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The Effect of Arsenic on Type 2 Diabetes and Inflammation

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Dedication

I would like to dedicate this project to my family. First, my mom for her unconditional love and support and being the strongest woman I know. Next, my grandparents who were the foundation for who I have become today with their unwavering encouragement. Finally, my husband – who has been my rock during this entire process. I would not have made it this far without all of your love and support.

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Abstract

Arsenic, a ubiquitous environmental contaminant, has been shown to cause a number of health effects. At high concentrations the inorganic form is a well-known toxin, but at lower concentrations the effects range from various cancers, to cardiovascular disease and type 2 diabetes. At higher concentrations of arsenic (500-1000µg/L) there have been epidemiological studies conducted demonstrating an increased risk in the development of type 2 diabetes with this exposure. At lower levels of arsenic exposure (<500 µg/L) the epidemiological results are inconclusive. Arsenic is also an immunotoxicant, meaning that it will cause changes in the immune response. The changes in the immune response will vary depending on a number of variables, including amount of arsenic exposure, forms of exposure and route of exposure. We wanted to determine if arsenic could modulate the immune system, and if this change could lead to an increase in susceptibility to type 2 diabetes development. We chose to examine this in C57BL/6 and db/+ mice – two non-susceptible strains. After 8 weeks (4 weeks old to 12 weeks old) of low dose inorganic arsenic exposure (50 µg/kg or 500 µg/kg) we evaluated changes in body composition, glucose tolerance and immune response. We saw that there were differences based on sex, genotype and treatment group present after the 8-week treatment period in body composition, while there were minimal changes in glucose tolerance. Finally, the immune response showed great variability depending on sex, genotype and treatment group. This project has demonstrated that while we are trying to compare differences in *in vivo* and epidemiological studies to find a link between arsenic

and type 2 diabetes, there may be deeper levels of complications based on individual variability to arsenic exposure.

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Chapter 1 - Introduction

1.1 Arsenic

Arsenic is a ubiquitous element found in the earth's crust that exists in organic and inorganic forms. Unlike other elements, organic arsenic is currently believed to have little impact on health, whereas inorganic arsenic is a known toxin. Typical arsenic exposure is about 50 µg/day, with about 3.5 µg/day coming from inorganic sources (ATSDR, 2013). Organic arsenic includes monomethylarsenous acid (MMA) and dimethylarsinous acid (DMA), which are the main forms of arsenic excreted by humans. Inorganic arsenic (iAs) is found in the pentavalent form iAs(V) or trivalent form iAs(III), with the trivalent being the most toxic form humans are exposed to (Jomova et al., 2011).

1.2 Arsenic exposure: synthetic sources

We are exposed to arsenic through both natural and synthetic routes. Arsenic has been used in medicine to treat a number of ailments including anemia, asthma, cholera and syphilis giving it the nickname 'Therapeutic Mule' (Jolliffe, 1993; Przygoda, Feldmann, & Cullen, 2001). To treat these ailments, several arsenic-containing solutions have been used, including Donavan's Solution (AsI_3), de Valagin's solution ($AsCl_3$) and Fowler's Solution (1% potassium arsenite) until more recent and less toxic medical advances were made (Przygoda et al., 2001; Scheindlin, 2005). Fowler's Solution, which is 1% potassium arsenite, was also used as an alternative to quinine for the treatment of malaria (Scheindlin, 2005). More recently, arsenic has returned to use in medicine to treat

Acute Promyelocytic Leukemia (APL) in the form of Trisenox (Scheindlin, 2005). APL has a very high relapse rate, and FDA-approved Trisenox is meant for these patients (Scheindlin, 2005). After treatment with Trisenox, 70% of previously relapsed patients were able to achieve complete remission (Scheindlin, 2005). *In vitro*, Chen et al. demonstrated that arsenic trioxide induces apoptosis in the NB4 cell line, which gives a potential mechanism for this treatment (G.-Q. Chen et al., 2003). However, future work is still required to determine whether arsenic trioxide is a treatment option for other cancers.

Arsenic-containing compounds have also been used in industry as pesticides and preservatives (Mandal & Suzuki, 2002). Chromated copper arsenate, or CCA, is the most widely used wood preservative in the world and is used to create pressure treated wood (ATSDR, 2013). While not allowed for residential use since 2003, CCA treated wood is still used for nonresidential purposes, including utility poles (Hughes, Beck, Chen, Lewis, & Thomas, 2011). However, with the ban in place it is currently unknown how CCA-treated wood contributes to total arsenic exposure (ATSDR, 2013).

In agriculture, arsenic compounds have been used in cotton fields and orchards (Hughes et al., 2011). Lead arsenate was used in apple orchards until the 1960's, when health effects in orchard workers came into question (Hughes, 2002). While lead arsenate is no longer used in agriculture, millions of acres of land are still contaminated with the pesticide and can potentially impact the health of individuals living on or near this land (Hughes et al., 2011) While inorganic arsenic can no longer be used in this capacity, organic arsenic is still widely used on cotton (ATSDR, 2013). In industry, individuals working in smelters are exposed to high levels of arsenic via inhalation. These individuals are subjected to OSHA limits of up to 10 $\mu g/m^3$ arsenic exposure, which is

orders of magnitude higher than normal daily exposure (ATSDR, 2013). The greatest health risk posed to these individuals is respiratory cancer.

1.3 Arsenic exposure: natural sources

Exposure to arsenic can occur naturally through contaminated soil, air and water/food. Arsenic in the soil is typically in the inorganic form, with exposures in the range of 0.1-40 mg/kg and average of 3-6 mg/kg (ATSDR, 2013; Mandal & Suzuki, 2002). This can differ greatly based on location, depending on the proximity to agriculture, smelteries and arsenic rich soil deposits (Hughes et al., 2011).Typically, arsenic in the soil does not cause a major problem unless the soil is being consumed. However, arsenic in the soil becomes an issue when arsenic-rich deposits are the sites of wells and water runoff into drinking water sources.

Humans are exposed to $1x10^{-3}$ to 2 $\mu g/m^3$ inorganic arsenic from air sources, but this can also vary based on location, weather, and industry presence (ATSDR, 2013). The normal exposure in an unpolluted area is 0.04-0.09 µg/day (Mandal & Suzuki, 2002). Arsenic exposure from air can also occur in certain work environments, like smelteries. In these locations, OSHA limits have been established at 10 $\mu g/m^3$, which is still orders of magnitude higher than normal daily exposure (ATSDR, 2013). The most common health concern for arsenic inhalation exposure is respiratory cancer. No other cancers have been attributed to air exposure to arsenic which could be expected as inhalational exposure also tends to begin later in life, unlike other exposures like through food and water, which can be lifelong (Enterline, Day, & Marsh, 1995).

Food is the primary route of exposure to organic arsenic as well as high levels of inorganic arsenic. The range of exposure from contaminated food is 20-140 ppb, which is

a combination of both inorganic and organic species (ATSDR, 2013). Seafood consumption is associated with increased levels or organic arsenic exposure, including arsenobetaine and arsenocholine, but has not been shown to be associated with carcinogenicity (Borak & Hosgood, 2007). Inorganic arsenic in food typically is highest in chicken, rice, and apples, and there are few to no regulations in place to limit the concentrations people are exposed to (Navas-Acien & Nachman, 2013). Apples and apple juice are susceptible to arsenic contamination because, while arsenic has been banned for use as a pesticide, persistent remnants in orchard soils from previous use are rapidly taken up by these fruit trees (D. Wilson, Hooper, & Shi, 2012). In a study surveying the amount of arsenic and lead in common grocery store apple juice brands, the range of arsenic was 3.5-24.8 µg/L, the upper range of which is above the acceptable range of arsenic in public drinking water. Rice can accumulate arsenic if it is grown in areas with high arsenic in the soil or if it is cooked in water with high arsenic contamination According to the Consumer Report, levels of inorganic arsenic per serving in rice and cereals can range from 1.3-9.6 ppb (Consumer, November, Reports, Agency, & York, 2012). This is especially dangerous as rice is frequently used in children's cereal and snacks where multiple servings are consumed daily. The health impacts from consuming arsenic in food are the same as those from other exposure sources, including cancer, cardiovascular disease (CVD) and possibly type 2 diabetes (Davis et al., 2012).

Arsenic in drinking water has been a part of major regulatory changes in the past decades. In the United States, arsenic levels in public drinking water have been regulated by the Environmental Protection Agency (EPA), with a reduction from 50 µg/L to 10 µg/L occurring in 2001 because of its carcinogenicity (ATSDR, 2013). However, the

range of arsenic exposure in drinking water in the United States is vast, from 0-10 µg/L in public drinking water to 800 µg/L in private wells. In other countries such as Bangladesh and Taiwan, the concentration of arsenic in drinking water can be well over 1000 µg/L. Arsenic at the levels in Bangladesh and Taiwan has contributed to the development of skin lesions (arsenicosis) and a number of cancers, including skin, lung, kidney bladder and liver (ATSDR, 2013); however, in the United States, a study found no clear association between drinking water levels of ~100 µg/L and bladder cancer (Steinmaus, Yuan, Bates, & Smith, 2003).

Besides cancer, high levels of arsenic have been attributed to a number of chronic diseases. Argos et al. demonstrated in a range of arsenic exposures (0.1-864 µg/L) approximately 22% of deaths associated with chronic disease could be attributed to arsenic exposure greater than 10 µg/L drinking water in Bangladesh (2010). This study used repeated measurements of total arsenic in urine over time in individuals, and thus were able to demonstrate that decreasing arsenic exposure for a short period of time did not reduce individual risks for disease. Sohel et al. examined non-accidental mortality in Bangladesh caused by arsenic exposure and the increase for non-accidental death was noted even at the lowest levels of exposure (10-49 µg/L) (2009). There was also an increase in death from arsenic associated cancer, CVD, and infection in this population.

Finally, a number of epidemiological studies have been conducted examining the association between arsenic and type 2 diabetes. In areas such as Bangladesh and Taiwan, where arsenic levels in drinking water are high, there have been a number of studies demonstrating a positive correlation between arsenic exposure and type 2 diabetes risk (Nabi, Rahman, & Islam, 2005; M. Rahman, Tondel, Ahmad, & Axelson, 1998; Tsai,

Wang, & Ko, 1999; C.-H. Tseng, Chong, Heng, Tseng, & Tai, 2000; S. L. Wang et al., 2003, 2007). At lower exposures, for example in the United States where public drinking water is heavily regulated, the literature is mixed; several studies that have found a correlation between arsenic exposure and type 2 diabetes (Kuo et al., 2015; Lewis, Southwick, Ouellet-Hellstrom, Rench, & Calderon, 1999; Meliker, Wahl, Cameron, & Nriagu, 2007; Navas-Acien, 2008), while several other studies have found a weak correlation between arsenic and type 2 diabetes (Gribble et al., 2012; James et al., 2013; N. H. Kim et al., 2013) or no correlation at all (Steinmaus, Yuan, Liaw, & Smith, 2009; Zierold, Knobeloch, & Anderson, 2004). Unfortunately, because of the wide range of variables between studies, no common results have been produced. These epidemiological studies, along with *in vivo* and *in vitro* studies attempting to find a link between arsenic and type 2 diabetes will be reviewed later in this section.

1.4 Arsenic metabolism

The first step of arsenic metabolism occurs in the blood. Absorbed iAs(V) is rapidly reduced to iAs(III) at least partially in the blood, from where it is distributed to tissues and taken up by cells (ATSDR, 2013). The primary source of arsenic methylation occurs in the liver; however, a couple of different theories exist to fully explain the methylation of inorganic to organic arsenic. Methylation can occur through the classical pathway [As(V)→As(III)→ MMA(V)→MMA(III)→DMA(V)→DMA(III)] which is catalyzed solely by Arsenic (III) methyltransferase (As3MT) (Thomas et al., 2009). Alternatively, iAs(III) is conjugated to glutathione (GSH), forming several arsenic:glutathione intermediates, however the end results are the same (Kumagai & Sumi, 2007). Reviewed by Hughes et al., a number of early experiments helped solidify

the function and location of S-adenosylmethionine (SAM) and GSH in the methylation of arsenic in the liver (Hughes et al., 2011). Healy et al. examined arsenite methyltransferase activity in B6C3F1 mice exposed to 0, 25 or 2500 µg/L arsenic for either 32 or 91 days in the liver, testis, kidney and lung (Healy, Casarez, Ayala-Fierro, & Aposhian, 1998). They found that the methyltransferase activity varied based on tissue, with the testes having the highest level of activity, followed by kidney, liver, and lung, respectively, independent of time or concentration of arsenic exposure.

The final step of arsenic metabolism is either storage in body tissues or excretion. Small quantities of arsenic are stored in body tissues along with keratin-rich tissues including hair and nails (Weir, 2002). The majority of arsenic is excreted in urine within 3 days, however a small percentage is excreted in breast milk (M Vahter, 2008) and/or feces (Mandal & Suzuki, 2002). General proportions of urinary arsenic metabolites are 40-75% DMA, 20-25% iAs and 15-25% MMA as demonstrated in Figure 1.1, however a number of variables will alter these amounts including exposure time, dose, route of exposure, and arsenic species; sex and age of the individual (ATSDR 2013). Calderon et al. examined changes in arsenic metabolism over a five-day period in a cohort consisting primarily of members of the Church of Jesus Christ of Latter-Day Saints (1999). Day-to-day variation in arsenic excretion was low and no sex differences were reported, but they did find differences in arsenic excretion based on age. This can be compared to the study by Concha et al. that examined blood and urine arsenic levels in children and women in three villages in northern Argentina (1998). Blood arsenic levels were 10x higher in both women and children living in exposed villages (~200 µg/L in water) compared to unexposed villages (1 µg/L). Urine arsenic levels were 30x higher in both women and

children in the exposed area. Differences were noted in one of the villages where children had a higher percentage of iAs and women had a much higher percentage of DMA, but MMA was similar. These differences between adults and children could be due to methylation deficiency, which will lead to age differences in arsenic metabolism

While the majority of studies have focused on MMA(V), Aposhian et al (2000) found a dose-response relationship for arsenic in drinking water and urinary MMA(III). MMA(III) is more toxic than inorganic arsenite, demonstrating that the methylation step may not be a detoxification process of iAs. MMA(V) reductase is a rate-limiting enzyme of the inorganic arsenite biotransformation pathway. When MMA(V) reaches critical levels in the cell, it will be converted to MMA(III), so higher levels of exposure will have higher levels of MMA(III) (Aposhian et al., 2000). This leaves a new variable for epidemiological studies to consider in the analysis of urinary arsenic metabolites.

1.5 Mechanisms of arsenic

There are a number of proposed mechanisms for arsenic toxicity, including enzyme inhibition, generation of reactive oxygen species (ROS) and epigenetic mechanisms (X. Wu et al., 2016). While pentavalent arsenic is thought to be less toxic, possible mechanisms include replacing phosphate in essential biochemical reactions (X. Wu et al., 2016). It can replace glucose-6-phosphate and 6-phosphogluconate *in vitro*, replace phosphate in the sodium pump, diminish formation of adenosine triphosphate (ATP) by replacing phosphate and deplete ATP in cellular systems (ATSDR, 2013). Arsenic (III) has the ability to inhibit multiple important enzymes by binding to the sulfhydryl group (X. Wu et al., 2016). This alone has many downstream implications, as

enzymes affected include but are not limited to kinases, phosphatases and caspases (Miller, Schipper, Lee, Singer, & Waxman, 2002).

Arsenic (III) exposure also causes production of different forms of oxygen species including H_2O_2 and OH radicals (X. Wu et al., 2016). The production of these reactive oxygen species can cause inhibition of a number of cellular processes including DNA repair, signal transduction and genotoxicity (X. Wu et al., 2016). The disruption of these cellular processes is thought to be one of the main mechanisms behind the carcinogenesis of arsenic exposure (X. Wu et al., 2016). Arsenic (III) can also cause DNA damage because of its ability to replace phosphate, causing errors in DNA repair (Mandal & Suzuki, 2002).

1.6 Type 2 Diabetes

Diabetes is a worldwide problem affecting 415 million people as of 2015, with the number expected to increase to 642 million by the year 2040 (Federation, 2015). Type 1 diabetes is characterized by a deficiency of the body to produce insulin, caused by an autoimmune disorder that causes pancreatic beta cell destruction (Federation, 2015; World Health Organization, 2016). Onset of the disease appears typically in childhood and is managed by lifelong insulin administration (Federation, 2015). Type 2 diabetes is the most common form, and is characterized by insulin resistance leading to high blood glucose (Federation, 2015). Perhaps the most challenging aspect of type 2 diabetes is that the disease can go undiagnosed for many years, with approximately 193 million people being affected and unaware (Federation, 2015). A number of different factors have been attributed to the development of type 2 diabetes, many of them being lifestyle management including poor nutrition, obesity, along with genetics and aging (Federation,

2015; World Health Organization, 2016). However, we are now noticing a greater increase in the number of type 2 diabetes cases than can't be attributed to these factors alone, making room for the possibility of environmental effects (Jeon, Ha, & Kim, 2015). A number of studies have examined the effects of environmental contaminants on the progression of type 2 diabetes, including effects of persistent organic pollutants, bisphenol A and metals (Jeon et al., 2015). There is growing evidence that areas exposed to high levels of arsenic also show increased incidence of type 2 diabetes, which will be reviewed in the next section.

1.7 Review of Arsenic and Type 2 Diabetes Epidemiological Studies

A number of epidemiological studies have examined the possible link between iAs exposure and type 2 diabetes, specifically iAs contamination of drinking water. In some areas of Bangladesh and Taiwan, people are exposed to greater than 1000 µg/L, and these areas not only have a high correlation between arsenic exposure and black foot disease but also type 2 diabetes (Abernathy et al., 1999; Kuo, Moon, Thayer, & Navas-Acien, 2013; Navas-Acien et al., 2006; W. Wang, Xie, Lin, & Zhang, 2014). As the levels of arsenic drop to low or moderate exposure, the correlation is not as clear, as is evident in Table 1.1. This can be because there is no clear mechanism discovered yet between arsenic exposure and type 2 diabetes, but also a number of studies have very different variables and exposure levels which makes drawing conclusions across the board impossible. In my review of the epidemiological studies available from 1998-2016, represented in Table 1.1, I excluded any studies that did not include original data, were not related to arsenic exposure in drinking water, and lacked outcomes related to diabetes or glucose metabolism.

The range of exposure between all of the epidemiological studies is vast, including regulated drinking water (0-10 μg/L) in the United States to the tube well exposure in Bangladesh and Taiwan as high as 1400μg/L. Some studies failed to report arsenic exposure, only reporting total urinary arsenic concentration as an indicator of exposure (N. H. Kim et al., 2013; Y. Kim & Lee, 2011a; Navas-Acien, 2008; Ruiz-Navarro, Navarro-Alarcon, Lopez Gonzalez-de la Serrana, Perez-Valero, & Lopez-Martinez, 1998; Steinmaus et al., 2009; S. L. Wang et al., 2007). While informative, these studies lack the appropriate information to determine how their outcomes were directly related to levels of arsenic exposure.

Urinary arsenic was the primary biomarker used for arsenic exposure in the epidemiological studies. Compared to blood arsenic, which has a half-life of 1-4 hours, urinary arsenic has a half-life of 4 days (NRC, 2001). Other possible biomarkers of arsenic exposure include hair and nails as arsenic has a high affinity for keratin, and can represent months to a year of exposure, respectively (NRC, 2001). It is difficult to speciate arsenic from hair and nails and arsenic contamination may lie on the surface of these biomarkers and must be removed before analysis is performed to avoid falsely elevated detections (ATSDR, 2013; Mandal, Ogra, & Suzuki, 2003; Maull et al., 2012). Mandal et al. did show that through the use of inductively coupled plasma mass spectrometry (ICP-MS) it is possible to determine arsenic metabolites in these tissues, including iAs(III), iAs(V), DMA(III) and DMA(V). However, urinary arsenic has been the preferred marker for the majority of studies.

To give an accurate representation of total urinary arsenic, dilution of urine is typically accounted for through the concentration of creatinine in urine. This can be

calculated two ways – either by measuring µg urinary arsenic per gram of urinary creatinine or by µg of urinary arsenic per liter of urine and measuring urinary creatinine as a separate variable (Maull et al., 2012). The second method is the preferred method because of the wide variety of individual variation in creatinine levels, however many studies use both of these methods (Maull et al., 2012). Another issue in the use of creatinine to determine urine dilution is that in diabetics, creatinine tends to be lower because of increased glomerular filtration and increased water intake that leads to increased urine dilution (Maull et al., 2012).

Studies also differ in the method used to diagnose diabetes. The options used include self-report through the use of diabetic medication, physician diagnosis, fasting blood glucose greater than 126 mg/dL or death certificate. Most studies use fasting blood glucose of >127 mg/dL, but fasting times varied from 8-16 hours, with some studies requiring dietary restrictions leading up to the exam (i.e. no seafood, as seafood is the main source of organic arsenic exposure).

A number of studies also differ in the variables considered when determining total urinary arsenic and diabetes. These variables include age, sex, ethnicity, body mass index, education, smoking, alcohol consumption and seafood consumption. These differences in population are very clear when comparing Meliker et al. and Lewis et al (2007). These studies appear to be relatively similar, both populations living in the United States, relying on death certificates to determine whether arsenic played a role in increased type 2 diabetes mortality, and both populations were exposed to below an average of 200 µg/L arsenic. However, the cohorts in each of these studies were very different, with over 2000 individuals included in the Lewis study who were all Mormon

whereas in the Meliker study, the cohort was significantly larger with over 80,000 individuals living in a more urban setting. These differences alone could contribute to the differences in results; Lewis reported few incidences of type 2 diabetes-related deaths and Meliker reported higher mortality rates associated with type 2 diabetes.

Two studies from the United States that used similar populations to evaluate different end points were Gribble et al. and Kuo et al. (2012, 2015). Each of these studies was based on populations from tribal areas of Arizona, Oklahoma, North and South Dakota as a part of the Strong Heart Study. These locations have similar populations that have low migration to other areas, which would keep the record of arsenic exposure consistent. They also all fall below 61 µg/L for the range of arsenic exposure. Both studies evaluated total urinary arsenic in relation to type 2 diabetes risk. Gribble found that there was no association between urinary arsenic and diabetes risk in patients without diabetes or with controlled diabetes. However, there was an association present in those who participated in the study with uncontrolled diabetes, which was defined as Hemoglobin A1c (Hb1Ac) ≥ 8%. Evaluating another endpoint, Kuo et al. determined that there was a difference in type 2 diabetes risk depending on the breakdown of arsenic metabolites in the urine. A higher percentage of MMA was associated with a decreased risk of type 2 diabetes.

Both Navas-Acien et al. and Steinmaus et al. used data from the 2003-2004 National Health and Nutrition Examination Survey (NHANES) study, so the populations were exactly the same (2008, 2009). However, each study accounted for inorganic arsenic in total urinary arsenic differently, which was heavily evaluated by Longnecker (2009). Steinmaus found no significant association in the same group of participants

when urinary metabolites that could come from seafood are excluded. Navas-Acien found a positive correlation between urinary arsenic and type 2 diabetes, and instead of simply subtracting organic urinary metabolites also noted that while DMA primarily comes from iAs exposure, seafood can also contribute to DMA levels.

The remaining studies from the United States had relatively low numbers of participants and were mixed in their results. Both Kim et al and James et al found mild associations for the risk of type 2 diabetes with low arsenic exposure (2013, 2013). Zierold et al. however found no association (2004). Both Kim et al. and James et al., required medical examination for participants, meaning individuals were diagnosed diabetic either by self-diagnosis with a medical follow up or by glucose tolerance test, where Zierold was only with self-diagnosed, lacking medical follow-up. These differences in methods could lead to inconsistencies in diabetes reports at the conclusion of studies.

Another issue with study design is that a number of the studies take place within the same area, meaning that population overlap can be a problem. For example, the studies that originated from Taiwan (Tsai et al., 1999; C.-H. Tseng et al., 2000; S. L. Wang et al., 2003, 2007) all came from populations in the same area, which may skew the only positive correlations between arsenic and diabetes found in these studies. The same could be said for the studies that came out of Bangladesh (Y. Chen et al., 2010; Islam et al., 2012; Nabi et al., 2005; M. Rahman et al., 1998). However, Chen et al. found no evidence that type 2 diabetes was associated with arsenice exposure in the Bangladesh population (2010). This study had large participation (n=11,319) and a wide range of arsenic exposures, but the number of diabetes cases present was approximately 2% of the

group. It is impossible to account for all of these variables within each study, however this is a clear example of how streamlined variables need to be accounted for in epidemiological studies.

1.8 Review of Arsenic and Type 2 Diabetes *in Vivo* Studies

Attempting to link the effects of arsenic and type 2 diabetes *in vivo* has had many of the same complications that are present in epidemiological studies. These include, but are not limited to: animal model used, differences in arsenic species, duration of exposure, route of exposure and sampling methods. A number of animal models have been used to investigate the potential role of arsenic on type 2 diabetes development, including mice, rats, hamsters and guinea pigs. However, each of these animals metabolize arsenic slightly differently from each other as well as humans. When evaluating urinary metabolite breakdown, mice are the most similar to humans and one of the better options for arsenic studies, with hamsters following up as a second choice (Mitchell, Ayala-fie, & Carter, 2000). Rats are not suitable models as these models sequester arsenic in red blood cells, which is very different from human metabolism. As such, only experimental studies evaluating arsenic exposure and diabetic endpoints in mice will be reviewed.

In Table 1.2, it is apparent that a wide range of arsenic concentrations have been used in mice, from 50 µg/L to $5x10^4$ µg/L, which is orders of magnitude higher than human exposures. However, higher concentrations of arsenic can be justified in mouse studies because mice are known to have an overall faster metabolisms compare to humans.

The most common strain of mice used was C57BL/6, which could be because these mice, when fed a high fat diet, are excellent models for obesity and diabetes. Also used were ICR mice, which is an outbred strain, B6C3F1 mice that are a hybrid between

C57BL/6 females and C3H males, and Swiss albino mice which are frequently used in toxicology studies. One study also used diabetic C57BKS/Lep^{db} (db/db) mice and the heterozygote (db/+) as a control (Liu et al., 2014). While the study by Huang et al. included a non-diabetic variable (estrogen), it was essential to include this in the analysis, as it is the only study that used female mice (2015). This is because it has been demonstrated that there are differences in metal toxicity between males and females in not only arsenic, but other metals as well (Marie Vahter, Åkesson, Lidén, Ceccatelli, & Berglund, 2007)

Each study had a difference range and duration of arsenic exposure, ranging from a single acute exposure to 20 weeks of chronic exposure. The most common route of exposure was drinking water, although Mitchell et al. used intraperitoneal injection (2000). A major problem with using arsenic in drinking water is that iAs(III) can oxidize, so water must to be changed frequently. Another problem with arsenic in drinking water is that depending on the number of mice in a cage, there was no way to definitively determine how much arsenic each individual mouse drank. Finally, in some studies the mice with arsenic in the drinking water actually consumed less water than the controls (D S Paul, Devesa, & Hernandez-Zavala, 2008; David S. Paul, Hernandez-Zavala, et al., 2007; David S. Paul, Walton, Saunders, & Styblo, 2011) which caused a reduction in the overall arsenic exposure. Paul et al. concluded that because of the reduced arsenic consumption in their low fat diet (LFD) treated mice, concentrations well above $5x10^4$µg/L would have to be used to produce enough arsenic accumulation in tissues to produce a change in glucose tolerance toward a diabetic phenotype (2011).

Methods to evaluate glucose tolerance also varied from study to study (Table 1.2) with fasting times ranging from 5 to 14 hours. Glucose bolus was either 1 g/kg or 2 g/kg body weight with either oral glucose tolerance test (OGTT) or intraperitoneal glucose tolerance test (IPGTT) was used to determine glucose tolerance, and glucose measurements were typically taken at 0, 15, 30, 60, 90, and 120-minutes post glucose bolus. The different conditions behind the oral glucose tolerance test have been tested to determine ideal conditions (Andrikopoulos, Blair, Deluca, Fam, & Proietto, 2008). Fasting for long periods of time (i.e. overnight) resulted in high fat and chow fed mice having no differences in basal glucose and insulin levels, but 6 hours of fasting resulted in a clear difference in glucose tolerance comparing high fat and chow fed animals (Andrikopoulos et al., 2008). When comparing IPGTT and OGTT, IPGTT has a 10-20% rate of error if the needle punctures the intestines or stomach, as well as differences in glucose profiles. Plasma glucose levels are lower in the OGTT and IPGTT plasma insulin increases at a slower rate (Andrikopoulos et al., 2008). Finally, they also examined the amount of glucose to give mice, and when comparing 0.5 g/kg, 1 g/kg and 2 g/kg glucose, only the 2 g/kg dose made a clear difference between chow and high fat diet.

