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CHARACTERIZATION OF METABOLIC EFFECTS OF DIRECT AND ANCESTRAL EXPOSURE TO NICOTINE IN MICE

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by

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Table of Contents

Abstract

CHARACTERIZATION OF METABOLIC EFFECTS OF DIRECT AND ANCESTRAL EXPOSURE TO NICOTINE IN MICE

Stephanie R. Aguiar

Metabolic diseases, such as obesity or type 2 diabetes, are affecting millions of individuals globally and are projected to continue impacting sixty percent of the world's population by 2050. Factors attributed to the development of metabolic diseases have often been identified as sedentary lifestyle and hypercaloric diets. In recent years the idea of the "exposome," or the sum of an individual's environmental exposures within their lifetime, has shed light on the importance of chemical exposure and incidence of metabolic disease. One environmental factor that can perturb metabolic function is the use of tobacco products. Specifically, exposure to nicotine can elicit metabolic disruption from direct, *in utero*, and ancestral exposures. Though global tobacco use rates among adults have declined, there are still communities that continue to use tobacco like adult men. Paternal smoking has also been associated with childhood overweight and obesity status in descendants and grandchildren. Paternal nicotine use has been shown to increase incidence of metabolic disruption in the next generation of male mice. However, further characterization of paternal nicotine exposure and metabolic outcomes in the next generation remains to be characterized. Investigation into the metabolic outcomes upon paternal nicotine exposure in females of the next generation remains to be elucidated. Also, investigation into dietary interventions upon paternal predisposition to nicotine exposure has not been previously explored. Referring to the idea of the exposome, we are exposed to multiple factors within our lifetime. It is important to understand the metabolic outcomes that arise upon paternal nicotine exposure and dietary intervention with a hypercaloric diet as we are exposed to multiple factors within ours and our ancestors' lifetime. It is also important to understand the metabolic outcomes of direct exposure to nicotine. Direct nicotine exposure in the F0 generation has been associated with increased risk of metabolic disruption in the form of metabolic syndrome.

Here we characterize the metabolic outcomes upon direct or ancestral exposure to nicotine in a rodent model.

In Chapter 1 of this dissertation, I thoroughly detail relevant information on various fields that mesh into my novel research topic. Specifically, I outline the recent prevalence and projections of metabolic syndrome and its associated disorders at the global level. I discuss the factors that are associated with the development of metabolic diseases and summarize some of the recent investigation into chemical exposures and metabolic disruption. Specifically, exposure to endocrine-disrupting chemicals (EDCs) during critical windows of susceptibility during development can lead to adverse metabolic outcomes. One EDC, nicotine, found in tobacco, can elicit metabolic disruption from direct or developmental exposure. Paternal tobacco smoking has been associated with metabolic outcomes in unexposed grandchildren. Paternal nicotine exposure can also elicit metabolic disruption in males of the next generation in rodents. Mechanisms underlying these alterations that arise upon paternal nicotine exposure are still being elucidated; however, one hypothesis is the alteration of sperm small non-coding RNAs leads to metabolic outcomes observed. Investigation into paternal nicotine exposure and a secondary challenge with a different metabolic disease risk factor in the next generation, such as diet, has not been studied. The next two data chapters detail findings of nicotine exposure at two different moments in life, adulthood and after paternal preconception exposure, and the metabolic outcomes that arise at the physiological and transcriptomic levels.

In Chapter 2 of this dissertation, I demonstrate that direct exposure of the F0 generation to nicotine leads to physiological and transcriptomic metabolic outcomes. Chronic nicotine exposure elicited cardiometabolic disruption at the physiological and molecular levels. Nicotine exposure elicited altered plasma metabolites, blood glucose levels during metabolic testing in both male and female rodents. Specifically, nicotine exposure in males was associated with impaired insulin tolerance and decreased body weights. Hepatic

transcriptomics reveal alterations in gene expression of biological processes involved with cardiovascular disease upon nicotine or tributyltin exposures. These alterations suggest that direct exposure to endocrine disrupting chemicals like nicotine or tributyltin elicits cardiometabolic alterations in mice. Furthermore, exposures to these endocrine disrupting chemicals may be associated with increased risk of cardiometabolic disease in humans.

In Chapter 3 of this dissertation, I demonstrate that paternal exposure to nicotine predisposes offspring to metabolic disruption that is further exacerbated in the presence of a hypercaloric diet. I also show that there is a sexually dimorphic phenotype observed in the F1 generation upon paternal preconception nicotine exposure. Specifically, there are different metabolic processes altered in the sexes upon paternal nicotine exposure, such as modifications to hepatic gene expression in gluconeogenesis in F1 females and glycogenolysis in F1 males. F1 males also had decreases plasma glucagon, an important metabolite involved in glycogenolysis. Although physiological outcomes were mild, hepatic transcriptomics reveal alterations in gene expression of biological processes involved with lipid and xenobiotic metabolism suggesting alterations in the fat metabolism. Transcriptomic alterations to the metabolically relevant liver tissue ultimately reveal that there was metabolic disruption that arises from paternal nicotine exposure and is further exacerbated by a hypercaloric diet.

Finally, in Chapter 4 of this dissertation, I summarize the findings from both data chapters and discuss how these findings shed light into the effects of direct and ancestral exposure to nicotine on adverse metabolic outcomes. The findings here further provide compelling evidence that paternal nicotine exposure can elicit long-lasting metabolic effects that can be further exacerbated by a hypercaloric diet that represents the diet 50% of the American population follows. This dissertation demonstrates that nicotine and hypercaloric diet, two types of environmental factors, can elicit cardiometabolic alterations at the

physiological and molecular levels. Future studies will investigate potential mechanisms that link paternal nicotine exposure and metabolic outcomes observed.

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CHAPTER 1

INTRODUCTION

1.0 Metabolic disease

The prevalence of metabolic diseases across the globe is increasing and projected to keep increasing by 2050 with obesity leading to the largest number of deaths, followed by hypertension, hyperlipidemia, and type 2 diabetes (T2D) (Chong et al., 2023). Cardiometabolic disease is defined as both metabolic and cardiovascular diseases, which include previously described metabolic diseases paired with conditions like high blood pressure, atherosclerosis, and stroke (National Academies of Sciences, 2021). Cardiovascular diseases are the leading cause of death globally, affecting about 1 in 12 people (Vaduganathan et al., 2022). Metabolic syndrome highlights the accumulation of three or more risk factor metabolic diseases, like obesity, T2D, and/or hyperlipidemia. Therefore, the incidence of obesity and/or T2D often parallels the incidence of metabolic syndrome. About one in three adults have metabolic syndrome in the United States (Alberti et al., 2009). Globally, about 20-25% of the world's adult population lives with metabolic syndrome (Saklayen, 2018). The prevalence of diagnosed T2D in adults in the United States was about 11.3% (Bullard et al., 2018). In the world's population, the prevalence of diagnosed T2D in adults was 6.28% in 2022 (Khan et al., 2020). Obesity rates in the adult population in the United States was 42.4% in 2018 (Hales et al., 2020). Globally, obesity rates in adults have doubled since 1990, and 43% of adults were overweight in 2022 (Boutari and Mantzoros, 2022). Worldwide, raised total cholesterol, an indicator of dyslipidemia or hyperlipidemia, was shown to affect about 39% of adults (Liu et al., 2022). Metabolic disease prevalence rates are rapidly increasing across the globe and are projected to increase by 50% and continue to affect 39 million individuals by 2030 (Rowley et al., 2017; Finkelstein et al., 2012). Global metabolic disease prevalence is rapidly increasing and is projected to continue to affect

millions of people in the future, and therefore it's important to investigate and combat potential causes of metabolic disruption.

Metabolic diseases pose an economic burden on health systems around the world and for individuals dealing with disease (Vaquero Alvarez et al., 2020). There are factors that are often attributed to metabolic disease incidence, including sedentary lifestyle and poor diet. More recently, environmental exposures have been accepted as risk factors for metabolic diseases (Khalil et al., 2023; Leonel-Javares et al., 2021; Balhara et al., 2012).

