fed within each sex. n=6, and *=p < 0.05 ETOH vs Con. Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male; NIAAA, National Institute on Alcohol Abuse and Alcoholism. Raw data files for data presented in Figure 2 are attached as supplemental data (S1).

Figure 3: Circulating Adipokines in the Chronic model of ethanol feeding.

Serum was collected from the mice after 6 weeks of ethanol feeding and circulating levels of adiponectin, CTRP1, CTRP2, and CTRP3 were determined by immunoblot analysis. Data reported as mean ± SD. Male and female blots were performed and analyzed independently, and values were normalized to control fed within each sex, n=6, and *=p < 0.05 ETOH vs Con. Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male. Raw data files for data presented in Figure 3 are attached as supplemental data (S2).

Figure 4: Tissue adipokine gene expression.

After 6 weeks gonadal adipose tissue samples were collected from the mice. There were no significant differences found in the gene expression of adiponectin, CTRP1, CTRP2, or CTRP3 (A-D). Data reported as mean ± SD and normalized to geometric mean Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male.

Figure 5: Gonadal adipose tissue adipokine protein content.

Gonadal adipose tissue samples were collected from the mice after ethanol feeding for 6 weeks or 10 days (NIAAA) and adiponectin, CTRP1, CTRP2, and CTRP3 and levels were examined by immunoblot analysis. Data reported as mean ± SD Male and female blots were performed and analyzed independently, and values were normalized to control fed within each sex, n=6, and *=$p < 0.05$ ETOH vs Con. Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male. Raw data files for data presented in Figure 5 are attached as supplemental data (S3).

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CHAPTER 3

METHODS

Adipose Tissue Lipid Analysis

Lipids were extracted as described by Bligh and Dyer and as previously performed (16, 18). Briefly, adipose tissue samples were weighed then homogenized in phosphate-buffered saline, 3.75 ml/ml of sample homogenate 1:2 (vol/vol) chloroform-methanol was added, followed by the addition of 1.25 ml/ml chloroform, followed by 1.25 ml distilled water. Samples were vortexed for 30 s between each addition. Samples were then centrifuged at 1,100x g for 10 min at room temperature to give a two-phase solution (aqueous phase on top and organic phase below). The lower phase was collected with a glass pipette with gentle positive pressure. Samples were then divided into 2 aliquots and dried under nitrogen gas at 60°C. To measure total triglyceride levels one aliquot from each sample was dissolved in tert-butyl alcohol-Triton X-100 (3:2 vol/vol) solution. Triglycerides were quantified via colorimetric assay according to manufactures directions (Infinity Triglycerides, Fisher Diagnostics, Cat# TR22421). The remaining aliquot was prepared for Fatty Acid Methyl Ester analysis.

Fatty Acid Methyl Ester Preparation and Analysis

Fatty acids (FA) of snap-frozen adipose tissue samples were extracted using the fatty acid methyl ester (FAME) method and measured with GC-MS. Briefly, boron trifluoride-methanol reagent (B1252; Sigma-Aldrich, St Louis, MO, USA) was added to a prepared aliquot of isolated adipose tissue lipids. The tube was then closed and heated in a block heater at 100°C for 1 hour before returning to room temperature. 1.5 mL distilled water was added and samples were centrifuged for 1 minute at 4000x g. FAMEs were extracted in the hexane phase, dried under nitrogen gas, suspended in 275 µL hexane, 5 µL C17 internal standard (1:9 hexane dilution) was added, and samples were stored at -80° C until further analysis. Gas chromatography (GC) using a flame-ionization detector, (Shimadzu GC- 2010; Shimadzu Corporation, Kyoto Japan) was performed on the samples using a capillary column (Zebron ZB-WAX, 30 m length, 0.25 mm i.d., 0.25 µm film thickness; Phenomenex, Torrance, CA, USA). The peaks were identified by comparison with Supelco 37 component FAME mix fatty acid standard (Sigma-aldrich Cat# 47885-U).