With all of these variables between *in vivo* studies, only Mitchell et al. failed to report arsenic having an impact on glucose tolerance (2000). A number of studies did report issues with water consumption in the arsenic treated groups (D S Paul et al., 2008; David S. Paul, Hernandez-Zavala, et al., 2007; David S. Paul et al., 2011), which resulted in lower overall exposures to arsenic. Finally, since the majority of studies use only male mice (Huang et al. is one exception since they used female mice), this did not allow for

an evaluation of whether there are sex-based differences in arsenic exposure, metabolism and glucose tolerance.

1.9 Review of Arsenic and Type 2 Diabetes *in Vitro* Studies

A number of *in vitro* studies have been conducted to provide a possible mechanism behind arsenic and type 2 diabetes in mouse adipocytes, focusing primarily on insulin-stimulated glucose uptake (ISGU). One problem caused by arsenic that has been evaluated by a number of studies is the inhibition of differentiation into adipocytes (Trouba, Wauson, & Vorce, 2000; Z. X. Wang et al., 2005; Wauson, Langan, & Vorce, 2002). This can be caused by exposure to arsenic reducing adipocyte protein 2 (aP2), peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein alpha (C/EBPα), which are all important adipocyte markers (Wauson et al., 2002). Also in 3T3-L1 adipocytes, 3uM arsenic trioxide was shown to inhibit PPARγ and retinoid x receptor alpha (RXRα), as well as inhibiting interactions between serine/threonine kinase Akt (or protein kinase B -PKB) and PPARγ, halting differentiation in a reversible manner (Z. X. Wang et al., 2005). Hou et al., 2013, also showed a reduction in adipogenesis caused by inducing the endoplasmic reticulum (ER) stress response and upregulated CCAAT/enhancer-binding protein homologous protein 10 (CHOP10), which will inhibit CCAAT/enhancer-binding protein beta (C/EBPβ) and suppress adipogenesis.

Another problem caused by arsenic is the inability of glucose transporter type 4 (GLUT4) to translocate to the cell membrane, which does not allow for glucose uptake by the cell (David S. Paul, Harmon, Devesa, Thomas, & Styblo, 2007; Walton et al., 2004; Xue et al., 2011). A number of possible arsenic targets have been identified,

including PKB/Akt, which can be reduced by arsenic exposure (Walton et al., 2004) and downregulation of GLUT4 expression (Xue et al., 2011).

All of these problems can also be attributed to the induction of oxidative stress in the adipocytes by arsenic. Salazard et al. demonstrated an increase in Heme oxygenase 1 in adipocytes treated with arsenic, which is involved in oxidative stress along with an increase in hypoxia-inducible factor 1 alpha (HIF1α) expression (2004). NAD-dependent deacetylase sirtuin-3 (Sirt3) expression and target proteins forkhead box P3a (FOXO3a), manganese superoxide dismutase (MnSOD), and peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) were also reduced in long term, low level arsenic exposure (Divya et al., 2015). These studies support the idea that arsenic can induce oxidative stress, which can possibly be a cause of insulin resistance.

While these studies are very promising at providing potential mechanisms between arsenic and type 2 diabetes, unfortunately none of them have been supported *in vivo* or in a human system.

1.10 Arsenic and Inflammation

Finally, we will examine the effects of arsenic on the immune system. Depending on the concentration and species of arsenic, very different impacts occur on the immune system. When taking this into consideration in the context of diabetes, a disorder that is normally associated with higher body mass index (BMI), another layer of inflammation is added. The baseline inflammatory profiles between lean and obese individuals are very different, so it would be expected that exposure to arsenic would impact these scenarios very differently (Ferrante, 2007). The effects of arsenic on inflammation in humans and mice have been extensively reviewed by (Dangleben, Skibola, & Smith, 2013; Mandal &

Suzuki, 2002; Tobergte & Curtis, 2013). According to these sources, arsenic can not only affect gene expression, but cell population, apoptosis, and ROS production in a number of different immunological cell types. Because of the role of macrophages in obesity and the differences in macrophages present in lean vs. obese individuals, we wanted to explore the effects of arsenic on these immune cells in greater detail.

In humans, the majority of the studies have examined the effects of arsenic on circulating peripheral blood mononuclear cells (PBMCs), not directly on macrophages. In these studies, a number of different results have been seen depending on the concentration of arsenic in question. In Bangladesh, a study examining 16 individuals, 5 control and 11 with skin lesions, reported downregulation of important immunological genes, such as tumor necrosis factor alpha (TNF-α), expressed by PBMCs in circulation (Argos et al., 2006). Another study in Mexico with 10 individuals, 5 control and 5 having urinary arsenic concentrations ranging from 117.23-435.12 mg/g creatinine, demonstrated that arsenic exposure correlates to suppression of a number of inflammatory genes (Salgado-Bustamante et al., 2010). Both of these studies are contradicted by a third study from Taiwan, with 24 individuals exposed to a range of different concentrations of arsenic up to 46.5 µg/L (M.-M. Wu, Chiou, Ho, Chen, & Lee, 2008). This study documented an increase in gene expression in interleukin 1-beta (IL-1β), interleukin 6 (IL-6), chemokine ligand 2 (CCL2) and cluster of differentiation 14 (CD14), all of which would indicate an increase in a pro-inflammatory immune environment.

When comparing the effects of arsenic on macrophages in mice and humans the results are very similar. Studies reported reduced macrophage adhesiveness, reduced

cytokine response, and reduced nitric oxide production (Banerjee et al., 2009; Bourdonnay et al., 2009; Sengupta & Bishayi, 2002; Srivastava et al., 2013). However, in one study with PBMC-derived macrophages exposed to arsenic, as little as 1 μM As_2O_3 caused an exacerbated response to LPS as measured by TNF-α and interleukin 8 (IL-8) mRNA production (Lemarie, Morzadec, Bourdonnay, Fardel, & Vernhet, 2006). Studies in mice were performed either in peritoneal macrophages that were subsequently exposed to arsenic, mice exposed to arsenic peritoneally then isolating macrophages from spleen, or from strict cell culture using RAW264.7 cells (Sakurai et al., 2004; Sengupta & Bishayi, 2002; Srivastava et al., 2013). In studies using sub-toxic levels of arsenic, diminished macrophages functions as well as decreases in cytokines were present including TNF-α, IL-1β, IL-6, transforming growth factor beta (TGF-β) and IL-10 (Sengupta & Bishayi, 2002; Srivastava et al., 2013). In another study, cytotoxic doses of arsenic were used and an increase in TNF-α was reported (Sakurai, Kaise, & Matsubara, 1998).

Overall, a number of the studies investigating the effect of arsenic on macrophages demonstrate the ability of the toxicant to reduce macrophage function, diminish cytokine production and reduce nitric oxide production. The greatest differences in study design occur in the concentration of arsenic used (cytotoxic vs non-cytoxic) or in the method of arsenic exposure (PBMCs exposed to arsenic *in vitro* or PBMCs exposed to arsenic *in vivo* then analyzed). As demonstrated in the epidemiological and *in vivo* studies examining effects on arsenic, any small change between conditions can produce very different results.

1.11 Our Study in Arsenic and Type 2 Diabetes

In our study, we chose to use C57BL/6 mice along with the diabetic C57BKS/Lep^{db} db/db and db/+ mice, however because of breeding issues we were left with a large number of db/+ mice. These are considered the control in a number of diabetes studies, so we wanted to see how these mice would compare to the background C57BL/6 mice. We also chose to use both males and females in this study to determine whether sex differences impacted the effects of arsenic exposure.

We chose to use $AsCl_3$ for safety reasons, as it was the only option in solution that was available. When added to water, it dissociates into iAs(III) and HCL at negligible amounts. We used 0, 50 or 500 µg/kg concentrations and chose to deliver this by oral gavage instead of in drinking water because we could guarantee each individual mouse would receive the desired amount of arsenic. These concentrations also represent low level exposure, as 50 µg/kg in a 20g mouse is a final exposure of 1 µg every other day – which according to Table 1.3 is lower than most *in Vivo* studies have examined. The second concentration, 500 µg/kg, is a final exposure of 10 µg every other day in a 20g mouse, which while high is still not out of the range of previously studied concentrations of arsenic in mice (Table 1. 3). Finally, for the *in vitro* study, we chose to use Raw 264.7 macrophages to determine the effect of arsenic on inflammation. Macrophages are an important component of obesity and a number of studies have examined the effects of PBMCs, but have not looked at the effect directly on macrophages.

Our goal for this study was to evaluate in a diabetic model the effects of low dose arsenic exposure on the progression of type 2 diabetes development. We could claim that we were measuring progression of the disease by the time point analysis of the dual

energy X-ray absorptiometry (DEXA) scan and OGTT, which would have told us if glucose tolerance was worsening over the period of arsenic treatment or if obesity was increasing. We wanted to start the mice at a young enough age that obesity and glucose dysregulation had not yet begun to be apparent. Our hypothesis based on the background information provided was that arsenic exposure would increase inflammation, leading to a diabetic phenotype in non-susceptible individuals.

Table 1.1 Summary of Studies Examining Arsenic Exposure in Humans.

Reference	Location, number of participants	Range of Exposure	Endpoint	Conclusion
(Y. Chen et al., 2010) **Cross-section**	Bangladesh n = 11,319	Range = 0.1-864 µg/L	Total urinary arsenic and HB1AC levels	No association between arsenic exposure and T2D
(Coronado-Gonzalez, Maria Del Razo, Garcia-Vargas, Sanmiguel-Salazar, & Escobedo-de la Pena, 2007) Case-Control	Mexico n = 400 cases = 200	Range 20-400 µg/L	Total urinary arsenic	No association at low levels of urinary arsenic High levels (>104 µg/g) have 2.65x higher probability of T2D development
(Del Razo et al., 2011) Cross-section	Mexico n = 258	Mean = 42.9 µg/L Range = 3.1-215.2 µg/L	Urinary arsenic metabolites, fasting blood glucose and 2-hour blood glucose	Found significant association with arsenic concentration in drinking water and fasting blood glucose and 2-hour blood glucose
(Feseke, Ayotte, Bouchard, & Levallois, 2015) Cross-section	Canada n = 3151	Municipal sources - <10 µg/L Private wells - undisclosed	Total urinary arsenic	Higher urinary arsenic levels present in those with T2D and prediabetes
(Gribble et al., 2012) Cross-section	US – Arizona, Oklahoma, North and South Dakota n total = 3925 Nondiabetic n = 1,986 Diabetic n = 1,939	Range = 1-61 µg/L	Total urinary arsenic HBA1C	No association between urinary arsenic and HBA1C or insulin resistance in people w/o diabetes Association restricted to patients with uncontrolled diabetes
(Islam et al., 2012) Cross-section	Bangladesh n = 1004	Mean = 159 µg/L Range = 10-1401 µg/L	Risk of T2D development	Increased risk for T2D development in >50 µg/L, greatest risk at >250 µg/L for greater than 10 years

(James et al., 2013) Cases-Control	United States – San Luis Valley Nondiabetic n =488 Diabetic n = 60	Mean 39 µg/L Range – undetectable - 752 µg/L	T2D Risks	Mild association for T2D risk at levels <100 µg/L
(Jovanovic et al., 2013) Cross-section	Serbia Unexposed n = 1,324,489 Exposed n = 195,190	No exposure vs 56.1 µg/L	Number of diabetic cases present	Higher number of diabetes cases in exposed region
(Y. Kim & Lee, 2011b) Cross-section	Korea n = 1677	No range given	Total urinary arsenic	Higher urinary arsenic levels present in those with T2D
(N. H. Kim et al., 2013) Case-control	United States n = 300	No range given	Risk of T2D development	Slight association between arsenic exposure and T2D risk
(Kuo et al., 2015) Case-Control	United States – Arizona, Oklahoma, North and South Dakota n total = 3925 Nondiabetic n = 1,986 Diabetic n = 1,939	Range = 1-61 µg/L	Urinary arsenic metabolites	Higher MMA% in urine associated with decreased T2D risk
(Lewis et al., 1999) Retrospective	United States – Utah n females = 961 n males = 1,242	<200 µg/L	Mortality analysis	Few incidence of death attributed to T2D
(Li et al., 2013) Cross-Section	China n = 669	Range = 0-760 µg/L	Risk of T2D development	No significant association between arsenic and T2D risk
(Meliker et al., 2007) Retrospective	United States – Michigan n females = 41,282 n males = 38,722	Average 11.0 µg/L	Mortality analysis	Higher mortality rates observed for T2D
(Nabi et al., 2005) Case-control	Bangladesh n = 235	Mean = 11.3 vs 218 µg/L Range = 3 - 875 µg/L	Risk of T2D development	Diabetes prevalence in exposed individuals 2.8x higher than unexposed
(Navas-Acien, 2008) Case-Control	United States n = 788	No range given	Total urinary arsenic	Total urinary arsenic levels are associated with increased T2D
(M. Rahman et al., 1998) Cross-section	Bangladesh Unexposed n = 854 Keratosis n = 251	I - <500 µg/L II – 500-1000 µg/L III – 1000 µg/L <	Number of diabetic cases present	Increase in T2D cases in patients with keratosis

(Ruiz-Navarro et al., 1998) Case-control	Spain n = 89	No range given	Risk of T2D development	No correlation to urinary arsenic and T2D incidence
(Steinmaus et al., 2009) Cross-section	United States Nondiabetic n = 697 Diabetic n = 98	No range given	Total urinary arsenic	No association between T2D and urinary arsenic
(Tsai et al., 1999) Retrospective	Taiwan n = 19,536	Mean = 790 µg/L Range = 250-1140 µg/L	Mortality analysis	Found correlation between arsenic exposure and T2D related mortality
(C. H. Tseng et al., 2000) Cohort	Taiwan n = 446	Range = 700-930 µg/L	Risk of T2D development	Correlation between long term arsenic exposure and T2D
(J. P. Wang et al., 2009) Cross-section	China n = 235	Range = 16-272 µg/L	Risk of T2D	Blood glucose is lower and urinary arsenic higher in arsenic exposed individuals
(S. L. Wang et al., 2003) Cross-section	Taiwan n = 27,543	Range >350 µg/L	Risk of T2D	Increased levels of T2D in arseniases areas
(S. L. Wang et al., 2007) Cross-section	Taiwan n = 660	No range given	Risk of T2D	Found correlation between arsenic in hair and increased blood glucose
(Zierold et al., 2004) Case-Control	US – Wisconsin n = 1885	<2µg/L 2-10 µg/L 10 µg/L<	Evaluate prevalence of chronic illness in population	No significant association found

MMA – monomethylarsonic acid T2D- type 2 diabetes

Table 1.2 Summary of *in Vivo* Studies Examining Arsenic and Type 2 Diabetes

	In Vivo Study Parameters				Glucose Tolerance Test		
Reference	**Strain**	**Exposure**	**Endpoint**	**Conclusion**	**Glucose Test**	**Fasting Time & Glucose Bolus**	**Measurement Times (min)**
(Huang et al., 2015)	ICR 7-13 week old females	Arsenic trioxide (0, 50, 500 µg/L) in drinking water + ovariectomized or sham	Glucose tolerance and estrogen	Arsenic increased blood glucose in sham mice, and caused an even greater increase in ovariectomized mice	OGTT	Overnight 1g/kg	0, 15, 45, 75, 105
(Liu et al., 2014)	C57BKS/Lep^{dr} (db/db and db/+) 7-23 week old males	Sodium Arsenite (0, 3000 µg/L) in drinking water	Glucose tolerance	Decreased glucose tolerance in db/db mice	OGTT	12 hours 2g/kg	0, 15, 30, 60, 90, 120
(Mitchell, Ayala-Fierro, et al., 2000)	B6C3F1 Male adult 1x exposure I.P.	Sodium Arsenite (0, 100, 1000 µg/L) Sodium Arsenate (0, 100, 1000 µg/L)	Blood arsenic Blood glucose	Blood arsenic not a sign of toxicity No changes in blood glucose	Blood Glucose	x	x
(David S. Paul, Hernandez-Zavala, et al., 2007)	C57BL/6 4-12 week old males	iAsIII (0, 2.5x10^4, 5x10^4 µg/L) in drinking water	Glucose tolerance	Decreased glucose tolerance in	IPGTT	5 hours 2g/kg	0, 15, 30, 60, 90, 120

				50ppm treatment group			
(D S Paul et al., 2008)	C57BL/6 4-12 week old males	iAsIII (0, $1x10^3$, $1x10^4$, $2.5x10^4$, $5x10^4$ µg/L) or MAsIII(0, 100, $1x10^3$, $2.5x10^3$, $5x10^3$ µg/L) in drinking water	Glucose tolerance	iAsIII impaired glucose tolerance at $5x10^4$ µg/L	IPGTT	5 hours 2g/kg	0, 15, 30, 60, 90, 120
(David S. Paul, Walton, Saunders, & Styblo, 2011)	C57BL/6 4-24 week old males	iAsIII (0, $2.5x10^4$, $5x10^4$ µg/L) in drinking water + high or low fat diet	Glucose tolerance	Higher blood glucose in HFD at $5x10^4$ µg/L compared to LFD	OGTT	Overnight 2g/kg	0, 15, 30, 60, 90, 120
(S. Rahman, Hossain, & Rahmatullah, 2016)	Swiss Albino Mice Male adult 1x exposure	Sodium Arsenite (0, 100, 200, 400 µg/20g body weight) orally	Blood glucose	Blood glucose elevated at all levels of arsenic exposure	OGTT	Overnight 2g/kg	120
(Rodríguez, Limón-Pacheco, Del Razo, & Giordano, 2016)	C57BL/6 Adult male 1 or 2-month exposure	Sodium Arsenite (0, $5x10^4$µg/L) in drinking water	Blood glucose	1 month did not alter serum glucose levels 2 months decreased glucose tolerance	IPGTT	12 hour 2g/kg	0, 30, 90, 150

OGTT- oral glucose tolerance test, IPGTT, intraperitoneal glucose tolerance test

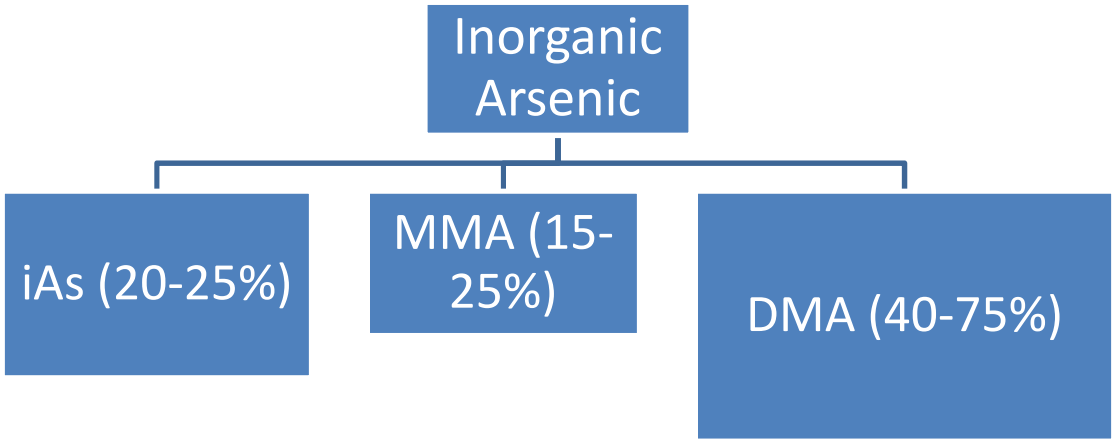


Figure 1.1. Urinary inorganic arsenic metabolites. Common metabolites found in urine that can be attributed to inorganic arsenic exposure are inorganic arsenic (iAs) (III) and (V), monomethylarsonous acid (MMA) (III) and (V) and dimethylarsonous acid (DMA) (III) and (V). Percentages are estimates of the concentrtion typically found in human urine samples.

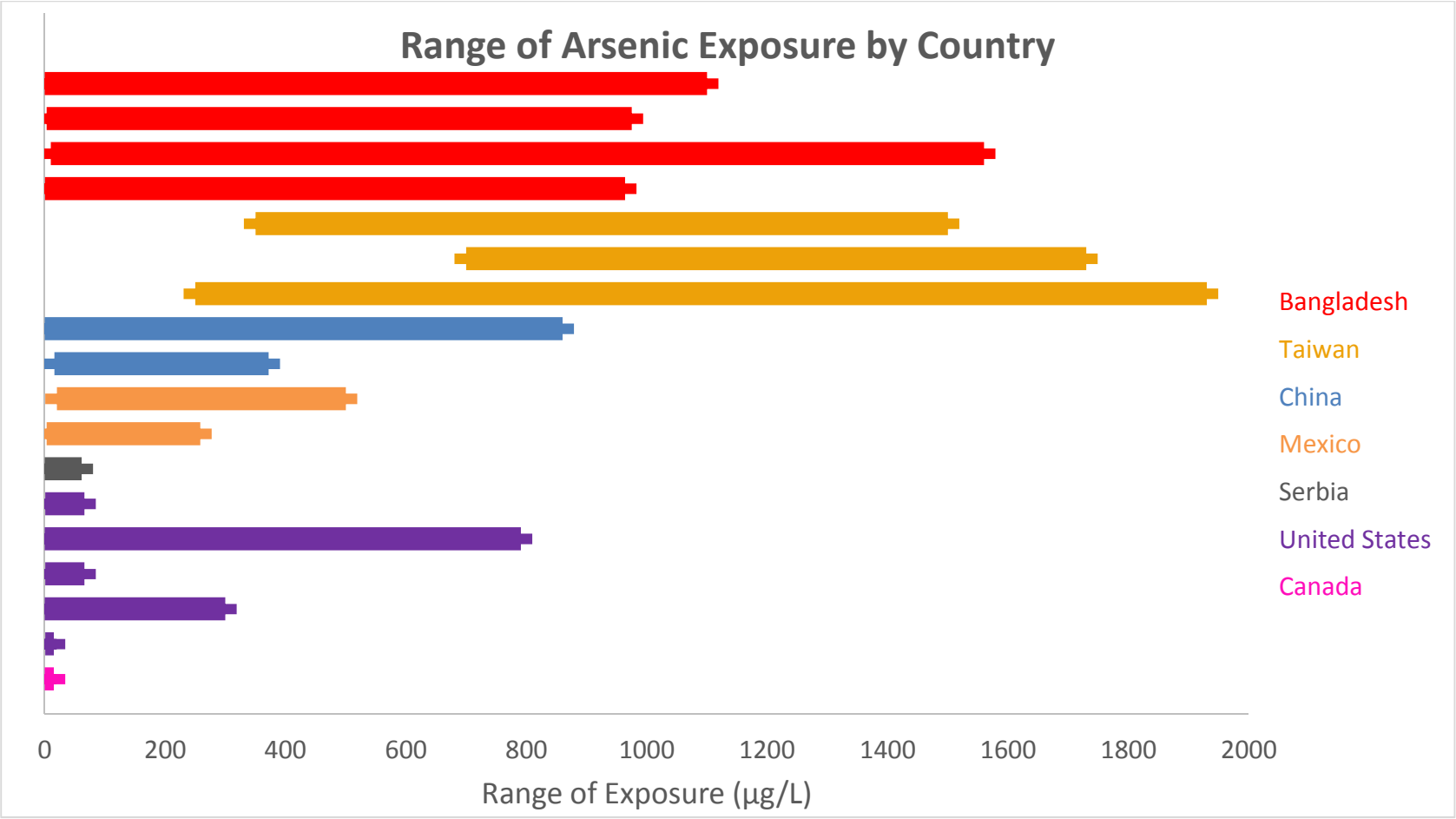


Figure 1.2. **Summary of arsenic exposure by country.** Arsenic exposure varies from country to country, and presented are the ranges of exposures that have been examined in epidemiological studies.

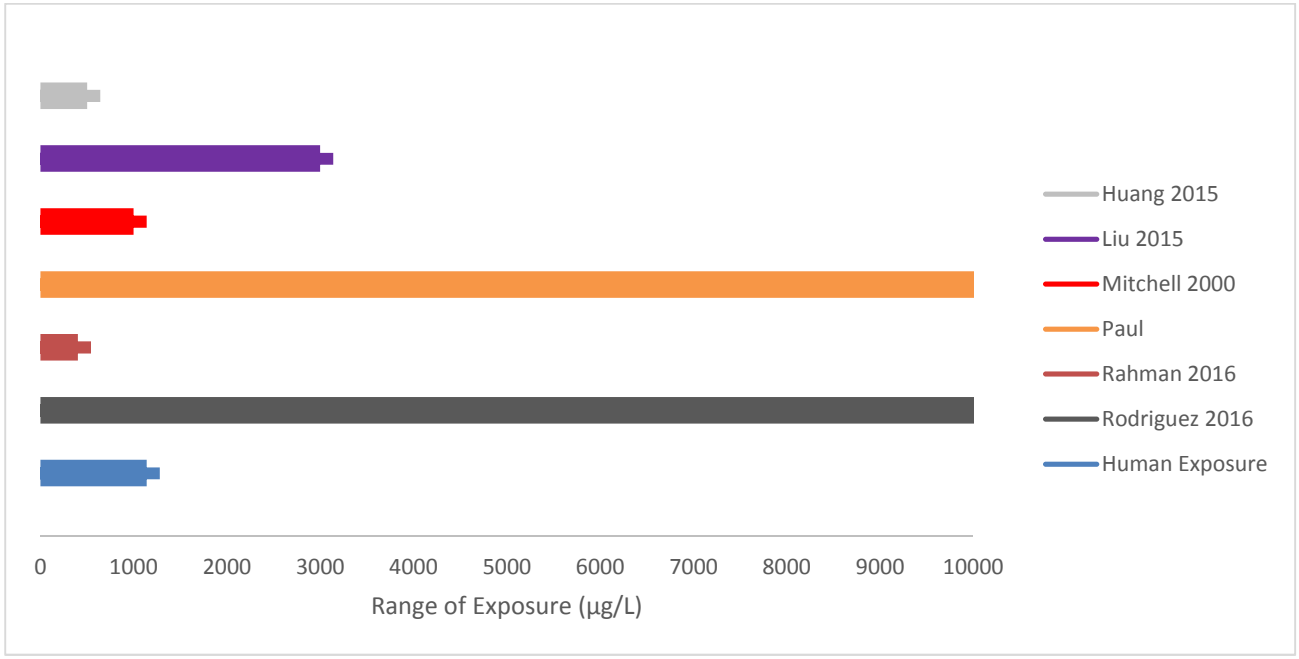


Figure 1.3. Range of *in Vivo* exposure compared to human exposure. *In Vivo* studies have attempted to provide a link between arsenic exposure and type 2 diabetes, however these animal exposures tend to be much higher than to human exposures.

Chapter 2 – Arsenic and Type 2 Diabetes in C57BL/6 Mice

2.1 Introduction

Arsenic is an element found in the earth's crust that can be found in organic and inorganic forms, with the inorganic form being a known toxin. Typical total arsenic exposure is about 50 µg/day from a variety of sources, with about 3.5 µg/day coming from the inorganic form and the rest coming for non-toxic organic sources such as seafood (ATSDR, 2013). The main sources of inorganic arsenic exposure are from food and water.

In the United States, the amount of arsenic in public drinking water is very highly regulated, having been reduced from 50 µg/L to 10 µg/L by the EPA in 2001 because it is a known carcinogen (ATSDR, 2013). In other countries however, the levels of drinking water are not regulated and individuals are exposed to up to ten times the amount of arsenic as in the United States. These include Bangladesh and Taiwan, where the well water can be on the order of 1000 µg/L, leading to increased risks for health issues including hyper-pigmentation, cardiovascular disease and type 2 diabetes, which have been heavily reviewed (Becker & Axelrad, 2014; Huang et al., 2011; Navas-Acien et al., 2006; Thayer, Heindel, Bucher, & Gallo, 2012; W. Wang et al., 2014). Other at risk areas include rural populations in the United States that rely on well water and do not regularly check for contaminants, as these sources of drinking water are not regulated. The concentration of arsenic in these wells can also reach critical levels of almost 800 µg/L (James et al., 2013).

Type 2 diabetes is a metabolic disease that results in high blood sugar and insulin resistance (Mathers & Loncar, 2006). This disease is typically considered a lifestyle disorder resulting from inactivity, poor diet and high body mass index (BMI), but more recently aging and genetic components are recognized to play a role in disease development (Federation, 2015). In 2015, 415 million people were affected by type 2 diabetes with the number expected to increase to 642 million by the year 2040 (Federation, 2015). This increase in population is much greater than can be attributed to lifestyle, age and genetics alone, which has led to research examining environmental contaminants in the development of the disease, including but not limited to cadmium, mercury and arsenic (Y. W. Chen et al., 2009).