1.1 Different types of metabolic diseases

Type 2 diabetes (T2D) is a condition in which the body does not produce enough insulin or cannot use insulin properly and results in high blood glucose levels (or hyperglycemia) (Goyal et al., 2024). In a healthy human or animal, insulin is released from the pancreas and signals to target tissues such as the liver or adipose tissue to uptake peripheral blood glucose (Nakrani et al., 2024). Hyperglycemia can lead to excessive thirst, hunger, and fatigue and overtime hyperglycemic episodes can elicit damage to the heart, kidneys, and nerves (Mouri and Badireddy, 2024). Glucose metabolism involves multiple biochemical processes including the production, transport, storage, and breakdown of glucose. Alterations to glucose metabolism, like inability to recognize or use insulin by the target tissues, can lead to increased blood glucose levels (Aronoff et al., 2004). Glucagon, another important signaling molecule in glucose metabolism, is a metabolite secreted from α-cells in the pancreas and once released and signals to the liver to breakdown stored glycogen into glucose that can be released into the blood (Venugopal et al., 2024). Impaired insulin and/or glucagon levels can elicit impaired glucose tolerance and lead to T2D (Aronoff et al., 2024).

There are other hormones/metabolites involved in metabolic processes such as glucose homeostasis or fat metabolism that include molecules like leptin, ghrelin, resistin, C-peptide 2 and GLP-1 (Genchi et al., 2021; Poher et al., 2018; Banerjee et al., 2004; Chen et al., 2023;

Wharton et al., 2022). Leptin is a hormone secreted by fat cells involving in the regulation of appetite. When the leptin pathway works properly, leptin is secreted during meals, to signal to the brain and reduce appetite (Morris and Rui, 2009). Disruption of the leptin signaling pathway can lead to “leptin resistance” in which the body becomes desensitized to the hormone leading to increased appetite and reduced energy expenditure (Genchi et al., 2021). Ghrelin, also known as the hunger hormone, is produced by cells in the lining of the stomach and plays a role in signaling to the brain, stimulating appetite and promoting fat storage (Young and Jialal, 2024). When ghrelin levels are elevated, appetite and fat accumulation are increased, leading to metabolic disruption and weight gain (Poher et al., 2018). Resistin is an adipokine, (i.e., hormone produced by adipose cells) that regulates glucose uptake and inflammatory processes (Tripathi et al., 2020). Elevated resistin levels decrease available peripheral blood glucose levels making it difficult for target tissues to uptake glucose thus leading to impaired glucose tolerance (Banerjee et al., 2004). C-peptide 2 is a hormone produced alongside insulin and released in equal amounts by the pancreas where it regulates the correct folding and production of insulin from proinsulin (Venugopal et al., 2024). Decreased C-peptide 2 levels would indicate decreased insulin production and secretion from the pancreas, and thus insulin levels would also be lowered leading to hyperglycemic and/or diabetic conditions (Chen et al., 2023). GLP-1, or glucagon-like peptide 1, is a hormone produced by cells in the small intestine that stimulates pancreatic insulin secretion and suppresses pancreatic glucagon secretion (Nachawi et al., 2022). GLP-1 also decreases gastric emptying leading to a feeling of satiety and thus decreased food intake and is recently being marketed as a treatment for weight loss (Jensterle et al., 2022). However, elevated GLP-1 levels can decrease food intake and dysregulate insulin and glucagon signaling thus leading to potential hypoglycemia and/or pancreatitis (Wharton et al., 2022). The levels and signaling of these specific metabolites play vital roles in maintaining processes like glucose homeostasis and weight gain that are involved in metabolic disruption (Chen et al., 2023; Wharton et al., 2022). It is important to consider the involvement of these metabolites in the

etiology of metabolic diseases as modifications to the levels of these hormones/metabolites are indicative of metabolic disruption and disease (Nachawi et al., 2022; Jensterle et al., 2022; Wharton et al., 2022; Chen et al; 2023; Poher et al., 2018).

Another metabolic disease that has increased global prevalence in the past several decades is obesity, which is defined as excessive accumulation of fat in the body (Safaei et al., 2021). The primary cause of accumulation of excessive amount of fat tissue is due to an imbalance between calories consumed and calories burned (Panuganti et al., 2024; Safaei et al., 2024). Obesity can lead to a variety of other diseases such as cardiovascular disease, T2D, and cancer (Tremmel et al., 2017). Obesity is often determined by body mass index (BMI) greater than 30, which is calculated as weight in kilograms divided by height in meters squared (Safaei et al., 2024).

Hyperlipidemia is a condition where there are high levels of lipids or fat in the blood and is ultimately a risk factor for cardiovascular issues and is linked with obesity (Huff et al., 2024). Cholesterol is a lipophilic molecule that assists in cell membrane structure and a precursor for the synthesis of other molecules into hormones (Zampelas and Magriplis, 2019). Hyperlipidemia is specifically an accumulation of lipids like triglycerides and cholesterol in the blood and can lead to other metabolic disruption conditions like obesity (Zhu et al., 2024).

These metabolic disorders are just some of the various forms of metabolic disruption in a living system, other conditions include hypertension and cardiovascular disease. There is an interplay between these metabolic disorders that can influence the risk of developing more than one cardiometabolic condition. Obesity leads to increased adipose tissue which can lead to inflammation and increase risk of developing T2D (Nicholas et al., 2024). Type 2 diabetes can also lead to dyslipidemia as there is overproduction of hepatic triglyceride-rich lipoproteins. Dyslipidemia can further be a risk factor for the development of cardiovascular disease (Taskinen and Boren, 2015).

1.2 Metabolic disease etiology in the liver

The liver plays an important role in metabolic disorders (Ding et al., 2018). In T2D, the liver contributes to the regulation of blood glucose levels by breaking down stored glycogen (Soon and Torbenson, 2023). High blood glucose levels trigger release of insulin from pancreatic β-cells into the blood which signals to target tissues, including the liver, to uptake and utilize peripheral glucose thus reducing glucose levels in blood (Thota and Akbar, 2024). Disruption towards the production and transport of insulin, glucagon, and glucose leads to diabetic conditions (Svendsen et al., 2018). The liver also plays a role in lipid metabolism via the uptake and release of lipids, and accumulation of fat in the liver is indicative of metabolic disruption that can lead to disorders like T2D, hyperlipidemia, and/or obesity (Lonardo et al., 2018). Disturbances in lipid metabolism in the liver influence insulin signaling and lead to impaired insulin secretion from the pancreas which consequently gives rise to insulin resistance and/or altered glucose metabolism (Savage et al., 2007).

In addition to the role of the liver in glucose and lipid homeostasis, it also plays an important role in blood detoxification and filters blood by removing both endogenous and exogenous substance byproducts (Grant, 1991). The liver contains many xenobiotic metabolism enzymes that breakdown or transform chemical molecules into water-soluble metabolites that can be excreted through urine and/or bile (Gu and Manautou, 2012). Disruption to xenobiotic metabolism processes in the liver can lead to liver injury and accumulation of molecules that cannot be converted to metabolites that can be removed from the body (Dienes and Drebber, 2010). Alterations to important metabolic and xenobiotic processes in the liver at the molecular or physiological level ultimately leads to increased susceptibility to metabolic disruption and disease (Lonardo et al., 2018; Savage et al., 2007; Dienes and Drebber, 2010).

2.0 Factors associated with metabolic disease

Traditional factors associated with metabolic disease incidence include genetics, sedentary lifestyle, and/or hypercaloric diets (Ali et al., 2023; Silva et al., 2019). Recently there has been increasing efforts in developing tools to examine the adverse health effects of environmental exposures in humans (Baccarelli et al., 2023; Boogaard et al., 2024). First and foremost, it is important to emphasize the importance of known causes of metabolic disease incidence.