Serum Evaluations

Serum glucose and triglyceride concentrations were determined using commercially available assays according to manufactures directions (Glucose: Calbiochem. Cat# CBA086; Triglycerides: Infinity Triglycerides, Fisher Diagnostics. Cat# TR22421). Serum ALT levels were measured using an enzymatic assay kit (Cat# TR71121) according to manufacturer's instructions. Serum AST levels were measured using an enzymatic assay kit (Cat# A7561) according to manufacturer's instructions.

CHAPTER 4

RESULTS

Effects of Ethanol on Circulating Glucose

The chronic ethanol feeding model showed no differences in levels of glucose in serum when comparing ETOH fed male and female mice to their respective controls (Fig 1).

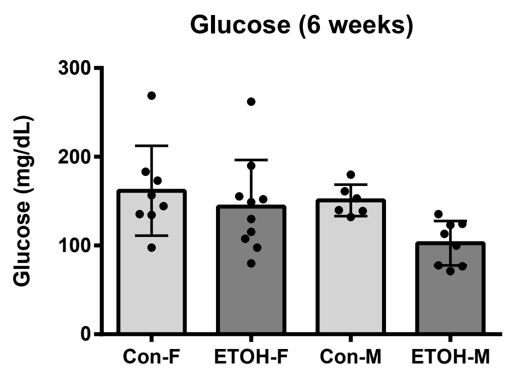

Figure 1.
Figure 4.1 Chronic Serum Glucose
Circulating levels of glucose were determined using a commercially available assay. Serum was collected from the mice after 10-day plus binge model of ethanol feeding (NIAAA) and after 6 weeks of ethanol feeding (Chronic). Data is reported as mean ± SD. * $p < 0.05$ ETOH vs. Control. Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male.

Effects of Ethanol on Circulating Triglycerides

The chronic ethanol feeding model showed no differences in circulating triglycerides when comparing ETOH fed male and female mice to their respective controls (Fig 2).

Figure 2.

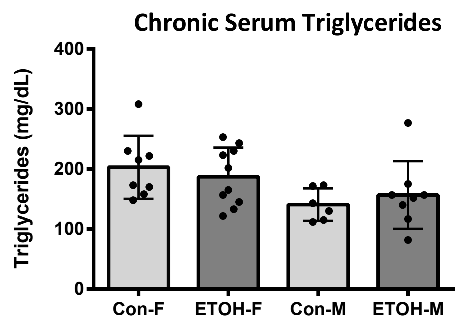

Figure 4.2 NIAAA and Chronic Serum Triglycerides
Circulating levels of triglycerides were determined using a commercially available assay. Serum was collected from the mice after the NIAAA model of ethanol feeding (A) and after the chronic model of ethanol feeding (B). Data is reported as mean ± SD. * $p < 0.05$ ETOH vs. Control. Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male.

Effects of Ethanol on Serum Transaminases

The NIAAA model showed no differences in ALT levels when comparing ETOH fed male and female mice to their respective controls (Fig 3 A). However, the chronic model showed a significant increase ($p < 0.05$) in ALT and AST levels in female ETOH fed mice compared to the female control group (Fig 3 B-C) while there was no difference found in ALT and AST levels for chronic male ETOH fed mice compared to the male control group (Fig 3 B-C).

Figure 3.