While at higher levels positive correlations between arsenic exposure and type 2 diabetes have been demonstrated in the epidemiological data, at mid and lower level exposure (<500µg/L) the results are inconclusive. Because of inconsistent methodologies, comparing and contrasting data between studies at these levels is nearly impossible. The same trend can be observed in animal studies, where at higher exposures glucose intolerance can be induced by arsenic either acutely or chronically (Kuo et al., 2013). While mice metabolize arsenic more rapidly than humans, the majority of these studies use doses well beyond what would be considered relevant exposures for human studies. In our study, we proposed to examine the diabetic effects of arsenic exposure on a non-susceptible mouse strain at a concentration of arsenic that is closer to low-level exposure in humans. The goal will be to determine if the lower level of arsenic we are exposed to in the United States, which is highly regulated, is still enough to be concerned about diabetes susceptibility. We will evaluate body composition, glucose tolerance and

adipokine expression to elucidate a possible mechanism behind arsenic exposure leading to the development of diabetes. We hypothesized that arsenic exposure would increase obesity and decrease glucose regulation allowing for progression toward a diabetic phenotype.

2.2 Methods and Materials

2.2.1 Animals

Male and female B6.BKS(D)*Lepr*^{db}/J heterozygote mice were bred and housed at the University of South Carolina Animal Resource Facility. Offspring of the heterozygote x heterozygote cross were genotyped by RT-PCR followed by a restriction digest for the Leptin gene at weaning (3 weeks of age). The primer sequences were forward: 5'-AGAACGGACACTCTTTGAAGTCTC-3'; and reverse: 5'-CATTCAAACCATAGTTTAGGTTTGTGT-3'. PCR products were digested using *RsaI* enzyme, which cleaved sequences into one 135 base pair band or 27, 108, and 135 base pair bands representing wildtype or heterozygous genotypes, respectively. Once genotyped, animals were randomly grouped into control, 1x inorganic arsenic ($AsCl_3$ - Alfa Aesar, 50 µg/kg body weight) or 10x inorganic arsenic (500 µg/kg body weight) and were treated every other day beginning at 4 weeks of age (Figure 2.1). These doses, relating to human exposure, are 0, 1 and 10 µg every other day, respectively in a 20-g mouse. This spectrum is broad enough to include a level of exposure lower and consistent with current literature (Huang et al., 2015; Liu et al., 2014; Mitchell, Ayala-Fierro, et al., 2000; D S Paul et al., 2008; David S. Paul, Hernandez-Zavala, et al., 2007; David S. Paul et al., 2011; S. Rahman et al., 2016; Rodríguez et al., 2016). Mice received 100 µl/20 g body weight of the appropriate dilution of arsenic (or diluent water for controls) via oral

gavage using a flexible gavage needle. Weights were recorded every other day, and every 4 weeks an oral glucose tolerance test (OGTT) and Dual Energy X-ray Absorptiometry (DEXA) scan were performed. Animals were maintained on a 12:12 hour light cycle and given food and water ad libitum. At 12 weeks, animals were euthanized for tissue collection using isoflurane overdose followed by cervical dislocation. Principles of laboratory animal care were followed and the Institutional Animal Care and Usage Committee of the University of South Carolina approved all protocols.

2.2.2 Body composition

Along with monitoring weight every other day, body composition was assessed every four weeks (weeks 4, 8 and 12 of age). Mice were lightly anesthetized with isoflurane and measurements were taken to determine lean mass, fat mass and body fat percentage for each mouse by dual-energy X-ray absorptiometry (DEXA) (Lunar PIXImus, Madison, WI).

2.2.3 Glucose tolerance

An oral glucose tolerance test (OGTT) was performed every four weeks following arsenic exposure starting at 4 weeks old (weeks 4, 8 and 12 of age). Mice were fasted for 4 hours during their sleep cycle before initiating the OGTT with a fasting blood glucose level. Glucose was given orally (2 mg/kg body weight) and blood glucose measurements were taken from tail vein prick at 15, 30, 60, 90 and 120 minutes using TRUE test strips and glucometer. Area under the curve (AUC) for the glucose tolerance test was calculated using the trapezoidal rule.

2.2.4 Tissue collection

Following 8 weeks of control or arsenic treatment, mice were euthanized for tissue collection. Reproductive fat pads were collected for histology, preserved for 24 hours in 4% paraformaldehyde and then transferred to 70% ethanol before histology, or snap frozen on dry ice and stored at -80ºC for triglyceride, protein and RNA analysis. Spleen, liver, kidney, quadricep muscle and heart were also snap frozen and stored at -80ºC for protein and RNA analysis.

2.2.5 Histology

Reproductive adipose tissue was sectioned and stained with hemotoxylin and eosin (H&E) to evaluate adipocyte size and diameter. Slides were evaluated using AxioVision LE 4.8 software and ImageJ plugin Adiposoft (ver. 1.13) to measure circumference and diameter for at least 2 fields of view for each section of tissue. Histological score was conducted by two blinded reviewers independently and averaged.

2.2.6 Real-time PCR

RNA was isolated from reproductive adipose tissue by Isolate RNA kit according to the manufacturer's protocol (Bioline). RNA was converted to cDNA using SensiFAST cDNA synthesis kit according to manufacturer's protocol (Bioline). Real-time PCR (qPCR) was then performed on 100 ng of cDNA using SensiFAST SYBR (Bioline) and 10 µM of designed and validated primers according to manufacturer's protocol. Custom primers (Table 2.1) were designed for each target using Primer Blast Search (NCBI) and purchased from Integrated DNA Technologies (IDT) for qPCR. Gene expression patterns

for each gene were normalized to the expression of the housekeeping gene RPLP0 and quantified using the $\Delta\Delta^{CT}$ method.

2.2.7 Triglycerides

Triglycerides were measured using saponification as previously described by Jouihan (2012) using reagents from Sigma. Briefly, tissues were weighed and then digested overnight at 55°C in ethanolic KOH. Digested tissue was diluted with 50% ethanol, and 200 µl added to 215 µl 1M $MgCl_2$. Samples were put on ice for 10 minutes. Glycerol Standard Solution (2.5 mg/ml) was used to create a standard curve, and Free Glycerol Reagent was used to spectrophotometrically determine triglyceride levels at 540nm based on the 8-point standard curve generated in triplicate.

2.2.8 Statistics

All statistical analyses were carried out using R (version 3.3.1) or GraphPad Prism 7 (San Diego, CA). The n for each treatment group were as follows: male wild-type control (n=5), male wild-type arsenic (n=4), male heterozygote control (n=11), male heterozygote arsenic 1x (n=8), male heterozygote arsenic 10x (n=3), female wild-type control (n=6), female wild-type arsenic (n=5), female heterozygote control (n=9), female heterozygote arsenic 1x (n=6), female heterozygote arsenic 10x (n=3), unless otherwise noted. Three mice were excluded from all analyses as they were outliers in more than 3 different categories analyzed. Data are expressed as mean ± SEM. Regression analysis were performed to compare exposure, genotypic and sex differences. A *p*-value of <0.05 was considered statistically significant.

2.3 Results

2.3.1 Weight change

To determine whether arsenic affects the development of type 2 diabetes which has an obesity component to the disease, we examined how exposure alters body composition. We weighed mice every other day during the dosing period, and found that when compared to the wild-type control, the slope of the male heterozygote control and 1x treatment group were significantly steeper showing greater change in weight (Figure 2.2a). This means that not only were there genotypic differences in weight gain, but treatment based differences as well. However, the increase in arsenic exposure in the heterozygotes from 1x to 10x did not further affect the weight change (Figure 2.2b). When comparing the same effects in the females, all slopes in the change in weight were significantly different from the wild-type control (Figure 2.3). This means that all groups gained weight faster than the control, demonstrating both genotypic and treatment based effects on weight.

2.3.2 Body composition analysis

From 4-8 weeks in males, there are no differences in body composition caused by exposure to arsenic (Figure 2.4a). After the full dosing period (4-12 weeks), there is a significant difference in the control wild-type and heterozygous males (Figure 2.4b). While not significant, it does appear that arsenic causes a slight increase in fat mass and percentage in wild-type males exposed to arsenic over the entire 12-week period (Figure 2.4b). In the heterozygote dose-response, arsenic appears to actually reduce the fat mass from 4-8 weeks (Figure 2.4c). This trend is no longer present after the full dosing period

(4-12 weeks), but the lean mass appears to be reduced in the 10x arsenic exposure group compared to the other two treatments and the percent fat increased (Figure 2.4d).

In females there are small genotypic differences present starting between 4-8 weeks (Figure 2.5a) demonstrated by the female heterozygous controls having slightly greater change in lean, fat and total mass compared to the wild-type control. These changes are exaggerated when evaluating differences from 4-12 weeks (Figure 2.5b), as the change in lean, fat and total mass are significantly greater in the heterozygote controls compared to wild-type controls. In the heterozygote dose response, from 4-8 weeks the 10x arsenic treatment has a significant effect on the change in lean and total mass (Figure 2.5c); however, these changes do not persist through the entire dosing period (Figure 2.5d).

2.3.3 Oral glucose tolerance test

We hypothesized that if arsenic increased the risk of developing type 2 diabetes, then we would see a decrease in glucose tolerance. Overall, arsenic did not appear to have a major impact on the OGTT in males (Figure 2.6) We chose to break down the glucose tolerance test into 2 measurable components – fasting blood glucose and area under the curve. Arsenic does not affect the fasting blood glucose in males when comparing wild-type to heterozygote over the 8-week dosing period (Figure 2.7). It appears that the male heterozygotes exposed to arsenic had a higher baseline fasting blood glucose, which was resolved at the end of the 8 weeks and no difference was observed (Figure 2.7a). While the baseline fasting blood glucose in our male heterozygote 10x treatment group started out lower than the other initial blood glucose measurements, this trend remained constant during the duration of exposure (Figure

2.7b). These results demonstrate that even with differences in baseline glucose tolerance, arsenic did not cause reduced glucose tolerance during the course of exposure.

We also measured change in glucose tolerance through analyzing the area under the curve of the glucose tolerance test (Figure 2.7c and 2.7d). There was no significant difference in any genotype based on treatment group, although the male heterozygotes begin with a slightly higher AUC and end with this intact. These results do not support our hypothesis that arsenic will decreased glucose tolerance.

In the female OGTT, there was a significant difference in the peak of the curve in the 12-week measurement between wild-type control and heterozygote controls (Figure 2.8c). Another significant difference occurred in the heterozygote dose response at the baseline glucose tolerance test (Figure 2.8d). The 10x treatment group was significantly lower than the 1x treatment group at the 30-minute time point. However, this did not continue and is no longer visible in the 8-week (Figure 2.8e) or 12-week (Figure 12.8f) curves. These finding show that while at certain time points, arsenic may cause slight changes in glucose tolerance, over the duration of chronic exposure this change does not persist and lead to sustained reduced glucose tolerance.

Breaking down the OGTT, we did see a significant difference in the fasting blood glucose at 12 weeks between the wild-type and heterozygote controls shown by an increase from 100 mg/dl in the controls to 120 mg/dl in the heterozygotes (Figure 2.9a). No changes in the fasting blood glucose in the heterozygote dose response were observed thereby supporting the idea that glucose tolerance is not altered by arsenic exposure (Figure 2.9b). While there appeared to be a slight difference in AUC measurements in the female heterozygote 1x treatment group at 4 weeks, this did not continue through to 12

weeks (Figure 2.9c). This change was also not exacerbated by increasing the exposure to arsenic (Figure 2.9d), further demonstrating that arsenic does not have an effect on glucose tolerance in the wild-type or heterozygote females.

2.3.4 Histology

We expected that because there were differences in weight change, then there would also be changes in adipose tissue morphology. Figure 2.10 presents representative histological images of each treatment group. We analyzed these images to quantitate adipocyte size and diameter in both males (Figure 2.11) and females (Figure 2.12). In the male heterozygotes exposed to arsenic, there was an increase in size and diameter of adipocytes analyzed, but this only applied to the 1x arsenic concentration and the change was not significant (Figure 2.11a and b). At 10x, the male heterozygotes had reduced size and diameter compared to the control heterozygotes, although again, the difference was not significant (Figure 2.11c and d). This differs from the females, where there were no changes in adipocyte size present (Figure 2.12). Overall, while we have demonstrated that there were changes in weight gain, fat mass and lean mass these results did not translate into the expected changes in adipose tissue morphology.

2.3.5 Gene expression

To examine the effects of arsenic on inflammation, which can alter the inflammatory state both locally in adipose tissue and systemically, we examined the expression of pro- and anti-inflammatory adipokines in males (Figure 2.13) and females (Figure 2.14).

In wild-type males, arsenic had no significant effect on adipokine expression of TNF-α, leptin or adiponectin However, it appears to increase adiponectin and decrease

TNF-α in the arsenic-treated group compared to controls. This differs from the heterozygotes, where arsenic significantly increases leptin expression in the 1x treatment group (Figure 2.13e), which is not exacerbated by increasing the arsenic exposure to 10x. In fact, the 10x treatment in heterozygotes appears to reduce the expression of adiponectin, leptin and TNF-α compared to the 1x treatment, showing that increasing arsenic exposure may actually diminish total immune response.

In female wild-type mice exposed to arsenic, there was a significant increase in leptin expression (Figure 2.14b). Also while not significant, TNF-α expression appears to be increased, (Figure 2.14c). In the heterozygotes, arsenic had no significant effect on adipokine expression, although the 10x increased leptin and TNF-α expression compared to both control and 1x exposures (Figure 2.14e and f). These results demonstrated that even if arsenic does not have a significant effect on the immune system, depending on individual genetics the immune response will be different.

2.3.6 Triglycerides

Because of the effects of arsenic on weight, we wanted to determine whether this also altered triglyceride levels in various tissues throughout the body. We chose to examine tissues that have been demonstrated to accumulate arsenic or be affected by arsenic exposure, including the heart, kidney, liver and muscle.

In the males, arsenic had no significant effect on triglyceride levels (Figure 2.15), however the 1x treatment group appeared to raise triglyceride levels slightly in the liver and muscle, the 10x treatment group was reduced to levels very similar to control. In the females, arsenic had no significant effect on triglyceride levels (Figure 2.16), while it appeared that in the heterozygote group, 1x arsenic treatment increases heart and kidney

triglycerides, the 10x treatment reduces these levels back to comparable to control. There are significant genotypic differences present in the females in both the liver and heart triglycerides (Figure 2.16c and d). The heterozygote females have higher triglyceride levels in both organs, which may be attributed to them having larger lean, fat and total mass compared to the wild-type mice, but overall arsenic had no impact on organ triglyceride levels.

2.4 Discussion

The current literature is undecided as to whether arsenic plays a role in the development of type 2 diabetes, especially at lower doses. We, therefore, sought to examine the effects of arsenic on body composition, glucose tolerance and cytokine expression in non-susceptible mice to determine whether these may contribute to increased risks for the development of type 2 diabetes. Our results demonstrated that there were no indicators of diabetes developing in these animals, but other changes that could lead to increased susceptibility were observed such as an increase in systemic and localized inflammation, which we will evaluate further in later studies.

Male and female wild-type mice are representative of a non-susceptible population; however, type 2 diabetes can be induced when fed a high fat diet. We examined whether arsenic would change the body composition in these mice, leading to increased fat mass, decreased glucose tolerance and increased inflammation – all of which could lead to the progression of diabetes. However, we found at the level of arsenic used in these studies was insufficient to induce changes in glucose tolerance, but it was enough to induce changes in weight gain and changes in inflammation in adipose tissue.

Arsenic alone is not a known inducer of obesity. In animal studies, arsenic exposure, especially at higher levels, caused reduced water and food intake leading to a slower, but not significantly different, increase in weight (D S Paul et al., 2008; David S. Paul, Hernandez-Zavala, et al., 2007). In our animal model, both males and females demonstrated that weight gain was increased during the period of arsenic exposure. One of the major differences when comparing our study to others is the route of arsenic exposure. Our mice were exposed every other day by oral gavage, where drinking water exposures were used for other studies. The prolonged exposure may cause a decrease in appetite that would damped weight gain during exposure periods.

The differences in body composition between female wild-type and heterozygote controls were very prominent compared to males. The heterozygote females had a greater increase in lean, fat and total mass from baseline to 12 weeks old when compared to the wild-type females The only significant change in body composition we have reported that is directly related to arsenic exposure is between the female heterozygote control and the 10x treatment group, where at this higher concentration of arsenic, we saw a decrease in lean and total mass from 4-8 weeks. This however was no longer present at the conclusion of the study, demonstrating that the body may be able to correct for some changes over time even when challenged with a toxicant such as arsenic at low levels.

When comparing these to the adipose tissue histology, there was no difference in adipocyte size or diameter in the arsenic treatment of males or females. Typically, in an obese individual, the adipose tissue morphology will change. Adipocytes become larger as obesity increases but because there was no difference in weight, only an increase in fat

mass, this may be the reason for no difference. Different results may have been obtained if other fat pads (visceral, subcutaneous) were examined for comparison.

While we may have reported differences in weight change in our treatment groups, there were no changes in fasting blood glucose over the 12-week period. We evaluated glucose tolerance by an OGTT, which we examined by analyzing the fasting blood glucose and area under the curve. Typically, in a diabetic individual the fasting blood glucose and area under the curve measurement will be much higher than a non-diabetic. In previous studies examining the effects of arsenic on glucose tolerance using mice, changes in blood glucose tolerance were reported, but the concentration and original form and route of arsenic exposure varied greatly (Huang et al., 2015; Liu et al., 2014; D S Paul et al., 2008; David S. Paul, Harmon, et al., 2007; David S. Paul et al., 2011; S. Rahman et al., 2016; Rodríguez et al., 2016). This may mean that the lower levels of arsenic we used might not alter glucose tolerance over a short period of time.

Because of the changes in body weight, we wanted to determine whether there was a concurrent affect in local inflammation in the adipose tissue. We examined three adipokines that have different effects on the local and systemic immune system: adiponectin (an anti-inflammatory cytokine), TNF-α and leptin, which are all pro-inflammatory cytokines. In the wild-type males, we observed a 2-fold increase in adiponectin, which shows that while there were no significant changes in body composition, arsenic induced a slight suppression in the local immune response. In the male heterozygotes, which did have changes in body composition, leptin expression was significantly upregulated 2-fold in the 1x treatment group or slightly increased in the 10x treatment group.

In females the inflammatory response was very different. In the wild-type exposed to arsenic, there was a significant 2-fold increase in adipose tissue expression of leptin accompanied by a slight increase in TNF-α, which can be attributed to this group having a larger change in weight over the exposure time. The female heterozygotes had a mixed immune response, with 1x arsenic treated mice having a 2-fold increase in adiponectin and TNF-α and the 10x treatment group having a 2-fold increase in leptin and TNF-α, although none of the changes were significant. Both of these treatment groups had very similar weight changes, so the differences in response must be the result of the concentrations of arsenic used. The increase in arsenic in the 10x group may have been sufficient to overstimulate the pro-inflammatory response and reduce the expression of adiponectin.

We chose to examine triglycerides because of the varying changes arsenic caused in body composition. All of the organ triglyceride levels measured in this experiment have been shown to be increased in diabetic cases (Christoffersen et al., 2003; Kelley & Goodpaster, 2001; Shimomura, Bashmakov, & Horton, 1999; Sun, 2002). While we saw minimal indicators of diabetes, the majority of the changes in these organ triglycerides can be attributed to the changes in fat or body weight over the duration of treatment. So by modifying body composition, arsenic also affected triglyceride content in different organs in the body.

Overall, our study demonstrated that the effects caused by arsenic differ, not only by concentration of exposure and sex but by genotype as well. None of the systems affected by arsenic responded similarly within these different models. In the future, adding in the db/db genotype will allow us to determine how arsenic affects an individual

that will genotypically develop type 2 diabetes at an early age. It would also be beneficial to compare this study to a diet-induced diabetes model using the same mice to determine whether our parameters would change as obesity increases. Adding these two pieces of the puzzle would allow us to not only compare the mechanisms by which arsenic affects a non-susceptible individual to a diet-induced obese individual but also affects in a genetically-induced obese individual.

Table 2.1. Real-time PCR Primer List.

ID	Forward 5'-3'	Reverse 5'-3'
Adiponectin	CTACTGCAACATTCCGGGAC	TGCGAATATTGTGAAGCCCC
Leptin	GACATTTCACACACGCAGTC	GAGTCATGCCTTTGGATGGG
TNF-α	AGCACAGAAAGCATGATCCG	CTACAGGCTTGTCACTCGAA
RPLP0	CCTGAAGTGCTCGACATCAC	GCGCTTGTACCCATTGATGA

Abbreviations: Tumor necrosis factor alpha – TNF-α, Ribosomal protein lateral stalk subunit P0 – RPLP0

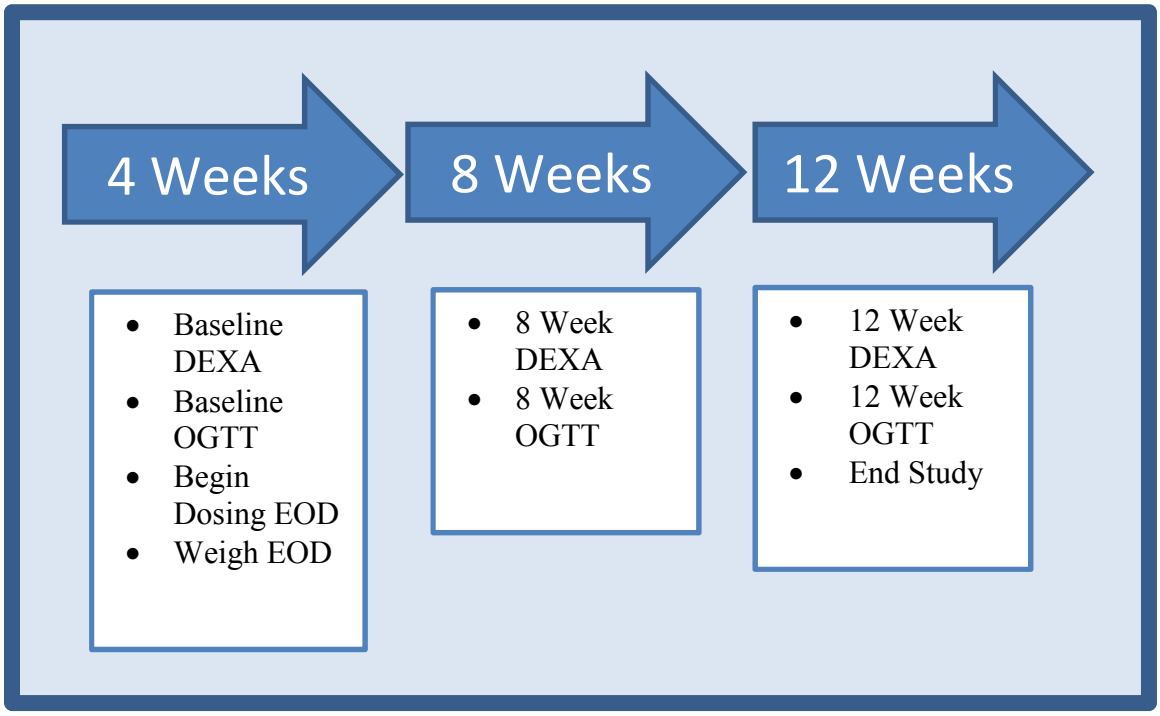


Figure 2.1 Experimental layout. Starting at the age of 4 weeks, mice will begin arsenic exposure and weighing every other day (EOD). This will also be the baseline oral glucose tolerance test (OGTT) and dual-energy X-ray absorptiometry (DEXA) scan. These tests will be repeated at week 8 and week 12, when the study will conclude.

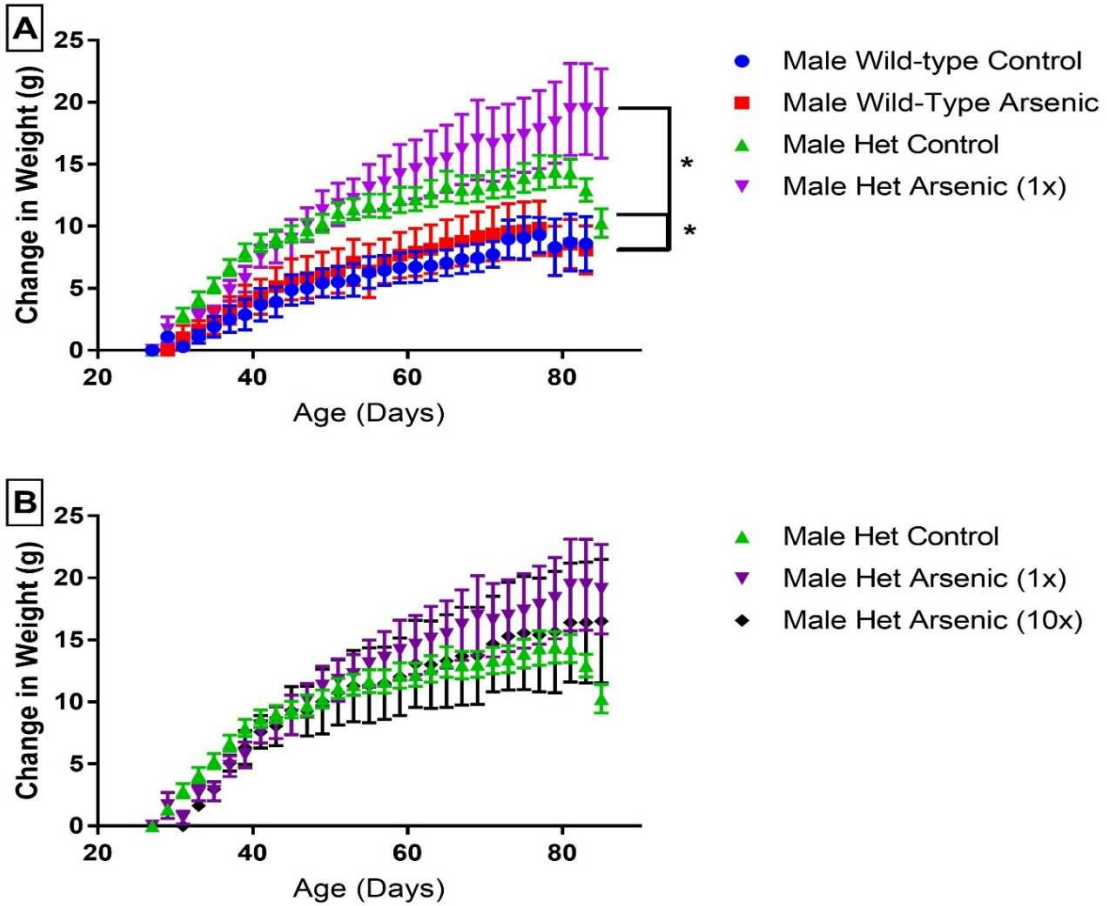


Figure 2.2. Change in weight in male wild-type and heterozygotes after 8 weeks of arsenic exposure. Mean weight change for each genotype (WT – wild-type, Het – heterozygote) over time. A) Compared to wild-type controls, the mean change in weight for het male controls and iAs 1x was significantly greater. B) Increase in arsenic exposure did not alter average change in weight for control, 1x, and 10x hets. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression and is indicated as * ($p<0.05$).

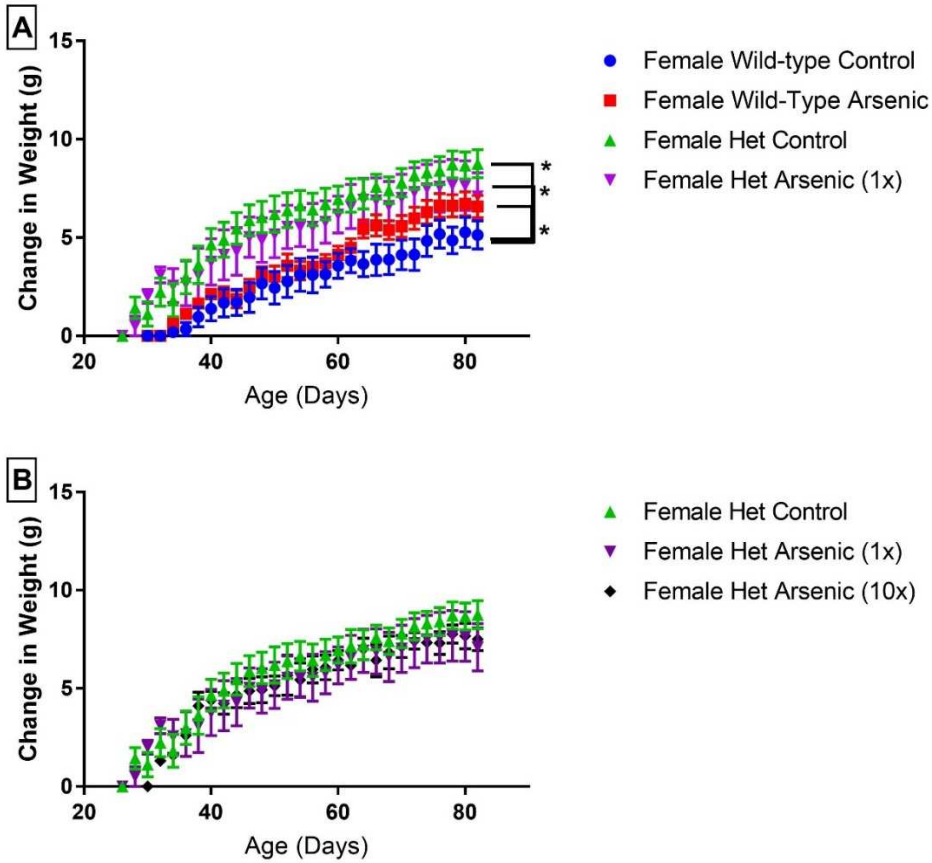


Figure 2.3. Change in weight in female wild-type and heterozygotes after 8 weeks of arsenic exposure. Mean weight change for each genotype (WT – wild-type, Het – heterozygote) over time. A) Compared to wild-type controls, the mean change in weight for iAs-treated WT and het females was significantly greater over the 8-week dosing period. B) Increase in arsenic exposure did not increase the mean change in weight for control, 1x, and 10x hets. Data are represented as the mean of each treatment group ± SEM Statistical significance was assessed using linear and is indicated as * ($p<0.05$).