2.1 Genetics and metabolic disease

Inherited metabolic disorders are often caused by alterations to specific genes that ultimately affect metabolism (Barroso and McCarthy, 2019). Genome-wide association studies (GWAS) have been used to identify gene variants that are associated with two or more metabolic traits (Ziki and Mani, 2016). Investigation into mutations that lead to inherited metabolic disorders vary, with some studies showing that polymorphisms to genes like low-density lipoprotein receptor (*Ldlr*), interleukin-6 (*Il-6*) and lipase C (*Lipc*) have been associated with increased risk of metabolic disruption and disease (Marc et al., 2007). Another study demonstrates that glucocorticoid receptor (*Gr*) and adiponectin (*Adipoq*) variations were associated with metabolic syndrome (Rana et al., 2022). However, only about 2.7% of the obesity cases can be explained due to genetic factors (Locke et al., 2015). The heritability of metabolic disorders like metabolic syndrome is suggested to be 10-30%, and genetic factors are estimated to contribute to 50% towards T2D risk (Phillips 2013). This suggests thatgenetic factors associated with the incidence of metabolic diseases are variable and often predispose individuals to disease that are exacerbated by introduction of additional factors.

2.2 Lifestyle and metabolic disease

Lifestyle factors such as exercise behaviors, sleep patterns, and stress levels can all influence the risk of developing metabolic diseases (Kim et al., 2021; Chasens et al., 2021). Inadequate sleep, both short and long periods of sleep, have been shown to affect

cardiometabolic processes and increase risk of metabolic syndrome (Che et al., 2021). Specifically, individuals that self-reported difficulty falling/staying asleep were at increased risk of developing hyperglycemia and low levels of circulating high-density lipoprotein cholesterol (Grandner et al. 2012). Chronic stress has been shown to increase cortisol levels which can lead to increased weight gain, fat storage, and insulin resistance (Hewagalamulage et al., 2016). A common main contributor to metabolic disease has been associated with lack of exercise and/or paired with a sedentary lifestyle (Macias et al., 2021). Sedentary individuals tend to have higher BMI, greater waist circumference, higher blood pressure and increased risk of insulin resistance when compared to more active individuals (Leon-Latre et al., 2014). Physical activity and exercise are often prescribed treatments for individuals experiencing symptoms associated with metabolic diseases (Sylow and Richter, 2019). While increased physical activity, adequate sleep, and decreased stress has been shown to alleviate the severity of metabolic diseases, there are other factors outside of lifestyle, such as diet, that contribute to prevalence of disease (Vancampfort and Stubbs, 2017; Grandner et al., 2012; Kivimaki et al., 2022).

2.3 Diet and metabolic disease

Diet plays a key role in the development of metabolic disease in that maintaining a diet rich in fats and carbohydrates can lead to increased prevalence of metabolic disruption and disease (Okube et al., 2020). Diets high in calories and processed foods have been linked to increased fat accumulation and storage and thus increased prevalence of diseases associated with metabolic syndrome (Suliga et al., 2015). Diets that contain more calories from fat than protein or carbohydrates have traditionally been a major contributing factor for the onset of metabolic diseases such as obesity and/or type 2 diabetes (Abiri et al., 2023). In rodent models, it's been shown that a diet containing 45% of calories from fat can lead to diet-induced obesity (Hintze et al., 2018). Famines (i.e., an extreme shortage of food) can also lead to incidence of metabolic disruption as inaccessibility to proper nutrition can cause

the body to lack of intake in calories which could also lead to fluctuations in blood glucose levels (Brandt et al., 2023).

3.0 Environmental factors of metabolic disease

Direct and/or indirect exposure to environmental factors like man-made or naturally occurring chemicals is associated with adverse health outcomes like metabolic disruption and disease (Khalil et al., 2023). Humans are exposed to substances in water, air, soil, and food in their daily environments that can lead to harmful health outcomes. Air pollution, chemicals in drinking water, pesticides in food, and cigarette smoking are just some examples of factors in the environment that can lead to adverse health effects to humans (Leonel-Javares et al., 2021; Balhara et al., 2012).

3.1 The Exposome

The exposome is a concept used to denote the health outcomes that arise from all environmental exposures an individual is exposed to in their lifetime (Wild, 2005). The term “exposome” was first coined by Dr. Christopher Wild and joins together the fields of environmental health, epidemiology, and genomics. In the exposome, there are external and internal factors that add up to the collection of environmental exposures in an individual’s lifetime that include: diet, behaviors, lifestyle, chemicals/substances, education level, and financial status (Miller et al., 2010). The external exposome specifically highlights diet, behaviors, lifestyles and chemical/substance exposures in the environment (D’Errico et al., 2023). Though the term “exposome” was recently coined, there are many studies that demonstrate that environmental exposures are directly linked to adverse health outcomes like cancer, immune-mediated disease, and cardiometabolic diseases (DeBord et al., 2016; Bloszies and Fiehn, 2018). In the 1950s, there were causal links established between smoking tobacco and direct adverse health effects like lung cancer, making the public aware of an environmental factor that impacts health (Doll and Hill, 1954). There are increasing

studies on environmental exposures to chemicals/substances and adverse health effects in the past several decades.

3.2 Chemical exposures and the exposome: adverse health outcomes

Environmental exposures to toxicants can interact with the genome and influence gene expression and response to environmental factors (Baccarelli and Bollati, 2009). Depending on the window of exposure, whether it is during critical developmental periods, during puberty, or in adulthood, functional alterations can lead to long-lasting adverse health outcomes (Harris et al., 2017; Yang et al., 2015). Embryonic development is a critical window of susceptibility to environmental exposures because the disruption of important processes like embryogenesis where cells are rapidly proliferating and differentiating into specialized tissues, can lead to long-lasting health impacts like improper organ function, negative neurobehavioral outcomes, cancer, and/or metabolic disease later in life (Bauer et al., 2021; Mork and Wilson, 2023; Russ and Howard, 2016; Mattison, 2010). The developmental origins of health and disease (DOHaD) hypothesis postulates that environmental exposures during early life can perpetually impact health and lead to increased risk of disease later in life (Lacagnina, 2019). This hypothesis was proposed by David J.P. Barker in 1986 whose findings proposed a direct link between prenatal nutrition and coronary disease later in life (Barker and Osmond, 1986). Air and water pollution, pesticides, and chemical exposures can lead to a myriad of human health effects including, but not limited to, cancers, autoimmune diseases, and inflammatory or metabolic diseases (Virolainen et al., 2023). Exposure to chemicals found in the workplace, at home, and elsewhere makes us increasingly unprotected and subject to interaction with harmful substances. There are many chemicals that people’s behaviors regularly expose themselves to, like alcohol, or tobacco that have been associated with adverse health outcomes (Grønbæk 2009; Meister et al., 2000; Mitchell et al., 1999). For example, exposure to tobacco-related chemicals can lead to adverse health effects including lung damage and cancer, cardiovascular disease, and increased

susceptibility to developing diabetes (Mitchell et al., 1999). These are also examples of environmental factors that can lead to metabolic disease (Zakhari, 2013; Jabeen et al., 2021).

3.3 Sexually dimorphic responses to chemical exposures

Exposures to certain chemicals, like endocrine-disrupting chemicals (EDCs), can lead to different phenotypes depending on sex (McCabe et al., 2017). Exposures to chemicals during critical windows of susceptibility, such as embryonic development, can elicit pronounced sexual dimorphic phenotypes as this is a period of rapid growth and development and is at high risk for gene expression alterations (McCabe et al., 2017; Palanza et al., 2015). Exposure to fungicide vinclozolin during embryonic development has led to a feminized male phenotype in rodents, specifically resulting in hypospadias, a birth defect where the urethra is improperly located on the underside of the penis (Gillette et al., 2014). Human studies on sex-dependent differences upon chemical exposure are limited, however once study on bisphenol A (BPA) exposure on pregnant women revealed that female offspring weighed less than male offspring (Veiga-Lopez et al., 2015). Males and females can exhibit different phenotypic outcomes due to distinct differences in hormone levels that have been determined during evolution and sex-selection processes that lead to pronounced differing physical attributes between the sexes (Chu and Lee, 2012). Of note, overall body fat content and fat distribution is significantly different between males and females. Fat accumulation in females tend to occur in subcutaneous depots such as those located in the in the gluteal-femoral region of the body while males accumulate visceral fat in the abdomen (Frank et al., 2018; Lumish et al., 2020). Subcutaneous fat is associated with regulation of body temperature (e.g., thermogenesis), while visceral fat is associated with more concerning metabolic outcomes such as hypercholesterolemia, lipid metabolism and cardiovascular disease (Ibrahim 2010). Given these baseline metabolic differences, it should come as no surprise that environmental exposures associated with metabolic disease led to such alterations in a sexually dimorphic manner affecting males more than females.

