The Effects of Arsenic Exposure on Fatty Liver Disease Development and the Glucocorticoid Response

By

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Abstract

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Arsenic, a naturally occurring metalloid, has been linked to a wide variety of metabolic disorders and diseases, including type II diabetes and non-alcoholic fatty liver disease. However, the exact mechanism of action of which arsenic leads to these maladies is not well understood. Research has elucidated the importance of other external factors, such as stress and diet, that may be increasing the development and severity of arsenic's negative impacts. This dissertation aims to explore the combinatorial effects of arsenic and the lifestyle factors: diet and stress. **Chapter I** is an overview of how ubiquitous arsenic is in the world and how one unique population located in Antofagasta, Chile, has become a natural experiment in epidemiology given their clear start and stop exposure time frame and subsequent arsenic-linked disease incidence. This population in addition to others around the world, most notably those in Mexico, Bangladesh, and India, are the inspiration for the studies included in this dissertation. **Chapter II** evaluates a high fructose diet in combination with both low and high dose arsenic in an *in vivo* model. Both fructose and arsenic are metabolized in the liver and when in surplus can lead to dysregulation of triglyceride processing and storage. This study contributes to existing research on dietary influence on the adverse effects of arsenic exposure, which until now has been focused solely on high fat diets. **Chapter III** seeks to evaluate if arsenic antagonizes the glucocorticoid receptor by using an *in vivo* model exposed to both arsenic and a synthetic glucocorticoid. . Furthermore, there is a large person-to-person variation in susceptibility, but the majority of the reasons are unknown, making these studies crucial to determine where that susceptibility may lie.

Dedication

I dedicate this piece of work to my best friend and inspiration, my mother.

Thank you for showing me how to be a fierce woman in science.

I will forever treasure all you have taught me.

Contents

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Lastly, I want to acknowledge all of those who showed me adversity, because of them I learned how to fight for what I want and to persevere.

Chapter I: The Risks of Arsenic – Antofagasta, Chile

The risks of arsenic

Arsenic is an abundant naturally occurring metalloid found in drinking water. In nature, arsenic can be found in both inorganic (iAs) and organic forms. More than 100 million people, predominately in India, Bangladesh, China, Chile, and Mexico, are at risk of an exposure to arsenic contaminated drinking water above the World Health Organization's (WHO) established limits of 10 μg/L (World Health Organization, 2011). In the United States (US), an estimated 12% of all public water systems have arsenic concentrations above the US standard of 10 μg/L (US EPA, 2001), this number does not account for private water well, which is estimated at more than 3 million additional people exposed to arsenic.

Once ingested, arsenic travels to the liver via the portal vein to be metabolized. In the liver, arsenite methyltransferase (AS3MT) with the help of glutathione (GSH) and S-adenosyl methionine (SAM) methylates arsenic to produce monomethylarsenous acid (MMA^{III}). MMA^{III} can then be further methylated to produce dimethylarsinous acid (DMA^{III}) or it can be oxidized to produce monomethylarsonic acid (MMA^{V}). Similarly, DMA^{III} can be oxidized to produce dimethylarsenic acid (DMA^{V}). The most toxic forms to human health are MMA^{III} and DMA^{III}; however, DMA^{III} is not often found in urine as it is produced at far fewer rate than the other forms. The most common metabolites excreted in urine are iAs^{III}, MMA^{III}, and DMA^{V}.

Chronic arsenic exposure increases the risk of cancer (most commonly are skin, lung, bladder, kidney, and liver) and noncancerous maladies such as cardiovascular (CVD), pulmonary diseases such as asthma, and metabolic dysfunction. Recent research is making advancements in the mechanism driving these increased risks. Time of exposure has proven to be a critical component in disease development. One study found that early life exposure is most detrimental to disease development; the odds of developing lung and bladder cancer increased significantly (OR = 5.24 and 8.11, respectively) if arsenic exposure occurred during fetal development or in early life (Steinmaus et al., 2015). Another similar study in mice found early life exposure increases susceptibility to hyperglycemia, hypercholesterolemia, and NAFLD later in life (Sanchez-Soria et al., 2014). Synergistic effects, such as arsenic coupled with increased obesity, are leading to an enhanced understanding of disease progression and severity (Steinmaus et al., 2015). Understanding arsenic's behavior in endocrine-related diseases will continue to guide US and WHO standards and reduce underestimating the overall burden of arsenic.

Due to the increased risk of cancer development following arsenic exposure, arsenic is considered a Group 1 carcinogen by the International Agency for Research on Cancer and ranked number one on the Environmental Protection Agency's US National Priorities List (ATSDR, 2017). By prioritizing arsenic, significant gains have been made in understanding the mechanisms of arsenic-induced cancers. However, scientific gaps exist in understanding arsenic exposure and the effects it has on different aspects of the metabolic system especially given the wide variety of lifestyles.

Epidemiological example – Antofagasta, Chile

In northern Chile lies the Atacama Desert, the driest habitable place on earth. Nearly everyone in this region resides in developed cities where they can access water provided by the municipal

water supply that flows down from a few rivers located in the Andes Mountains. Due to an increasing population in 1958, the largest city, Antofagasta, began to supplement the only water source with two other rivers. Unknowingly, these rivers were heavily contaminated with arsenic and concentrations increased from 90 μg/L to near 1000 μg/L. This exposure lasted for nearly 15 years when a treatment plant was installed and dramatically reduced arsenic levels in the water; today, arsenic water concentrations in most cities meet the WHO guideline of 10, although levels in a few areas still lie above the WHO's recommendation of 10 μg/L. Everyone residing in Antofagasta during this time was exposed as there was no other water source. This is rare epidemiological study with a clear start and end of exposure and a large population from which to examine the latency of arsenic exposure on an outcome of interest. People who were born during the exposure period provide a rare opportunity to investigate the long-term consequences of early-life exposure and of high arsenic exposures on disease development and progression.

In May of 2017, I travelled to Antofagasta, Chile to conduct a 150 subject human study. Over the course of three months, we collected data and specimen from both males and females ages 45-56 years of age (**Figure 1**). There were 75 exposed and 75 unexposed controls. To participate, the subjects had to be 10 years of age or younger during the major exposure time period (1958-1972). A control was defined as someone in the same age group who did not live in Antofagasta during the exposure period but moved to Antofagasta later in life. Subjects were recruited from workers at the local hospital. They were to be fasted for 12 hours before their sample collection appointment and had to come in first thing in the morning, before 9:30 AM. Data collected included lifetime residential and work history, tobacco smoke exposure, socioeconomic status, diet, and medical history and medication (**Table 1**). Once the subject completed the questionnaire, preliminary data and samples was collected including temperature, weight, height, saliva, buccal swab, and blood pressure. The subject brought in a urine sample with them and approximately 45 mL of blood was collected. The blood was then processed to obtain peripheral blood mononuclear cells, granulocytes, red blood cells, serum, plasma, clots, and white blood cells. All processed blood samples were aliquoted, stored at -80°C and then shipped back to the United State overnight on dry ice. Additionally, a small aliquot of blood was sent to a laboratory for metabolic analysis including high-density lipoprotein (HDL), low-density lipoprotein, very-low-density lipoprotein, triglycerides, and blood glucose (**Figure 2**).

This study was done as a follow-up to a population-based case-control study conducted between 2007 and 2010 in Antofagasta, which found increased odds of type II diabetes, defined as self-reported physician diagnosed or currently taking hyperglycemic medications, with arsenic exposure (Steinmaus et al., 2013). A basic analysis of the 2017 cohort data did not find a similar trend when comparing Hb1Ac or blood glucose levels (**Figure 3**). These data were not adjusted for smoking status, cancer, gender, or age. Reported diabetes prevalence for the 2017 data collection was 10.67% and therefore was not prevalent enough to include in the analysis (**Figure 4**).

While no immediate associations were seen based on these data, these human samples will prove to be critically important as more data becomes available to further evaluate mechanism of actions of arsenic. For example, arsenic has been shown to affect glucose metabolism given a

high fat diet (Ditzel et al., 2016); however, no other diets in combination with arsenic exposure have been explored. Additionally, arsenic has been linked to increasing cortisol levels, the body's stress hormone. Increased cortisol levels then lead to a greater susceptibility to arsenic and its associated diseases (Cohen et al., 2018). To explore these possible connections between organ systems and arsenic, animal models are a valuable resource to provide data for further study in humans. In animal studies, exposure conditions can easily be manipulated, and genetic and environmental factors can also be controlled. In particular, mice metabolize arsenic in a similar way to humans and therefore are often used in arsenic mechanistic studies; however, an important consideration is the differences in toxicokinetics of mice and humans; mice are able to metabolize arsenic more efficiently than humans requiring different doses to equivalate those of human concern (Stýblo et al., 2019). The following chapters delve into two independent experiments exploring arsenic exposure and the effects it has on glucose homeostasis and lipid metabolism under different conditions in a C57Bl/6, a proven mouse model for arsenic toxicity studies (Kenyon et al., 2008; Wu et al., 2017).

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Figures

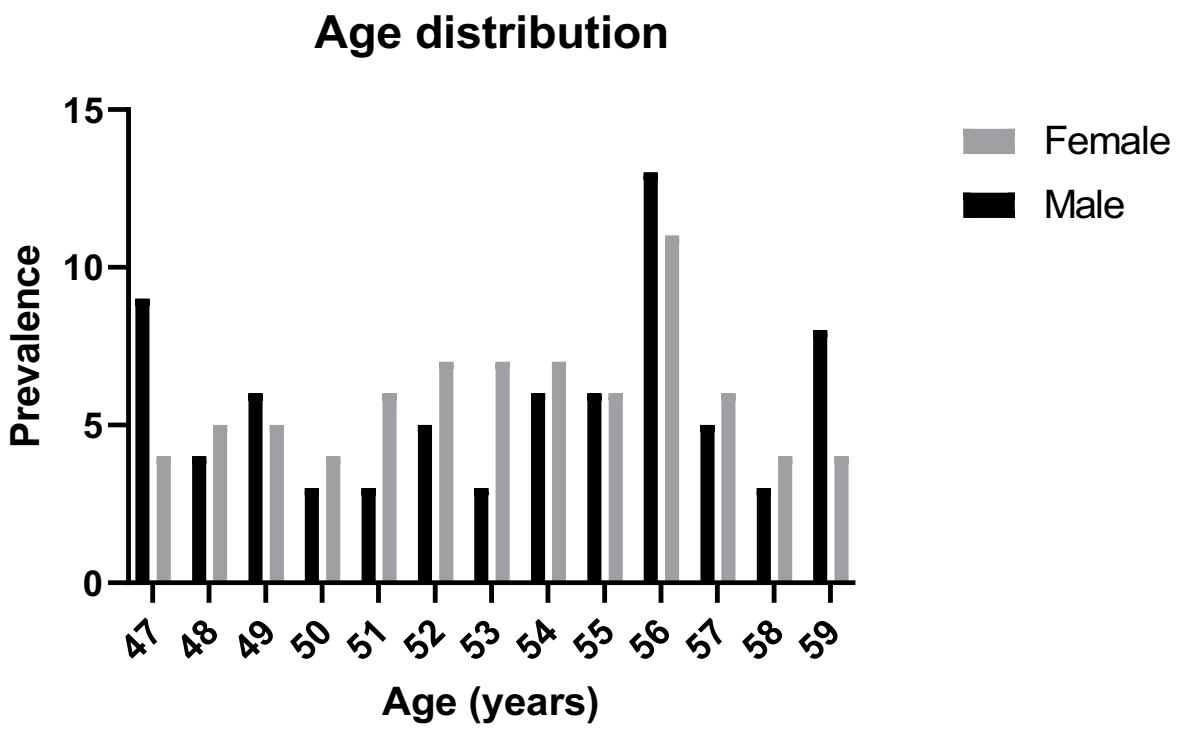

Figure 1: Age distribution of all study participants by sex.