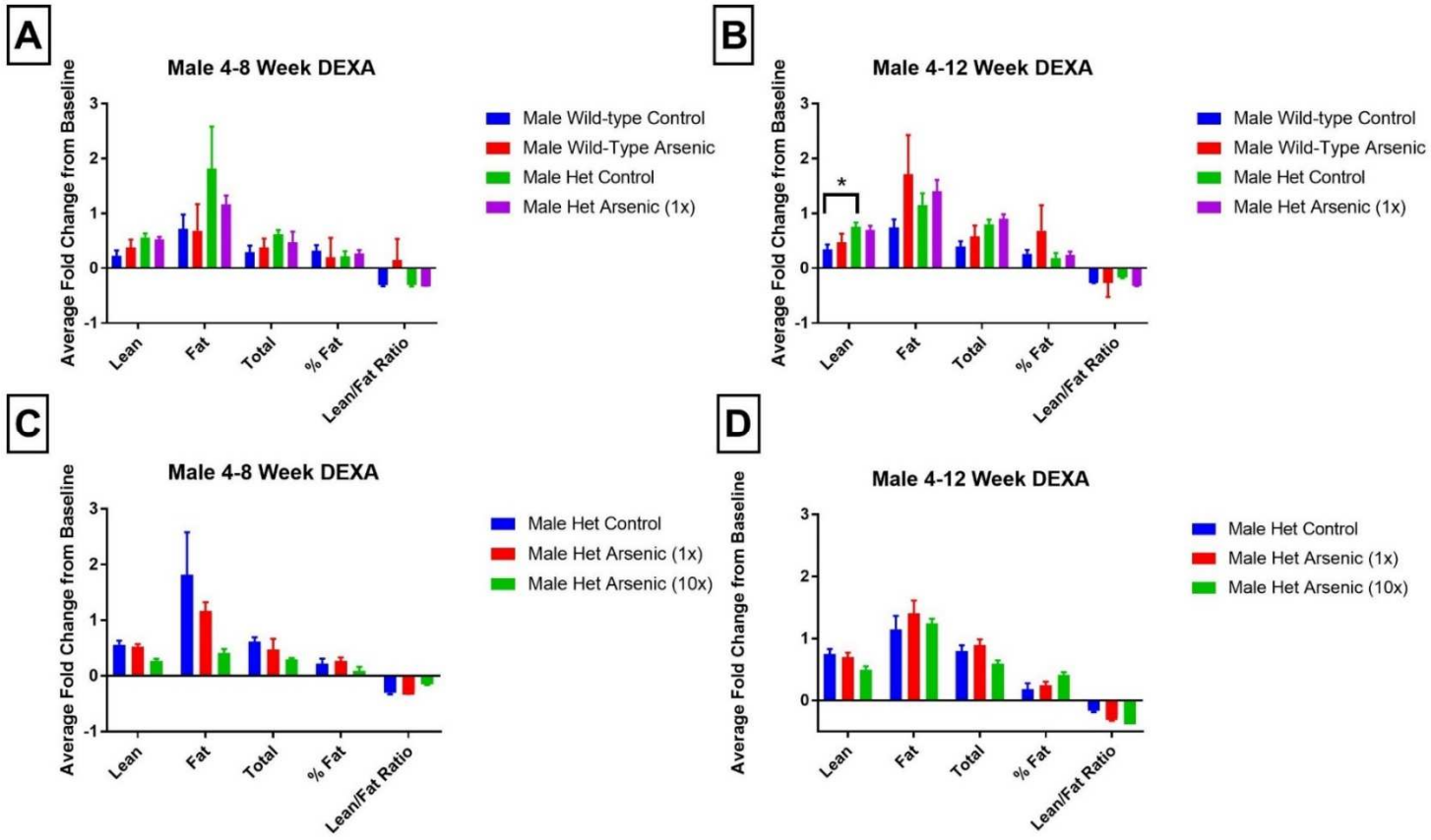


Figure 2.4. Mean change in body composition in males. Change in DEXA scan results from male WT and hets at 4-8 weeks (A and B) and from 4-12 weeks (C and D) after arsenic exposure. Arsenic had no effect on body composition, however the lean mass in the male het control from 4-12 weeks had a significantly greater increase compared to wild-type control. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

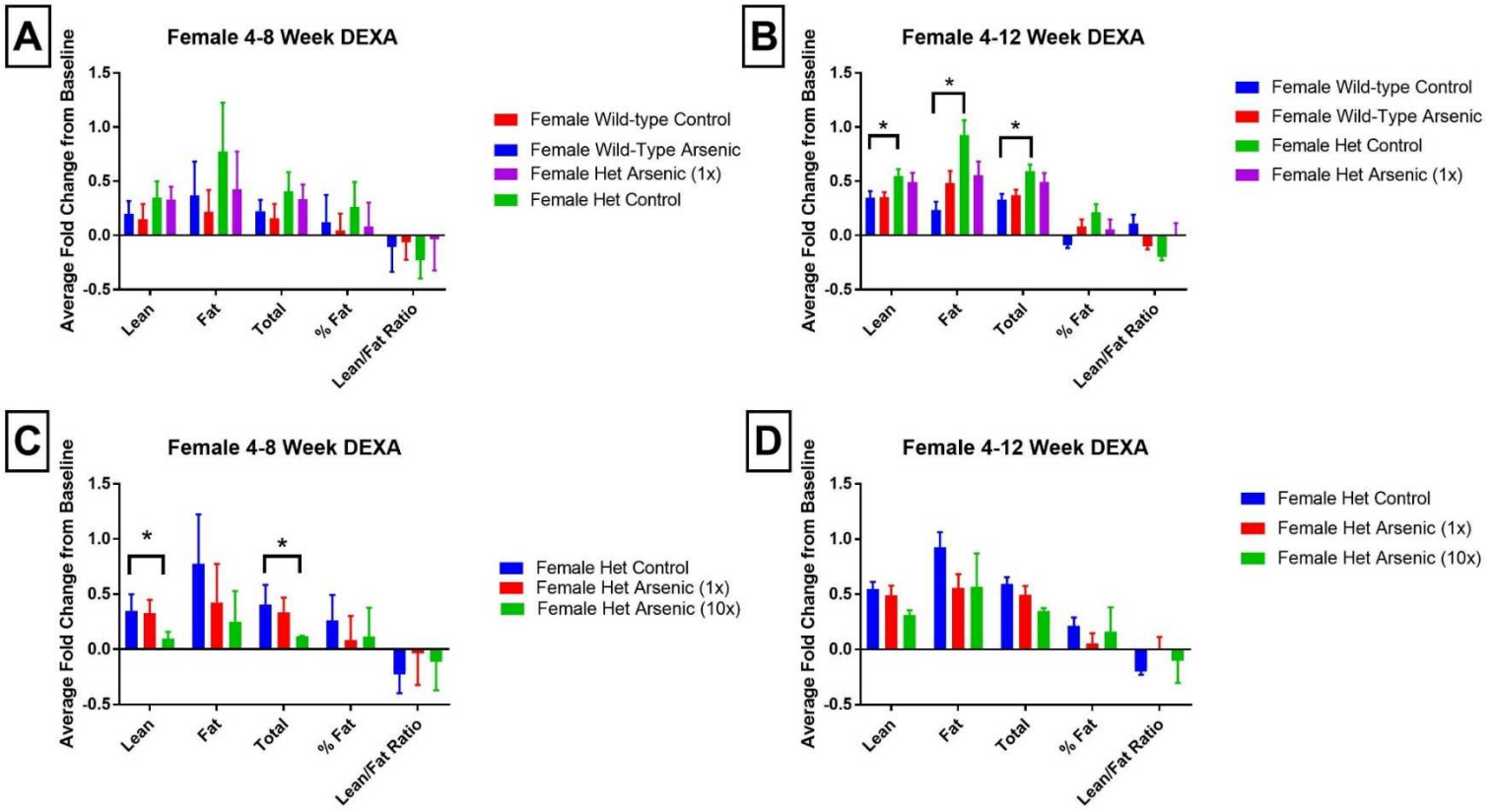


Figure 2.5. Mean change in body composition in females. Change in DEXA scan results from female WT and heterozygotes at 4-8 weeks (A and B) and from 4-12 weeks (C and D) after arsenic exposure. From 4-8 weeks, 10x arsenic significantly reduced the change in lean and total mass in female hets compared to control, however this did not persist until 12 weeks. From 4-12 weeks, arsenic had no effect on body composition, however the control female hets had a greater increase in lean, fat and total mass compared to wild-type controls. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

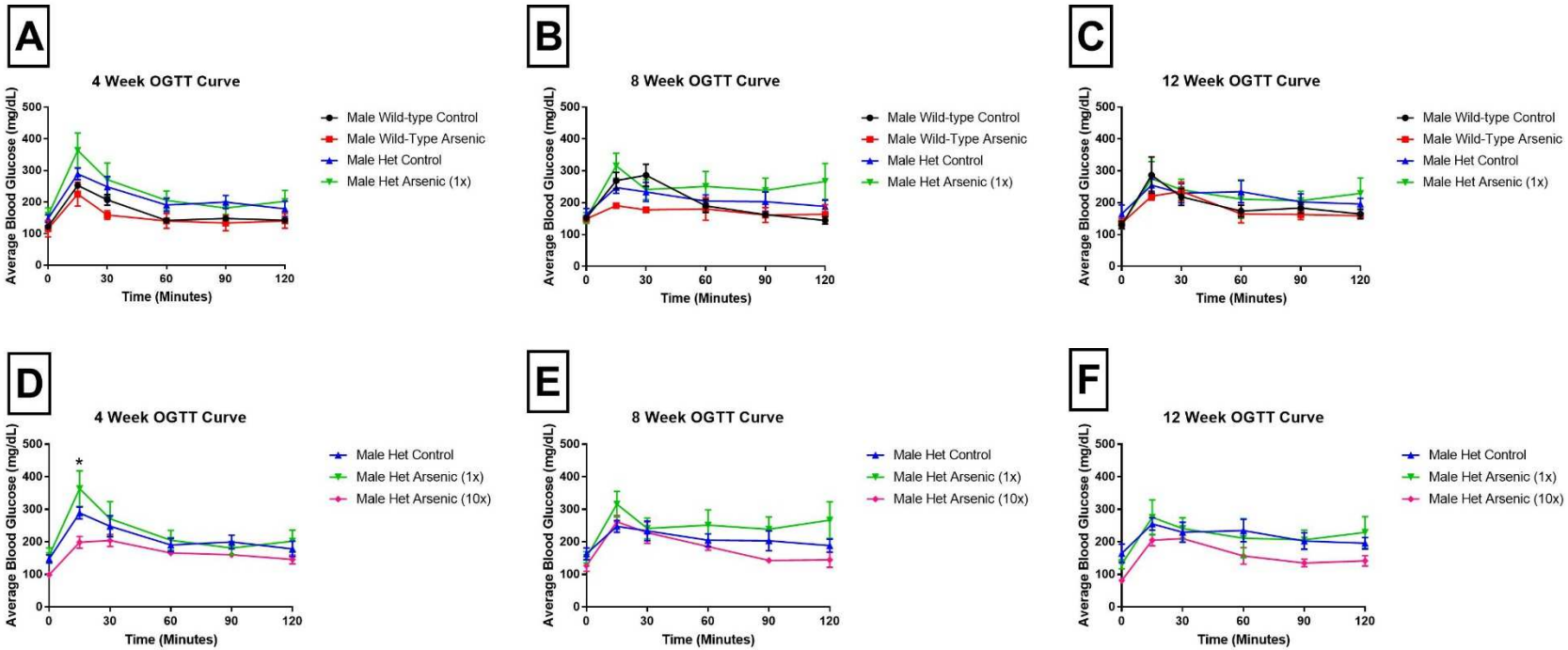


Figure 2.6. Male oral glucose tolerance test (OGTT) curves. Change in glucose tolerance during oral glucose tolerance test in male wild-type and heterozygotes at 4 (A, B), 8 (C, D) and 12 (E, F) weeks old after arsenic exposure. While significant differences were present at the 15-minute time point in the male hets at the baseline OGTT measurement, no changes were persistent through the entire dosing period. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using ANOVA followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

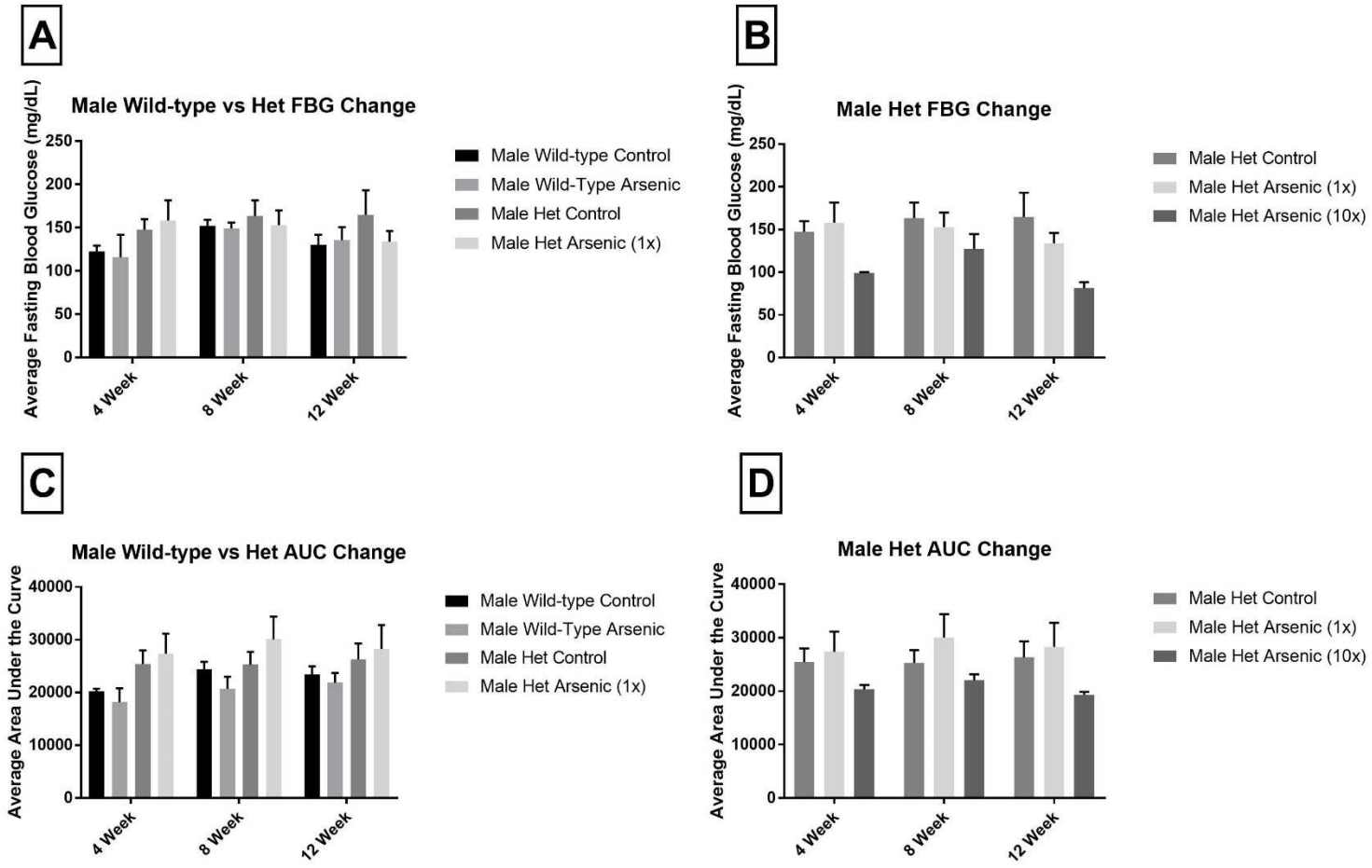


Figure 2.7. Changes in male glucose tolerance. Change in fasting blood glucose (A and B) and area under the curve (C and D) in male wild-type and heterozygous mice after 8 weeks of arsenic exposure. Neither arsenic nor genotype significantly affected FBG or AUC measurements. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

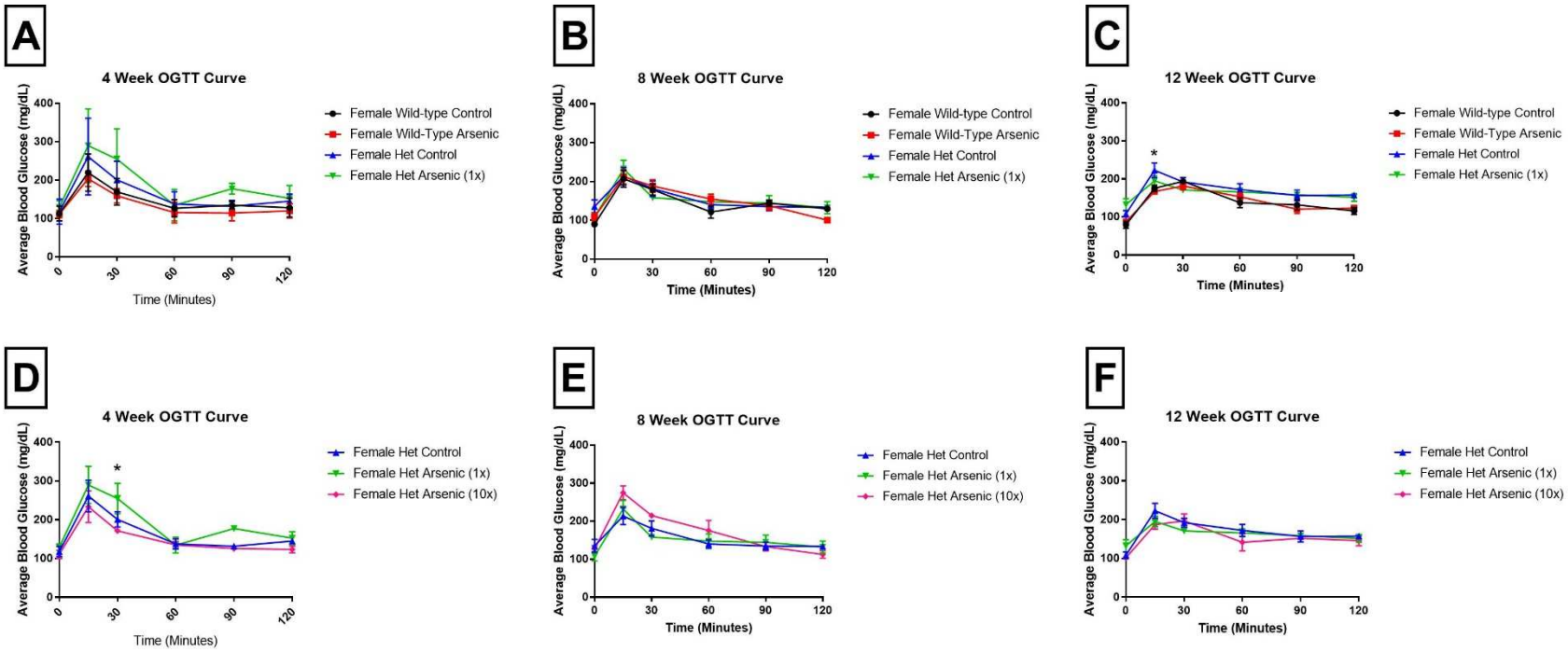


Figure 2.8. Female oral glucose tolerance test (OGTT) curves. Change in glucose tolerance during oral glucose tolerance test in female wild-type and heterozygous mice at 4 (A, B), 8 (C, D) and 12 (E, F) weeks old after arsenic exposure. While a significant increase in blood glucose are present at the 30-minute time point in the female 1x hets at baseline, this difference is not exacerbated by arsenic over the dosing period. Arsenic did significantly increase the peak at 15 minutes in the 12 week OGTT in female het control compared to wild-type control. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using ANOVA followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

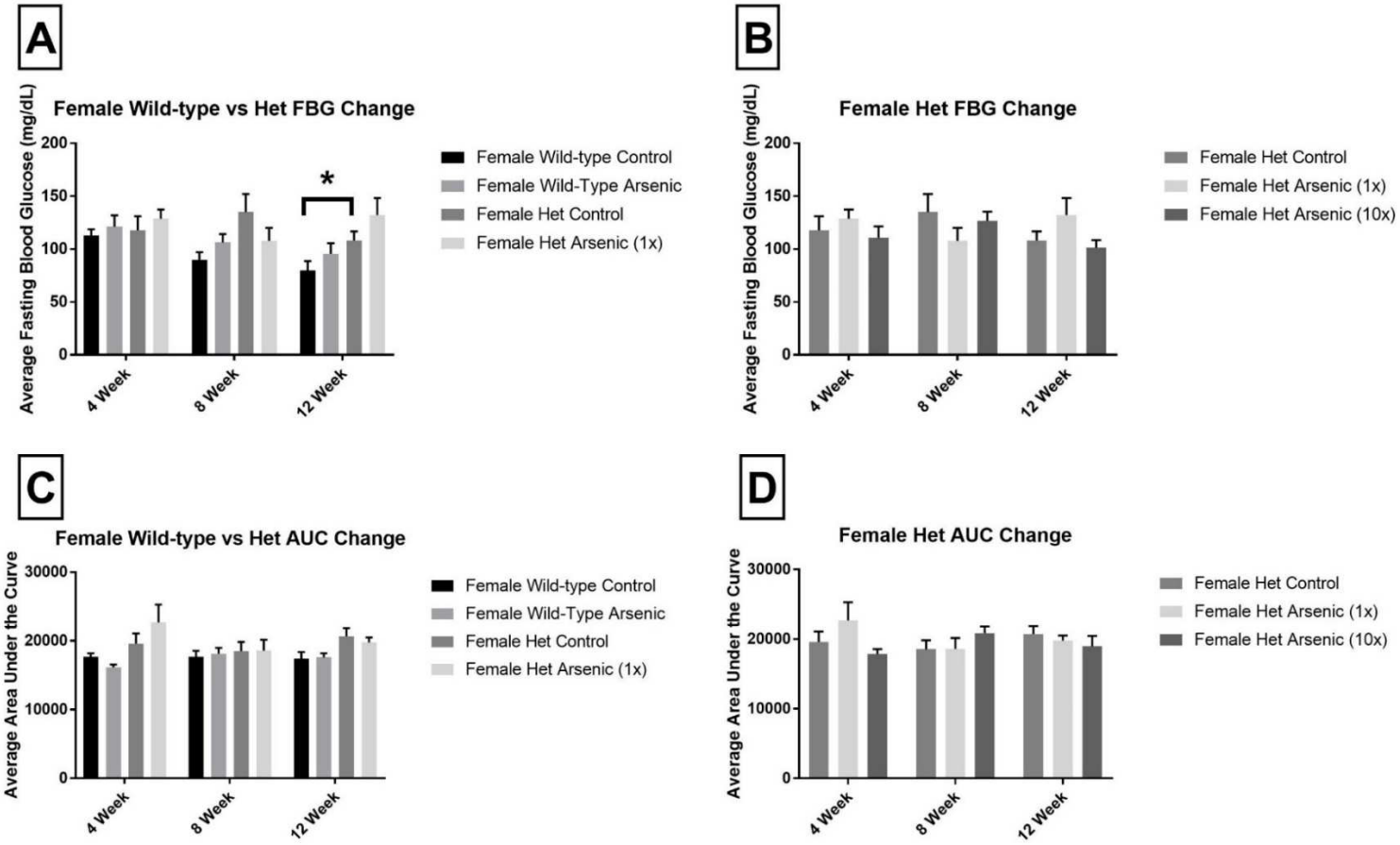


Figure 2.9. Changes in female glucose tolerance. Change in fasting blood glucose (A and B) and area under the curve (C and D) in female wild-type and heterozygous mice after 8 weeks of arsenic exposure. While arsenic exposure did not significantly affect FBG or AUC measurements, wild-type controls had a lower fasting blood glucose at 12 weeks compared to het controls. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

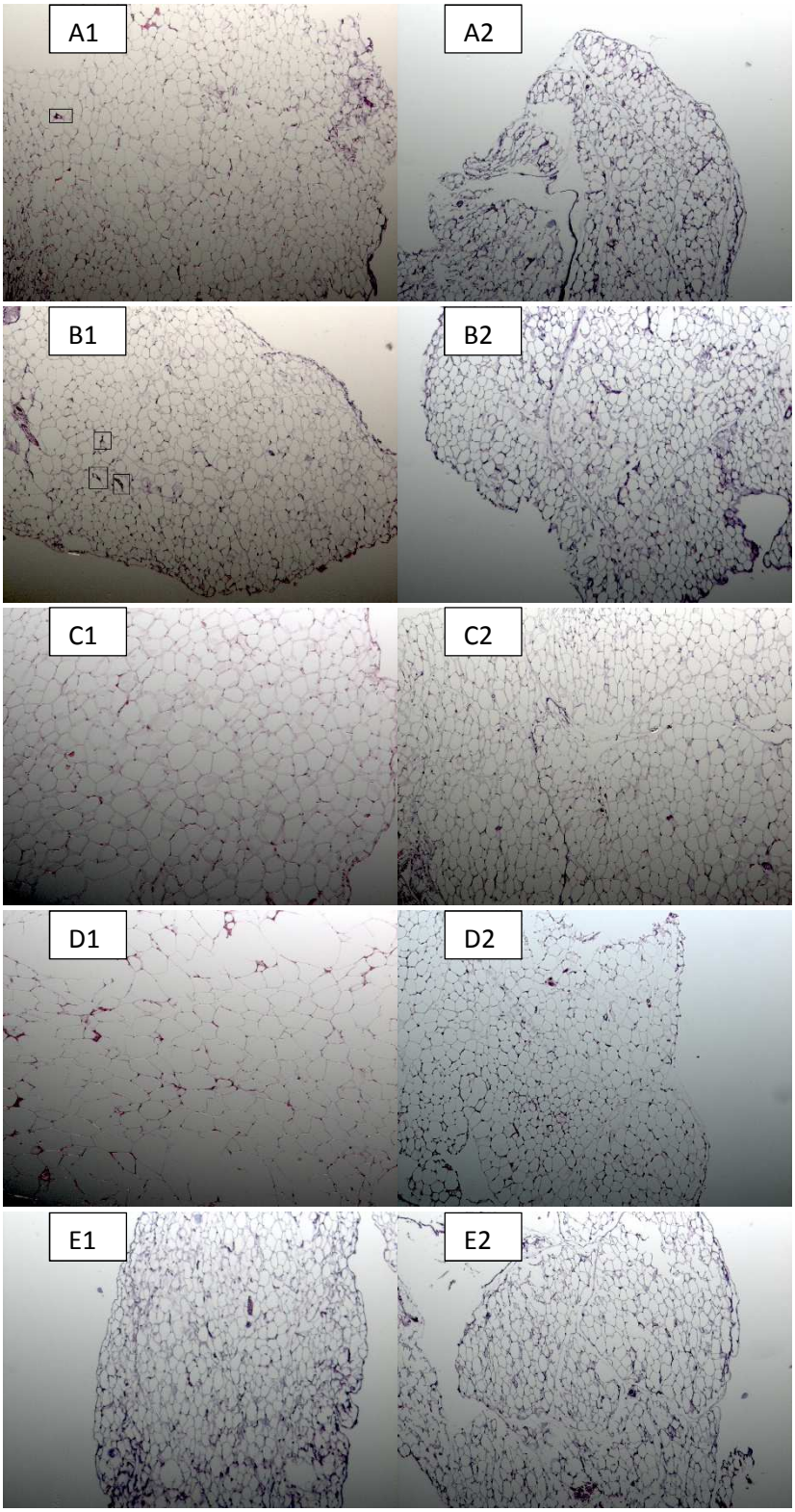


Figure 2.10. Reproductive adipose tissue histology. H&E sections from males (column 1) and females (column 2). A) WT Control B) WT iAs C) Het Control D) Het iAs (1x) E) Het iAs (10x). Images are at 4x magnification. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