3.4 Endocrine-disrupting chemicals (EDCs)

Endocrine-disrupting chemicals (EDCs) interfere with endocrine system function, modulating hormone expression and altering metabolic pathways in humans (Yilmaz et al., 2020). The National Endocrine Society defines EDCs as “exogenous chemicals, or mixtures of chemicals, that interfere with hormone action” (Gore et al., 2014). These EDCs are typically considered “non-mutagenic,” and exposure to them is prevalent in the environment whether through manufactured processes, such as pesticides, phthalates, and perfluoroalkyl substances (PFAS), or through natural occurrence, like some heavy metals (Kahn et al., 2020; Fatoki and Badmus, 2022). Environmental chemical production has increased rapidly in the past several decades and research into chemical exposure and adverse health effects has subsequently risen (Onyeaka et al., 2024; Wilson and Schwarzman, 2009). Some EDC exposures are associated withincreased susceptibility of metabolic diseases such as obesity and/or type 2 diabetes (Heindel et al., 2022). Tributyltin (TBT) is a highly toxic biocide that has been used extensively in the past to prevent the growth of marine organisms on ships. It has been shown to lead to endocrine disruption and subsequently increased risk of weight gain/obesity and impaired glucose/insulin homeostasis in mice and salmonids (Grün et al., 2006; Zhan et al., 2020; Meador et al., 2011). In humans, TBT exposure studies are limited, however exposure to trace amounts of TBT elicits skin irritation and vascular dysfunction through increased oxidative stress pathways (Ronconi et al., 2018). This is possible due to trace amounts of TBT being found in house dust, and in human blood and urine (Fromme et al., 2005). There is much documentation of exposure to chemicals/substances and adverse health outcomes that lead to metabolic disruption and disease. Recently, there has been investigation into environmental chemical exposures and multigenerational disease that affects unexposed descendants and leads to adverse health outcomes in future generations in the form of metabolic disease incidence (King and Skinner, 2020; Chamorro-Garcia et al., 2017; Guo et al., 2018; Rebuzzini et al., 2022).

3.5 Chemical exposure and multigenerational disease

Environmental stressors have been shown to lead to alterations in the genome that result in transgenerational epigenetic inheritance in unexposed generations. There are limited epidemiological studies demonstrating transgenerational epigenetic inheritance, as it is difficult to follow multiple generations and have individuals consent to participate in these multigenerational studies. A major epidemiological study followed the Dutch Famine of 1944-1945, where children that were born from mothers that experienced famine during pregnancy experienced increased risk to disease such as obesity, diabetes, and cardiovascular issues (De Rooij et al., 2022). This study ultimately demonstrated that reduction of calorie intake in pregnant mothers led to epigenetic alterations in the next generation that increased risk of metabolic disease (De Rooij et al., 2022; Vaiserman and Lushchak, 2021). Another example of a multigenerational study after ancestral environmental exposure is work done with the Överkalix cohort, that highlights that those paternal grandfathers that experienced nutrition deficits or surpluses during puberty had effects on descendants' cardiovascular health and susceptibility to diabetes mortality (Kaati et al., 2012). The findings of this study are limited; however, the Uppsala Birth Multigeneration Study found that an abundance of food during prepubertal periods in males led to increased cancer mortality rates in their male grandchildren descendants (Kaati et al., 2002; Vågerö et al., 2018). These epidemiological cohort studies shed light on the significance of parental environmental exposures and transgenerational effects on male descendants.

More information is available from studies performed in animal models. The anti-androgenic fungicide vinclozolin was found to impact sperm fertility in subsequent generations upon maternal in utero exposure in rats (Anway et al., 2005). In a different study, gestating female mice that were exposed to dioxin, a byproduct of pesticide manufacturing, led to transgenerational phenotypes, such as increased risk to kidney disease, and sperm epimutations into the third generation (Manikkam et al., 2012). Prenatal exposure to TBT

elicited a transgenerational phenotype like non-alcoholic fatty liver disease (NAFLD) with increased fat depot size and overall increased of fat storage through the F3 and F4 generations in mice (Chamorro-Garcia et al., 2013; Chamorro-Garcia et al., 2017). Specifically, these F4 descendants experienced a predisposition to obesity when their dietary fat was increased (Chamorro-Garcia et al., 2017). These studies reveal that EDC exposures can have multigenerational effects on health, more specifically metabolic disruption in unexposed descendants. However, little is known about the mechanisms through which non-mutagenic alterations that occurred in one generation, could be propagated across multiple generations without further exposure to the corresponding environmental factor.

4.0 Epigenetics

The term “epigenetics” was first introduced by Conrad Waddington in 1942, and he defined the field as the study of complex developmental processes between genotype and phenotype (Deichmann, 2016). Epigenetics is considered the study of heritable traits without changes to the DNA sequence (Dupont et al., 2009). Environmental exposures to factors like stress, diet, and chemicals can alter gene expression and function without making changes to the DNA sequence (Pinel et al., 2019). Humans are continuously exposed to a myriad of environmental factors that shape health and risk of disease (Mitchell et al., 1999). The canonical model of inheritance supports that the genetic material from maternal and paternal chromosomes is passed onto their offspring. In the past several decades, the field of epigenetics has shown that alterations to genetic code (i.e., DNA sequence) is not the only mode of inheritance of traits into the next generation (Lacal and Ventura, 2018). Transgenerational epigenetic inheritance (TEI) is the process of transferring epigenetic signatures from one generation to the next and beyond without altering DNA sequence (Bošković and Rando, 2018). This type of inheritance links environmental exposures and acquisition of adverse health effects that are passed onto future unexposed generations. TEI can alter phenotypes by altering gene expression patterns through several, well-established,

mechanisms: DNA methylation, histone modifications, small non-coding RNAs (ncRNAs), and higher order chromatin organization (Al Aboud et al., 2024).

4.1 Chromatin architecture.

Alterations to higher order chromatin organization entail the 3-dimensional architecture of chromatin, including DNA wrapped around histones, or nucleosomes, that are further folded into larger chromatin structures (McGinty and Tan, 2014). There are two compartments in chromatin referred to as euchromatin (compartment A) and heterochromatin (compartment B) that represent functionally different types of chromatin that are separated by location (Girelli et al., 2020). Heterochromatin has lower gene density and thus transcribed at decreased rates when compared to euchromatin (Penagos-Puig and Furlan-Magaril, 2020). Each compartment has different enriched base compositions, with heterochromatin being AT-rich and euchromatin being GC-rich (Akilli et al., 2024). GC-rich DNA interacts with transcription factors and is associated with gene expression, whereas AT-rich DNA is associated with gene silencing (Padeken et al., 2022). When euchromatin and heterochromatin are established, there are histones and chromatin proteins that contribute to maintaining each compartment separated (Du et al., 2022). Histone modifications may also be involved in the establishment of different chromatin states. Alterations to chromatin organization can alter gene expression which can lead to risk of disease (Constanze and Cockerill, 2013).