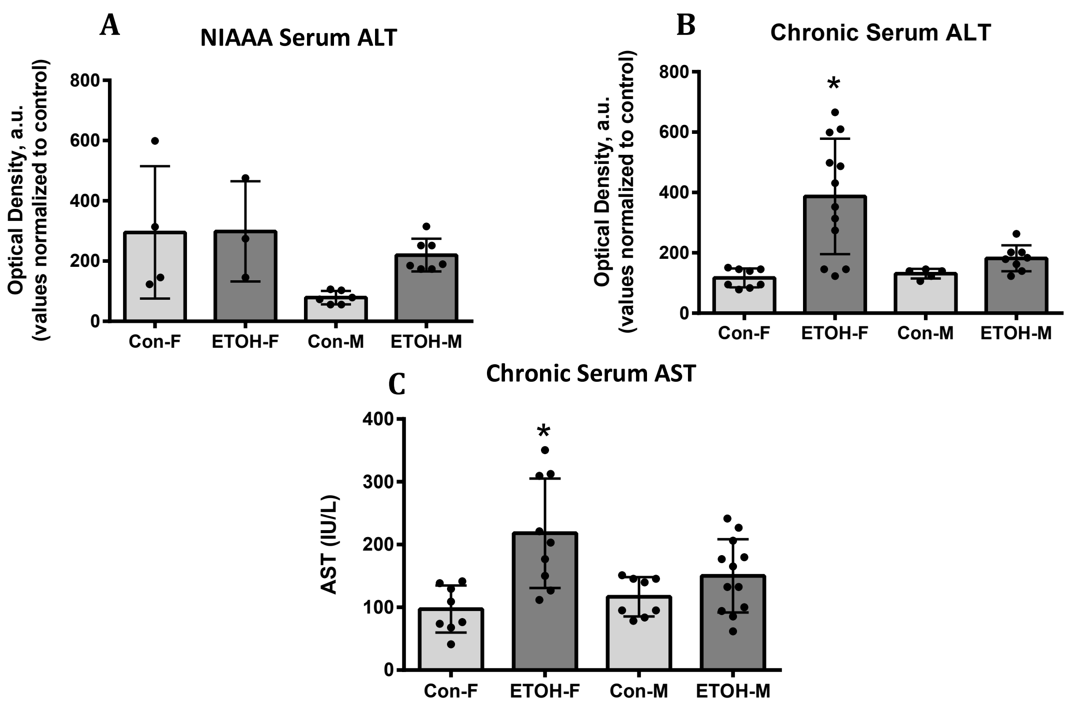

Figure 4.3 NIAAA and Chronic Serum Transaminases
ALT (A-B) and AST (C) levels were measured in the serum using a commercially available assay. Serum was collected from the mice after the NIAAA model of ethanol feeding and after the chronic model of ethanol feeding. Data is reported as mean ± SD. * $p < 0.05$ ETOH vs. Control. Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male.

Chronic Adipose Tissue Lipid Analysis

The chronic model male mice showed no significant differences between the ETOH fed male mice and the control fed male mice. However, the ETOH fed female mice showed a significant increase in levels of oleic acid with ETOH consumption compared to the control fed female mice.

Figure 4.