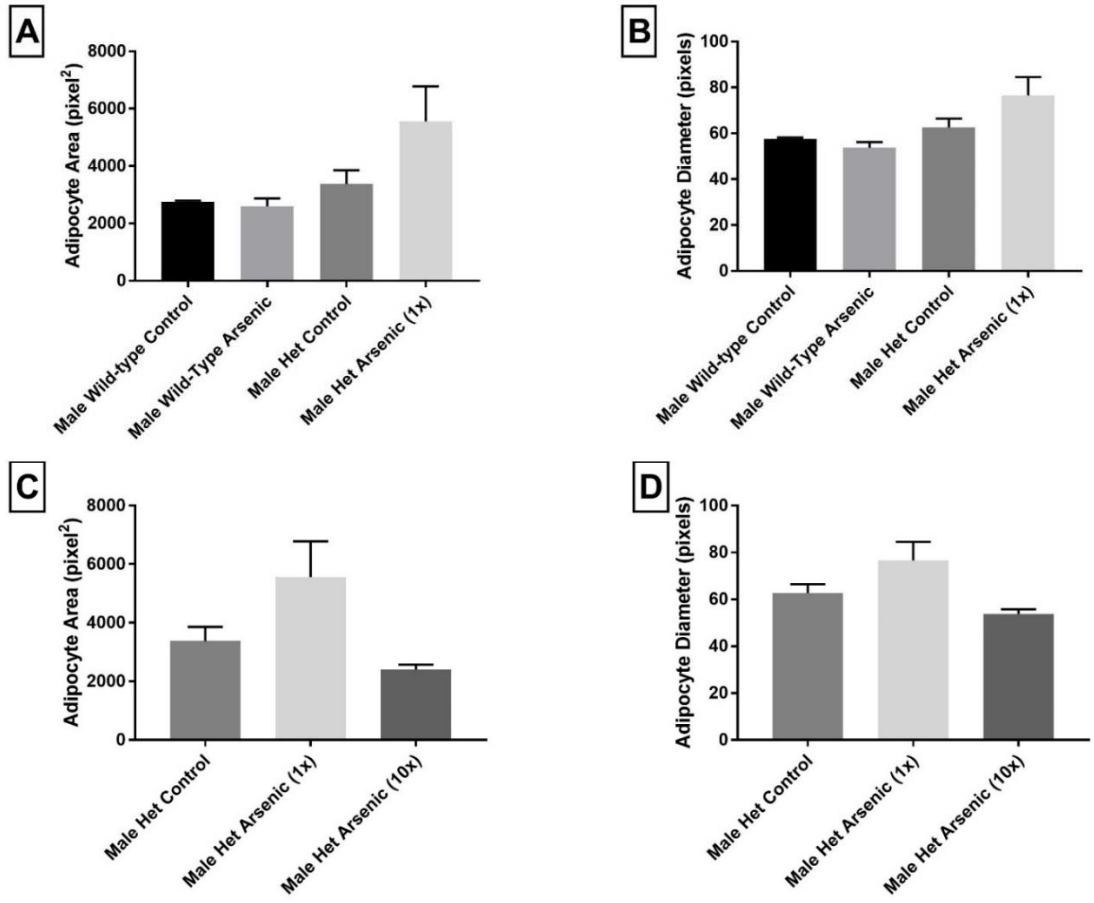


Figure 2.11. Arsenic does not significantly alter male reproductive adipose tissue histology. Software analysis of reproductive fat histological sections reveal that there are no significant differences in adipocyte diameter or area in wild-type or het males. Graphs represent the male adipocyte area (A and B) and diameter (C and D) analyzed using Adiposoft software and data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

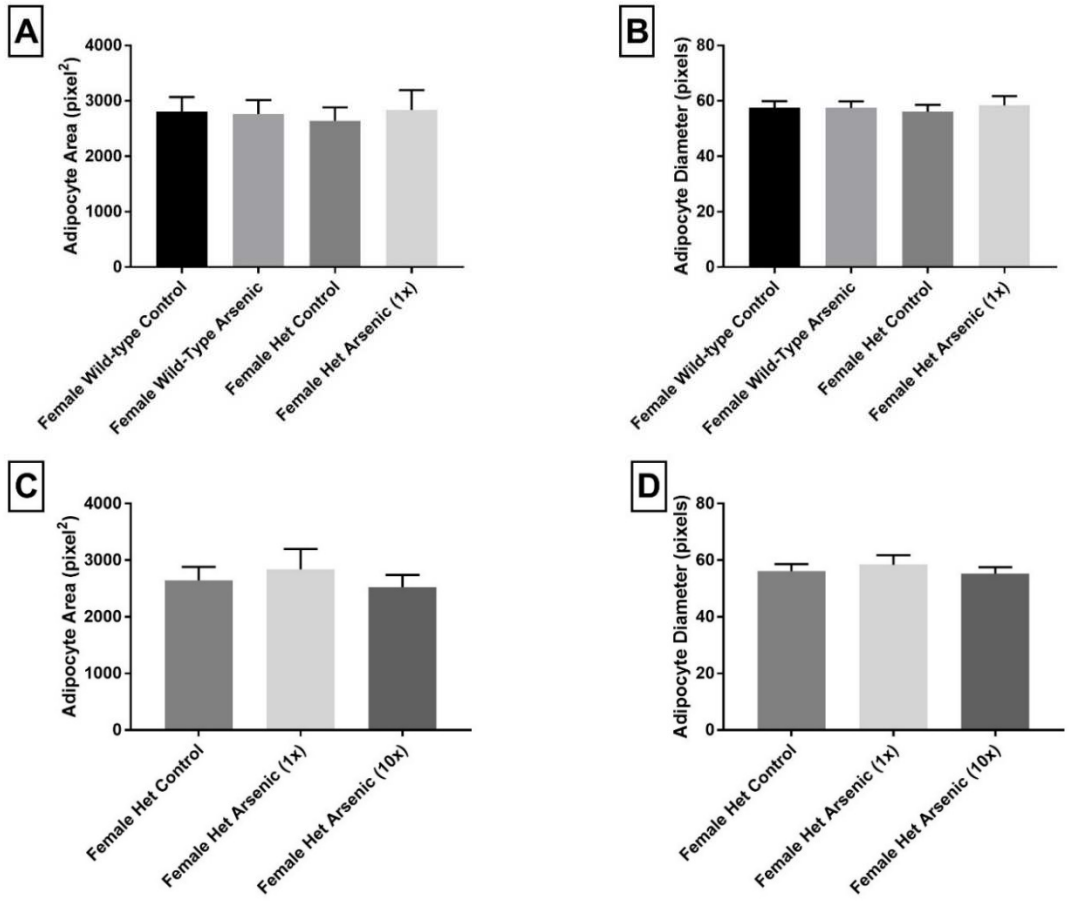


Figure 2.12. Arsenic does not significantly alter female reproductive adipose tissue histology. Software analysis of reproductive fat histological sections reveal that there are no significant differences in adipocyte diameter or area in wild-type or het females. Graphs represent the female adipocyte area (A and B) and diameter (C and D) analyzed using Adiposoft software and data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

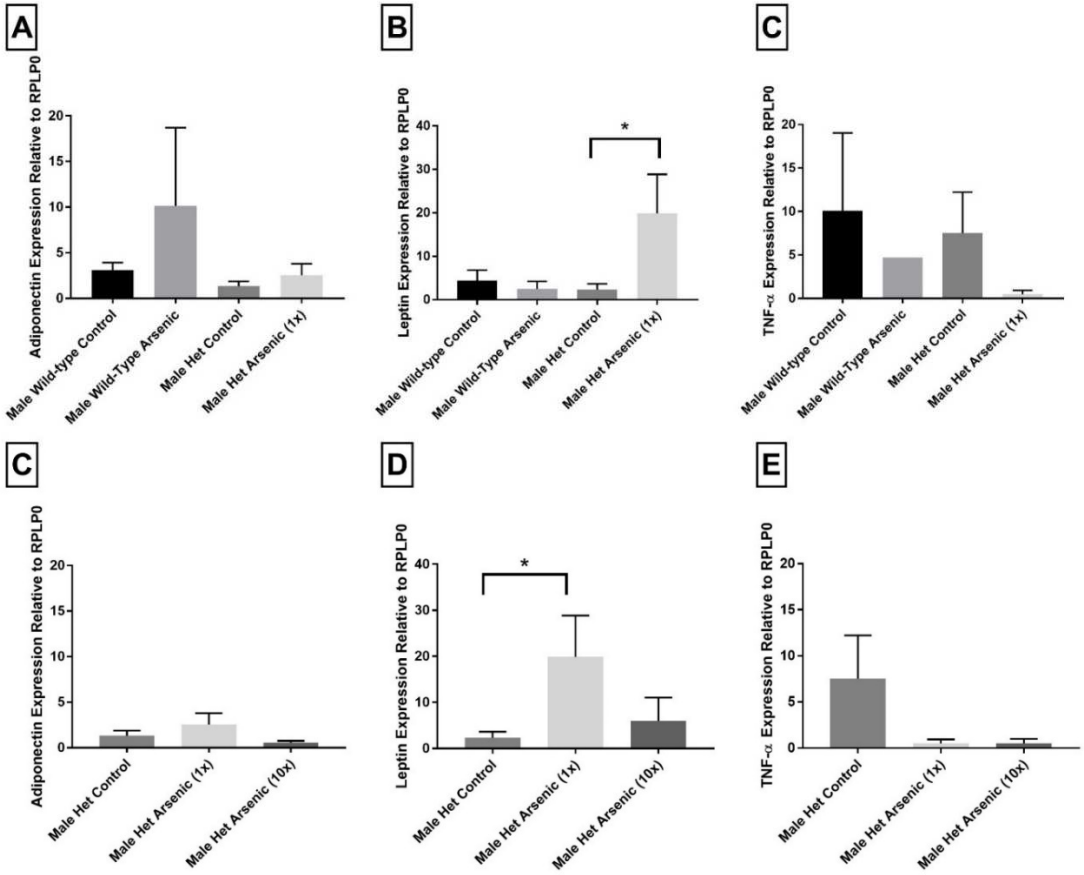


Figure 2.13. Arsenic significantly increases leptin expression in male heterozygote reproductive adipose tissue. Graphs A, B and C represent wild-type vs heterozygote gene expression and graphs D, E and F represent heterozygote dose response gene expression. Arsenic exposure significantly increased leptin expression in reproductive adipose tissue compared to control, but this was not exacerbated by 10x exposure. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

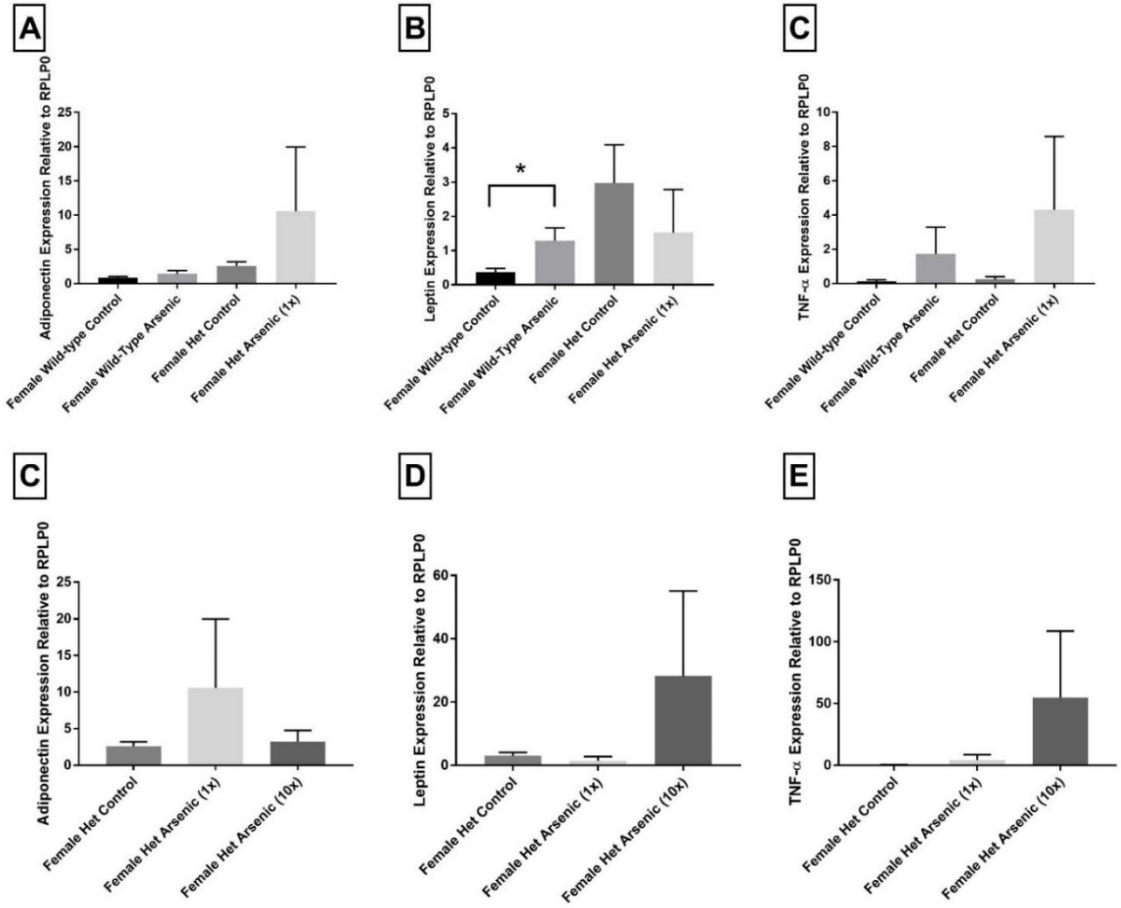


Figure 2.14. Arsenic significantly increases leptin expression in female wild-type reproductive adipose tissue. Graphs A, B and C represent wild-type vs heterozygote gene expression and graphs D, E and F represent heterozygote dose response gene expression. Arsenic exposure increases expression of leptin in wild-type females exposed to arsenic compared to controls. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

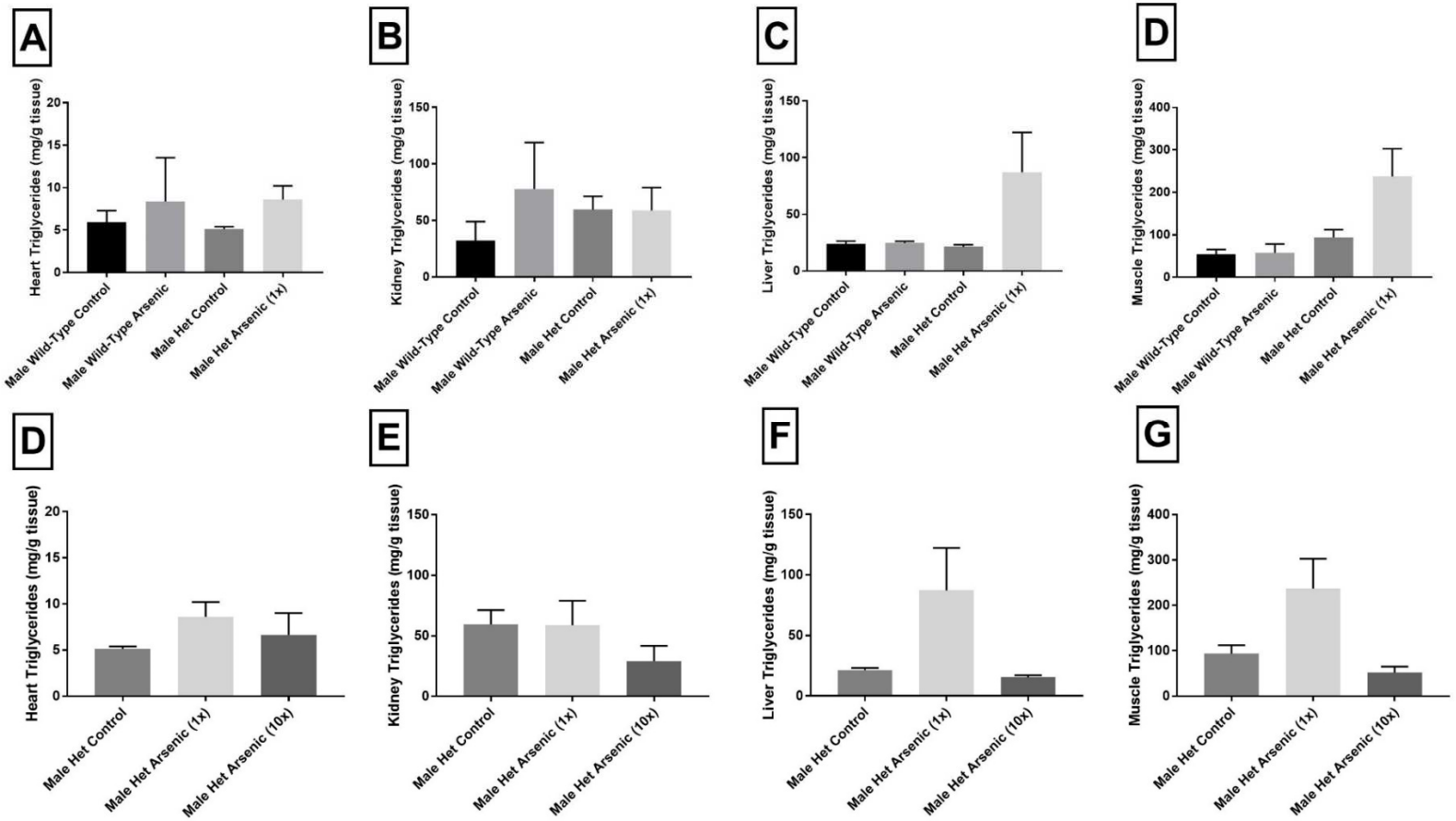


Figure 2.15. No differences present in male organ triglycerides. Graphs A-D represent wild-type vs heterozygote triglycerides in different organs and graphs E-H represent heterozygote dose response triglycerides in different organs. There are no significant changes in triglyceride levels in the heart, kidney, liver or muscle caused by arsenic exposure or genotype differences. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

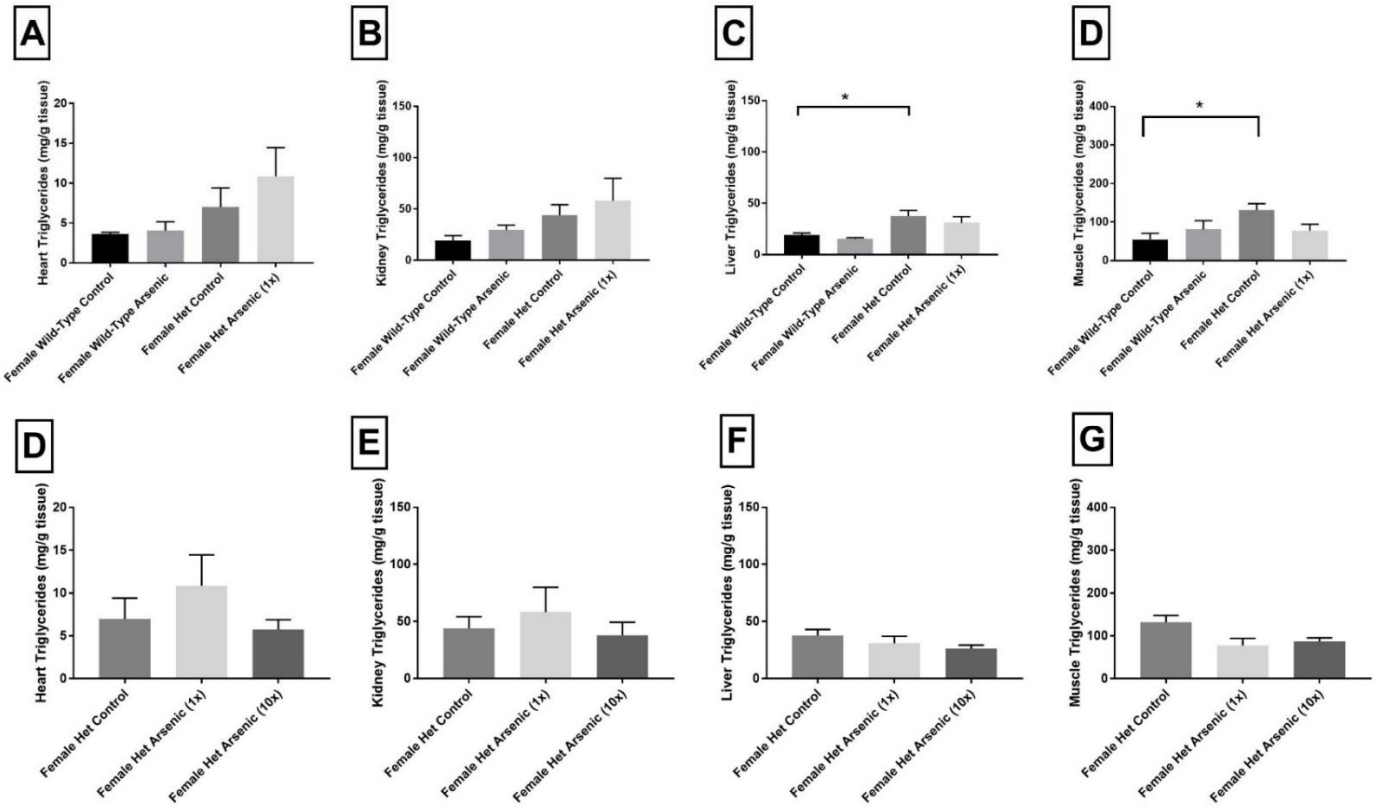


Figure 2.16. Female heterozygote controls have higher levels of liver and muscle triglycerides compared to wild-type controls. Graphs A-D represent wild-type vs heterozygote triglycerides in different organs and graphs E-H represent heterozygote dose response triglycerides in different organs. Arsenic exposure did not change triglyceride levels in the heart, kidney, liver or muscle. However, female het controls had significantly higher levels of liver and muscle triglycerides compared to wild-type controls. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

Chapter 3 – Arsenic and Inflammation

3.1 Introduction

Arsenic, a ubiquitous element, is a known toxin in its inorganic form. Humans are exposed to about 50 µg/day, 3.5 µ/day coming from inorganic sources in food and water (ATSDR, 2013). Because arsenic is a well-known carcinogen, in an attempt to reduce levels of exposure the amount of arsenic in public drinking water is very highly regulated, with a relatively recent reduction in action level from 50 µg/L to 10 µg/L by the EPA in 2001 (ATSDR, 2013). Besides cancer, arsenic is known to affect a number of different organ systems and has been linked to cardiovascular disease, type 2 diabetes and immunological disorders (Mandal & Suzuki, 2002). It has been demonstrated that at higher concentrations (>500 µg/L), arsenic may increase susceptibility to type 2 diabetes, but at lower concentrations the results are inconclusive (Huang et al., 2011; Navas-Acien et al., 2006). The effects of arsenic on inflammation in humans and mice have been extensively reviewed by Dangleben et al., Mandal and Suzuki, and Tobergte and Curtis (2013; 2002; 2013). According to these sources, arsenic can alter gene expression, immune cell population, and ROS production that can lead to either a range of different consequences from a suppressed immune system to a pro-inflammatory immune state, however the results will vary based on the concentration of arsenic in question. While the majority of studies have focused on higher levels of arsenic exposure and the effects on the immune system, there is still a lot unknown about how low-level arsenic exposure can affect the immune response.

When considering the effects of arsenic on inflammation, the baseline inflammatory profile is an important component to be considered. In lean individuals, there is a balance in the inflammatory state. However, as BMI increases, the inflammatory profile shifts towards a more pro-inflammatory state that is both local in the adipose tissue and systemic (Mosser & Edwards, 2008). Thus, it is important to determine whether arsenic affects metabolic diseases, such as type 2 diabetes, where changes in inflammation may play a vital role in the progression of the disease. In our study we propose to examine the effects of arsenic on the immune system in both sexes of two lean mouse strains – C57BL/6 and db/+ at low (50 µg/kg) and high (500 µg/kg) levels of arsenic. This will allow us to begin to determine whether there are sex differences in the immune response induced by arsenic exposure and to examine the role of genotype in these lean mice. We will evaluate inflammation by examining systemic cytokine profiles and organ-specific gene expression. This will be compared to the overall change in body composition in these mice to determine whether the inflammatory profile changes as a result of arsenic exposure or due to changes in the mouse fat mass. Based on current literature and the results at high levels of arsenic exposure, we hypothesize that in these lean mice, arsenic will increase systemic and local inflammation.

3.2 Methods

3.2.1 Animals

Male and female B6.BKS(D)*Lepr*^{db}/J heterozygote mice were bred and housed at the University of South Carolina Animal Resource Facility. Offspring of the heterozygote x heterozygote cross were genotyped by RT-PCR followed by a restriction digest for the

Leptin gene at weaning. The primer sequences were forward: 5'-AGAACGGACACTCTTTGAAGTCTC-3'; and reverse: 5'-CATTCAAACCATAGTTTAGGTTTGTGT-3'. PCR products were digested using *RsaI* enzyme, which cleaved sequences into one 135 base pair band or 27, 108 and 135 base pair bands representing wildtype or heterozygous genotypes, respectively. Once genotypes, animals were grouped into control, 1x inorganic arsenic ($AsCl_3$ - Alfa Aesar, 50µg/kg) or 10x inorganic arsenic (500µg/kg) and were gavaged every other day. Weights were recorded every other day starting when the mice were 4 weeks old, and every 4 weeks an oral glucose tolerance test (OGTT) and Dual Energy X-ray Absorptiometry (DEXA) scan were performed. Animals were maintained on a 12:12 hour light cycle and given food and water ad libitum. At 12 weeks, animals were sacrificed for tissue collection using isoflurane overdose followed by cervical dislocation. Principles of laboratory animal care were followed and the Institutional Animal Care and Usage Committee of the University of South Carolina approved all experiments.

3.2.2 Tissue collection

Mice were euthanized at 12 weeks of age for tissue collection. Blood was collected by cardiac puncture and serum separated for cytokine analysis. Reproductive adipose tissue was collected for histology, preserved for 24 hours in 4% paraformaldehyde and then transferred to 70% ethanol before histology, or snap frozen on dry ice and stored at -80ºC for triglyceride, protein, and RNA analysis. Spleen, liver, kidney, thigh muscle and heart were also snap frozen and stored at -80ºC for protein and RNA analysis.

3.2.3 Cytokine analysis

Spleen and serum cytokines were measured by multiplex bead-based assay (Bio-Plex, Bio-Rad) according to manufacturer's protocol for the following cytokines: interleukin (IL)-1β, IL-4, IL-6, IL-10, IL-13, interferon (IFN)-γ, granulocyte macrophage colony-stimulating factor (GMCSF) and tumor necrosis factor (TNF)-α. Limits of detection were as follows in the spleen (in pg/ml): IL-1β=28.11, IL-4=3.59, IL-6=1.01, IL-10=11.03, IL-13=53.43, IFN-γ=14.09, GMCSF=9.37 and in the serum (in pg/ml): IL-1β=60.52, IL-6=1.27 and TNF-α=53.82. Standard curves were generated in triplicate. Cytokine measurements below the limit of detection were assigned a value of the limit of detection/√2 for statistical analysis and plotting. TNF-α levels were measured in homogenized spleen using ELISA kits (eBioScience) according to the manufacturer's instructions, with a limit of detection of 8 pg/mL.

3.2.4 Gene expression

RNA was isolated from reproductive adipose tissue according to the manufacturer's protocol (Bioline Isolate RNA kit). RNA was converted to cDNA using SensiFAST cDNA synthesis kit according to manufacturer's protocol (Bioline). Real-time PCR was then performed on 100ng of cDNA using SensiFAST SYBR (Bioline) and 10μM of designed and validated primers according to manufacturer's protocol. Custom primers (Table 3.1) were designed for each target using Primer Blast Search (NCBI) and purchased from Integrated DNA Technologies (IDT) for qPCR. Gene expression patterns for each gene were normalized to the expression of the housekeeping gene RPLP0 and quantified using the $\Delta\Delta^{CT}$ method.

3.3 Statistics

All statistical analyses were carried out using Stata for linear regression and Tukey's post-test. The n for each treatment group were as follows: male wild-type control (n=5), male wild-type arsenic (n=4), male heterozygote control (n=11), male heterozygote arsenic 1x (n=8), male heterozygote arsenic 10x (n=3), female wild-type control (n=6), female wild-type arsenic (n=5), female heterozygote control (n=9), female heterozygote arsenic 1x (n=6), female heterozygote arsenic 10x (n=3), unless otherwise noted. Three mice were excluded from all analyses as they were outliers in more than 3 different categories analyzed. Data are expressed as means ± SEM. Regression analysis were performed to compare exposure, genotypic, and sex differences. A p-value of <0.05 was considered statistically significant.