4.2 Histone modifications

DNA coils around an octamer of histones making the nucleosome. Histone tails interact with DNA grooves and are targets for chromatin binding proteins like transcription factors and epigenetic modifiers that can alter the chemical residues associated to amino acids in the histone tails, such as the addition or removal of methyl or acetyl groups, contributing to altering chromatin accessibility (Cutter and Hayes, 2015). There are two types of heterochromatin: constitutive and facultative. Constitutive heterochromatin is transcriptionally

silent, while facultative heterochromatin is cell type specific and represses key genes whose expression is not needed during embryonic development (Rang et al., 2023). Histone modifications are posttranslational alterations to histones that ultimately affect gene expression and chromatin structure (Bannister and Kouzarides, 2011). The processes associated with histone modifications include acetylation or methylation of lysine residues, phosphorylation, and ubiquitination. These molecular processes alter the affinity of histones to DNA permitting transcription factors to bind to DNA thus altering gene expression (Blakey and Litt, 2015). Histone modifications can compact or loosen chromatin via electrostatic interactions which increases or reduces accessibility of transcription to specific regions; thus, altering gene expression (Rang et al., 2023; Cutter and Hayes, 2015).

4.3 DNA methylation

DNA methylation marks heterochromatin and organizes chromatin structure (Klein and Costa, 1997). DNA methylation occurs on CpG residues by forming 5 methyl-cytosine. DNA methylation is a process in which there is addition of methyl groups that attach to specific locations on the 5-methylcytosine position via DNA methyltransferases. Methylation can silence genes by recruiting proteins or by preventing transcription factors from binding to specific regions in DNA (Moore et al., 2013). As cells divide, the DNA methylation alteration patterns are copied into new DNA, which is further passed onto daughter cells and eventually these marks are transmitted to future generations (Kiselev et al., 2021; Alegria-Torres et al., 2011). DNA methylation marks can escape reprogramming events during development by binding to unique protein binding sites that protect them from enzymes involved in demethylation, such as ten-eleven translocation methylcytosine dioxygenase (TET) (Moore et al., 2013). In mammals, about 1% of genes can escape epigenetic reprogramming through “imprinting,” where genes are only expressed from one of the two parental copies in embryos. During imprinting, there is DNA methylation of imprint control regions (ICR), where parental-

specific methylation imprints are maintained in the developing zygote and behave as epigenetic marks that then control gene expression (Tomizawa and Sasaki, 2012).

4.4 Small non-coding RNAs

Small non-coding RNAs are a type of RNA molecule that are less than 200 nucleotides long and can regulate gene expression via binding to messenger RNA (mRNA) through complementary base pairing and inhibiting or activating transcription of mRNA (Shimoni et al., 2005). Small non-coding RNAs (ncRNAs) like microRNAs (miRNAs) specifically bind to the 3' untranslated region (UTR) of target mRNAs inhibiting translation (MacFarlane and Murphy, 2010). Inhibition of translation of specific mRNAs results in decreased expression of a particular gene that is encoded by the altered mRNA (Shimoni et al., 2005; MacFarlane and Murphy, 2010). Piwi-interacting RNAs (piRNAs) are another example of small ncRNAs that can regulate gene expression through complementary binding of mRNA leading to gene silencing (Zhang et al., 2023). Another small ncRNA include tRNA fragments or tRFs that bind to the 3' untranslated region (UTR) and inhibit translation thus silencing target gene expression (Xie et al., 2020).

4.5 Epigenetic signatures, reprogramming and inheritance

Epigenetic marks regulate gene expression during early developmental stages like embryogenesis (Kim and Costello, 2017; Wilkinson et al., 2023). Germ cells carry all the necessary information the embryo needs after fertilization before maternal-to-zygotic transition, including RNAs and proteins (Alberts et al., 2002). The sperm and the oocyte also carry epigenetic elements that may contribute to regulation of gene expression in the zygote (Guthmann et al., 2019; Hammoud et al., 2009). During embryogenesis, however, many epigenetic marks are erased, reset and re-established via epigenetic reprogramming events and thus inherited epigenetic patterns might not be maintained through development (Ben Maamar et al., 2021). For an epigenetic mark to pass to the next generation they must evade

erasure during reprogramming and in mammals only one percent of genes can escape this event (Chong and Whitelaw, 2004). Epigenetic marks carried via germ cells might become targets of environmental exposures and regulate gene expression and thus phenotype in the next generation (Tiffon, 2018).

4.6 Environmental exposures and epigenetic marks

Active or repressive epigenetic marks can be altered by environmental exposures (Ho et al., 2012). Diet is a well-studied factor that has been shown to alter epigenetic marks that are passed onto future generations (Barker and Clark, 1997). As previously mentioned, data obtained from the Överkalix study showed paternal lack or excess of nutrition had a negative impact on cardiovascular and metabolic health in descendants (Kaati et al., 2012). Metals, like arsenic, have also been shown to modulate DNA methylation patterns that activate oncogene expression and development of cancer (Reichard and Puga, 2010). Arsenic exposure has also been associated with increased incidence of metabolic and cardiovascular disease in humans in the Strong Heart Study (Kuo et al., 2022). Exposure to EDCs like TBT can lead to transgenerational metabolic disruption via alterations to chromatin organization that was propagated across multiple generations (Chamorro-Garcia et al., 2017; Diaz-Castillo et al., 2019). Exposures to environmental chemicals, like acrylamide or phthalates, have led to alterations in expression of small ncRNAs in sperm (Trigg et al, 2021; Oluwayiose et al.,2023; Ferrero et al., 2024). These sperm small ncRNAs are then transferred to developing embryos upon fertilization, leading to alterations in gene expression. These are all examples of agents that lead to alterations of epigenetic marks potentially contributing to adverse metabolic disease outcomes in future descendants (Reichard and Puga, 2010; Chamorro-Garcia et al., 2017).

5.0 Paternal contributions to the next generation

Paternal exposures to environmental factors such as chemicals, diet, or stress have been shown to lead to epigenetic alterations that affect not only individuals directly exposed but also unexposed future generations (Sales et al., 2017; Kaati et al., 2007; Yang et al., 2023). However, the specific epigenetic mechanisms through which these alterations can be propagated across generations are still being elucidated.

5.1 Sperm and small non-coding RNAs

Sperm are the male reproductive germ cells that fertilize the oocyte, the maternal germ cell, leading to development of a zygote (Alberts et al., 2002). Sperm small ncRNAs are an example of an epigenetic modulator of gene expression (Sharma, 2019). Sperm small ncRNAs species mixtures and levels can be altered by exposures to environmental toxicants, including nicotine (Zeid and Gould, 2023). Though the connection between nicotine exposure and alterations in sperm small ncRNAs and investigation into metabolic disruption in the next unexposed generation are limited, there is evidence that nicotine alters sperm small ncRNAs species and loads that lead to heritable alterations in the next generation (Zeid and Gould, 2023; Wang et al., 2022). The heritable alterations introduced to the next generation involved behavioral changes and increased tolerance to nicotine when given to F1 animals (Vallaster et al., 2017). Small non-coding RNAs can also alter biological processes important in paternal health like spermatogenesis (Joshi and Rajender, 2020).

5.2 Spermatogenesis and small non-coding RNAs

Spermatogenesis is the process of sperm development in the seminiferous tubules of the testes to produce haploid spermatozoa (Suede et al., 2024). There are three stages in spermatogenesis: mitosis, meiosis, and spermiogenesis (Suede et al., 2024). During mitosis, spermatogonia stem cells divide and differentiate into spermatogonia; at meiosis spermatocytes undergo two meiotic divisions thus reducing the number of chromosomes in each cell into a haploid state; and finally, during spermiogenesis round spermatids eventually

mature into spermatozoa (Kotaja, 2013). The entire process of spermatogenesis takes up to 74 days in humans and 35 days in mice and results in the constant production of mature sperm (Griswold and Hogarth, 2022; Chen et al., 2016).

Recent investigation into paternal contributions to next generation's health has revealed that sperm small ncRNAs species and loads are altered upon environmental exposures and transferred to the embryo eliciting altered gene expression in offspring (Sharma,2019). Small ncRNAs are delivered to the zygote at fertilization where they regulate embryonic development and can lead to increased susceptibility of disease later in life (Kumar et al., 2013). Sperm small ncRNAs can be delivered to the embryo via vesicles known as epididymosomes that sequester the small RNAs and deliver them to maturing sperm as it makes the journey from the epididymis to the embryo upon fertilization (Liu and Sharma, 2023).