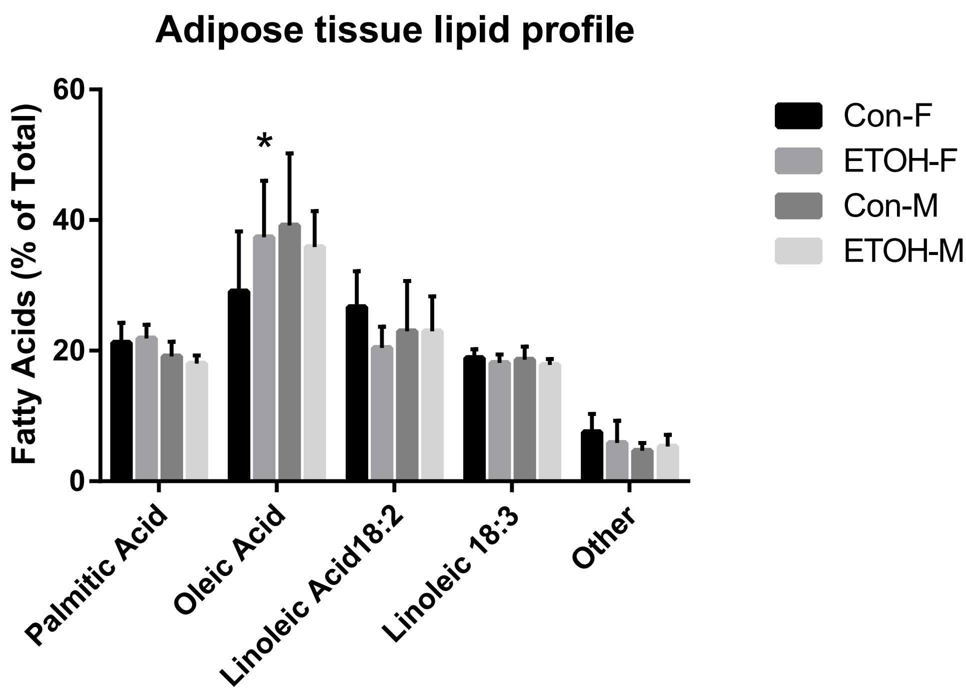

Figure 4.4 Chronic Adipose Tissue Lipid Analysis
Adipose tissue lipids were isolated, extracted using the FAME method, and measured with gas-chromatography mass spectrometry. Data was analyzed using a 1-way ANOVA and is represented by mean + S.D. * $p < 0.05$ ETOH vs. Control. Abbreviations: Con-F, pair-fed female; ETOH-F, ethanol fed female; Con-M, pair-fed male; ETOH-M, ethanol fed male.

CHAPTER 5

DISCUSSION

The aim of this study was to determine the effect alcohol consumption has in mice on levels of adipokines: CTRP1, CTRP2, and CTRP3. Because adipokines have lipid-regulating functions, their disruption could contribute to ectopic fat deposition, which is a major contributor to the secondary injury caused to tissues due to excessive alcohol consumption (Lang and Steiner 2017; Parker 2018). The major finding of this study is that excessive alcohol consumption affects adipokine levels in a sex specific manner, as female mice appear to be more sensitive to ethanol consumption. Alcoholic cirrhosis occurs at a higher rate in female alcoholic patients, at an earlier age, with a lower proportional amount of alcohol consumption (Rehm et al. 2010; Mandrekar and Fulham 2016; Nagy et al. 2016). In this study, male and female mice consumed similar amounts of ethanol, however, ethanol fed female mice showed significant inflammation (elevations in TNF and IL-6) and a significantly higher mortality rate when compared to control-fed mice. Where as, there was no difference in TNF, IL-6, or mortality between control and ethanol fed male mice. One potential reason for this is because women have less body water than men, so they exhibit a higher concentration of alcohol in the blood when similar amounts of alcohol are consumed (Rehm et al. 2010; Ghosh and Vaughan 2012). It is important to note that ethanol consumption led to increased levels of PAI-1, indicating in both sexes alcohol consumption leads to damage tissues and disruptions to overall tissue homeostasis (Ghosh and Vaughan 2012) . The levels of alanine transaminase (ALT) and aspartate transaminase (AST) were analyzed in both the NIAAA and chronic models. Transaminases are liver enzymes that can be measured in the serum to determine the degree of injury to the liver (Barone et al. 2016; Kim et al. 2016). In the chronic model, female ETOH fed mice showed a significant increase in both ALT and AST levels, twice as much as the male ETOH fed mice. This contributes to our finding that female mice were more sensitive to the ETOH than the male mice.

Leptin and Adiponectin

It has been documented that circulating levels of leptin and adiponectin are affected by alcohol consumption. Our findings demonstrate that leptin levels can vary depending on the feeding model, as leptin levels were increased in the NIAAA model (which includes a final ethanol bolus) but showed no difference after 6 weeks of chronic ethanol feeding. Considering the chronic model showed no difference in leptin levels between control and ETOH groups, the increase in leptin levels found in ETOH fed mice in the NIAAA model could be a result of the final ethanol gavage given to the mice before serum and tissue samples were collected. The chronic model showed that alcohol consumption did not affect the circulating levels of leptin, suggesting that over a longer period of time, leptin levels will be unchanged by alcohol consumption. This variability could be due to the time of the last alcohol exposure, or possibly dependent upon the feeding model used in the study.