3.4 Results

3.4.1 Effect of arsenic on fat mass

We predicted that arsenic exposure would cause changes in body composition that would affect systemic inflammation. In order to determine whether the effects on the immune system were caused by change in body fat or by arsenic, we first plotted the change in body fat from baseline (4 weeks) to 12 weeks of age for both males and females (Figure 3.1). While the male heterozygous animals appear to have a greater change in body fat after 8 weeks of 1x arsenic exposure, the change was not significant. The only difference found to be significant based on genotype was between female wild-type control and female heterozygotes, indicated by an increase in fat mass from baseline in the heterozygotes compared to wild-type (Figure 3.1).

3.4.2 Effect of arsenic on serum cytokines

To determine the effect of arsenic on systemic inflammation, we chose to examine the serum cytokines. Both TNF-α and IL-1β were below the limit of detection, and therefore excluded. There was no significant difference in IL-6 in circulation based on treatment group or genotype for males or females (Figure 3.2), however there were sextypic differences in the heterozygotes. In the males exposed to 10x arsenic, there is a large increase in IL-6 in circulation, but in females as arsenic exposure increases in the heterozygotes IL-6 in circulation decreases, demonstrating that males may have more proinflammatory cytokines in circulation in response to arsenic exposure.

3.4.3. Effects of arsenic on spleen cytokines

To further examine the effects of arsenic on systemic inflammation, and because cytokines are short-lived in circulation, we also evaluated cytokine levels in the spleen. In the male heterozygotes, IFN-γ was decreased in the 1x arsenic treatment group, but this is not present or amplified in the 10x treatment group (Figures 3.3 and 3.4). This differed greatly from the response in the females, where in the wild-type mice IFN-γ and IL-1β were significantly increased (Figure 3.5). In the female heterozygotes, IL-13 was significantly decreased between 1x and 10x treatment groups but IL-10 and IL-4 were significantly increased from 1x-10x treatment groups (Figure 3.6). This demonstrated that in females, depending on genotype, arsenic can either induce a more proinflammatory response in circulating cytokines or anti-inflammatory response.

3.4.4 Effect of arsenic on local inflammation

We wanted to examine the effects of arsenic exposure on local inflammation, and to do this we chose to evaluate changes in macrophage expression. With changes in body composition, there are changes in macrophage populations. As fat mass increases, there is a shift from balanced M1/M2 macrophage phenotypes to a pro-inflammatory M1 phenotype. In adipose tissue, this is typically correlated with an increase in macrophage accumulation in tissue (Kathryn, Wellen, & Gokhan, 2003). In Figures 3.7 and 3.8, we did not see a change in expression of either inducible nitric oxide synthase (iNOS) (M1), arginase (M2), or CCL2 (macrophage recruitment) in either males or females after arsenic exposure. However, there were overall differences in the heterozygous males exposed to 10x arsenic; while not significant, arginase, iNOS, and CCL2 were reduced compared to the 1x treatment group (Figure 3.7). There were also non-significant sextypic differences, as CCL2 in 1x arsenic-exposed heterozygous males was increased from control, where in 1x arsenic-exposed heterozygous females CCL2 was reduced.

We chose the same method of assessment in the spleen, however, unlike in the adipose tissue, male heterozygotes in the 10x treatment group showed an increase in expression in both iNOS and arginase (Figure 3.9). There were minimal changes in expression in either gene in the wild-type males, indicating there were no changes in macrophage population. Females had no significant change in either iNOS or arginase based on exposure to arsenic (Figure 3.10). However, when examining differences between the two genes, female wild-type mice had a decrease in arginase and an increase in iNOS when exposed to arsenic.

Finally, we wanted to evaluate changes in macrophage populations in the liver, as this is the primary organ for detoxification. We saw no significant changes in male (Figure 3.11) or female (Figure 3.12) liver expression of arginase or iNOS with arsenic exposure. While not significant, there is a large increase in the expression of iNOS in the male heterozygotes exposed to 10x arsenic. This differs from the female heterozygotes exposed to 10x arsenic, where there is a decrease in iNOS expression.

3.5 Discussion

Overall, there were differences in inflammation caused by arsenic exposure in both sexes, genotypes and treatment groups. Most studies focusing on the effects of arsenic exposure are limited in scope to the effects on the development of type 2 diabetes. Here, we present a case for low-dose arsenic exposure, similar to exposure levels in the United States, wherein arsenic is not diabetogenic but induces changes in the immune response. We also present novel male and female data, since the majority of studies examining the effects of arsenic have been limited to only males.

In circulating cytokines, males and females exhibited very different responses depending on genotype. Where male heterozygotes had a decrease in IFN-γ when exposed to 1x arsenic, female wild-type mice had an increase in IFN-γ. In female heterozygotes, when increasing arsenic exposure from 1x to 10x arsenic there is a drastic change in cytokine profile with an increase in IL-4 and IL-10 and a decrease in IL-13, which demonstrates changes in the anti-inflammatory environment. Overall, the circulating inflammatory environments for each of these treatment groups is very different. Where wild-type males were not affected, the change in male heterozygotes indicates a decrease in inflammation. In the female wild-type mice, arsenic increased

both IFN-γ and IL-1β, which are both pro-inflammatory cytokines. For female heterozygotes, as arsenic concentration increased, there was an increase in anti-inflammatory cytokines. This is opposite of what we would have expected based on the change in fat mass in the females, as the heterozygotes had the greatest increase in fat mass. Based on current literature, this increase in fat mass in this genotype should have had more pro-inflammatory cytokines in circulation, suggesting that the changes exhibited in systemic inflammation were arsenic driven, not obesity driven.

To examine inflammation at a local level, we chose to evaluate gene expression changes in organs that are affected by arsenic exposure: adipose tissue, liver and spleen. These three tissues accumulate arsenic, and are vulnerable to exposures (ATSDR, 2013). We hypothesized that since arsenic is typically an inducer of inflammation, the macrophage populations in these tissues would shift from a balanced M1/M2 population toward a primarily M1 population. To test this hypothesis, we chose three genes, arginase, iNOS and CCL2 to determine the effects of arsenic on macrophage populations. The primary organ affected was the spleen of heterozygote males. In this group, as arsenic exposure increased, the expression of iNOS and arginase increased. This could be due to an increase in total macrophage population. Female spleens, despite the increased pro-inflammatory cytokines in circulation, were not affected at the tissue level.

Here we have presented that, depending on sex and genotype, arsenic has very different effects on the immune system. Both of these mouse models represent a non-susceptible population for diabetes, and while there was a significant difference in fat mass in females based on genotype, all of the changes observed are correlated with arsenic exposure. These findings will inform future analysis of arsenic and the

development of diabetes in a susceptible model. While we cannot link these low levels of arsenic to diabetes in our models, we can say that low-dose arsenic exposures can have significant impacts on the immune system that warrant further exploration.

Table 3.1. Real-time PCR Primer list.

ID	Forward 5'-3'	Reverse 5'-3'
CCL2	CAGCAGGTGTCCCAAAGAAG	ACCTTAGGGCAGATGCAGTT
iNOS	GAAGTTCAGCAACAACCCCA	TTCAAGATAGGGAGCTGCGA
Arginase	CCCTGCCAATCATGTTCCTG	GAGAAAGGGGCTCCGACTAT
RPLP0	CCTGAAGTGCTCGACATCAC	GCGCTTGTACCCATTGATGA

Abbreviations: Chemokine ligand 2 – CCL2, Inducible nitric oxide synthase – iNOS, Ribosomal protein lateral stalk subunit P0 – RPLP0

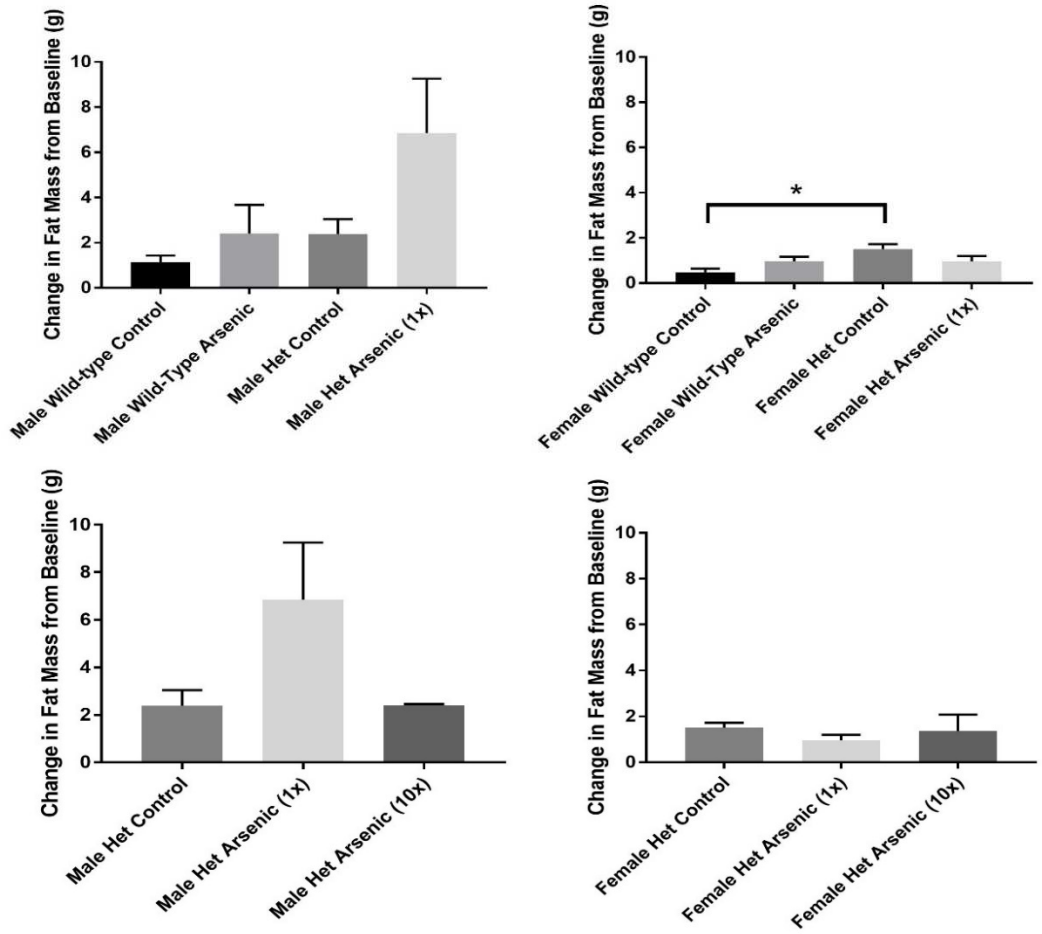


Figure 3.1. Arsenic does not significantly alter change in fat mass. Change in fat mass from 4 weeks to 12 weeks old in male and female mice. Female wild-type mice have a significantly lower change in fat mass than female heterozygotes without arsenic exposure. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

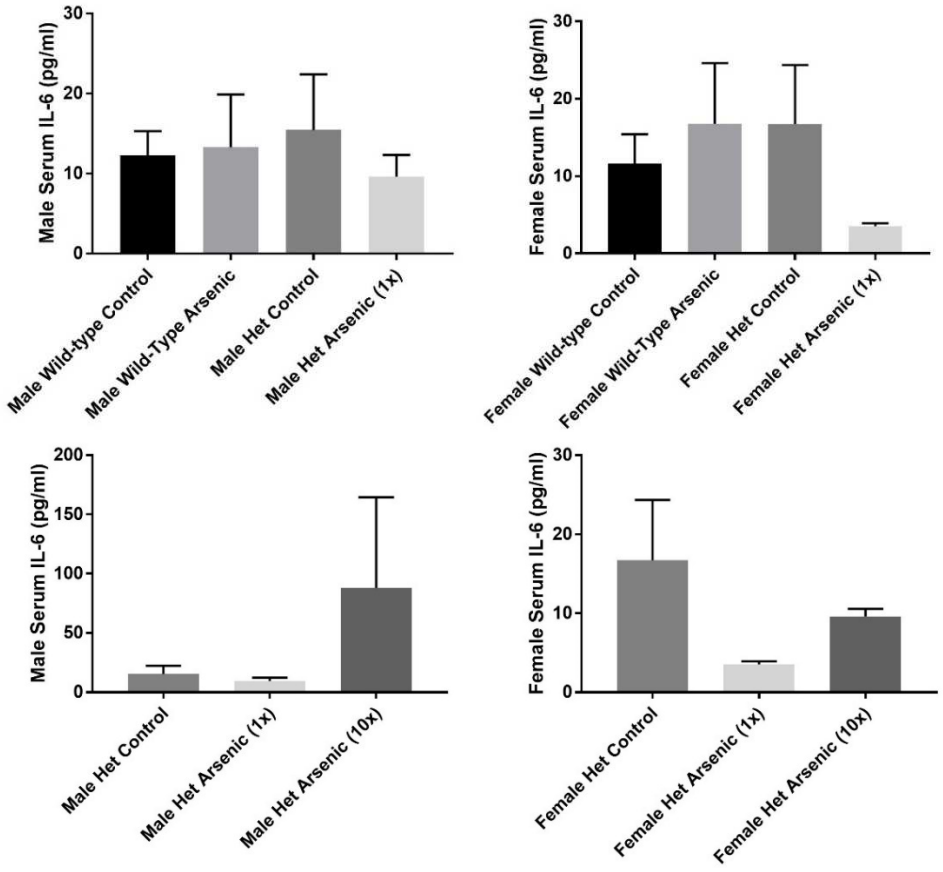


Figure 3.2. Arsenic has no effect on serum IL-6 levels. Serum cytokine profile of male and female wild-type and heterozygous mice. There were no significant differences present in IL-6 levels in circulation in either males or females. Cytokines were measured by Bio-Plex. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

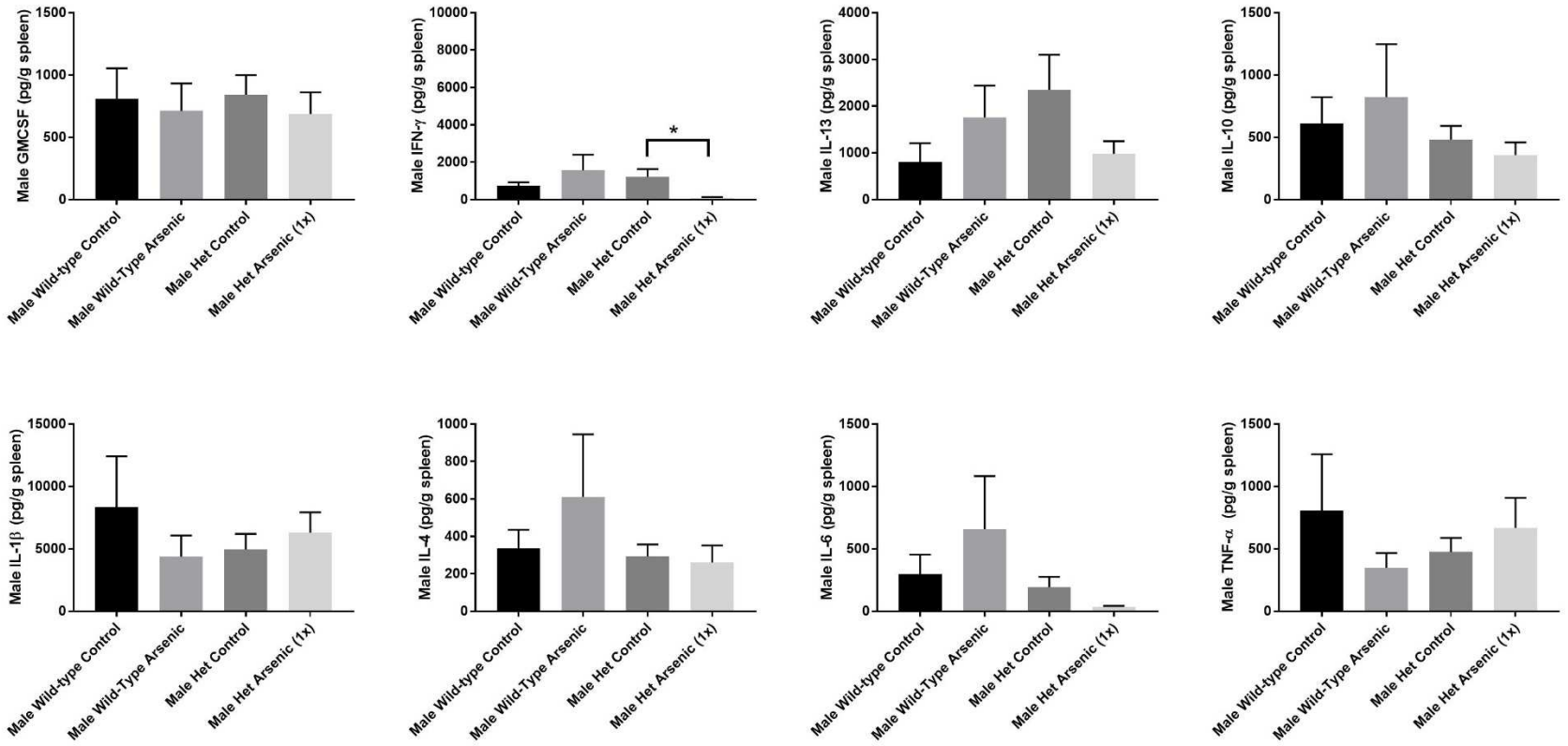


Figure 3.3. Arsenic reduces IFN-γ in spleen of male heterozygotes. Spleen cytokine analysis of male wild-type and heterozygotes after 8 weeks of arsenic exposure. IFN-γ was significantly reduced in male heterozygotes. Cytokines were measured by elisa and the results calculated as pg/g spleen tissue. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

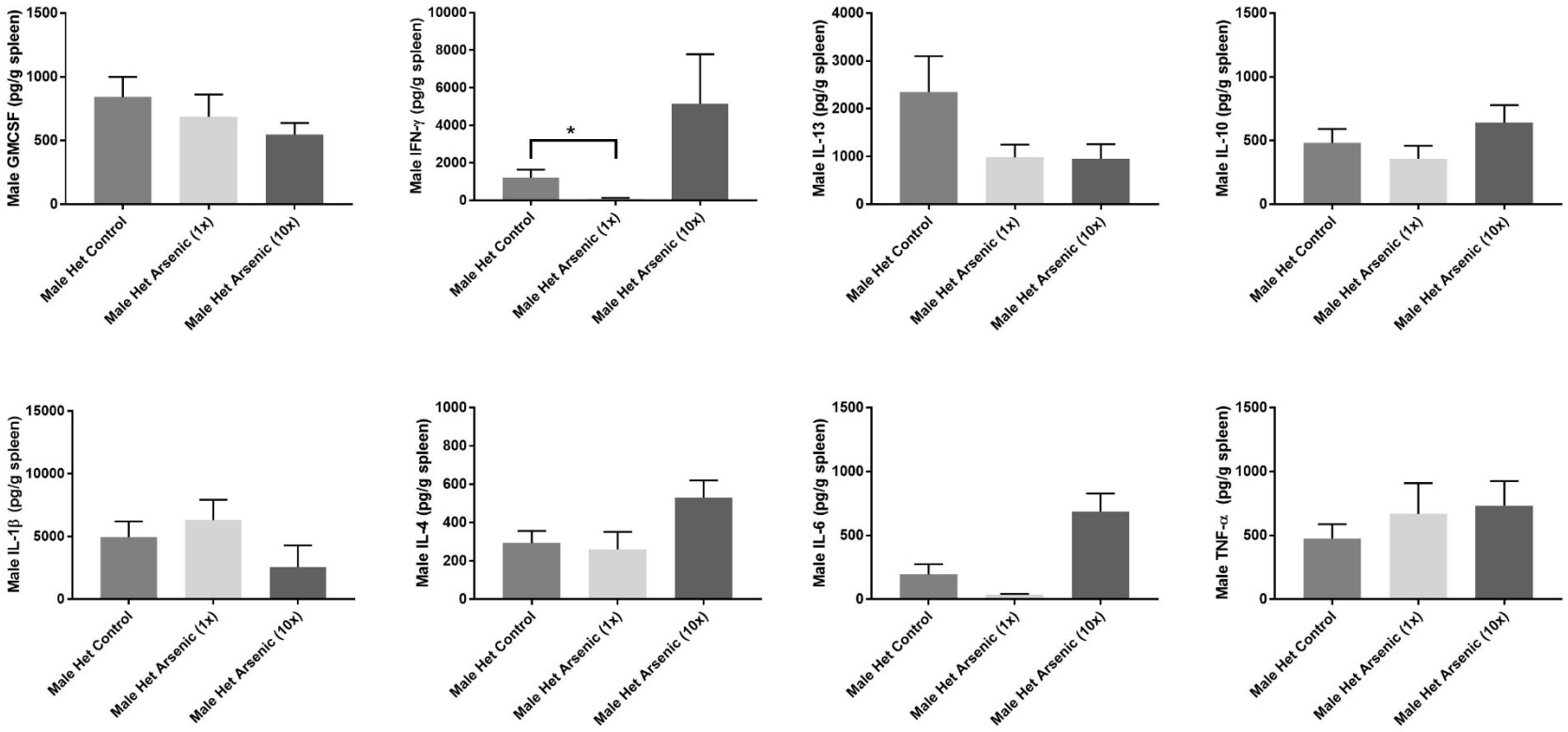


Figure 3.4. Increase in arsenic exposure does not affect IFN-γ in male heterozygotes. Spleen cytokine analysis of male heterozygotes after 8 weeks of arsenic exposure. IFN-γ was reduced in male heterozygotes in the 1x arsenic treatment group, but this was not exacerbated by increasing exposure levels. Cytokines were analyzed by elisa and the results calculated as pg/g spleen tissue. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

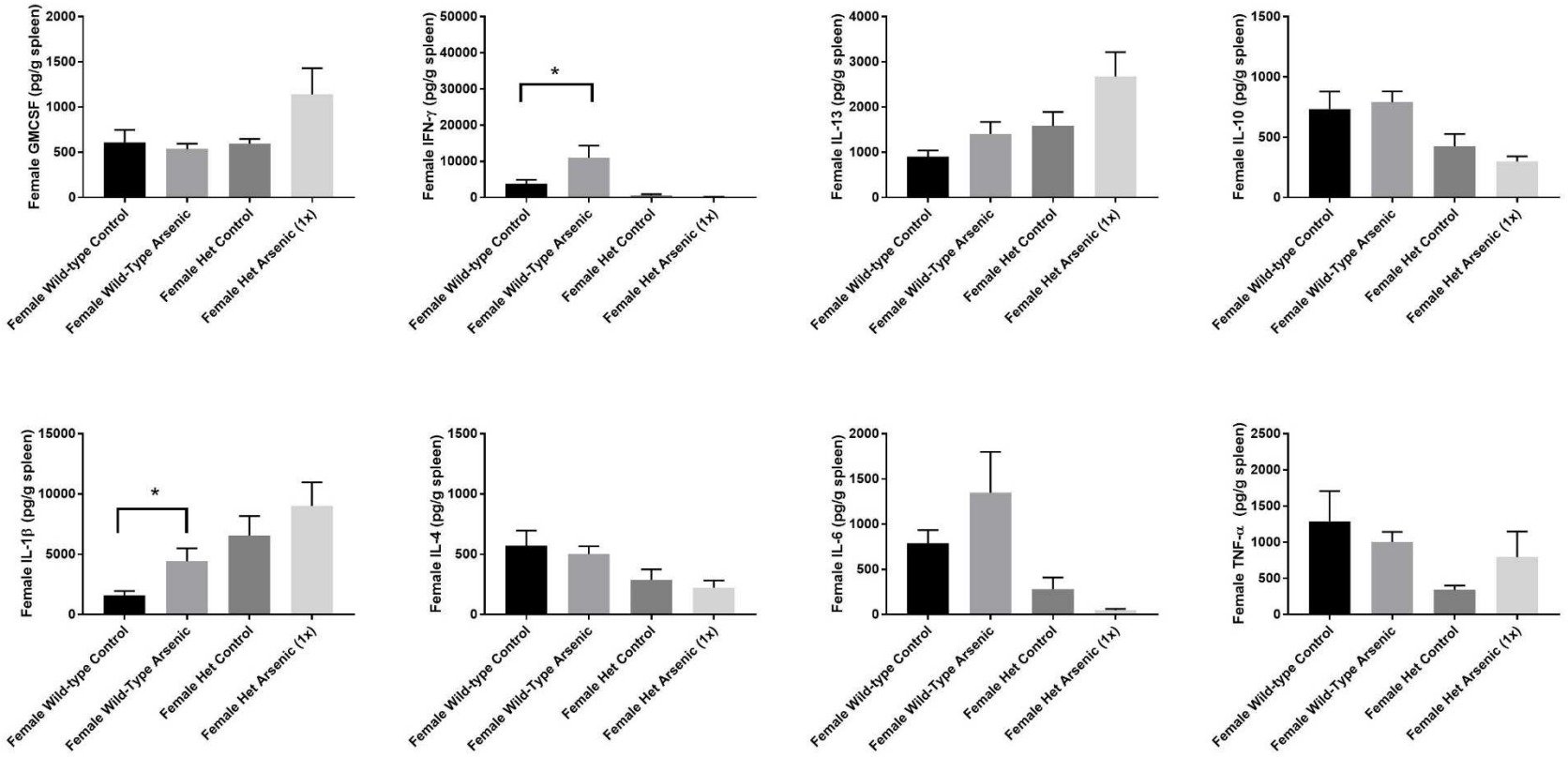


Figure 3.5. Arsenic exposure increases IL-1β and IFN-γ in wild-type females. Spleen cytokine analysis of female wild-type and heterozygotes after 8 weeks of arsenic exposure. Female wild-type mice had a significant increase in IFN-γ and IL-1β caused by arsenic exposure. Cytokines were measured by elisa and the results calculated as pg/g spleen tissue. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

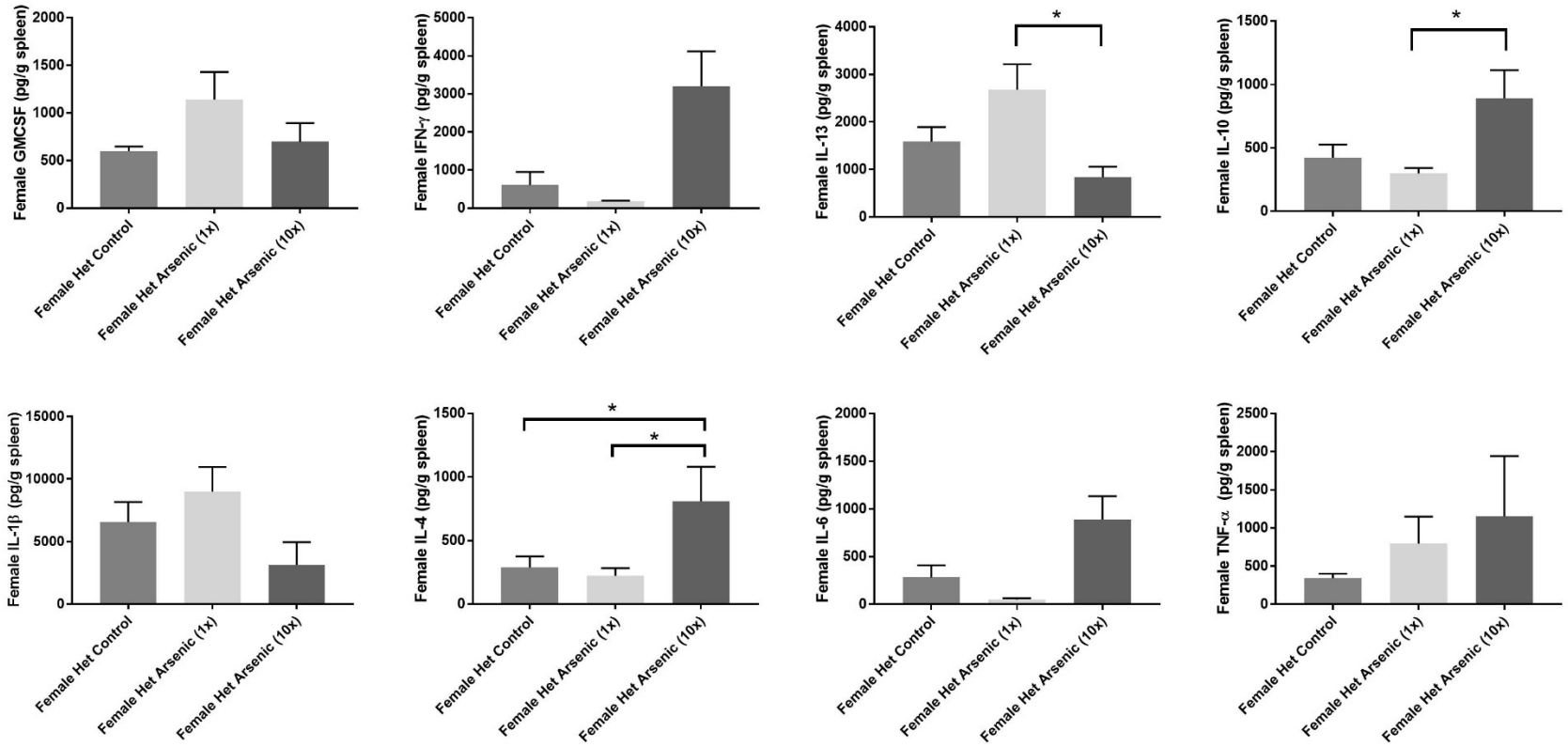


Figure 3.6. Increase in arsenic exposure affects female heterozygote spleen cytokines. Spleen cytokine analysis of female heterozygotes after 8 weeks of arsenic exposure. Increase from 1x to 10x arsenic exposure increased IL-10 and IL-4 concentration, but decreased IL-13 concentration. Cytokines were measured by elisa and the results calculated as pg/g spleen tissue. Data are represented as a mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