Sperm-borne small ncRNAs have multiple roles in sperm function and development by targeting and down-regulating specific transcripts. Sperm can carry small ncRNAs in epididymal vesicles that are transferred into developing zygotes upon fertilization and can influence embryonic development (Sharma, 2019). Environmental exposures can alter sperm small ncRNAs that are transmitted to future generations and modulate risk of disease (Sharma et al., 2019; Chen et al., 2016; Skinner et al., 2018).

5.3 Paternal environmental exposures and next generation's health

Different windows of exposure during an individual's lifetime can have differing adverse health effects. Developmental exposures to chemicals like alcohol can elicit severe adverse health outcomes, such as cognitive and neurobehavioral disorders (Subramoney et al.,2018). Another example, nicotine exposure during pregnancy can elicit neurodevelopmental alterations in developing offspring, whereas nicotine exposure during adulthood increases risk of cardiovascular diseases and lung cancer (Ren et al., 2022). Exposure *in utero* to nicotine can elicit several adverse health outcomes in the developing offspring including

prematurity, stillbirth, impairments to neurodevelopment, skeletal and lung development (Wells and Lotfipour, 2023). One window of exposure that is not well studied is parental preconception exposure windows and adverse health outcomes in the next generation. Paternal exposures to environmental factors like high-fat or a low-protein diets have led to metabolic alterations, like abnormal triglyceride metabolism and altered hepatic transcriptomic profile (Aizawa et al., 2022; Carone et al., 2010). There are also studies showing that chemical exposures like pesticides can elicit fetal malformations and childhood leukemia in the next generation (Patel et al., 2020). Paternal nicotine exposure demonstrated neurobehavioral alterations in the next generation (McCarthy and Bhide et al., 2021). Paternal preconception nicotine exposure in mice resulted in locomotor hyperactivity and attention deficits in the next unexposed generation (Zhang et al., 2020). Researchers have previously investigated paternal nicotine exposure paradigms and neurobehavioral alterations and have not focused on potential heritable alterations regarding metabolic disruption. Vallaster et al. showed that paternal nicotine exposure was associated with alterations in glucose homeostasis (Vallaster et al., 2017) in mice. Next generation phenotypes regarding metabolic disruption, like alterations in glucose homeostasis, or lipid metabolism have not been fully characterized in the paternal preconception exposure paradigm. Mechanisms underlying these heritable alterations have not been determined and are still being elucidated; however, one central hypothesis is that paternal environmental exposures alter epigenetic marks on sperm that are then transmitted to the developing zygote upon fertilization (Sharma et al., 2019; Skinner et al., 2018).

6.0 Tobacco use and tobacco-related chemicals

Tobacco is a plant, *Nicotiana tabacum* or *Nicotiana rustica*, that contains the highly addictive substance nicotine (Leone et al., 2010). Tobacco has been used for centuries for religious, cultural, and ceremonial purposes as early as the first century BC (Mishra and Mishra, 2013). Smoking tobacco exposes the user to a myriad of harmful chemicals including nicotine,

arsenic, carbon monoxide and formaldehyde (Engstrom et al., 2003). Global tobacco use has decreased in recent decades thank to policies and advertisements against tobacco as a harmful substance to human health (Fu and Xiao, 2023). Though global tobacco use has declined there are still many people in specific communities/groups that continue to use tobacco (Dai et al., 2022). Unfortunately, impoverished communities are targeted by tobacco companies to use tobacco which keeps individuals in a cycle of tobacco use and consequentially costly adverse health issues (Brown-Johnson et al., 2014). It is important to continue research into tobacco-related adverse health issues and consequences to further establish evidence that supports anti-tobacco policies and efforts to reduce tobacco campaigning. Specifically, research into chemicals found in tobacco products highlights how detrimental substances like nicotine are on development, lung health, and cardiovascular health.

6.1 Global male tobacco use

In 2020, the prevalence of global male smokers was 36.7%, while female smokers was 7.8% (WHO, 2021). In the United States, 24.1% of men used tobacco compared to 10% of women (CDC, 2023). There are several reasons men might smoke at higher rates than women, including gender role expectations that view female-smokers negatively and male-smokers neutrally (Waldron, 1991). Whatever the reason for smoking, paternal smoking has been shown to not only lead to direct health outcomes in the user, but also adverse neurobehavioral and metabolic outcomes in the next generation (Accordini et al., 2021; Zhang et al., 2020). Investigation into paternal cigarette smoke exposure has been shown to alter DNA methylation marks on sperm that is transmitted to the F1 generation and led to elevated liver fat and altered glucose levels during glucose tolerance testing in mice (Liu et al., 2022). There is a myriad of harmful chemicals found in tobacco and cigarette smoke that can individually lead to adverse health outcomes both through direct or ancestral exposure, like arsenic and nicotine (Lazarevic, et al.,2012; McCarthy and Bhide, 2021).

6.2 Chemicals in tobacco: arsenic and arsenic metabolism

Tobacco smoke produces several endocrine disrupting chemicals that can lead to endocrine disruption (Tweed et al., 2012). Arsenic, a naturally occurring element, can leach into groundwater and be absorbed into the tobacco plant with trace amounts of arsenic being found in the finished tobacco product (Marano et al., 2012). Arsenic-tainted groundwater is the main source of unhealthy exposure to arsenic and affects thousands of humans across the world (Shankar et al., 2014). Arsenic is a ubiquitous element that leaches into groundwater and is then consumed by humans leading to acute toxicity and disease, like skin cancer, chronic inflammation, and metabolic disruption (Marano et al., 2012; Shankar et al., 2014).

Ingested arsenic is metabolized in the liver where pentavalent arsenic (iAsV) is reduced to trivalent arsenic (iAsIII), then iAsIII is methylated by arsenic methyltransferase, and the resulting monomethylarsonic acid (MMAV) and dimethylarsinic acid (DMAV) are excreted in the urine (Watanabe and Hirano, 2013). Glutathione plays a vital role in arsenic metabolism as presence of this molecule aiding in the reduction of pentavalent arsenic, and then binding to the newly reduced trivalent arsenic species forming a complex that permits methylation (Doerge et al., 2020; Watanabe and Hirano, 2013). It is estimated that 70% of arsenic that is ingested is excreted through the urine, while the remaining arsenical species either are slowly filtered through the kidneys for long periods of time, or deposited onto skin, hair, and nails (Water 1999). Arsenic was a chemical of interest as it is found in tobacco products and there is literature demonstrating that arsenic exposure elicits adverse health outcomes in regards to metabolic disruption, specifically alterations to glucose homeostasis (Kirkley et al., 2018).

6.3 Chemicals in tobacco: nicotine and nicotine metabolism

Another chemical of interest, nicotine, the highly addictive chemical in tobacco products, has been shown to elicit various adverse health effects based on the route of exposure (Gibbs et al., 2016; Olds, 1997). Direct nicotine exposure has been shown to lead to decreased body

weight, various types of cancer, and alterations to glucose homeostasis (Minna, 2003; Bruin et al., 2007). Developmental, or in utero, exposure to nicotine has been shown to elicit neurodevelopment alterations, behavioral issues, and increased risk of miscarriage or stillbirth in humans and mice (Wells and Lotfipour, 2023; Pauly et al., 2004). Developmental exposure to nicotine has also elicited impaired glucose tolerance in male offspring in their postnatal life (Bruin et al., 2007).