Table 1: Demographic Information	
Total	150
Sex	
Male	74 (49.33%)
Female	76 (50.67%)
Age (years old)	
45-50	40 (26.67%)
51-55	56 (37.33%)
≥56	54 (36.00%)
Cancer case-control status	
Control	143 (95.33%)
Cancer cases: all	7 (4.67%)
Mining work	
No	123 (83.67%)
Yes	24 (16.33%)
Average cigarettes (per day)	
>0-5	52 (34.67%)
6-20	28 (18.67%)
>20	70 (46.67%)
Cumulative pack years	
Low	63 (42.00%)
Middle	20 (13.33%)
High	67 (44.67%)
BMI current (kg/m^2)	
<25	25 (16.67%)
25-30	65 (43.33%)
≥30	60 (40.00%)
BMI age 20 (kg/m^2)	
<25	116 (77.33%)
25-30	25 (16.67%)
≥30	9 (6.00%)
Hypertension	
No	114 (76.00%)
Yes	36 (24.00%)
Hyperglycemia (≥126 mg/dL)	
No	132 (88.00%)
Yes	18 (12.00%)
Diabetes	
No	134 (89.33%)
Yes	16 (10.67%)
Cholesterol (≥240 mg/dL)	
No	136 (90.67%)
Yes	14 (9.33%)
LDL (≥160 mg/dL)	
No	136 (90.67%)
Yes	14 (9.33%)
HDL (≥60 mg/dL)	
No	119 (79.33%)
Yes	31 (20.67%)
Triglycerides (≥200 mg/dL)	
No	111 (74.00%)
Yes	39 (26.00%)
CVD	
No	134 (89.93%)
Yes	15 (10.07%)

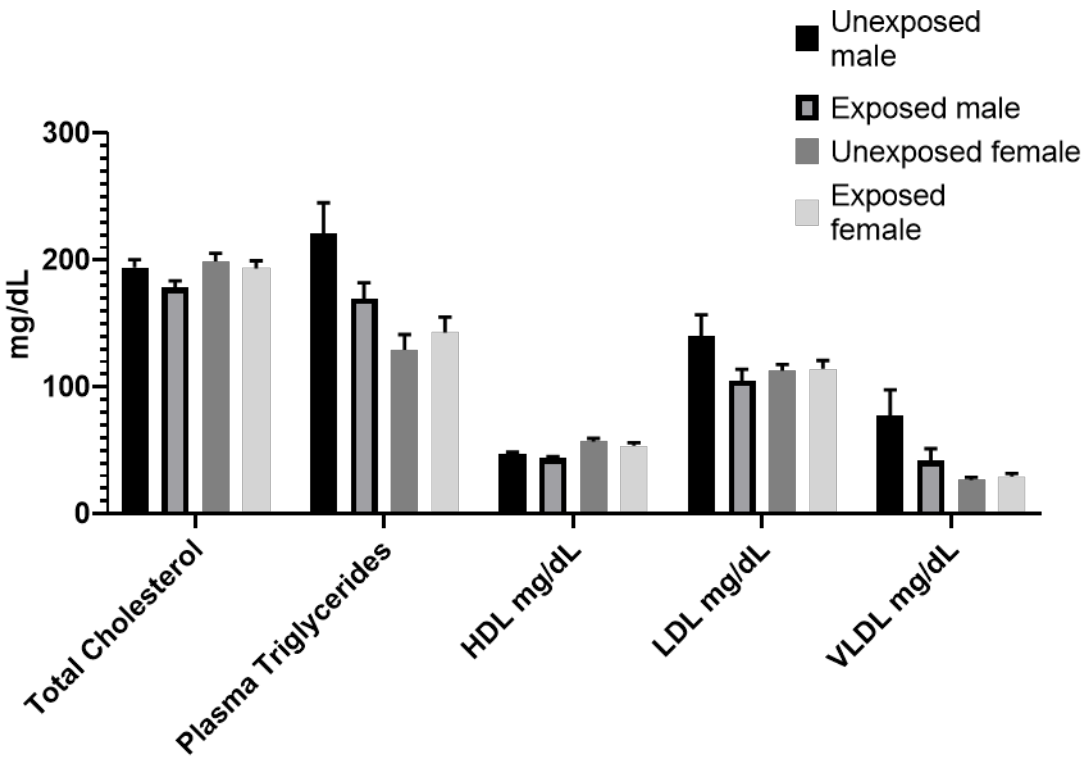

Figure 2: Metabolic analysis by sex. High-density lipoprotein (HDL); Low-density lipoprotein (LDL); Very-low-density lipoprotein (VLDL). Exposure defined as being born or under the age of 10 years old during the exposure period (1958-1970) in Antofagasta. Exposure levels at this time are estimated at 860 µg/L.

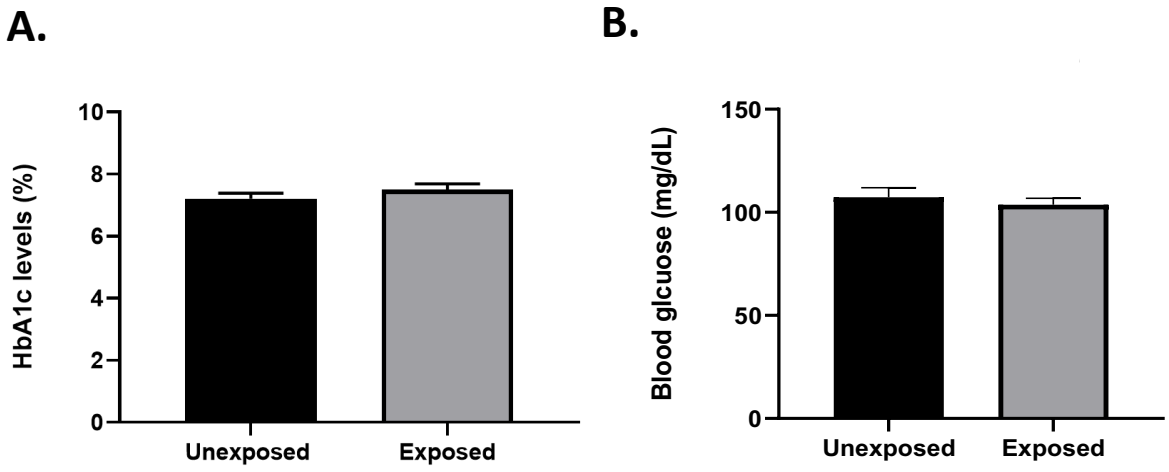

Figure 3: Hemoglobin A1c (**A**) and blood glucose (B) levels by exposure. n= 75.

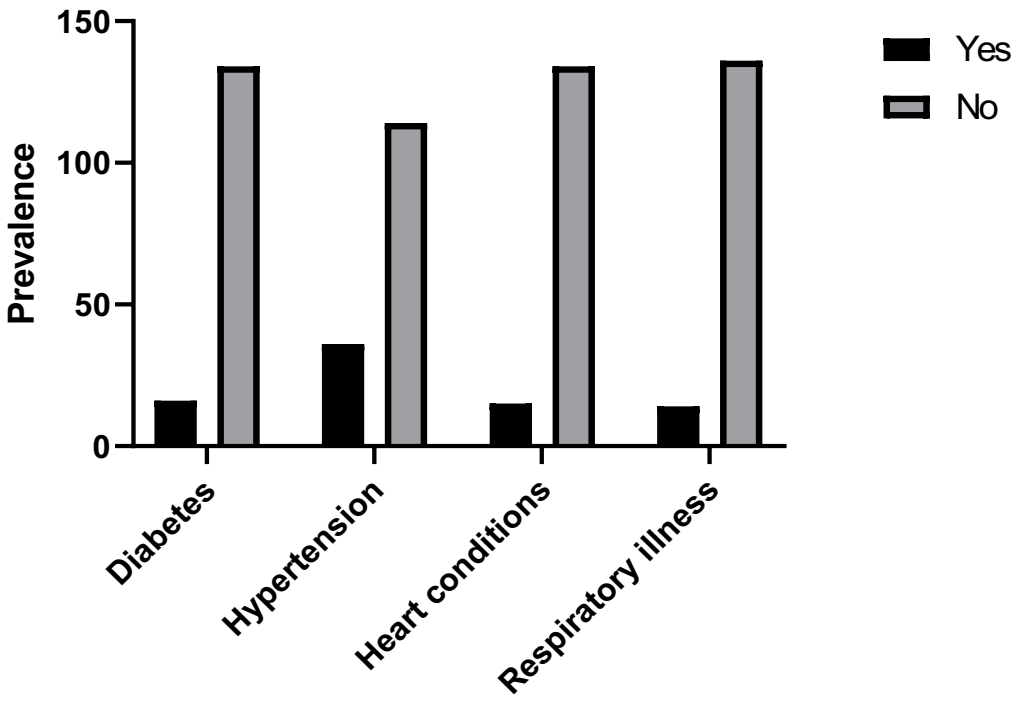

Figure 4: Preexisting health conditions

Chapter II: High dose arsenic exposure and a high fructose diet impact insulin tolerance and triglyceride composition

Abstract

Metabolic dysregulation is a complex and multifactorial condition that demands the consideration of multiple factors. Therefore, there is a strong need to assess multiple exposures and the possible interaction that may exist between them. Research thus far has found a significantly increased risk for non-alcoholic fatty liver disease (NAFLD) on a high-fat diet with co-exposure of arsenic. However, there is no mechanistic research considering the impacts of a high-fructose diet with arsenic exposure. Given the evidence that both arsenic and fructose are metabolized in the liver and hinder hepatic metabolic processes, the objective of this study is to determine the degree to which chronic arsenic exposure and a high-fructose diet act to induce liver injury. To determine if a co-exposure of arsenic and fructose create a greater than additive effect in promoting liver damage and NAFLD development with possible progression to more severe liver complications. Over 12 weeks, five-week-old C57Bl/6 mice were given a 60% fructose-based chow diet and treated with 300 or 1000 ppb arsenic. Glucose and insulin tolerance were measured at 12 weeks of exposure as well as body composition. Following euthanasia, plasma and liver triglycerides were measured as well as hepatic expression of genes involved in lipid processing and gluconeogenesis via qPCR. We found elevated hepatic triglycerides in the high dose but not low dose arsenic exposed treatment group. Congruently, a high dose of arsenic lowered circulating triglycerides compared to the other treatment groups. Upon analyzing gene expression associated with triglyceride metabolism, we found altered expression in *ApoA5* and *CO1*. Overall, our results suggest arsenic in combination with a high fructose diet leads to increased plasma triglycerides and decreased hepatic triglyceride secretion through ApoA5 mediated mitochondrial and lipoprotein dysregulation.

Background

Arsenic and NAFLD

NAFLD, known as the liver manifestation of metabolic syndrome, is increasing globally at alarming rate. NAFLD is denoted by the accumulation of fat in the liver and while the cause remains largely unknown, links have been made to obesity and T2DM. Recent evidence has also linked NAFLD to environmental pollutants, including arsenic(Das et al., 2010; Frediani et al., 2018; Islam et al., 2011; Liu et al., 2014). One study in particular conducted by the National Health and Nutrition Examination Survey (NHANES), found a positive association between arsenic exposure and NAFLD risk (Frediani et al., 2018). Other studies conducted in mice and zebra fish have reached similar conclusions and have begun to observe changes in gene expression and protein levels related to lipid metabolism (Bambino et al., 2017; Carlson & Van Beneden, 2014; Li et al., 2016; Xu, Lam, Shen, & Gong, 2013). Disruption of lipid metabolism is a well-established contributor to NAFLD, and arsenic is considered to be a metabolic disrupting chemical capable of prompting altered lipid metabolism (Sargis et al., 2017). Liver histology exams support this and found morphological changes including increased steatosis, hepatocellular ballooning, and increased lipid droplets following arsenic exposure (Sanchez-Soria et al., 2014). Lastly, arsenic can trigger inflammatory responses through an increase in reactive oxygen species (ROS) and a depletion glutathione (GSH), an antioxidant. Further inflammatory responses following arsenic exposure come from the increased expression of pro-

inflammatory cytokines (such as tumor necrosis factor-alpha, TNF-α) and a blunting of anti-inflammatory cytokines (such as interlekin-6, IL-6) (Tan et al., 2011). This imbalance of cytokines plays a crucial role in NAFLD and in the progression to non-alcoholic steatohepatitis (NASH), cirrhosis, and liver failure (Tilg, 2010; Xue Shi et al., 2014). Insulin resistance is known as a hallmark of NAFLD. Upon arsenic exposure, insulin resistance occurs in multiple tissues including liver and adipose. Arsenic can hinder insulin signaling pathways in multiple ways: it can alter insulin signaling in adipocytes and hepatocytes resulting in impaired glucose uptake, it can lead to an increase in important regulators of energy metabolism and inflammation, and it can alter adipocyte cytokine (adipokine) release that help regulate lipid metabolism. Arsenic not only disrupts insulin signaling but has also been shown to mimic insulin action causing further insulin dysregulation (Hamann et al., 2014; Zhang, Fennel, Douillet, & Styblo, 2017). Additionally, arsenic can be taken up into white adipose tissue. Here, arsenic downregulates PPARγ, peroxisome proliferator-activated receptor γ, which protects against insulin resistance and is considered to be the master regulator of adipogenesis. If adipogenesis is blocked, there will be fewer adipocytes available for TG storage. A decrease in TG storage coupled with arsenic induced lipolysis leads to an increase in circulating FFAs that mostly accumulate ectopically in the liver and skeletal muscle (Garciafigueroa, Klei, Ambrosio, & Barchowsky, 2013).