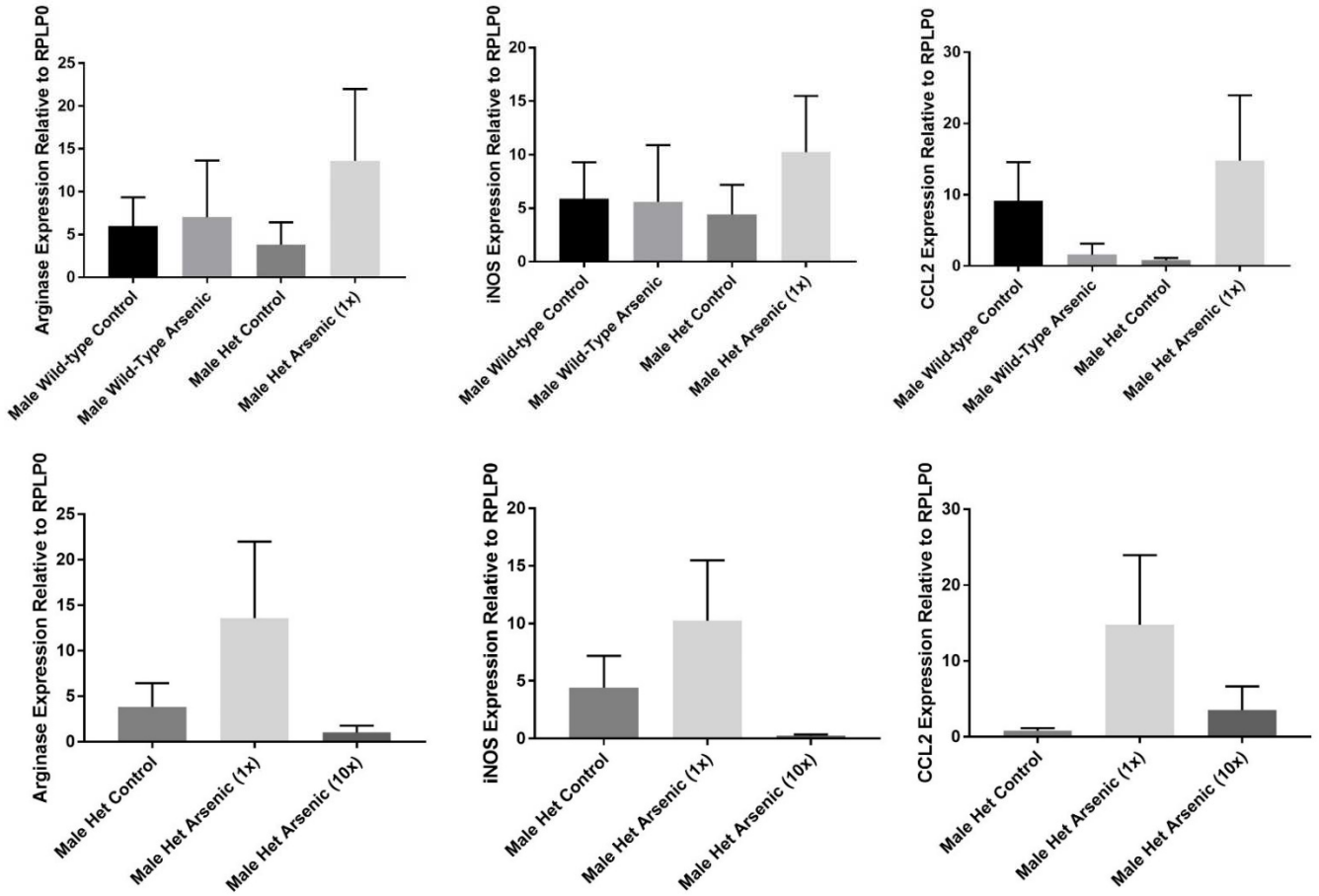


Figure 3.7. Arsenic exposure does not affect adipose tissue cytokine expression in males. In male adipose tissue, arginase, iNOS, and CCL2 expression was determined by RT-PCR. Arsenic did not have an effect on the expression of any analyzed genes. All data were normalized to RPLP0. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

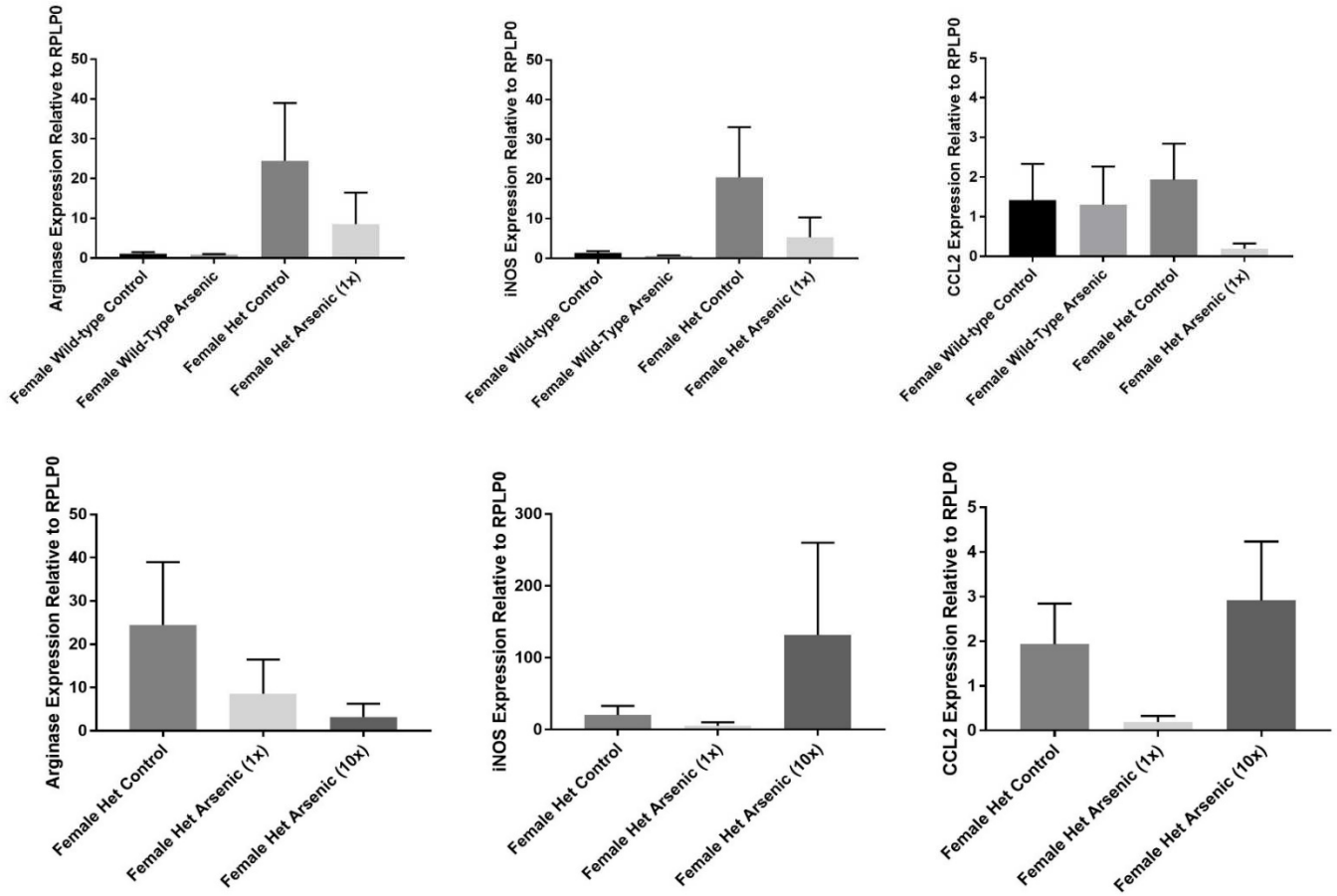


Figure 3.8. Arsenic exposure does not affect adipose tissue cytokine expression in females. In female adipose tissue, arginase, iNOS, and CCL2 expression was determined by RT-PCR. Arsenic did not significantly alter any of the analyzed genes. All data were normalized to RPLP0. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

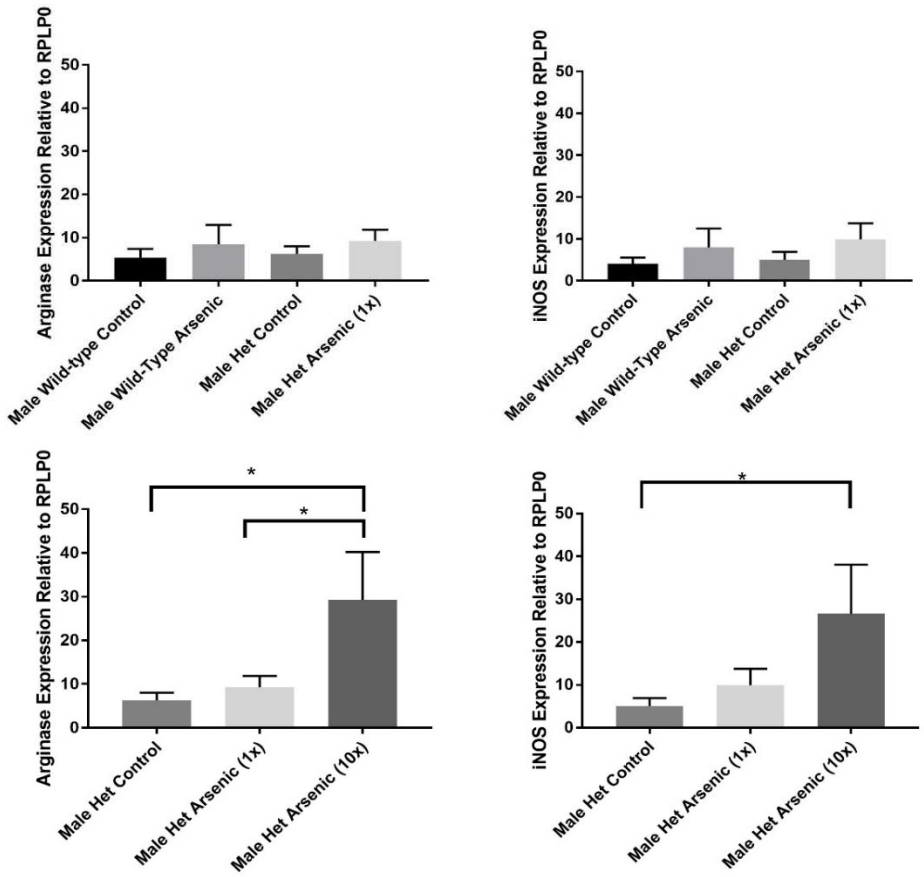


Figure 3.9. Increase in arsenic exposure affects macrophage gene expression in male heterozygote spleens. Male heterozygotes had an increase in arginase and iNOS expression in the spleen after 8 weeks of 10x arsenic exposure. Arginase and iNOS expression was determined by RT-PCR. All data were normalized to RPLP0. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

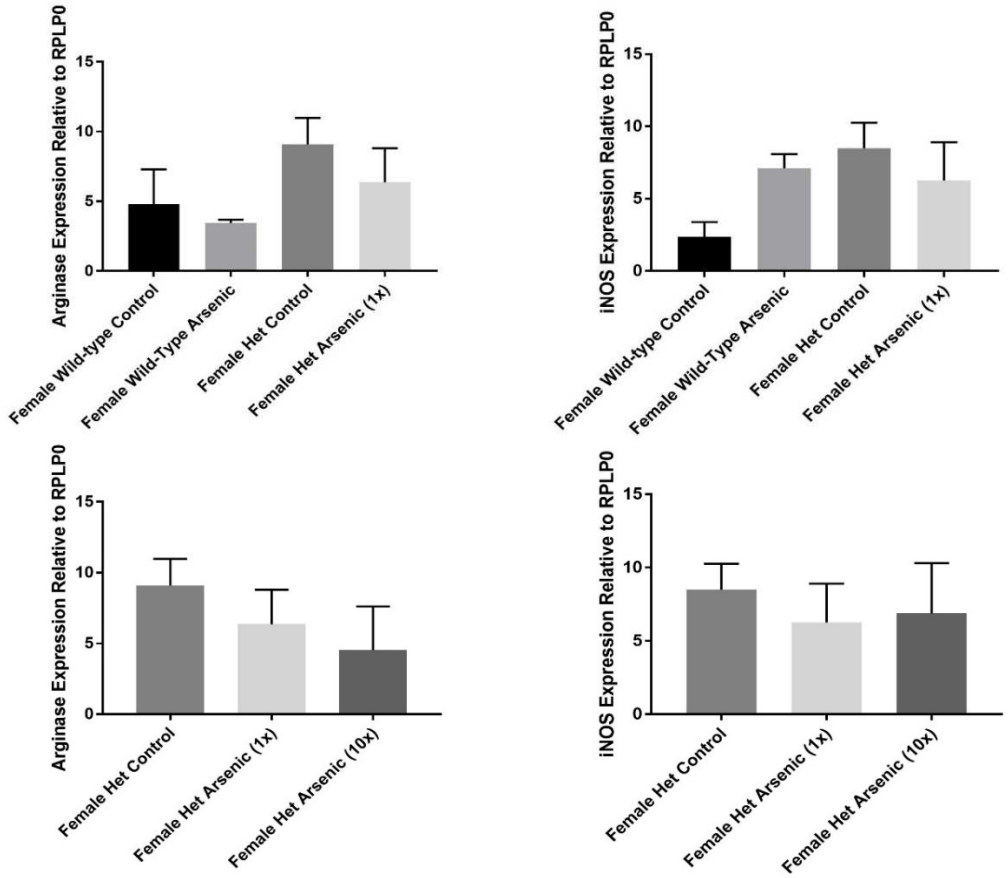


Figure 3.10. Arsenic exposure does not affect spleen cytokine expression in females. In females no change was observed in the spleen after 8 weeks of 10x arsenic exposure. Arginase and iNOS expression was determined by RT-PCR. All data were normalized to RPLP0. Data are represented as a mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

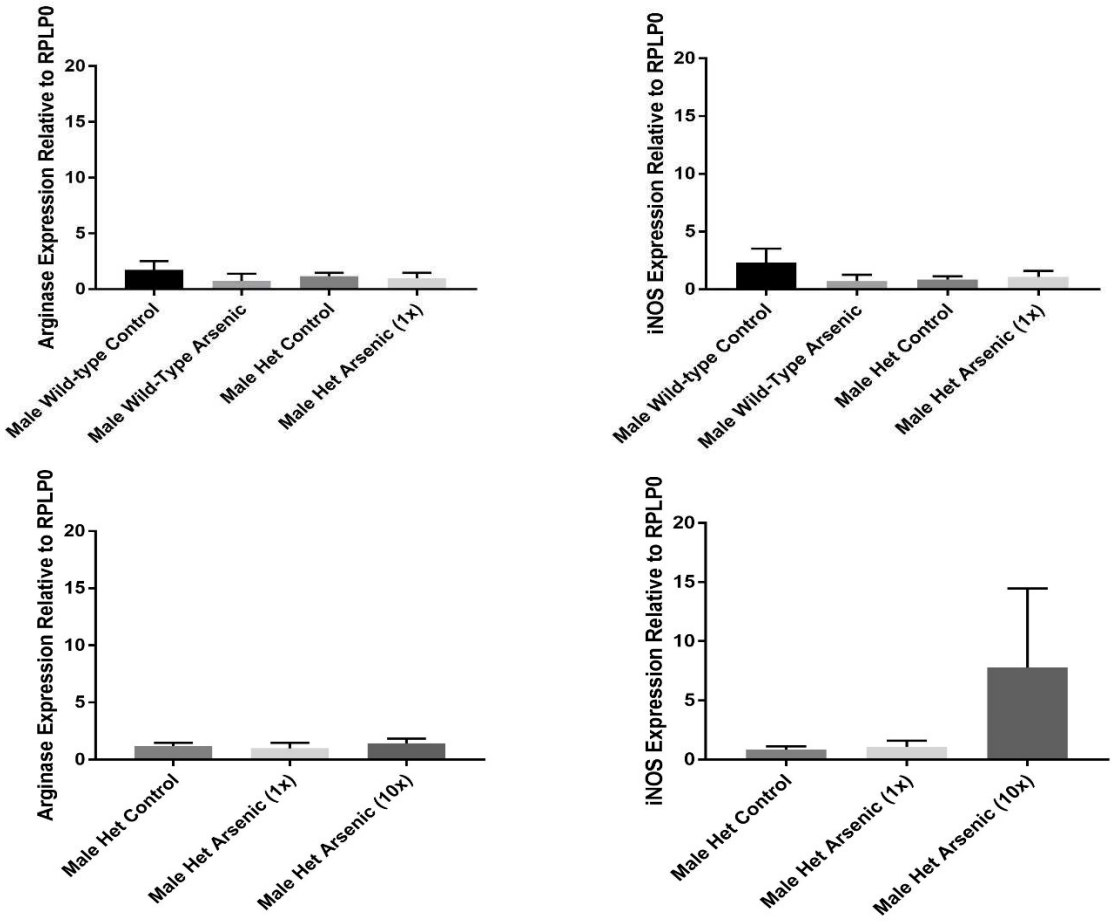


Figure 3.11. Arsenic exposure does not affect male macrophage gene expression in the liver. In males, there was no change in gene expression in the liver after 8 weeks of 10x arsenic exposure. Arginase and iNOS expression was determined by RT-PCR. All data were normalized to RPLP0. Data are represented as a mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

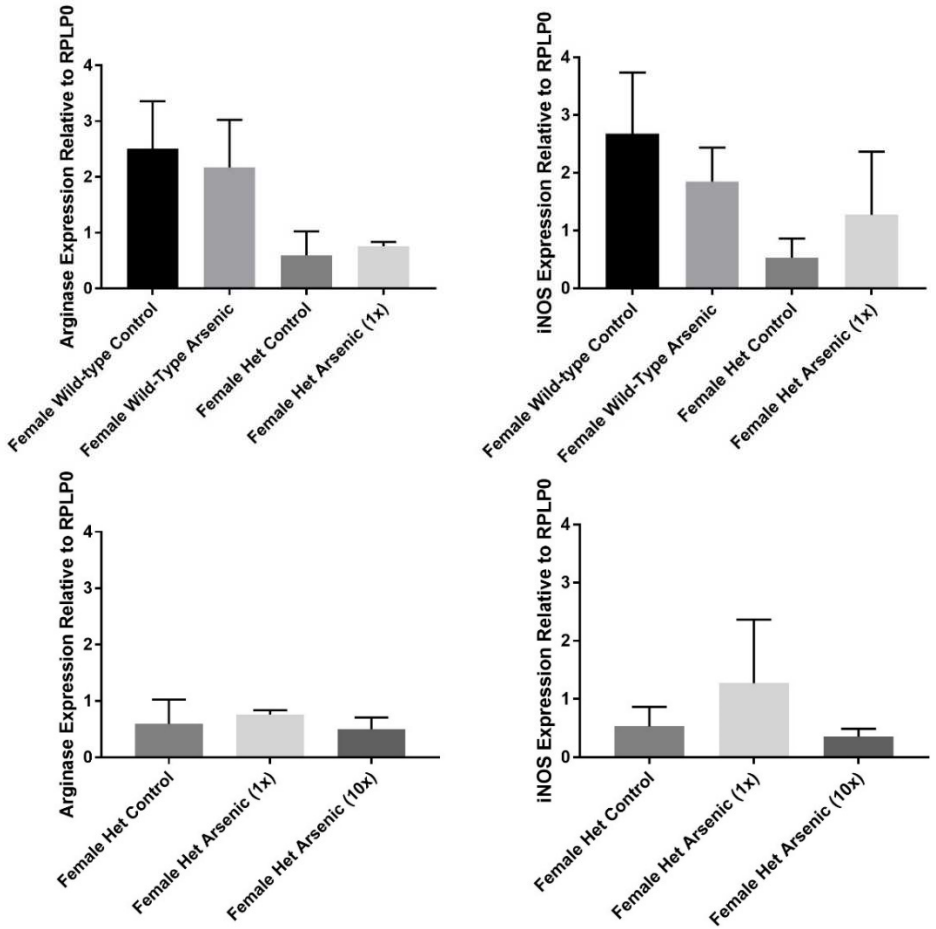


Figure 3.12. Arsenic exposure does not affect female macrophage gene expression in the liver. In females, there was no change in gene expression in the liver after 8 weeks of 10x arsenic exposure. Arginase and iNOS expression was determined by RT-PCR. All data were normalized to RPLP0. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using linear regression followed by Tukey's multiple comparison test and is indicated as * ($p<0.05$).

Chapter 4 – Arsenic and Macrophages

4.1 Introduction

Arsenic is a ubiquitous element found in the earth's crust that can be naturally occur in organic and inorganic forms. Typical arsenic exposure is about 50 µg/day, with about 3.5 µg/day from inorganic sources and the remainder from non-toxic organic sources (ATSDR, 2013). The main exposures to inorganic arsenic occur from ingestion of contaminated food and water. In the United States, the amount of arsenic in public drinking water is very highly regulated, and was recently reduced from 50 µg/L to 10 µg/L by the EPA in 2001 based on its carcinogenicity (ATSDR, 2013). Besides cancer, arsenic has been linked to a number of diseases, including cardiovascular disease, type 2 diabetes and immunological disorders (Mandal & Suzuki, 2002).

When considering arsenic and type 2 diabetes, inflammation must be addressed. The inflammatory profile of a non-diabetic is very different from that of a diabetic individual, and arsenic as an immunotoxic agent can exacerbate this situation. Arsenic, has previously been found to modulate the overall immune profile in both lean and obese individuals by affecting gene expression, cell population and ROS production (Dangleben et al., 2013; Mandal & Suzuki, 2002; Tobergte & Curtis, 2013). However, in obese individuals, the systemic and local inflammatory profile contains more pro-inflammatory components, such as M1 macrophages, whereas lean individuals will have a balance of M1 to M2 macrophages (Mosser & Edwards, 2008). Arsenic has been shown

to affect macrophages (Lemarie et al., 2006), therefore it could be suggested that arsenic would have different effects on the immune system in lean and obese individuals.

In this study we proposed to examine the effects of sub-cytotoxic levels of arsenic on Raw 264.7 macrophages in both normal and high glucose environments, to represent non-diabetic and nondiabetic individuals, respectively. By varying glucose concentration, we were able to model the effects of arsenic in both normal and high blood glucose scenarios, similar to a normal and hyperglycemic individual and the stress that is put on the macrophages in each scenario. We evaluated gene expression changes in these macrophages by real-time PCR to determine whether they are more pro-inflammatory after an immune challenge following exposure to arsenic. We chose to evaluate these gene expression changes after either 3 or 24 hours of arsenic exposure to demonstrate differences in acute and chronic exposure, as arsenic has a half-life in the blood of approximately 3 hours (ATSDR, 2013) We hypothesized that arsenic exposure and higher concentrations of glucose will increase inflammatory cytokine expression in macrophages, especially over a period of chronic exposure.

4.2 Methods

4.2.1 Cell culture

Raw 264.7 macrophages (ATCC) were grown to confluence in high or low glucose Dulbecco's Modified Eagle Medium (DMEM, Hyclone) with 2% FBS (Hyclone) and 1% penicillin/streptomycin (ThermoFisher) at 37°C and 5% CO_2. Cells were stimulated with lipopolysaccharide (Sigma-Aldrich) at 50ng/ml for 4 hours after exposure to inorganic arsenic ($AsCl_3$ - Alfa Aesar,) in a serial dilution ranging from $1x10^{-7}$µM to

100µM for 3-24 hours to determine sub-cytotoxic concentrations to be used in future experiments

4.2.2 Cell viability

Cell viability was determined using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's instructions. Cells were grown to confluence and then incubated with arsenic (0-100 µM) for 3 hours to determine sub-cytotoxic doses to be used in subsequent experiments.

4.2.3 Gene expression

RNA was isolated from cells using Isolate II kit (Bioline) according to manufacturer's protocol with quantity and quality measured spectrophotometrically via 260/280 ratio. Real-Time PCR was then performed using SensiFast 1-Step Sybr Green kit (Bioline) for PCR according to manufacturer's protocol with 1ng RNA and 10 µM of designed and validated primers. Custom primers (Table 4.1) were designed for each target using Primer Blast Search (NCBI) and purchased from Integrated DNA Technologies (IDT) for qPCR. Gene expression patterns for each gene were normalized to the expression of the housekeeping gene RPLP0 and quantified using the $\Delta\Delta^{CT}$ method.

4.2.5 Statistics

All statistical analyses were carried out using GraphPad Prism 7 Software (San Diego, CA). Results are expressed as the mean ± SEM, and all experiments were performed in triplicate to test reproducibility of results. Multiple comparisons were made

using one-way analysis of variance (ANOVA), followed by *post hoc* analysis using Tukey's method. A p-value of <0.05 was considered statistically significant.

4.3 Results

4.3.1 Cell viability

We wanted to establish sub-cytotoxic doses of arsenic to be used in the RAW264.7 macrophage experiments. The MTT assay (Figure 4.1), which determines cell viability colormetrically, demonstrated that any concentration greater than 0.1 μM had significantly reduced survival, as absorbance in the assay dropped from 2.74 to less than 0.5.

4.3.2. Three-hour arsenic exposure in low glucose

Based on our hypothesis that sub-cytotoxic exposure to arsenic will modify cytokine gene expression in macrophages, we examined the changes in gene expression that occur at 3 hours of exposure in normal glucose concentrations. As arsenic concentrations increase from low (0.001μM) to high (0.01μM), there were no changes in cytokine expression in IL-10, TNF-α, STAT3 or TGF-β (Figure 4.2). These findings do not support our initial hypothesis that higher levels of arsenic exposure are enough to drive a pro-inflammatory response in macrophages.

4.3.3. Three-hour arsenic exposure in high glucose

In a diabetic state, as blood glucose increases we would expect the inflammatory state of macrophages to change from neutral to pro-inflammatory. Therefore, by adding in the inflammatory modulator of arsenic should cause a change in macrophage state, most likely pushing them toward a pro-inflammatory state. Here, we demonstrated that as

arsenic concentration increases, the expressions of IL-10 significantly decreased (Figure 4.3). These findings support our hypothesis of increasing arsenic exposure increases a pro-inflammatory response as IL-10 is an anti-inflammatory cytokine that inhibits Th1 immune responses (Couper, Blount, & Riley, 2008).

4.3.4. Twenty-four-hour arsenic exposure in low glucose

We wanted to determine whether increasing the exposure time, similar to a more chronic level of arsenic exposure, would affect expression of cytokines by macrophages differently than short-term exposure. As expected, in low glucose, there was no significant changes in gene expression induced by arsenic exposure (Figure 4.4), demonstrated by no significant changes in gene expression in IL-10, TNF-α, STAT3 or TGF-β. This does not support our hypothesis that increasing the duration of arsenic exposure (acute vs chronic) would increase the expression of pro-inflammatory cytokines in macrophages.

4.3.5. Twenty-four-hour arsenic exposure in high glucose

Finally, we wanted to determine the effects of chronic arsenic exposure on macrophages cultured in a high glucose environment, modeling a diabetic individual being exposed to the additional stressor of arsenic. After stimulation with LPS, we saw no significant changes in gene expression induced by arsenic exposure in IL-10, TNF-α, STAT3 or TGF-β (Figure 4.5), suggesting that chronic arsenic exposure does not induce inflammatory changes in macrophages.