Ingested nicotine is metabolized by the liver and kidneys and generates a primary metabolite known as cotinine (Murphy 2021). The formation of cotinine occurs in two steps: first, cytochrome P450 catalyzes the 5’ oxidation to an iminium ion, then that ion is oxidized into cotinine (Nakajima et al., 1996). The kidney filters nicotine and its metabolites and excretes these molecules through urine. Cotinine can be detected in blood and has a half-life of about 16 hours and is often used as a biomarker of nicotine use (Benowitz et al., 2009). Nicotine can also accumulate in breastmilk of nursing smoking mothers and can be detected in the blood and urine of their infants (Benowitz et al., 2009; Calvaresi et al., 2016). Nicotine exposure elicits a myriad of adverse health effects and paternal nicotine exposure has been shown to elicit hepatic alterations and altered glucose homeostasis in mice (Vallaster et al., 2017). Maternal *in utero* nicotine exposure paradigms have been well studied (Bruin et al., 2008; Bruin et al., 2007; Zhang et al., 2018), and paternal preconception nicotine exposure studies are increasing but limited.

Investigation into nicotine exposure on sperm small ncRNAs is limited. Chronic nicotine exposure alters sperm small ncRNAs in C57BL/6J mice altering the F1 generations neurobehavioral outcomes and nicotine metabolism (Zeid and Gould, 2023). Investigation into sperm small ncRNAs upon paternal nicotine exposure and phenotypes of metabolic disruption in the next generation have not been investigated.

As stated briefly throughout this introduction, direct nicotine exposure has been shown to elicit adverse health outcomes such as metabolic syndrome and lung disease in humans

(Chen et al., 2023; Momayyezi et al., 2024; Bermudez et al., 2019). Tobacco use is a global health concern that contributes to metabolic disruption and disease and may partly explain the steady increase of metabolic disease prevalence (Bermudez et al., 2019; Rehman et al., 2021). Though global tobacco use is on the decline, there are still groups of people, like men, that continue to use tobacco products. Investigation into paternal nicotine exposure and metabolic disruption endpoints are limited, with Vallaster et al., highlighting in a mouse model that paternal nicotine induced hepatic transcriptomic alterations and altered glucose homeostasis in male mice (Vallaster et al., 2017). There also have been studies that demonstrate that paternal nicotine exposure alters sperm epigenetic marks, like DNA methylation profiles, or small non-coding RNAs (Liu et al., 2022; Zeid and Gould, 2023). Two key knowledge gaps that I will be addressing in my dissertation refer to 1) the interaction between paternal exposure to nicotine and the consumption of a hypercaloric diet in their offspring and 2) the analysis of the sexually dimorphic response to such exposures.

7.0 Ancestral and direct nicotine exposure is associated with metabolic disruption phenotypes in a mouse model

In this dissertation, the metabolic disruption endpoints that were focused on include glucose homeostasis, whose disruption is associated with T2D, excessive weight gain (i.e., obesity), alterations to plasma metabolites associated with metabolic processes, and functional analyses of the liver via transcriptomics. In data chapter # 1, I investigate the direct physiological and hepatic transcriptomic metabolic effects upon chronic nicotine exposure in adult male and female mice. In data chapter #2, I investigate the metabolic alterations in the next generation upon paternal preconception nicotine exposure and F1 dietary challenge with a hypercaloric diet. When investigating environmental exposures and impacts to human health and future generations' health, researchers typically investigate one environmental exposure to study that exposures' effects. Under the exposome paradigm, humans are exposed to a multitude of environmental factors throughout life that influence health and

disease. Investigation into the effects of nicotine use paired with hypercaloric diets are limited in animal models and in epidemiological studies.

As we are exposed to multiple environmental factors in our lifetime, it is pertinent to understand the interplay between diets and nicotine use on not only direct health, but also future generations' health. Future research in the lab will determine the epigenetic mechanism underlying the phenotypes we observed in experimental findings in Chapter 3 of this dissertation.

My dissertation provides valuable insight into the metabolic effects of nicotine exposure in two experimental paradigms: direct exposure and paternal exposure. Chapter 2 of this dissertation highlights how direct exposure to nicotine elicits metabolic disruption and exacerbates risks associated with metabolic disease. In the paternal exposure paradigm, described in chapter 3, the introduction of a hypercaloric diet on F1 animals paternally exposed to nicotine will shed light on the effects of secondary "challenges" that are also part of the exposome and that can exacerbate underlying alterations of paternal exposure to nicotine. Taken together, these two data chapters highlight the combined effects of nicotine on a direct exposure and paternal nicotine exposure paradigm. These studies provide the stepping stones for determining how paternal nicotine exposure might be leading to these metabolic disruption phenotypes in the next generation, and the combinatorial effect of adding a second environmental factor, such as a hypercaloric diet, to metabolic disease incidence.

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CHAPTER 2

CHRONIC NICOTINE EXPOSURE ELICITS HEPATIC TRANSCRIPTOMIC ALTERATIONS ASSOCIATED WITH CARDIOMETABOLIC HEALTH IN ADULT MICE

ABSTRACT:

Cardiometabolic diseases are a leading cause of mortality in humans on a global scale. Risk factors to developing either cardiovascular or metabolic diseases have often been attributed to sedentary lifestyle, hypercaloric diets and ultra-processed foods. Recent literature highlights that exposure to chemicals found in our environment may also be a contributing risk factor to development of cardiometabolic diseases. Humans are exposed to a variety of chemicals in their daily lives whether by accident or by their own behaviors. Exposure to anti-fouling agents like tributyltin (TBT) and chemicals found in tobacco, like nicotine, can lead to adverse health effects. Specifically, direct exposure of each individual to chemicals is associated with cardiometabolic disruption in the exposed organism. Here we demonstrate that chronic exposure to nicotine can elicit cardiometabolic alterations at the physiological and molecular level in adult mice. These findings highlight the effects of chronic nicotine exposure in an animal model and further characterize the cardiometabolic alterations that are elicited upon exposure. Specifically, these findings demonstrate the health risks of direct exposures to endocrine-disrupting chemicals like TBT and nicotine.

INTRODUCTION:

Cardiometabolic diseases are a group of conditions that include cardiovascular diseases (CVD), such as heart disease and stroke, and metabolic diseases, including type-2 diabetes, non-alcoholic fatty liver diseases (NAFLD) and other endocrine diseases. Prevalence of cardiometabolic diseases have reached epidemic proportions globally (Janssen, 2023; National Academies of Science, 2021; Roth et al., 2020). CVD is the leading cause of death globally with about one-third of global deaths in 2021 being attributed to CVD

(Vaduganathan et al., 2022). By 2050, CVD is projected to affect about 60% of the American population, with risk factors like obesity, hypertension, T2D, also expected to increase to affect more than half of the US population (Joynt Maddox et al., 2024). Causes that contribute to incidence of CVD include air pollution, tobacco use, poor diet, and lack of physical inactivity (Brown et al.,2024).

Within a lifetime, humans are exposed to a plethora of environmental factors that can affect our health. The exposome is the measure of all environmental exposures, from physical and chemical to social and lifestyle factors, a person experiences in their lifetime (Wild, 2005; Vermeulen et al., 2020; Wright, 2020). There are chemicals and factors within our environment that we are exposed to in our daily lives, like air pollution, cigarette smoke, and our diets that negatively influence our health by leading to outcomes such as lung cancer and/or cardiometabolic diseases (Saha et al., 2007; Elizabeth et al., 2020; Azimi and Rahman, 2024). Cigarette smoke exposure has been shown to lead to CVD, cancer, and metabolic disorders such as T2D and/or weight gain in humans (Saha et al., 2007; Dai et al., 2022). Investigation into individual exposure to environmental factors like tobacco products have been explored in both epidemiological and animal studies and demonstrated that exposure to cigarette smoke elicits cardiometabolic alterations (Wali et al., 2020; Saha et al., 2007).