Fructose and its contribution to NAFLD

Fructose is a hexose sugar naturally present in fruits and vegetables. Recently, fructose has been more commonly used to sweeten foods and beverages in the form of sucrose (fructose bound to glucose), or high-fructose corn syrup (HFCS). Unlike glucose, fructose is not an essential sugar required by the human body to sustain biological functions. Instead, fructose undergoes unregulated metabolism in the small intestine. A fructose metabolism threshold of 1g/kg exists in the small intestine and can therefore easily undergo saturation, causing fructose to enter the portal vein and travel to the liver (Jang et al., 2018). In the liver, fructose contributes to metabolic dysregulation through various pathways (Lustig, Mietus-Snyder, Valente, Schwarz, & Lim, 2010). Since fructose does not have a regulatory mechanism equivalent to the adenosine triphosphate (ATP) and citrate inhibition of phosphofructokinase in glycolysis, hepatic uptake of dietary fructose is not regulated, leading to increased hepatic gluconeogenesis, dysregulation of insulin signaling, disruption of free fatty acid (FFA) oxidation, and increased de novo lipogenesis (DNL) (Samir Softic et al., 2017). Fructose is considered a major risk factor for the development and progression of liver diseases, specifically NAFLD (Alwahsh & Gebhardt, 2017; Basaranoglu, Basaranoglu, & Bugianesi, 2015; Ouyang et al., 2008; Prasanthi Jegatheesan & Jean-Pascal De Bandt, 2017). In the past few decades, there has been an increased incidence of this metabolic disorder, which parallels the doubling of fructose consumption worldwide in the past 30 years. NAFLD has also become the most common liver disease worldwide (Hall & Finck, 2017; Lustig et al., 2010) . While NAFLD is not a fatal disease, it can lead to the eventual progression of cirrhosis and liver cancer, conditions which contribute to both increased morbidity and mortality. NAFLD is also not considered to be a criteria of metabolic syndrome,

but rather a hepatic manifestation of metabolic syndrome (Manco, 2017) . These conditions result in very similar hallmarks, namely insulin resistance, altered triglyceride and cholesterol levels, and increased blood pressure (Armstrong, Adams, Canbay, & Syn, 2014) . The precise mechanism of action by which NAFLD progresses to the more severe disease states of NASH, fibrosis, and cirrhosis, is much debated. Previously, a "two-hit" hypothesis was proposed. This theory postulates the first hit to be associated with the development of hepatic steatosis via the accumulation of triglycerides in hepatocytes, sensitizing the liver to various possible "second hits", which promote advancement to NASH and cirrhosis. The "two-hit" model has however, been proven to be too simplistic (Buzzetti, Pinzani, & Tsochatzis, 2016) . NAFLD is a complex disease that is the product of multiple factors that simultaneously act to advance disease development and progression. The most current theory is a "multiple hit" model in which steatosis is considered to be an epiphenomenon of several distinct damaging mechanisms. Increased hepatic DNL, altered adipose tissue, and diet lead to increased FFA infiltration and accumulation in hepatocytes. Consequently, hepatocytes become more vulnerable to any additional hits, such as oxidative stress, mitochondrial dysfunction, and increases in proinflammatory cytokine release. The combined dietary and environmental exposure increase the liver's susceptibility to further liver damage, increasing risk of developing NAFLD, and progression to NASH, and liver cancer.

Human relevance

Recent studies have reported that Chile is the largest consumer of and fastest growing in sales of sugar-sweetened beverages (Gustavo Cediel et al., 2018; Popkin & Hawkes, 2016). Not only are their consumption and sales the highest but they also have the highest per person daily calorie consumption from sugar- sweetened beverage**s** (Popkin & Hawkes, 2016). While Chile does not have the highest caloric consumption per capita, this nation does have one of the highest prevalence of NAFLD, with the current prevalence estimated at 24 percent compared to the US which has a prevalence of 25 percent (Rinella & Charlton, 2016; Riquelme et al., 2009). This contrast may indicate an alternative explanation behind the high prevalence of NAFLD that is not explained solely by calorie consumption. Chile, specifically Antofagasta natives, provide an ideal epidemiological study to investigate my hypothesis that NAFLD development could potentially be due to the prevalence of fructose sweetened beverages combined with a susceptible liver from a previous high arsenic exposure.

Methods

Animals and treatment

Wildtype C57Bl/6J male mice (*Mus Musculus*) were purchased at four weeks of age and from Jackson Laboratory (Bar Harbor, Maine). Previous literature has demonstrated them as a well-accepted model of arsenic toxicity and metabolic processes.

Upon arrival, mice were housed in small groups in sterile ventilated cages with Sani-chip wood bedding (Lab Supply, Fort Worth, Texas). They were kept on a 12-hour light/dark cycle at 23°C

with *ad libitum* access to food and water. The study included an initial cohort of 14 mice (n = 7/treatment) and a subsequent study of 12 (n = 6/treatment). Following a seven-day acclimation period, mice were administered 300 ppb of sodium (meta)arsenite ($NaAsO_2$, Sigma-Aldrich) in autoclaved water for 12 weeks. Using an allometric scaling approach to calculate the human equivalence dose approximates the 300 ppb in mice to 58.5 ppb in humans (FDA, 2005). Both arsenic-treated water and control autoclaved water were freshly prepared and changed three times per week to reduce oxidation of trivalent and pentavalent arsenical species. Water consumption was measured by weight at the time of water changes. Mice were fed *ad libitum* purified 60% fructose custom diet chow (TD.89247, Teklad Adjusted Vitamin Diets; Harlan Laboratories, Inc.). Due to the hygroscopicity of the fructose, chow was changed every few days and consumption by weight was measured at the time of changing.

All mice were euthanized at 12.5 weeks of arsenic treatment with carbon dioxide exposure followed by removal of vital organs. Immediately following carbon dioxide exposure, cardiac punctures were performed to remove up to 500 µL of blood. The blood was centrifuged at 4°C and serum was removed and stored at -80° for future use. Twelve tissues were removed, weighed, aliquoted and flash frozen in liquid nitrogen.

All methods were carried out in accordance with the Animal Use Protocol (#2015-06-7681-1) approved by UC Berkeley's Animal Care and Use Committees (ACUC). All personnel working with mice were properly trained and followed all standard operating procedures (SOPs) as required by both ACUC and National Institute of Health (NIH).

Glucose and insulin tolerance

Glucose tolerance tests (GTT) were performed following 12 weeks of arsenic exposure. Mice were injected with a 10% glucose-PBS solution. Blood was collected from a tail clip and blood glucose levels were measured before injection, 15 minutes following injection and then every 30 minutes over the span of 120 minutes. Blood glucose was measured on a Bayer Contour glucometer with the corresponding glucose strips (Bayer AG, Leverkusen, Germany). Blood was collected using Microvette CB300 Capillary Blood Collection Tube (Sarstedt, Nümbrecht, Germany). Tubes were kept on ice until centrifugation at 13.1 RPM, 4°C for 12 minutes. The serum was removed and stored at -80°C for future use. Following data collection, area under the curve was calculated to evaluate changes in blood glucose.

Insulin tolerance tests were performed during the 12^{th} week of arsenic exposure. Mice were injected intraperitoneally with 0.4 units/kg insulin. Glucose levels were measured before injection, 15 minutes following injection and then every 30 minutes over the span of 120 minutes using a Bayer Contour glucometer with the corresponding strips (Bayer AG, Leverkusen, Germany). Baseline insulin levels were calculated and then area under the curve was evaluated.

HOMA-IR was calculated following serum insulin ELISA measurements. Plasma insulin was measured using Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Elk Grove, IL, USA) per manufacturer's low range assay protocol. Once plasma insulin levels were determined,

HOMA-IR was calculated using the following equation:

$$\frac{\left(plasma\ insulin\left(\frac{mU}{L}\right)*23.09\right)*fasting\ blood\ glucose\ at\ time\ 0}{405}$$

Triglycerides

Liver and serum triglycerides were measured using the Serum Triglyceride Determination Kit (Sigma Aldrich, St. Louis, MO). Livers were cut into 100 mg, put into a 3:5 ratio of 3M potassium hydroxide and 100% ethanol, and incubated in a heated water bath for 24 hours. Following centrifugation, 1M magnesium chloride was added to the supernatant. Mixtures were then evaluated following the aforementioned kit's protocol. Liver triglyceride concentration was adjusted for weight and size of the tissue and well size. Serum was used as suggested per the kit's protocol

RNA isolation and Reverse Transcriptase Quantitative Polychain Reaction (RT-qPCR)

Using RNeasy Mini Kit (Qiagen, Hilden, Germany), total RNA was extracted from approximately 30 mg of flash frozen liver following the manufacturer's protocol. Total RNA was measured using Cytation 5 image reader's (BioTek Instruments Inc, Winooski, VT) nucleic acid quantification. All samples were evaluated for RNA quality ($A_{260/280} \geq 2$) and diluted to 5 ng/uL for RT-qPCR. Samples were then converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Following cDNA conversion, gene expression was evaluated using reverse transcription polymerase chain reaction (PCR) on a CFX96 Real-Time PCR C1000 thermal cycler (BioRad, Hercules, CA). iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA) was used as the reaction master mix following the manufacturer's protocol. Three genes were selected for analysis apolipoprotein A5 (ApoA5, F – TCC TCG CAG TGT TCG CAA G; R – CGA AGC TGC CTT TCA GGT TCT), lipoprotein lipase (*LPL*; F – GGG AGT TTG GCT CCA GAG TTT; R – TGT GTC TTC AGG GGT CCT TAG), and mitochondrially encoded cytochrome c oxidase I (*mt-CO1*; F – TCAACATGAAACCCCCAGCCA: R – GCGGCTAGCACTGGTAGTGA). The housekeeping gene used was mitochondrial ribosomal protein L19 (*mRPL19*; F – TCC TTG GTC TTA GAC CTG CG: R – ATG GAG CAC ATC CAC AAG C) and all data were normalized for efficiency of amplification.

Results

Chronic arsenic exposure has no effect on body composition, weight, or food and water consumption

No significant differences were seen in body mass over the 12-week period in comparison to the control cohort (**Figure 1**). Interestingly, there were also no differences seen in body composition including percent fat and percent lean muscle (**Figure 2**). On average, the control group gained 0.75 grams per week, the low dose gained 0.672 grams per week, and the high dose cohort gained 0.46 grams per week.

Additionally, no significant differences were seen food consumption across the three cohorts (approximately 5g/week) (**Figure 3**). Water consumption differed slightly for the high exposure

group as on average they consumed 12g/mouse/week of water while the control mice consumed approximately 16.5 g/mouse/week of water. However, this difference of weekly averages is not statistically significant.

High dose arsenic exposure leads to an impaired insulin response

A 16-hour fast was used to evaluate glucose homeostasis and tolerance following 12-weeks of arsenic exposure. Interestingly, no significant differences were seen in blood glucose levels over the 120-minute glucose tolerance test (**Figure 4A**). At time 0, control mice had an average fasting blood glucose (FBG) of 67.5, low dose an average FBG of 68.73, and high dose an average FBG of 66.33. All exposure groups had an appropriate blood glucose response of a rapid rise in blood glucose follow injections that continued through the 30-minute mark and then a gradual decrease in blood glucose following the one-hour mark

Alternatively, the insulin tolerance test using baseline glucose levels did show significant differences between the control group and high dose exposed mice (**Figure 4B**). All mice had an appropriate response following insulin injection with a significant decrease in blood glucose. However, while the control and low dose cohorts began to recover their blood glucose levels at time 30, the high dose exposure group maintained decreased blood glucose levels over the entire 120-minute time span. The significant differences came at the one-hour mark and maintained significantly different through the end of the measurements at 120 minutes. The control group had an average of 43.03 mg/dL at 60 minutes, 60.24 mg/dL at 90 minutes, and 68.95 mg/dL at 120 minutes compared to the high dose exposure group which had an average of 30.94 mg/dL at 60 minutes, 30.89 mg/dL at 90 minutes, and 34.67 mg/dL at 120 minutes.

HOMA-IR was lower in the high dose exposure group (HOMA-IR = 2.077) compared to the control (HOMA-IR = 4.183) (**Figure 5**), however this result was not statistically significant (p-value = 0.1292). There was no difference found when comparing low dose exposure to control with both values being around 4.