Adiponectin levels have also been shown to be unaffected (Tan et al. 2012), suppressed (Chen et al. 2007; Yu et al. 2010), or increased (Sierksma et al. 2004; Pravdova et al. 2009; Mandrekar and Fulham 2016) with the consumption of alcohol. The cause of elevated levels of serum adiponectin seen in many alcohol feeding protocols has not been established. However, chronic alcohol abuse has demonstrated associations between circulating adiponectin levels and the severity of liver damage and elevated adiponectin levels in cases of cirrhosis (Xu et al. 2003; Tietge et al. 2004; You et al. 2005; Lang and Steiner 2017). Contrary to our hypothesis, our data supports the finding that circulating adiponectin levels increase with both NIAAA and chronic alcohol consumption. As circulating adiponectin levels increase with ethanol exposure, activating adiponectin and adiponectin mediated signaling pathways may not be a successful strategy for the prevention/treatment of ALD, as its levels are already increased with chronic ethanol exposure.

CTRP1

We hypothesized that the alcohol-induced loss of CTRP1 may enhance the effects of alcohol abuse due to decreased skeletal muscle lipid oxidation (Scherer and Shapiro 1998), resulting in excessive circulating lipid levels, thus promoting ectopic lipid accumulation. In our study, the NIAAA model showed increased circulating levels of CTRP1 in female ETOH fed mice and no difference in the circulating levels of CTRP1 in the male ETOH fed mice. This could be due to the increase levels of CTRP1 found in the NIAAA adipose tissue for ETOH fed female mice. After 6 weeks of ethanol feeding CTRP1 levels were elevated in the female ethanol fed mice, compared with control fed mice; indicating that chronic alcohol consumption does not inhibit CTRP1 levels in females. On the other hand, after 6 weeks of ethanol feeding there was a significant decrease in CTRP1 levels in ETOH fed male mice. The mechanism responsible for the dimorphic effect of alcohol is unknown. This effect may be secondary to the observed increase in pro-inflammatory cytokines (TNF and IL-6). Alcohol-induced reduction in circulating CTRP1 levels requires further analysis to determine if similar results are shown in human alcoholics and whether restoration of CTRP1 would generate any protective effect in male mice.

CTRP2

As predicted, CTRP2 levels decreased with ETOH feeding in both male and female mice during the 10-day plus binge (the NIAAA model) ethanol feeding protocol. However, there was no long-term alcohol-induced difference in CTRP2 in either sex. Therefore, the data does not support that alcohol-induced changes to circulating CTRP2 levels contribute to the adverse effects of chronic alcohol abuse.

CTRP3

Ethanol feeding reduced circulating CTRP3 levels in female, but not male mice, in both the NIAAA model and chronic feeding model. In fact, in human subjects CTRP3 levels are reduced with diet-induced hepatic steatosis, or non-alcoholic fatty liver disease (Zhang et al. 2017). We have previously shown that CTRP3 suppresses lipid-induced elevations in pro-inflammatory cytokines, such as TNF. However, the effects of CTRP3 on reducing alcoholic fatty liver disease are yet to be explored. Our data, combined with the literature, identifies CTRP3 as an ideal candidate to develop novel treatments for alcoholic fatty liver disease.

CHAPTER 6

CONCLUSION

In conclusion, alcohol consumption does have an effect on novel adipokines: CTRP1, CTRP2, and CTRP3. Chronic alcohol consumption disrupts lipid synthesis, which leads to the buildup of hepatic lipids, resulting alcoholic fatty liver and eventually alcoholic cirrhosis (Nagy et al. 2016; Parker 2018; Rehm et al. 2010), the leading causes of liver failure and a leading cause of death in the United States (Nagy et al. 2016; Rehm et al. 2010). Based on our research, there are some differences in the effects of excessive alcohol consumption between males and females. Females are more sensitive to excessive alcohol consumption, but CTRP3 could provide an ideal candidate to develop novel treatment. Because CTRP3 levels are selectively reduced in ETOH fed female mice, this provides a novel mechanism to explore the increase susceptibility of females to alcoholic cirrhosis. Overexpressing CTRP3 in female mice fed an ETOH diet would prove to be a beneficial trial for the effects of CTRP3 on ameliorating AFLD in females. Based on our findings for the male ETOH fed mice, with more research, CTRP1 could prove to be a target for treating AFLD in males.

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