Discussion

We expected to see changes in cytokine expression in macrophages exposed to arsenic as arsenic trioxide has been shown to increase TNF-α and IL-8 secretion and expression significantly in macrophages isolated from human peripheral blood mononuclear cells (PBMCs). (Lemarie et al., 2006). We found that the lower concentrations of arsenic had the greatest impact on macrophage cytokine expression, specifically IL-10, at 3 hours in high glucose. IL-10 is an anti-inflammatory cytokine, and an increase in production would result in diminished M1 and Th1 (pro-inflammatory) responses (Couper et al., 2008), resulting in a suppressed immune response. In high glucose conditions, both low and high arsenic concentrations lead to a reduction in IL-10 expression, but this did not correlate with longer exposure, therefore supporting the possibility that acute arsenic exposure has a greater effect on immunity than chronic exposure Validation of increased protein secretion would solidify these results. Due to the importance of IL-10 in arsenic mediated macrophage changes, we also examined one of the downstream targets of IL-10, STAT3, as it is known transcription factor for anti-inflammatory macrophages (Martinez & Gordon, 2014) While there was a slight decrease in expression at the highest arsenic concentration in the 3 hour high glucose treatment group, it was not significantly affected, suggesting while gene expression is increased, IL-10 protein in suspension may not be increased. However, this result could further be explained by examining protein concentration of IL-10, as well as additional upstream regulators targeting STAT3 such as IL-6, which is something we could explore in further studies (H. M. Wilson, 2014). Also affected in this treatment, although not significantly, was TGF-β. This is another anti-inflammatory cytokine, and with the combined decrease

in expression in TGF-β and IL-10, we would have expected to see an increase in TNF-α regulation, as TNF- α expression is negatively regulated by these cytokines. However, this was not the case in our model demonstrated by no change in TNF-α expression regardless of arsenic concentration, glucose concentration or time of exposure.

To gain better insight into the effects of arsenic exposure on M1 vs M2 macrophages, we would have to first differentiate our cell culture toward one of these phenotypes prior to exposure. By doing so, we would be able to determine whether arsenic is in fact modulating the entire immune response (both pro and anti-inflammatory) by having our cells already skewed toward a specific phenotype. It is interesting that the shorter arsenic exposure in high glucose appeared to have a greater effect on gene expression in macrophages. This could indicate that for human exposure to arsenic, the important changes to the immune system are more visible after acute exposure.

Table 4.1. Real time PCR Primer list.

ID	Forward 5'-3'	Reverse 5'-3'
TGF-β	TTGCCCTCTACAACCAACACAA	GGCTTGCGACCCACGTAGTA
IL-10	TGGACTCCAGGACCTAGACA	AGTGTGGCCAGCCTTAGAAT
STAT3	TACCATTGACCTGCCGATGT	TCCGAGGTCAGATCCATGTC
TNF-α	AGCACAGAAAGCATGATCCG	CTACAGGCTTGTCACTCGAA
RPLP0	CCTGAAGTGCTCGACATCAC	GCGCTTGTACCCATTGATGA

Abbreviations – Transforming growth factor beta – TGF-β, Interleukin 10 – IL-10, Signal transducer and activator of transcription 3 – STAT3, Tumor necrosis alpha – TNF-α, Ribosomal protein lateral stalk subunit P0 – RPLP0

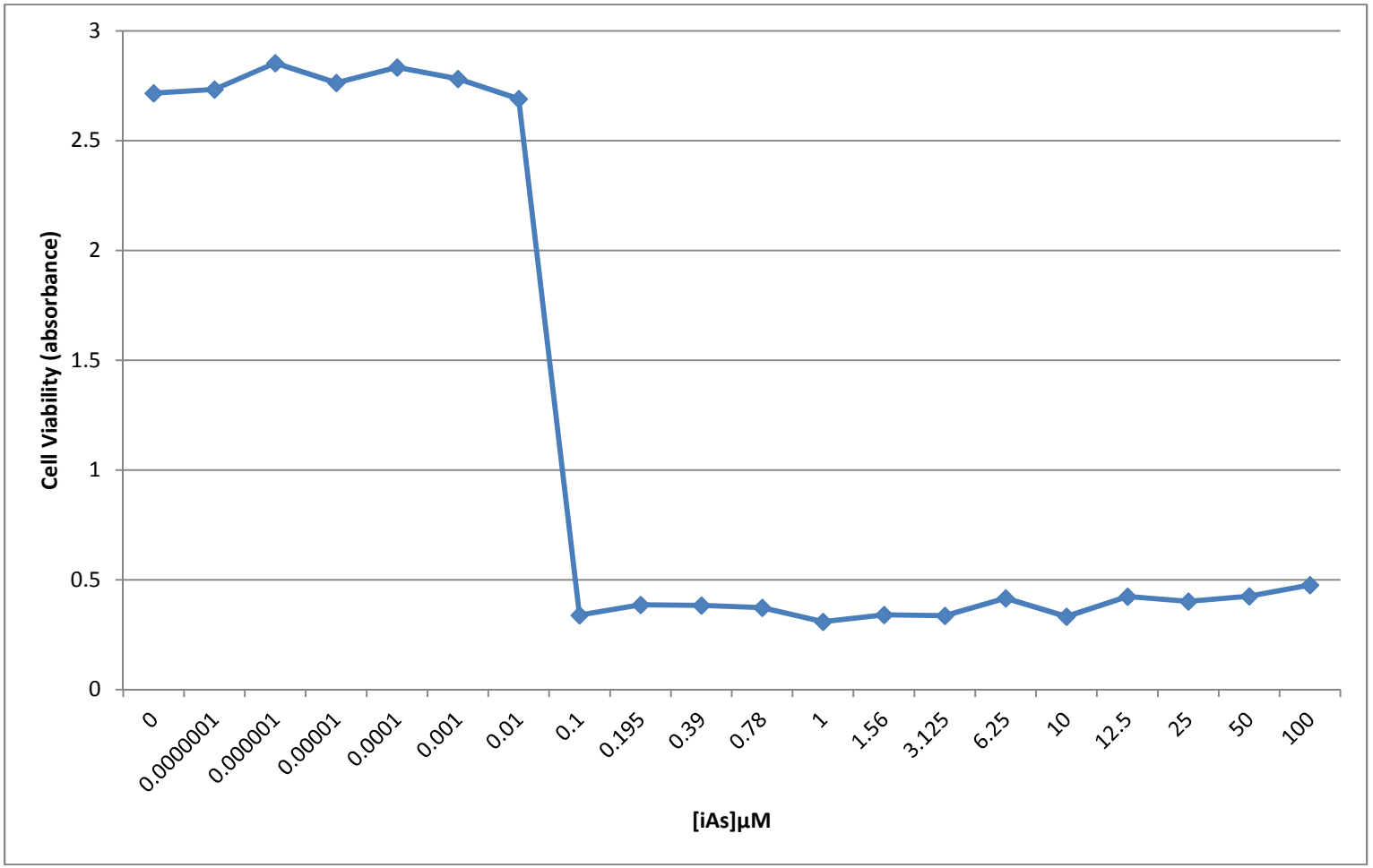


Figure 4.1. Raw 264.7 macrophage cell viability after arsenic exposure. Raw 264.7 macrophage viability after 3 hours of inorganic arsenic exposure at concentrations ranging from 0-100µM.

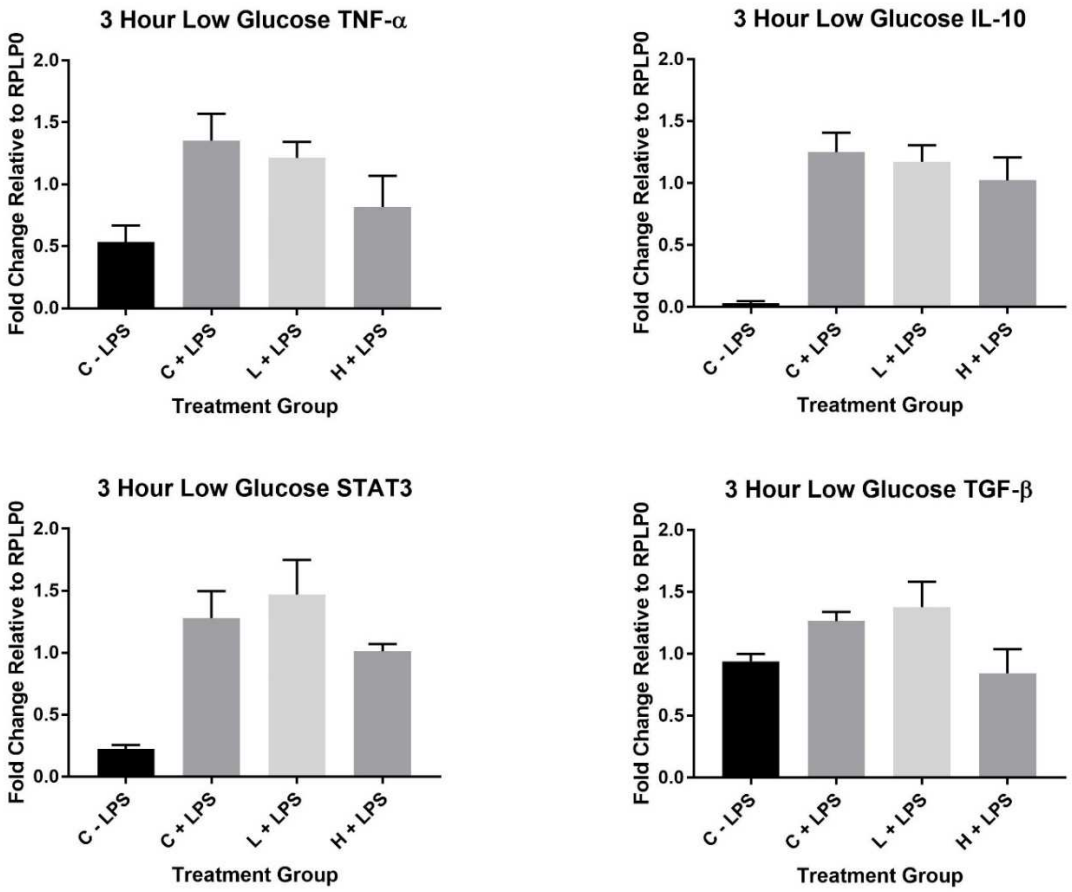


Figure 4.2. Short term arsenic exposure has no effect on macrophage cytokine expression in low glucose environment. Raw 264.7 macrophage gene expression after 3 hours of exposure in low glucose DMEM. mRNA levels are expressed relative to RPLP0. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using ANOVA, followed by Tukey's multiple comparison test.

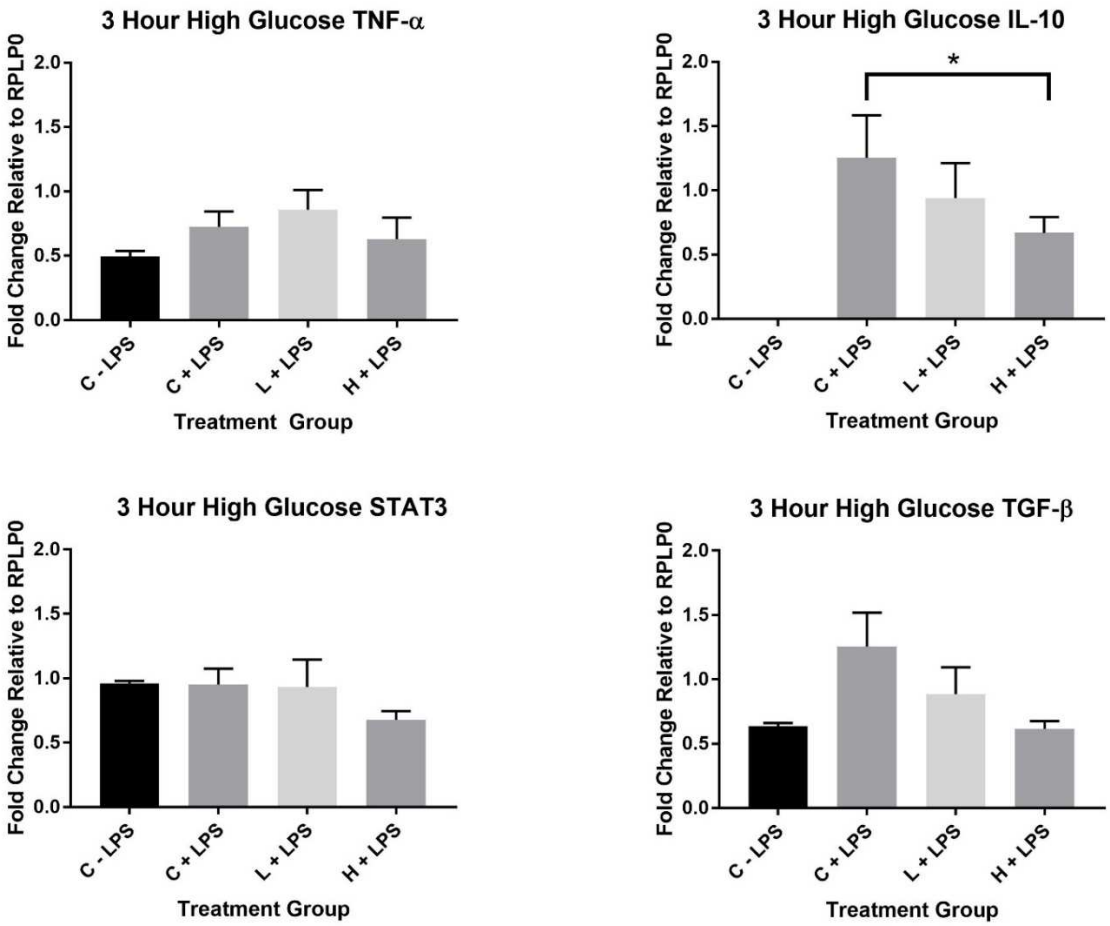


Figure 4.3. Short term arsenic exposure suppresses macrophage IL-10 expression in high glucose environment. Raw 264.7 macrophage gene expression after 3 hours of exposure in high glucose DMEM. mRNA levels are expressed relative to RPLP0. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using ANOVA followed by Tukey's multiple comparison test, and is indicated as * ($p<0.05$).

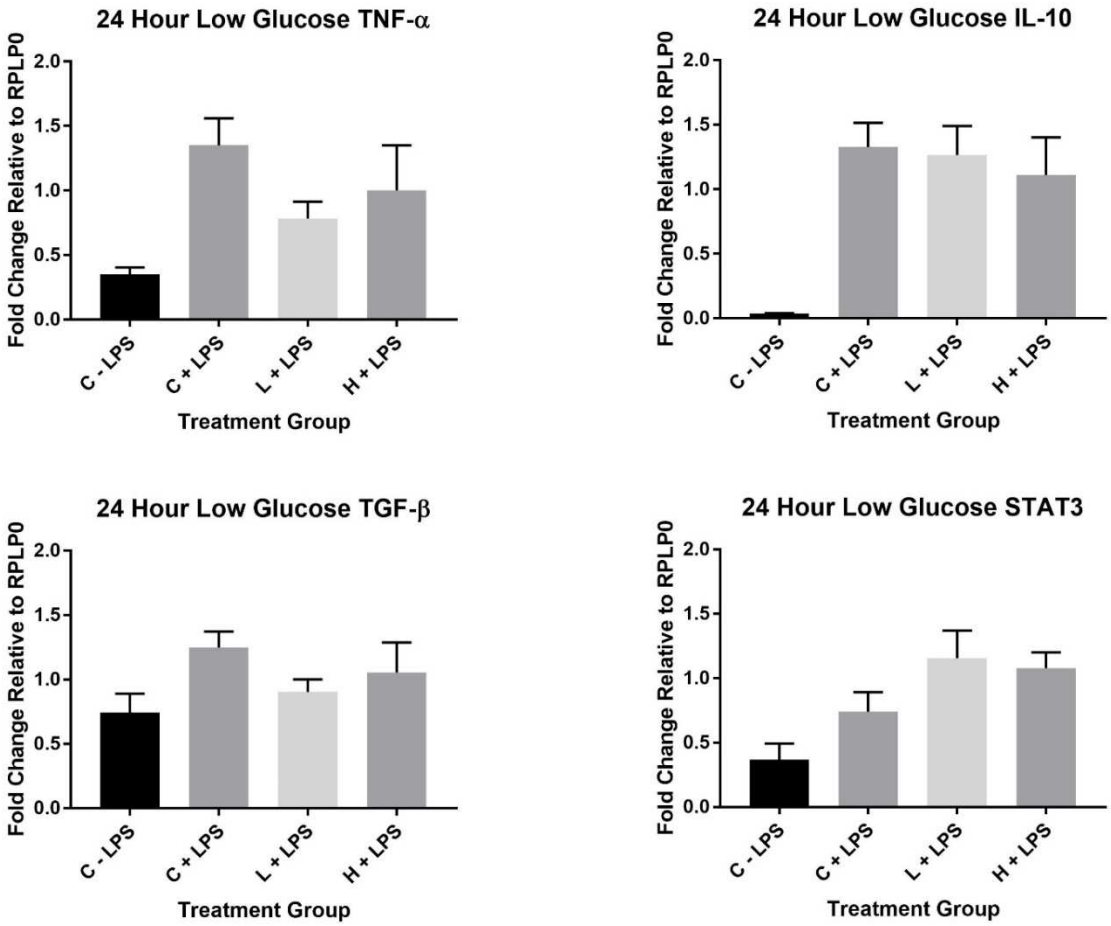


Figure 4.4. Long term arsenic exposure has no effect on macrophage cytokine expression in low glucose environment. Raw 264.7 macrophage gene expression after 24 hours of exposure in low glucose DMEM. mRNA levels are expressed relative to RPLP0. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using ANOVA followed by Tukey's multiple comparison test, and is indicated as * ($p<0.05$).

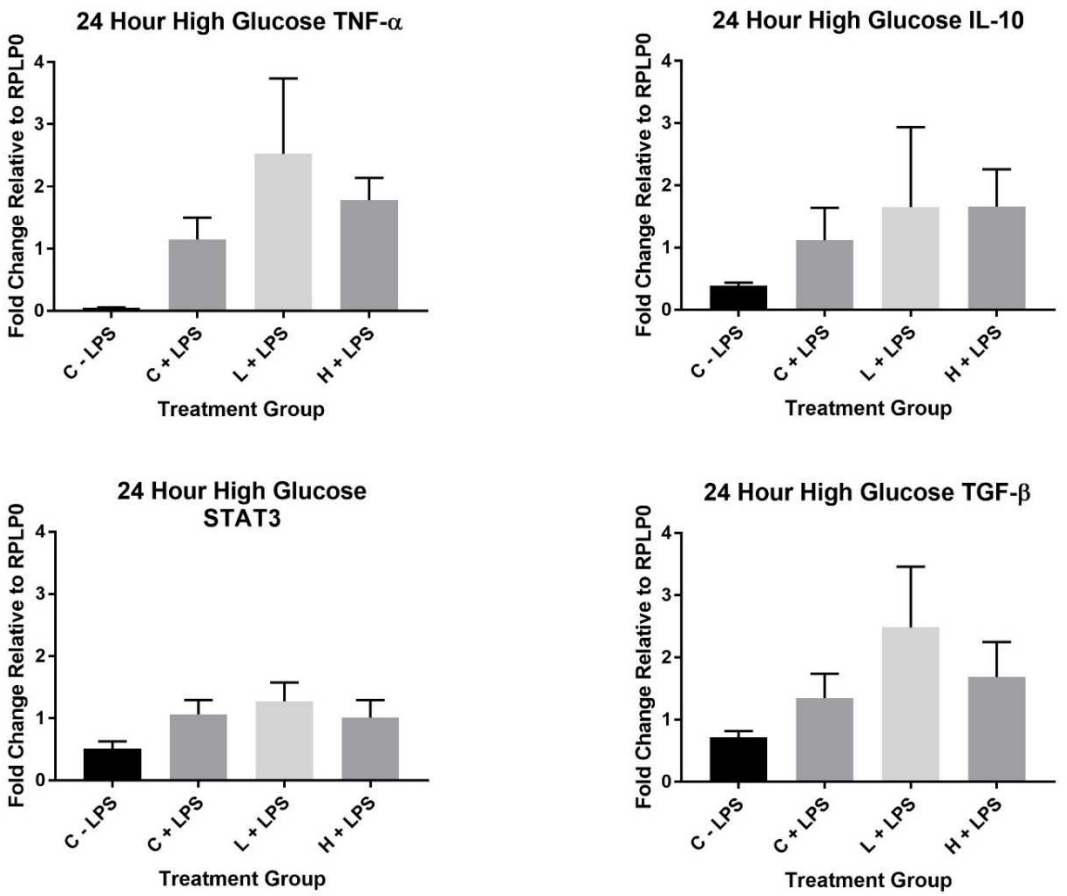


Figure 4.5. Long term arsenic exposure has no effect on macrophage cytokine expression in high glucose. Raw 264.7 macrophage gene expression after 24 hours of exposure in high glucose DMEM. mRNA levels are expressed relative to RPLP0. Data are represented as the mean of each treatment group ± SEM. Statistical significance was assessed using ANOVA followed by Tukey's multiple comparison test.

Chapter 5 – Conclusions and Future Directions

5.1 Baseline differences in genotype

In our research, we used two different mouse models (C57BL/6 and db/+) that are both representative of controls used in various diabetic experiments. Both are considered to be lean mice that do not develop type 2 diabetes under normal circumstances, but the C57BL/6 mouse will develop diabetes slowly when fed a high fat diet. In many studies that use the db/db mouse strain that become obese and diabetic around 4 weeks old, the db/+ mouse strain is the control. However, based on our study the C57BL/6 and db/+ mouse strains have several differences before the introduction of an environmental contaminant.

The evaluation of body composition in our C57BL/6 vs db/+ mice show the db/+ mice gain weight at a faster rate than our wild-type in both males and females. This includes a higher body fat percentage in the females at 12 weeks of age and wild-type males had slightly higher lean mass compared to the db/+ genotype (Table 5.1). Either of these conditions would result in a change in the expected inflammatory profile (Table 5.2 and Table 5.3). This was validated by male db/+ having slightly higher concentration of IL-13 in the spleen, and db/+ females having higher levels of IL-10 in spleen, TNF-α and IL-1β in serum. So at baseline, the male heterozygotes are already demonstrating a higher level of anti-inflammatory cytokines in circulation and females are more pro-inflammatory. For the chosen cytokines analyzed by gene expression the wild-type and db/+ mice demonstrated no differences, but IL-13 was not among the chosen cytokines

The female db/+ mice exhibited a wide range of gene expression changes in adipose tissue and spleen that would point toward increased macrophage activity and inflammation. Female db/+ mice also had higher levels of triglycerides in all organs examined except for the liver (Table 5.4). There were no notable differences in males or females in glucose tolerance.

Overall, besides body composition these mouse strains had varying differences in local and systemic inflammation and triglyceride levels. This makes them very different controls in experiments, so depending on the experiment certain changes may be magnified or lessened depending on the measured outcome.

5.2 Effects of low dose arsenic on diabetes development

The development of diabetes in humans is complex, with only 1/3 of individuals that develop insulin resistance along with obesity will become diabetic (Donath & Shoelson, 2011). A family history may indicate future susceptibility to the disease, but this alone does not guarantee an individual will develop diabetes. It is a combination of factors that causes disease to occur. Our mouse models represented two different strains that are not susceptible to obesity or diabetes, which gives us information needed to infer ways in which the general public could respond to arsenic exposure. We observed that low-dose arsenic exposure alone is not enough to induce diabetes development, although it may cause slight changes in body composition and organ triglycerides (Table 5.5 and Table 5.6). It would be beneficial to continue this study in a diabetes-susceptible model to determine whether arsenic could impact the development of disease or shift disease onset, now that we have defined the baseline response.

We found that low-dose arsenic exposure causes varying effects to the immune system that differ based on genotype and sex (Table 5.7 and Table 5.8). It was previously demonstrated in C57BL/6 male mice that exposure to arsenic at 100µg/L reduced immune response to influenza infection (Kozul, Ely, Enelow, & Hamilton, 2009). This differs from what we observed in our male C57BL/6 mice, which demonstrated 1-fold increase in circulating IFN-γ, IL-13 and IL-6 (Table 5.7).

Depending on the tissue examined, expression of these cytokines varied greatly (Table 5.8). We chose to further determine what the effect of arsenic exposure on macrophages *in vitro,* as these immune cell types are particularly affected as obesity increases. We report here that at acute exposure in high glucose, arsenic significantly reduced IL-10, an important anti-inflammatory mediator. While we did not see an overall suppression of the macrophage cytokine response due to arsenic exposure, we can say that depending on the stress placed on the cells (i.e. higher glucose concentration), there will be a different immune response. This is important in the context of human exposures, as no two individuals will have the same baseline stress levels, supporting the idea that the effect of arsenic on the body will vary greatly from person to person.

We originally hypothesized that arsenic exposure would decrease glucose tolerance in a non-susceptible mouse model, leading to an increase in type 2 diabetes susceptibility. Instead, we have demonstrated through these experiments that low-dose exposure to arsenic in a non-susceptible individual is not enough to drive diabetes, but it is enough to cause changes to the immune response.

Table 5.1. Summary of genotypic differences in body composition.

	Lean	Fat	Total	Fat %
Males				
Females				

Lines represent no change, or a fold change from control less than 1. One small arrows represent a fold change between 1-2 and 2 small arrows represent a fold change greater than 2.

Table 5.2. Summary of genotypic differences in systemic cytokines.

	Spleen								Serum		
	TNF-α	GMCSF	IFN-γ	IL-13	IL-10	IL-1β	IL-4	IL-6	TNF-α	IL-6	IL-1β
Males	—	—	—	↑	—	—	—	—	—	—	—
Females	—	—	—	—	↑↑	—	—	—	↑↑	—	↑↑

Lines represent no change, or a fold change from control less than 1. One small arrows represent a fold change between 1-2 and 2 small arrows represent a fold change greater than 2

Table 5.3. Summary of genotypic differences in immune-related gene expression.

	Adipose Tissue						Liver		Spleen	
	Adiponectin	Leptin	TNF-α	Arginase	iNOS	CCL2	Arginase	iNOS	Arginase	iNOS
Males	—	—	—	—	—	—	—	—	—	—
Females	↑↑	↑↑	↑	↑↑	↑↑	—	—	—	—	↑↑

Lines represent no change, or a fold change from control less than 1. One small arrows represent a fold change between 1-2 and 2 small arrows represent a fold change greater than 2.

Table 5.4. Summary of genotypic differences in organ triglycerides

	Muscle	Kidney	Liver	Heart
Males	–	–	–	–
Females	↑	↑	–	↑↑

Lines represent no change, or a fold change from control less than 1. One small arrows represent a fold change between 1-2 and 2 small arrows represent a fold change greater than 2.

Table 5.5. Summary of arsenic effects on body composition.

	Lean	Fat	Total	Fat %
Male WT				
Male Het				
Female WT				
Female Het				

Lines represent no change, or a fold change from control less than 1. One small arrows represent a fold change between 1-2 and 2 small arrows represent a fold change greater than 2. Light blue represents 1x arsenic exposure and dark blue represents 10x.

Table 5.6. Summary of arsenic effects on organ triglycerides.

	Muscle	Kidney	Liver	Heart
Male WT				
Male Het				
Female WT				
Female Het				

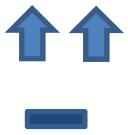
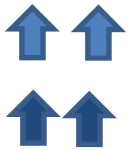
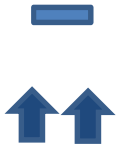
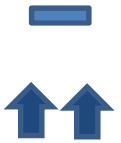
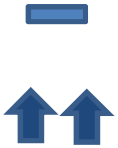
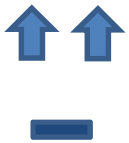
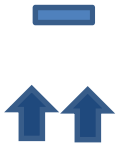
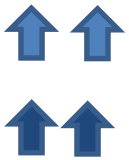
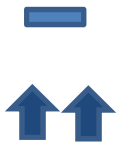
Lines represent no change, or a fold change from control less than 1. One small arrows represent a fold change between 1-2 and 2 small arrows represent a fold change greater than 2. Light blue represents 1x arsenic exposure and dark blue represents 10x.

Table 5.7. Summary of arsenic effects on cytokines.

	Spleen								Serum		
	TNF-α	GMCSF	IFN-γ	IL-13	IL-10	IL-1β	IL-4	IL-6	TNF-α	IL-6	IL-1β
Male WT	—	—	↑	↑	—	—	—	↑	—	—	—
Male Het	=	=	↑↑	=	=	=	=	=	=	=	=
Female WT	—	—	↑	—	—	↑	—	—	—	—	—
Female Het	=	=	=	=	↑	=	↑	↑↑	=	=	=

Lines represent no change, or a fold change from control less than 1. One small arrows represent a fold change between 1-2 and 2 small arrows represent a fold change greater than 2. Light blue represents 1x arsenic exposure and dark blue represents 10x.

Table 5.8. Summary of arsenic effects on immune-related gene expression.

	Adipose Tissue						Liver		Spleen	
	Adiponectin	**Leptin**	**TNF-α**	**Arginase**	**iNOS**	**CCL2**	**Arginase**	**iNOS**	**Arginase**	**iNOS**
Male WT										
Male Het										
Female WT										
Female Het										

Lines represent no change, or a fold change from control less than 1. One small arrows represent a fold change between 1-2 and 2 small arrows represent a fold change greater than 2. Light blue represents 1x arsenic exposure and dark blue represents 10x.

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