High dose arsenic exposure alters hepatic triglyceride accumulation and circulating triglyceride levels

There were significant differences observed for the triglyceride make-up between the control and high dose exposure group (**Figure 6**). As expected, the high dose exposure group had a significantly higher number of liver triglycerides (52.23 mg/dL) compared to the controls (37.68 mg/dL) (**Figure 6A**). Congruently, the plasma triglycerides were significantly decreased in the high dose exposure (0.0783 mg/dL) group in comparison to the control group (0.163 mg/dL) (**Figure 6C**). No differences were seen in the low dose group which had a liver triglyceride level of 37.85 mg/dL and a plasma triglyceride of 0.174 mg/dL.

Interestingly, the liver weights do not reflect this increase in triglyceride content in the high dose exposure group as there is no significant difference in liver weight as a percentage of total body weight (**Figure 7**). All groups have a relative liver weight of approximately 5.4%.

Triglyceride metabolism genes are affected by high dose arsenic exposure

Following the observed differences in triglyceride distribution and insulin tolerance, gene expression in liver was measured in relevant genes (**Figure 8**). Significant differences in ΔΔCq values between the control and high dose exposure cohorts were observed in *ApoA5,* 1.04 vs 1.90 respectively, with a p-value of 0.01 Similarly, when evaluating expression of *co1*, the control group had significantly higher expression than the high dose group, 1.025 versus 0.652 with a p-value of 0.02. When evaluating *LPL*, a significant difference was not found but there was a trending difference with higher expression in controls (1.02) compared to the high dose exposure group (0.818).

Discussion

In this study, we aimed to characterize the effects of inorganic arsenic combined with a high fructose diet on the development of NAFLD. Overall, we found that low dose arsenic has no effect on glucose metabolism and TG synthesis, whereas high dose arsenic shows increased insulin sensitivity and a change in TG distribution.

This study highlights the differences between specific diets and the effects of arsenic on metabolic processes. As many have showed, a high fat diet coupled with low dose arsenic (100 ppb) results in a metabolic profile similar to that of diabetes (Ditzel et al., 2016; Paul, Walton, Saunders, & Stýblo, 2011); however, this is not the case with a high fructose diet. While there is some alteration in glucose homeostasis, more evident is the alteration of lipid metabolism in the liver. The decrease in plasma triglycerides in the high dose arsenic cohort compared to the control led to the evaluation of possible altered genes in the liver. Namely, *ApoA5*, which is mainly synthesized in the liver and plays a role in maintaining normal plasma levels of circulating TGs and is a main component of hepatic lipid droplets. We found a significant increase in hepatic expression of *ApoA5* in the high dose arsenic cohort compared to the control cohort. This increase in *ApoA5* expression could help explain the increased insulin sensitivity in the high dose arsenic treated cohort as seen in a previous study of an *ApoA5* knock down mouse model finding protection from insulin resistance and in a human study where insulin resistant subjects had elevated levels of plasma ApoA5 (Camporez et al., 2015; Pennacchio et al., 2001). Furthermore, a previous study found that decreased *ApoA5* expression resulted in decreased lipid uptake by the liver, which is consistent with our results where high dose arsenic led to an increase in *ApoA5 expression* and increased liver TGs (Shu, Nelbach, Ryan, & Forte, 2010). However, we found arsenic exposure is associated with slight decrease in *LPL* expression, which is contradictory to some studies mentioned above where they all found a positive correlation between *ApoA5* and *LPL* expression.

Future experiments should include a repeat study of the high dose arsenic exposure group as well as an *ApoA5* knockout mouse model to determine if *ApoA5* is truly affected. Blood collection during the ITT would also be help in exploring the mechanism behind the increased insulin sensitivity observed in the high dose arsenic exposure group. Additionally, a western blot to analyzed apoA5 protein prevalence would also be beneficial to understand the observed altered gene expression.

Limitations of this study include the small sample size and the lack of a second repeat study with the high dose arsenic exposure group. Additionally, the exposure groups were limited to fructose

diet only and a future study including all exposure scenarios would be useful in determining the full effects of high dose arsenic exposure coupled with a high fructose diet. This study did include a female cohort; however, no differences in glucose tolerance or lipid metabolism were seen.

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Figures

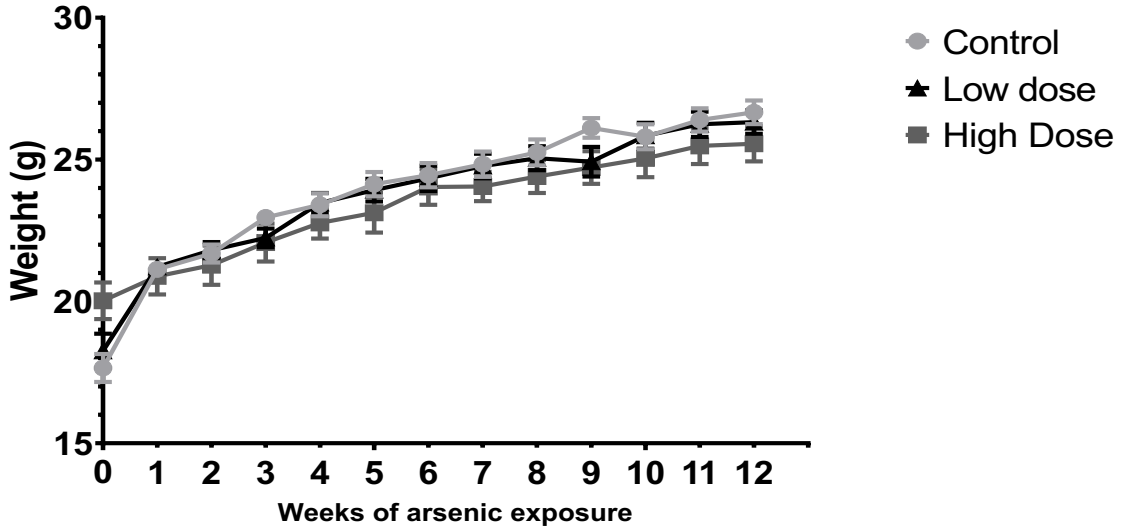

Figure 1: Average weekly body weight over length of the study (12 weeks).

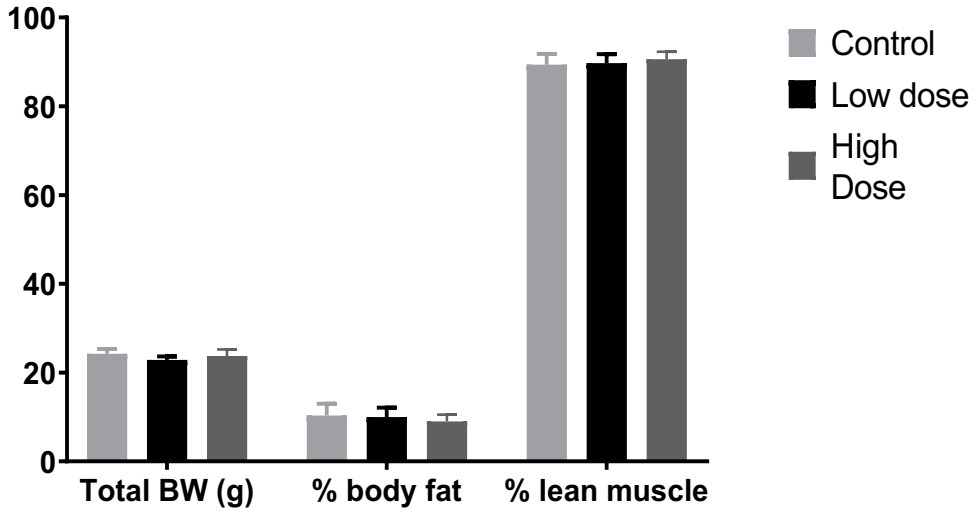

Figure 2: Body composition broken into three categories: mean body weight (BW), % body fat, and % lean muscle. Control and low dose: n = 25; high dose: n = 6.

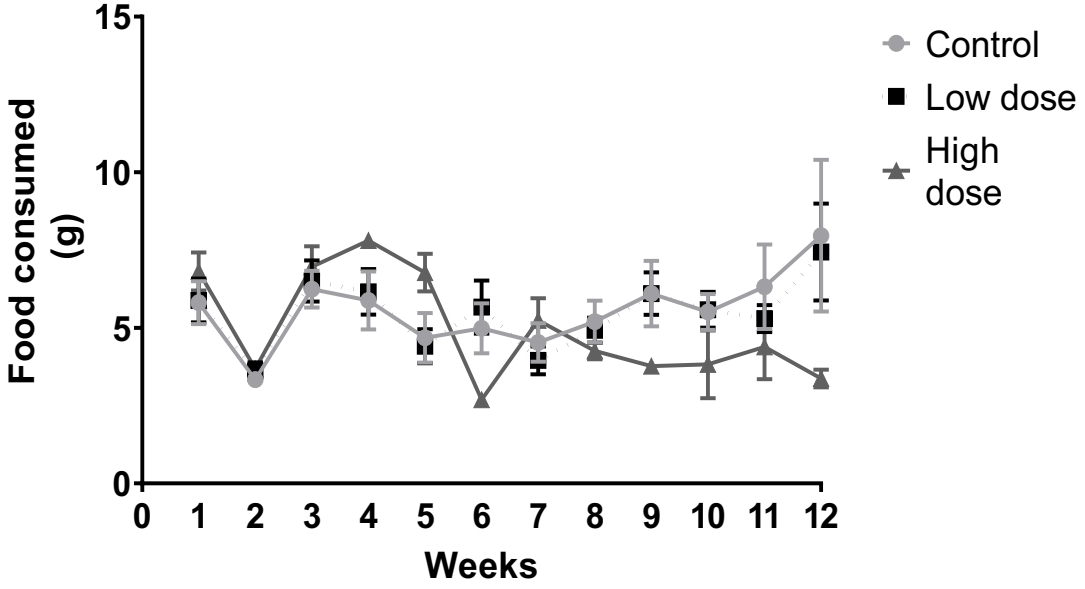

Figure 3: Average weekly food consumption by exposure group. Calculated based on food consumption recorded from a cage of 3 mice to represent weekly average consumption per mouse. For control and low dose: n = 8 (cages); for high dose: n = 2 (cages).

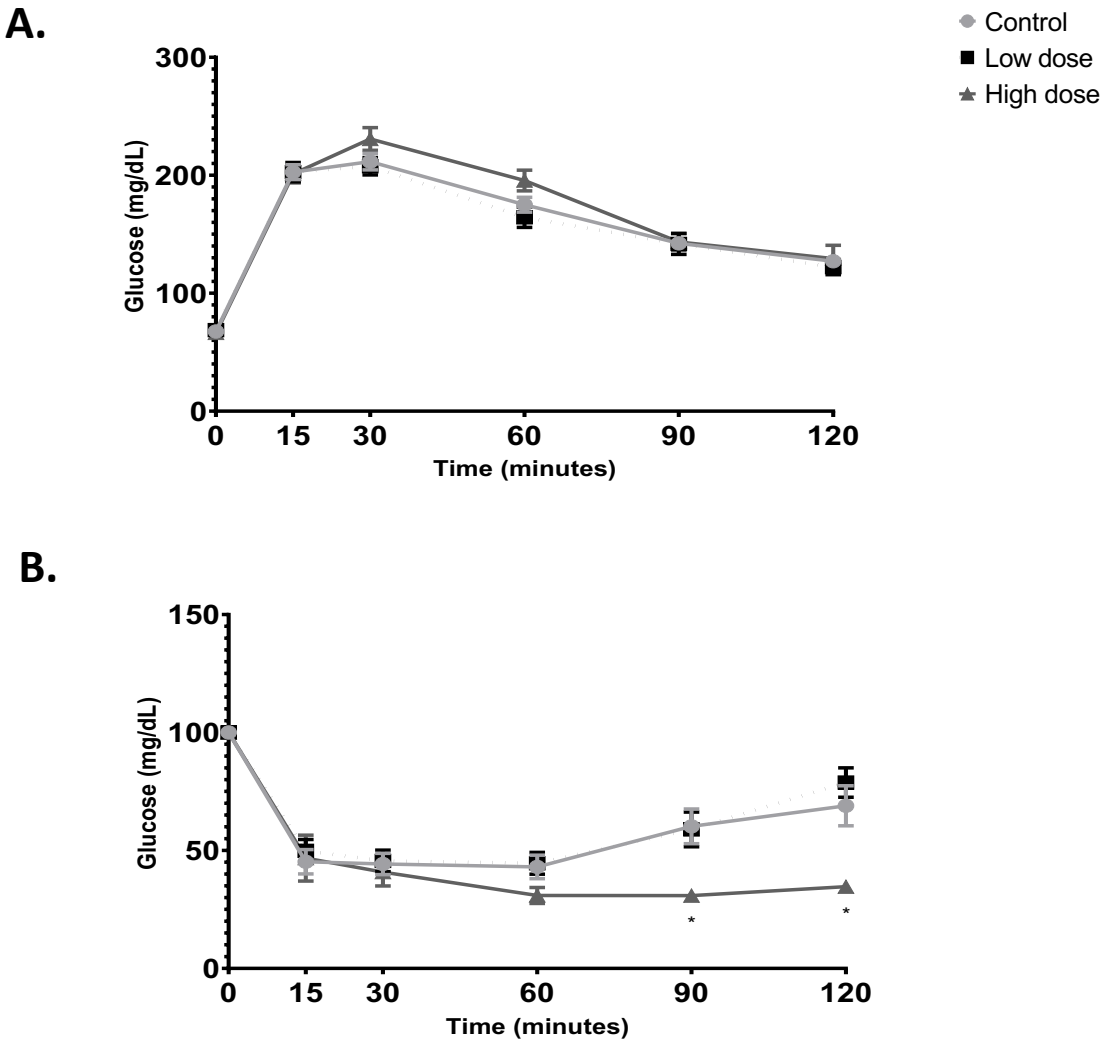

Figure 4: Following 12 weeks of arsenic exposure blood glucose was measured before **A.** 10% glucose **B.** 0.4 units/kg insulin intraperitoneal injection and blood glucose was measured following injection at 15 minutes, 30 minutes and then every 30 minutes over 120 minutes. For **A**, control and low dose: n = 24; high dose: n = 6. For **B**, control: n = 16; low dose: n = 15; high dose: n = 6. * p-value ≤ 0.05.

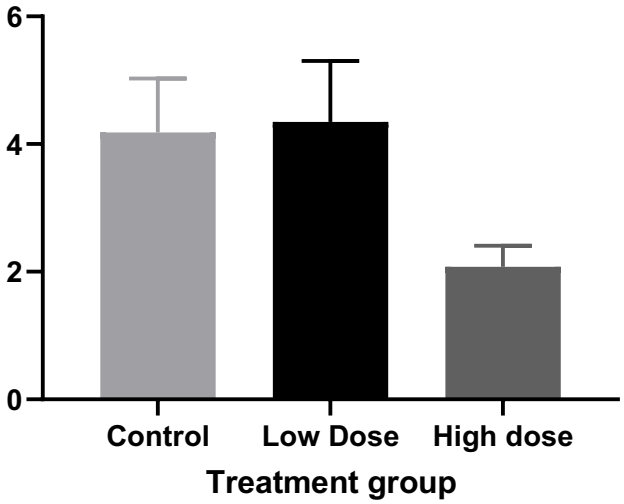

Figure 5: Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) by treatment group. For control: n = 14; low dose: n = 13; high dose: n = 6.

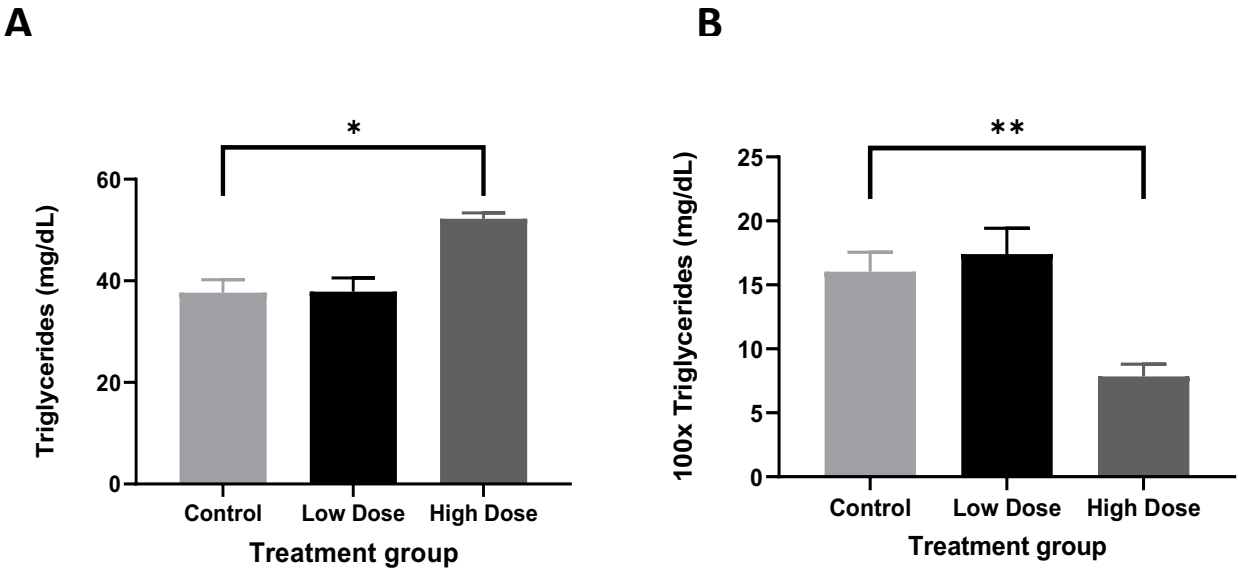

Figure 6: Liver (**A**) and plasma (**B**) triglycerides. Plasma triglycerides have been multiplied by 100 to be on same scale as liver triglycerides. For **A**, control and low dose: n = 25; high dose: n = 6. For **B**, control and low dose: n = 23; high dose: n = 5.

Figure 7: Percent liver weight calculated based on total body weight. For control and low dose: n = 25; for high dose: n = 6.

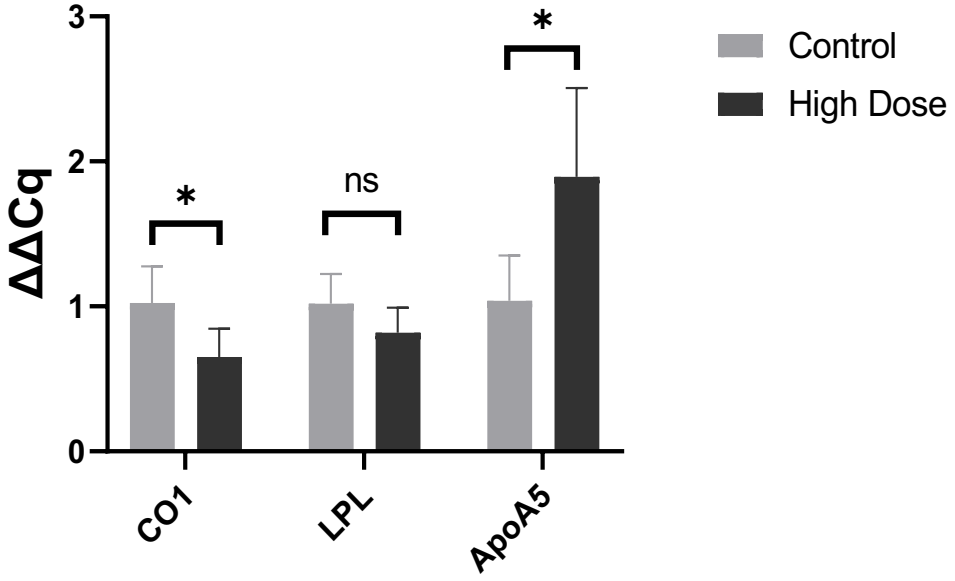

Figure 8: ΔΔCq gene expression using reference gene, mRPL19. CO1: mitochondrially encoded cytochrome c oxidase I; LPL: lipoprotein lipase; ApoA5: apolipoprotein A5. *p-value ≤ 0.05; ns = not significant. For CO1: control: n =6; high dose: n = 5. For LPL and ApoA5: n = 6 for all.

Chapter III: Arsenic alters PCK1 expression through interaction with the glucocorticoid receptor

Abstract

Background: Arsenic, a ubiquitous, naturally occurring metalloid, has been discovered to be an endocrine disruptor in particular interacting with the glucocorticoid receptor in vitro. However, whether arsenic disrupts glucocorticoid functions *in vivo* is not well understood.

Hypothesis: We hypothesize that arsenic will antagonize the glucocorticoid receptor. Arsenic exposure will antagonize chronic glucocorticoid exposure induced hyperinsulinemia, hypertriglyceridemia and fatty liver, glucose and insulin resistance, and immunosuppression.

Results: Arsenic combined with dexamethasone led to increased insulin resistance in a dose dependent manner. No difference in glucose tolerance was observed. Upon looking at altered gene expression associated with gluconeogenesis, we found altered expression in *PCK1*. Dexamethasone treatment led to overexpression of *PCK1* whereas high dose, but not low dose, arsenic exposure suppressed basal and dexamethasone induced *PCK1* induction.

Conclusions: Arsenic attenuated dexamethasone induced expression of the gluconeogenic gene, *PCK1,* in mouse liver. However, arsenic surprisingly exacerbated dexamethasone induced insulin resistance. These results suggest that while arsenic likely reduces glucocorticoid promoted hepatic gluconeogenesis, it modulates glucocorticoid actions on other aspects of glucose metabolism to enhance insulin resistance.

Background

Glucocorticoids (GC) are a class of steroid hormones secreted from the adrenal glands often in response to stress. They play a critical role in stress-related homeostasis, embryo development, blood glucose levels, blood vessel function, and lung and skin development. A delicate balance exists between the short- and long-term effects of stress hormones; short term effects can be beneficial whereas long-term exposure can lead to phenotypes such as insulin resistance, hypertriglyceridemia, and muscle atrophy. One of the most common GCs is cortisol in humans or corticosterone in rodents. Synthetic forms, such as dexamethasone (dex), are often used for medical treatments due to their anti-inflammatory and immunosuppressant effects.

GCs bind to the glucocorticoid receptor (GR) and once bound, a nuclear localization signaling motif and DNA binding domain are unmasked. The GR-ligand complex translocates via active transport into the nucleus where it interacts with DNA. The GR complex binds as a homodimer to a DNA recognition element or creates protein-protein interactions with cofactors leading to activation or suppression of glucocorticoid-response genes. These interactions lead to expression of glucocorticoid-responsive genes, which represent 3-10% of the human genome.

Recent research has found environmental contaminants, such as arsenic, can act as endocrine disruptors (Meakin, Martin, Szilagyi, Nylander-French, & Fry, 2019). Arsenic, in particular, has been reported to disrupt the GC complex-mediated gene regulation leading to the decreased expression of glucocorticoid-activated hepatic genes. However, the broader GR-related transcriptional effects of arsenic in different cell contexts and tissues are unknown. A few hypotheses have been developed. The first is epigenetics based as arsenic is a known disruptor of DNA methylation through its suppression of DNMT DNA methyltransferase), a family of enzymes responsible for catalyzing the transfer of a methyl group to DNA. There is evidence that

arsenic induces CPG methylation changes in the GR-signaling pathway (Allan et al., 2015). This is often seen more during fetal arsenic exposure as the fetus is more susceptible to epigenetic alterations(Perera & Herbstman, 2011). Additionally, male fetuses are disproportionally affected compared to females. Another potential mechanism arises from arsenic's affinity for dithiol groups. Arsenic binds with the thiol groups found on the GR leading to a variety of scenarios: the dissociation of unactivated receptor-steroid complexes, partial inhibition of activated complexes, or more uncommonly blocking the steroid from binding to the receptor (Bodwell, Gosse, Nomikos, & Hamilton, 2006). Most studied is arsenic's ability to alter the ligand-GR complex, disrupting its ability to interact with the DNA response elements and therefore preventing the complex's ability to induce transcriptional activity.

While research has elucidated potential mechanisms exploring the effects arsenic has on the GR, no studies have explored the metabolic effects of an impaired GR following arsenic exposure. We hypothesize that arsenic antagonizes the GR exacerbating dexamethasone effects including disrupted glucose and insulin homeostasis, hypertriglyceridemia, and altered body composition.

Methods

Animals and treatment

Wildtype C57Bl/6J male mice (*Mus Musculus*) were purchased at three weeks of age from Jackson Laboratory (Bar Harbor, Maine). Previous literature has demonstrated them as a well-accepted model of arsenic toxicity and metabolic processes. The study included an initial cohort of 24 mice (n = 6/treatment) and an identical repeat study. Upon arrival, mice were divided into groups of three and housed in sterile ventilated cages with Sani-chip wood bedding. They were kept on a 12-hour light/dark cycle at 23°C with *ad libitum* access to food and water. Following, a seven-day acclimation period, mice were administered 100 ppb or 1000 ppb of sodium (meta)arsenite ($NaAsO_2$, Sigma-LADrich) in autoclaved water. After 6 weeks of arsenic exposure, one group of mice from each arsenic dose as well as one set of control mice were administered 2mg/kg of dexamethasone via the same water source for seven days.

All water was freshly prepared and changed three times per week to reduce oxidation of trivalent and pentavalent arsenical species and reduce photodegradation of dexamethasone. Water consumption was measured at the time of water changes. Mice were fed *ad libitum* standard chow diet and food was changed weekly.

Body composition (body fat, lean muscle, and water weight) was measured using EchoMRI™ (Echo Medical Systems, Houston, TX) at 7 weeks of arsenic exposure and 7 days of dexamethasone treatment.

All mice were euthanized with carbon dioxide exposure at week 8 of arsenic treatment and day 13 of dexamethasone treatment. Immediately following carbon dioxide exposure, cardiac punctures were performed to remove up to 500 µL of blood. The blood was centrifuged at 4°C and the serum was removed and stored at -80° until future use. Twelve tissues were removed, weighed, aliquoted and flash frozen in liquid nitrogen.

All methods were carried out in accordance with the Animal Use Protocol (#2015-06-7681-1) approved by UC Berkeley's Animal Care and Use Committees (ACUC). All personnel working with mice were properly trained and followed all standard operating procedures (SOPs) as required by both ACUC and National Institute of Health (NIH).

Glucose and insulin tolerance tests

All mice underwent a 16-hour fasted GTT during the 7th week of arsenic exposure and one week of dexamethasone. Mice were injected with a 10% glucose-PBS solution. Glucose levels were measured before injection, 15 minutes following injection and then every 30 minutes over the span of 120 minutes. Following data collection, area under the curve was calculated. Blood glucose was measured on a Bayer Contour glucometer with the corresponding glucose strips (Bayer AG, Leverkusen, Germany). Blood was collected using Microvette CB300 Capillary Blood Collection Tube (Sarstedt, Nümbrecht, Germany). Tubes were kept on ice until centrifugation at 13.1 RPM, 4°C for 12 minutes. The serum was removed and stored at -80°C for future use. Following data collection, area under the curve was calculated to evaluate changes in blood glucose.

Three days later, mice underwent an ITT. Mice were injected intraperitoneally with 0.4 units/kg insulin. Glucose levels were measured before injection, 15 minutes following injection and then every 30 minutes over the span of 120 minutes using a Bayer Contour glucometer with the corresponding strips (Bayer AG, Leverkusen, Germany). Baseline insulin levels were calculated and then area under the curve was evaluated.

HOMA-IR was calculated following serum insulin ELISA measurements. Plasma insulin was measured using Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Elk Grove, IL, USA) per manufacturer's low range assay protocol. Once plasma insulin levels were determined, HOMA-IR was calculated using the following equation:

$$\frac{\left(plasma\ insulin\left(\frac{mU}{L}\right)*23.09\right)*fasting\ blood\ glucose\ at\ time\ 0}{405}$$

Triglycerides

Liver and serum triglycerides were measured using the Serum Triglyceride Determination Kit (Sigma LADrich, St. Louis, MO). Livers were cut into 100 mg, put into a 3:5 ratio of 3M potassium hydroxide and 100% ethanol, and incubated in a heated water bath for 24 hours. Following centrifugation, 1M magnesium chloride was added to the supernatant. Mixtures were then evaluated following the aforementioned kit's protocol. Liver triglyceride concentration was adjusted for weight and size of the tissue and well size. Serum was used as suggested per the kit's protocol

RNA isolation and Reverse Transcriptase Quantitative Polychain Reaction (RT-qPCR)

Using RNeasy Mini Kit (Qiagen, Hilden, Germany), total RNA was extracted from approximately 30 mg of flash frozen liver following the manufacturer's protocol. Total RNA was measured using Cytation 5 image reader's (BioTek Instruments Inc, Winooski, VT) nucleic acid quantification. All samples were evaluated for RNA quality ($A_{260/280} \geq 2$) and diluted to 5 ng/uL

for RT-qPCR. Samples were then converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Following cDNA conversion, gene expression was evaluated using reverse transcription polymerase chain reaction (PCR) on a CFX96 Real-Time PCR C1000 thermal cycler (BioRad, Hercules, CA). iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA) was used as the reaction master mix following the manufacturer's protocol. Primer sequences used are as follows: PECK (forward, CTGCATAACGGTCTGGACTTC; reverse, CAGCAACTGCCCGTACTCC) and G6pase (forward, ATG ACT TTG GGA TC C AGT CG; reverse, TGG AAC CAG ATG GGA AAG AG). RPL19 (forward, TCC TTG GTC TTA GAC CTG CG; reverse, ATG GAG CAC ATC CAC AAG C) was used as the housekeeping gene and all data were normalized for efficiency of amplification.

Results

Arsenic and dexamethasone increase percent body fat independently

To examine if arsenic and dexamethasone affect body weight, body composition was measured after 7 weeks of arsenic exposure and one week of dex and food and water consumption were measured weekly. Over the entirety of the experiment all mice steadily increased in body weight. Despite these differences, no major differences in food consumption or water intake was observed (data not shown). However, at week 6 of arsenic treatment and the start of dex treatment, there is a decrease in body weight in the dex treated groups only while dex null groups continue to gain weight (about 0.5 g). The control group with dex had the most severe weight loss with about 1.4g difference, the low dose arsenic with dex lost about 0.8g, and the high dose arsenic with dex lost almost 0.87 grams.

Significant differences were observed in body fat percentage between the null group and all treatment groups (**Figure 1B**). Mice treated with dexamethasone show an increased body fat percentage compared to their non-treated counter parts. The largest increase is seen in the dex treated groups with an increase of approximately 3%. No difference was observed when increasing arsenic dosage (CD: 12.8%, ALD: 12.4%, and AHD: 11.4%). Alternatively, in the absence of dex, arsenic exposure linearly increases body percent fat (CND: 6.3%, AL: 9.1%, AH: 10%).

When evaluating lean muscle percentage there is significant decrease in lean muscle in the arsenic unexposed, dexamethasone treated group ,CND: 89.65% and CD: 86.7%, and even greater decrease in the low dose arsenic treated with dexamethasone group, ALD: 86.8% However, this is unexpectedly not seen in the high dose arsenic with dexamethasone group,, AHD: 88.3% (**Figure 1C**).

Dexamethasone but not arsenic increases brown adipose tissue

Brown adipose tissue weight (by percent body fat) increased two-fold in dexamethasone treated groups compared to the null group (CD = 0.9962%, ALD = 1.023%, AHD = 1.035% versus CND = 0.4997%). This increase was not seen in arsenic treated groups without dexamethasone. (**Figure 2**).

Dexamethasone impairs insulin homeostasis, worsened by high dose arsenic

To determine the effects of arsenic on the GR, insulin response was measured by ITT (**Figure 3**). All dexamethasone treated groups showed significant impaired insulin response compared to the non-treated groups. HAD showed the most significant insulin resistance demonstrated by the lack of change in blood glucose levels over the 120 minutes. A correlation between arsenic dose and dexamethasone was observed as CD and LAD also showed insulin resistance but not as severe as the HAD. No differences were seen with arsenic exposure alone with dex compared to the null group. All three of these cohorts had an expected response of an initial decrease of blood glucose followed by a steady, slow increase over the 120-minute observation period.

Despite the differences seen in insulin sensitivity no significant differences were seen in pancreas size (**Figure 4).** There is a slight increase in the dexamethasone treated groups compared to their non-dex counterparts, with the largest difference being between the null cohort and the dex only cohort (CND: 0.61%, CD: 0.72%)

Dexamethasone induces PCK1, but arsenic suppresses this induction

Following our observations with insulin resistance, we sought to determine if genes transcribed by the GRE would help support this. We looked at the expression of two genes, *PCK1* and *G6PC*. While G6PC did not yield any comprehensive results, *PCK1* did show some trends (**Figure 5**). Dex increased *PCK1* as expected with a near 2-fold-increase in the ΔΔCq of CD (1.92) cohort compared to CND. Arsenic had contrasting results based on dose: low dose arsenic (AL) had a slight increase in expression with a ΔΔCq of 1.27 whereas high dose arsenic (AH) had suppressive effect (ΔΔCq = 0.68). When dex was in combination with arsenic, we saw a close to 2-fold increase in ΔΔCq in the low dose arsenic group (ΔΔCq = 1.8), but in the high dose arsenic group there appears to be two combative mechanisms leading to almost no change (ΔΔCq = 0.90).

Dex but not arsenic affects spleen size

As arsenic and dex both independently affect the immune system, we measured spleen size to evaluate if the GR could play a role in this effect (**Figure 6**). We found reduced spleen size in the CD, ALD, and AHD cohorts with weight percentages of 0.123%, 0.112%, and 0.163% respectively. In comparison to the double negative control, CND (0.29%), these results were statistically significant. No difference was seen with the arsenic only treatment groups compared to CND. Interestingly, the high dose arsenic with dex treatment group did see a slight increase in spleen size compared to the other dex treated groups but it was not significant.

Arsenic has no effect on circulating on corticosterone

To determine if arsenic increases circulating glucocorticoids, corticosterone was measured every 4 hours over a 24-hour period. As expected, the dex null cohorts had an appropriate corticosterone rhythm with increased levels at 7 am and 7 pm, which are in line with the light and dark cycles of the animal housing facility (**Figure 7**). This trend is lost amongst the three cohorts that are treated with dexamethasone. A severe depression of circulating corticosterone is observed in the dex treated cohorts; at the light cycle time periods (7 am and 7 pm) the difference in circulating corticosterone is significantly different for the dex treated cohorts when compared to the null control group. No difference was observed between the arsenic with dex treated mice in comparison to the dex only treatment group. Measuring corticosterone can be very difficult as

corticosterone levels vary significantly between individual mice as seen by the large standard error mean bars in the figure. Therefore, while it appears that LA and CD may be different at the 7 pm time, the variability in the cohort's measurements makes this difference insignificant (p-value, 0.232). Similarly, for the HA group at 7 am when compared to the null group, CD, the difference is not significant due to the large variability in the measurements of each group (p-value, 0.181).

Arsenic may affect circulating triglycerides, but not through inhibition of GR

Triglycerides were measured in both plasma and liver following euthanasia to determine if arsenic impairs triglycerides processing. Apart from the low dose arsenic group, no difference in liver triglycerides was seen between a dex verse no dex treatment group (i.e CND versus CD) (**Figure 8A**). The low dose arsenic group had a mean liver triglyceride level that was approximately 5 mg/dL lower than the control, although this difference was not found to be statistically significant (**Figure 8B**). A trending difference did emerge between high dose arsenic and the null treatment groups in plasma triglyceride levels (p-values, HAD: 0.0705; HA: 0.0793). While it was just shy of significant, it indicates a possible link between circulating triglycerides and arsenic exposure levels as there was no correlation seen in the lower dosing groups.

Discussion

This study is the first to measure the dose-dependent effects of arsenic on the transcriptome of liver and other tissues and biomarkers of GR activity (i.e. spleen size, plasma and liver lipids and glucose). Overall, we found a high dose arsenic exposure exacerbates a dexamethasone induced insulin resistance. Likely, this is attributed to altered expression glucogenic genes transcribed by the GRE; therefore, we measured hepatic expression of *PCK1* and *G6PC* (Hanoch Cassuto et al., 2005; Kuo, McQueen, Chen, & Wang, 2015). Dexamethasone alone led to an over expression of *PCK1* expression, but high dose arsenic led to a basal and dexamethasone induced suppression of *PCK*. These results are supportive of previous research that found low-dose arsenic on expression *PCK1* were strongly associated the GR-mediated regulatory pathway (Kaltreider, Davis, Lariviere, & Hamilton, 2001).

The body weight and fat percentage differences following arsenic exposure are in concordance with previous studies that show arsenic leads to an obesogenic phenotype (Castriota et al., 2020). While a decrease in body weight was observed following dex exposure, fat percentage was higher in the dex groups indicating a shift in fat storage. This is supported by the differences observed in brown adipose tissue, which was higher in dex exposed mice over no dex treatment. It does appear that arsenic has some effect on adiposity as the arsenic only treatment group had higher body fat percentage than the other arsenic null groups. There was also the least amount of change between the high dose arsenic and the high dose arsenic with dex indicating a possible ameliorating affect with the high dose arsenic and dexamethasone group. A future study should focus on adipose specific gene expression and possibly the beiging process, such as UCP-1, as that has been shown to be affected by arsenic and dexamethasone (Bae et al., 2019; Castriota et al., 2020; Luijten, Cannon, & Nedergaard, 2019; Zuo et al., 2019).

Dex has been shown to impact the immune system which is consistent with our observed decreased spleen size in the dex treated groups (Coutinho & Chapman, 2011; R Rooman, G Koster, R Bloemen, R Gresnigt, & SC van Buul-Offers, 1999). There are no differences observed between dex treatment and dex treatment with arsenic exposure, indicating that arsenic is not suppressing the immune system through the GR. Although arsenic does not affect spleen size, the spleen has an important role in the immune system and arsenic has been shown to alter the function of the immune system (Bellamri, Morzadec, Fardel, & Vernhet, 2018). A future study evaluating the robustness of the immune system with arsenic and dexamethasone would be interesting.

Interestingly, there was no difference seen in circulating corticosterone levels between the arsenic exposure plus dexamethasone groups when compared to the dexamethasone only treated mice. This would indicate that arsenic may not be antagonizing the GR as we would expect a significant increase in circulating corticosterone in the arsenic treated plus dexamethasone groups compared to the dexamethasone only treated group. However, because corticosterone is known to have high level of intra-variability (Jimeno, Hau, & Verhulst, 2018); therefore, this measurement would be beneficial to repeat to corroborate the lack of difference.

The next steps would be to confirm that the observed insulin resistance is a result of arsenic antagonization of GR. Additional exploration of the relationship with arsenic, dex, and adipose tissue function could also prove to be quite interesting. With these future studies and the data presented in this chapter, valuable insight into arsenic's impact on regulation of glucogenic genes and contribution to metabolic disease development.

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Figures

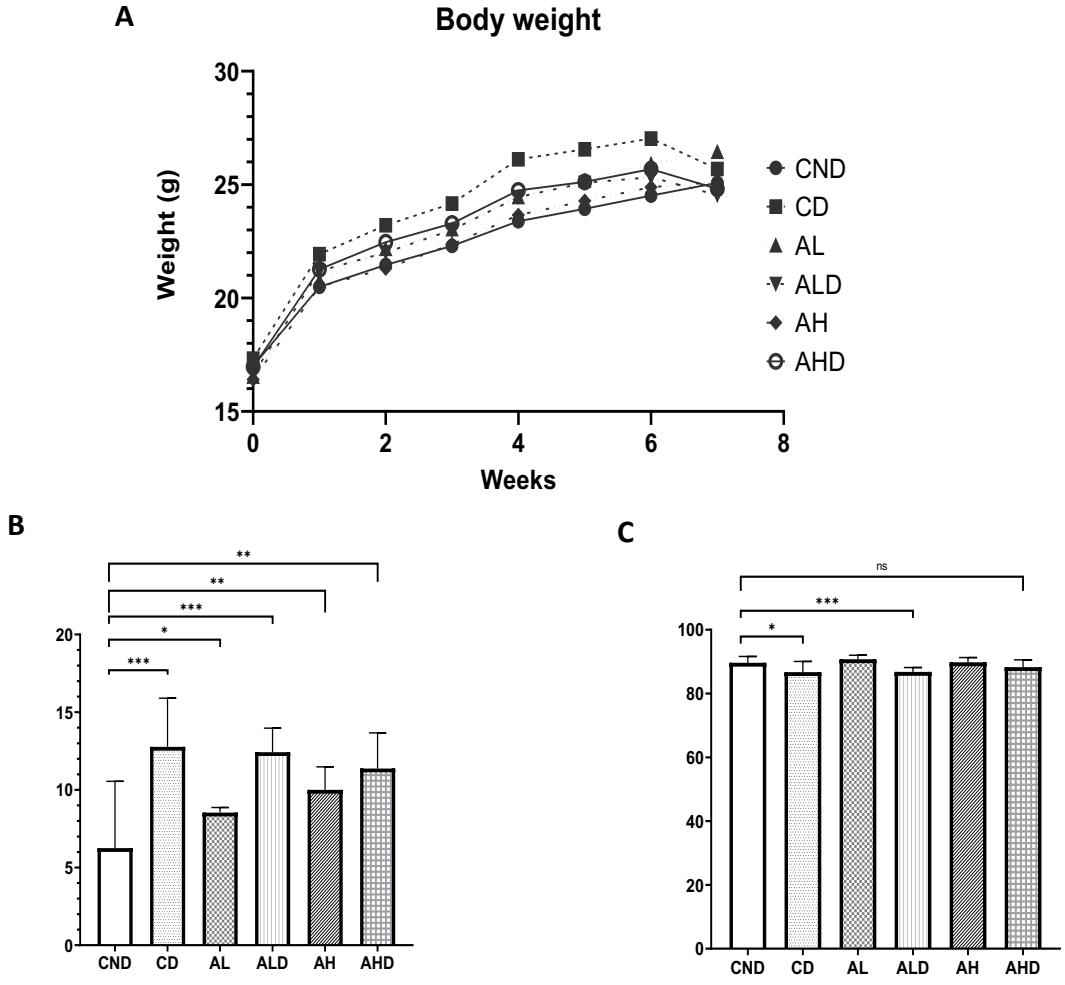

Figure 1: A: Average weekly weight. **B:** Total fat mass and **C.** lean muscle mass based on EchoMRI measurements including lean muscle and total water weight. CND: control no dex; CD: control with dex; AL: low dose arsenic; ALD: arsenic low dose with dex; AH: high dose arsenic; n = 12 for all treatment groups. *p-

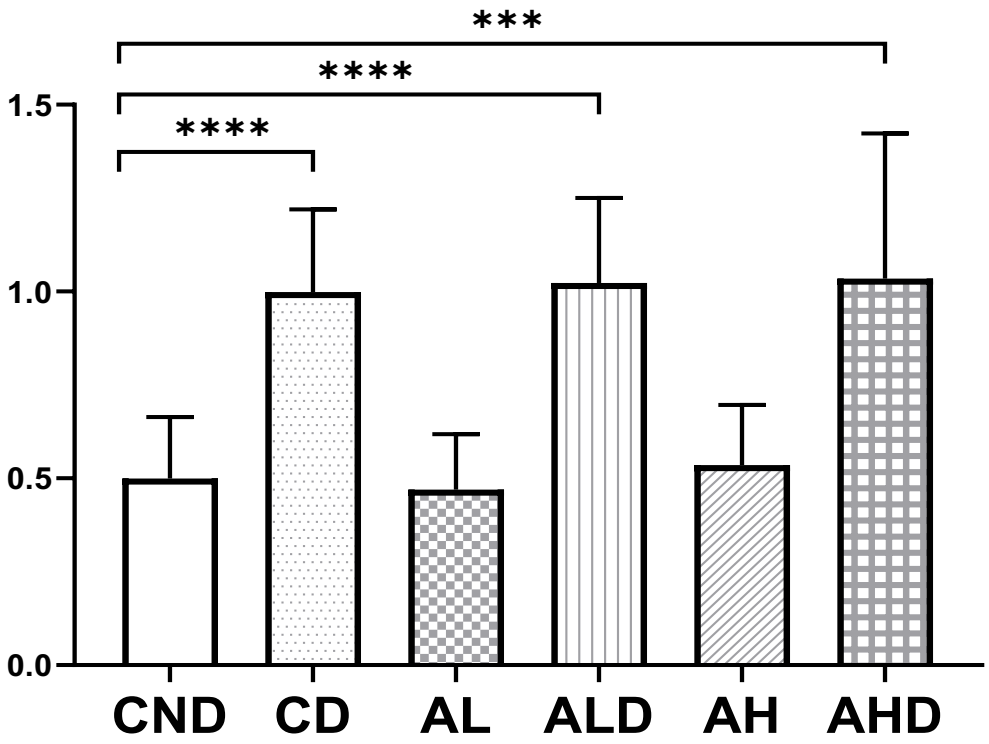

Figure 2: Brown adipose tissue as weight percentage of total body weight. CND: control no dex; CD: control with dex; AL: low dose arsenic; ALD: arsenic low dose with dex; AH: high dose arsenic; AHD: high dose arsenic with dex. For CND, CD, AH, AHD: n = 12; for AL, ALD: n = 11. ***p-value < 0.005; ****p-value < 0.0001

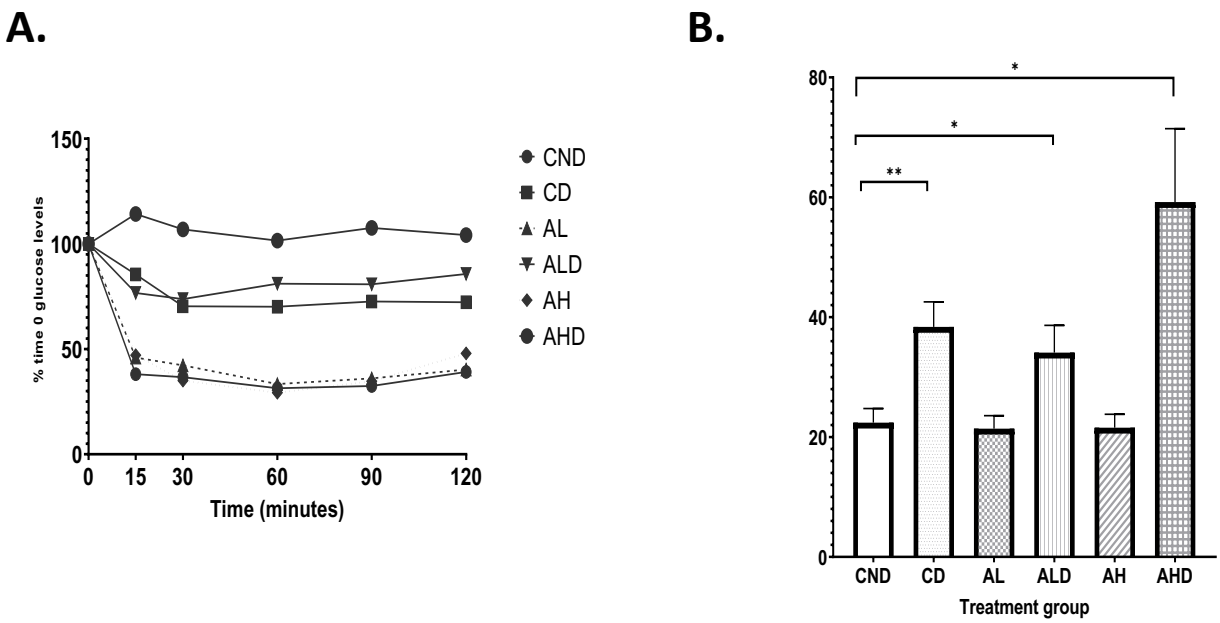

Figure 3: Insulin tolerance test (**A**) and its area under the curve (**B**)**.** CND: control no dex; CD: control with dex; AL: low dose arsenic; ALD: arsenic low dose with dex; AH: high dose arsenic; AHD: high dose arsenic with dex. **A**: for CND, AL, AH: n = 12; for CD: n = 11; for AHD: n = 10; for ALD: n = 9. **B**: n = 6 for all exposure groups. *p-value <0.05; **p-value < 0.01.

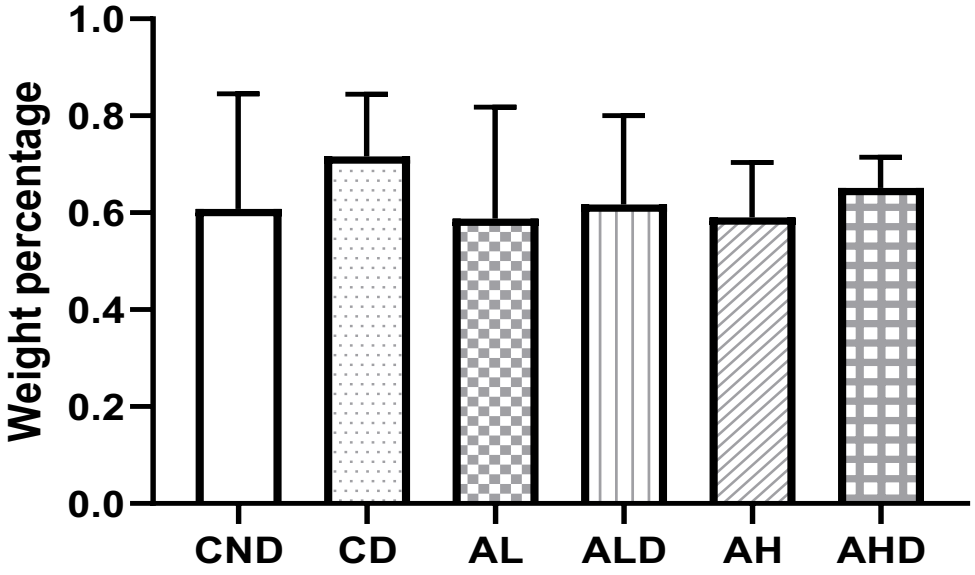

Figure 4: Pancreas tissue as weight percentage of total body weight. CND: control no dex; CD: control with dex; AL: low dose arsenic; ALD: arsenic low dose with dex; AH: high dose arsenic; AHD: high dose arsenic with dex. For CND, CD, AL, AH, AHD: n = 12; for ALD: n = 11.

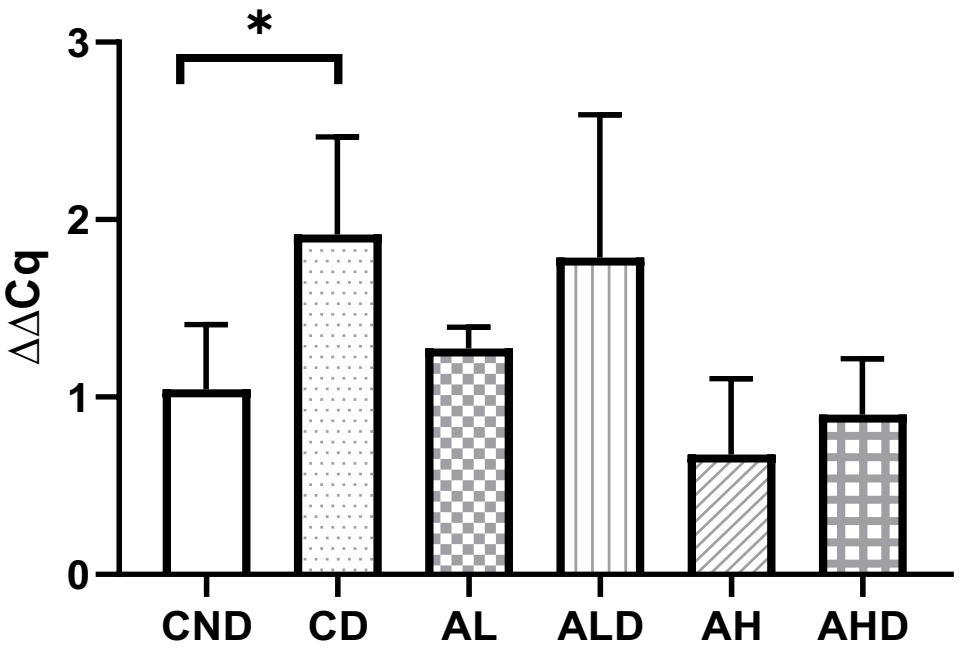

Figure 5: Gene expression by ΔΔCq CND: control no dex; CD: control with dex; AL: low dose arsenic; ALD: arsenic low dose with dex; AH: high dose arsenic; AHD: high dose arsenic with dex. For CND and AH: n = 6; for CD, AL, ALD, AHD: n = 5. groups. *p-value <0.05.

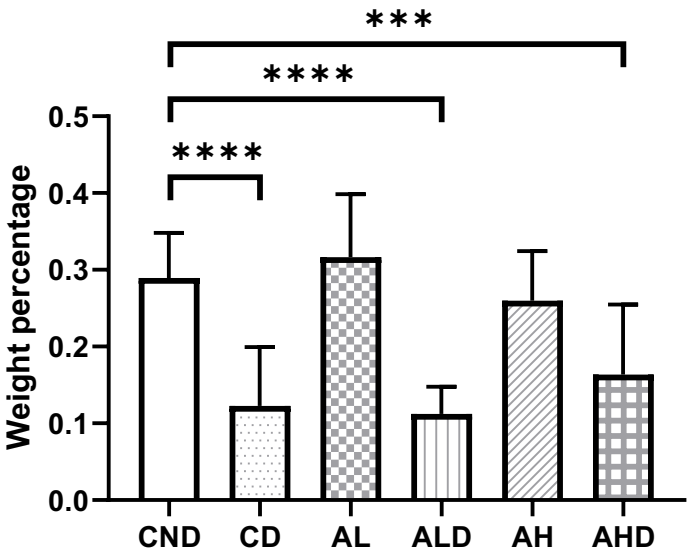

Figure 6: Spleen tissue as weight percentage of total body weight. CND: control no dex; CD: control with dex; AL: low dose arsenic; ALD: arsenic low dose with dex; AH: high dose arsenic; AHD: high dose arsenic with dex. For CND, CD, AL, AH, AHD: n = 12; for ALD: n = 11. ***p-value < 0.005; ****p-value < 0.0001

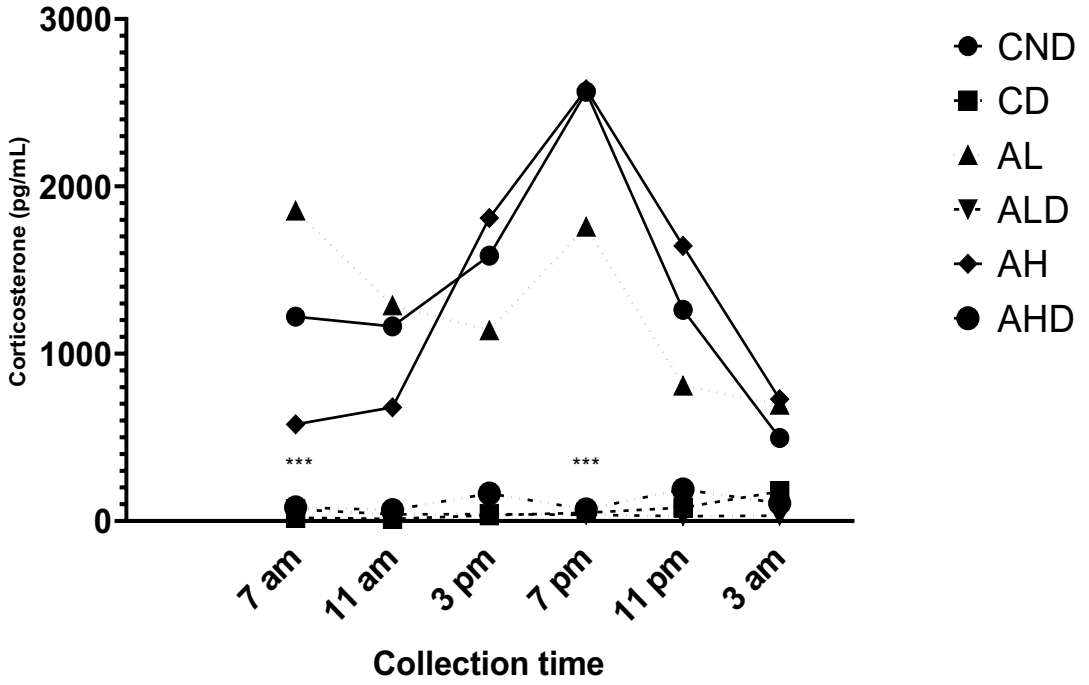

Figure 7: Corticosterone measurements every four hours over a 24-hour period. CND: control no dex; CD: control with dex; AL: low dose arsenic; ALD: arsenic low dose with dex; AH: high dose arsenic; AHD: high dose arsenic with dex. **A**: for CND, AL, AH: n = 12; for CD: n = 11; for AHD: n = 10; for ALD: n = 9. **B**: n = 6 for all exposure groups. ***p-value < 0.005

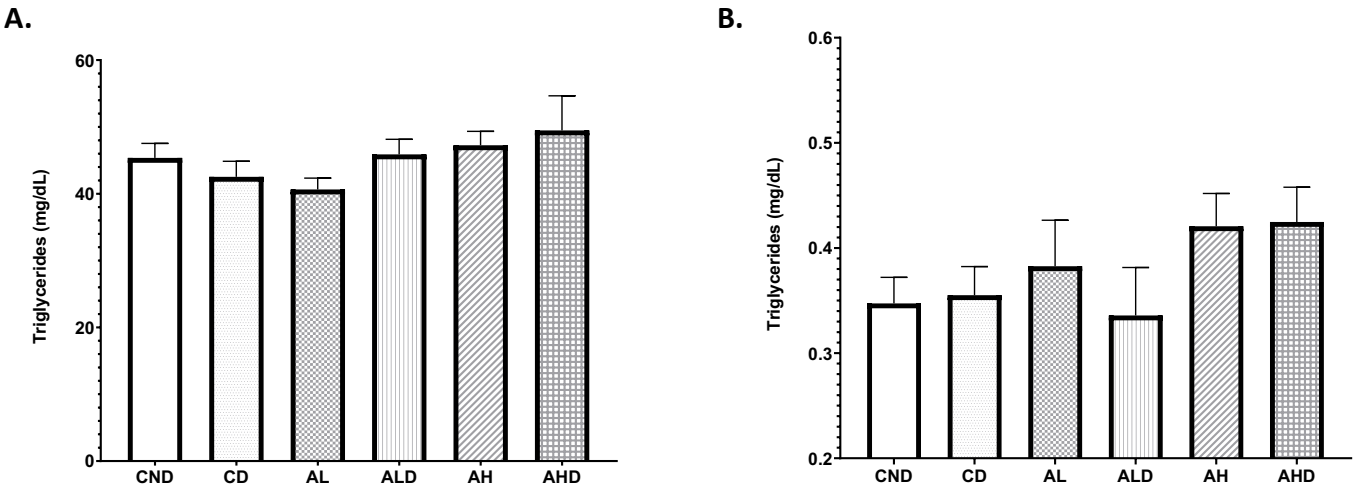

Figure 8: Plasma (**A**) and liver (**B**) triglycerides. CND: control no dex; CD: control with dex; AL: low dose arsenic; ALD: arsenic low dose with dex; AH: high dose arsenic; AHD: high dose arsenic with dex. **A**: for CND, AL, AH: n = 12; for CD: n = 11; for AHD: n = 10; for ALD: n = 9. **B**: n = 6 for all exposure groups.

Chapter IV: Conclusions

The objective of this dissertation is to explore the combinatorial effects of arsenic and the lifestyle factors: diet and stress.

Chapter I serves as the purpose for the subsequent studies. The past arsenic exposure in Antofagasta, Chile combined with current exposures across the globe of all exposure levels are the inspiration to study arsenic and its negative effects on the human body's processes. Because it is not always feasible to use a human model to study arsenic's interactions with various organs, the mouse model serves as a apt alternative, one that is more adaptable and controllable. The subsequent chapters explore arsenic in two different exposure scenarios: one with a co-exposure of a high fructose diet and their combinatorial effects on fatty liver progression and the other explores arsenic's interaction with the glucocorticoid receptor and its ability to antagonize the receptor.

Chapter II demonstrates the importance of diets and negative health effects. While we did not see our predicted results of worsening glucose homeostasis as other studies had (Ditzel et al., 2016; Paul et al., 2011) and that is often associated with fatty liver diseases, we did see hepatic hypertriglyceridemia. We found this is attributed to dysregulation of the *ApoA5* gene as well as the mitochondrial gene, *CO1*. Further studies should use knockout models to determine the full effects these two genes have in this context.

Previous research has shown arsenic interact the glucocorticoid receptor, a major regulator in the body's circulating stress levels as well as a transcription fregulator of a vast number of genes (Bodwell et al., 2006; Hanoch Cassuto et al., 2005; Ronald C. Kaltreider, Alisa M. Davis, Jean P. Lariviere, & Joshua W. Hamilton, 2001). Chapter III of this dissertation further researched the developed hypothesis that arsenic possibly antagonizes the glucocorticoid receptor. To accomplish this, we added in the synthetic glucocorticoid, dexamethasone and measure markers of glucose homeostasis, altered body composition, and inflammation. While we were not able to definitively determine if arsenic antagonizes the glucocorticoid receptor, we did find altered gene expression in *PCK1*, a glucocorticoid receptor complex mediated gene. This will help direct future studies and will lead to advances in understanding one aspect of how arsenic influences the endocrine system.

Overall, this dissertation demonstrates advanced methods and knowledge in environmental, molecular toxicology combined with metabolic biology. This research will contribute to the advancement of environmental health research through its interdisciplinary approaches. The mechanistic exploration of this work will help broaden the knowledge of arsenic's ability to negatively impact the human body and adds value to research that seeks to better affected communities past and present across the globe.

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