Endocrine-disrupting chemicals (EDCs) are substances found in the environment that either are naturally occurring or manmade that when exposed to can lead to harmful endocrine dysfunction leading to cardiometabolic disruption (Diamanti-Kandarakis et al., 2009; Zoeller et al., 2012). There are several known EDCs that cause alterations that lead specifically to metabolic disruption like nicotine (Panico et al., 2022; Xie et al., 2009; Huang et al., 2018). Nicotine exposure leads to endocrine disruption via interaction of nicotinic acetylcholine receptors, which leads to the secretion of stress hormones, like cortisol (Tweed et al., 2012). Another EDC that has been identified as a disruptor of metabolism is the anti-

fouling agent tributyltin (TBT). TBT exposure elicits endocrine disruption via activation of retinoid X receptor (RXR) and peroxisome proliferator-activated receptor gamma (PPARγ), which are key players in the development of fat tissue (Shoucri et al., 2017; Shoucri et al., 2018; Grun et al., 2006). Exposure to EDCs can lead to increased risk of metabolic disease prevalence due to endocrine disruption (Tweed et al., 2012; McGinnis and Crivello, 2011).

With rates of metabolic diseases rapidly increasing and projected to continue to increase in the next several decades, investigation into other factors that contributes to metabolic diseases have revealed that certain chemicals, like EDCs, can elicit cardiometabolic disruption (Heindel et al., 2017; Haverinen et al., 2021; Joynt Maddox et al., 2024; Miranda et al., 2019). The endocrine system plays a role in the metabolism of macromolecules and is regulated by hormones. Endocrine disruption can also impact cardiovascular processes via modifications to hormone signaling which influences heart rate and blood pressure (Gordan et al., 2015). EDC exposure can modulate the endocrine system and elicit increased adipocyte or fat cell function and lead to increased adiposity (Darbre, 2017). Exposure to EDCs such as pesticides like dichlorodiphenyltrichloroethane (DDT) have been shown to modulate endocrine function and lead to weight gain, one form of cardiometabolic disruption (Hovinga et al., 1993). Exposure to plastics such as phthalates have also been shown to lead to weight gain and adverse metabolic outcomes in rodents (Ema et al., 1990; Field et al., 1993). Environmental exposures to harmful chemicals can lead to cardiometabolic alterations and increase risk of disease in humans (Pena and Rollins, 2017; Shrivastav et al., 2024). As humans are exposed to a myriad of factors in their lifetime, it is important to investigate exposures to these varying factors to understand and characterize the adverse cardiometabolic health outcomes.

Nicotine, the main psychoactive ingredient in tobacco, can cause increases in blood pressure, plasma fatty acids, and metabolically disruptive increased level of catecholamines in the blood (Dani and Heinemann, 1996). In animal models, nicotine exposure elicits

decreased body weight over time and impaired glucose homeostasis (Bergman et al., 2012). Among other pathological implications, like neurobehavioral outcomes, nicotine exposure can also lead to metabolic alterations in unexposed generations (Vallaster et al., 2017). Nicotine can also elicit cardiovascular diseases like increased blood pressure, angiogenesis or new blood vessel growth, and atherosclerosis or plaque buildup in arteries in rodents (Fried et al., 2022).

TBT, a biocidal chemical that used to be produced as a fungicide, is found in dust and human blood (Antizar-Ladislao, 2008). TBT was primarily used in ship paints to prevent mollusks from attaching to the ship hulls (Beyer et al., 2022). However, TBT leaches into the sea water exposing marine animals. TBT has been shown to alter marine animals, specifically gastropod snails, by inducing pseudo hermaphroditism in females that develop male sexual characteristics upon exposure (Abidli et al., 2009). Exposure to TBT leads to metabolic disruption and increases the risk of inappropriate fat accumulation and adiposity in zebrafish and mice (Canny et al., 2021; Grün et al., 2006). In mice, we showed for the first time that ancestral TBT exposure induced transgenerational metabolic disruption (Chamorro-Garcia et al., 2013).

This study aims to investigate the adverse cardiometabolic effects upon chronic exposure to nicotine in adult male and female mice. Since TBT is a well-known metabolic disruptor, we used TBT as a positive control and to further characterize its effect of direct exposure in adult mice. Here we characterized metabolic alterations in C57BL/6J mice that were exposed to nicotine and TBT. Specifically, we hypothesized that direct nicotine or TBT exposure would elicit metabolic disruption at both the physiological and transcriptomic levels. Using a chronic exposure paradigm where animals were exposed for sixteen weeks and treatment ended at twenty-two-weeks of age of animals; we analyzed metabolic endpoints such as glucose and insulin tolerance, plasma metabolite levels, weekly body weight gain, and hepatic transcriptomics. These experiments show the association of chronic nicotine or

TBT exposures and related cardiometabolic health outcomes in an animal model. It is important to note that animals in this chronic toxicant exposure study were placed on a hypercaloric diet while exposed to treatments. This exposure paradigm is relevant to human exposure models as humans are exposed to a myriad of environmental factors within their lifetime, not just chemical exposures. The novelty of these experiments highlights the adverse cardiometabolic effects of chronic nicotine exposure and the physiological and molecular level.

METHODS:

Chemicals and reagents

(-)-Nicotine (#N3876), Tributyltin chloride, 96% (#T50202), D-(+)-glucose (#G8270), and human recombinant insulin (dry powder, #91077C) were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific, LLC. Nicotine was stored out of light and in a desiccator. Glucose and insulin stocks for glucose and insulin tolerance tests were prepared fresh the day of metabolic testing.

Animal care and maintenance

Mice were purchased from Jackson Laboratories. C57BL/6J mice, both males and females aged 3 weeks-old were housed in microisolator cages in a temperature-controlled room (21-22℃) with a 12 h light/dark cycle and provided the following diet from ENVIGO: the total western diet (New Total Western Diet VI, Irradiated, #TD.110919) at 120 g/4 animals per week for 10 animals per treatment and sex (total 60 mice). Diets were supplemented with fresh food pellets weekly. Animals received chemical treatments in fresh milliQ water in bottles twice a week. Treatments were prepared at concentrations of 32.4 M Nicotine, 50 nM Tributyltin, or the vehicle control 0.1% dimethyl sulfoxide (DMSO). Animals were treated humanely and with regard for alleviation of suffering following guidelines through the Institutional Animal Care and Use Committee of the University of California (IACUC). All

procedures conducted in this study were approved by the IACUC Santa Cruz approval number Chamr2208dn.

Animals began chemical exposures via drinking water at 7 weeks old until they were 22 weeks old, exposed for a period of 16 weeks. There were 10 animals per treatment per sex. At 22 weeks old, animals were sacrificed via isoflurane overdose. Blood was drawn from direct heart puncture into a heparinized syringe and placed into a clean tube containing protease inhibitors (Protease Inhibitor Cocktail, EDTA-free, Sigma-Aldrich #S8830). Blood was centrifuged for 10 min at 3,075 x g at 4º C. Plasma was transferred to a clean tube, snap-frozen in liquid nitrogen and preserved at -80º C. Samples were shipped to Eve Technologies Corporation (Calgary, AB) for analysis of a panel of plasma metabolites: amylin, c-peptide 2, ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PP, PYY, resistin and secretin (Mouse/Rat Metabolic Hormone Discovery Assay® 11-Plex, MRDMET). Liver and gonadal white adipose tissue was harvested and preserved at at -80º C for future analyses. All tissue harvesting was performed with the dissector blinded to which groups the animals belonged. At the moment of euthanasia, each mouse was assigned a code, known only to the lab member not involved in dissections.

Glucose and insulin tolerance tests

For nicotine and tributyltin treated animals, there were 5 animals tested per treatment group and per sex. Animals were maintained on a 12 hour light/dark cycle. Animals were tested during their light phase (inactive, not eating). Glucose and insulin stocks were prepared fresh daily and mixed with 0.9% saline. Animals were given 2 g of glucose/kg body weight (b.w.) or 0.75 IU of insulin/kg b.w. via intraperitoneal injection after 4H of fasting (from 8:30am-12:30pm). Blood glucose levels were measured with Contour® blood glucose meter (BAYER) and Contour® blood glucose strips (BAYER) every 30 minutes for 120 minutes after injection of glucose or insulin. Blood glucose levels were measured via tail prick with a 25 gauge needle. After tests were completed, animals were given their respective diets.

RNA isolation and sequencing

For nicotine and tributyltin experiments, gonadal white adipose tissue (gWAT) and liver tissue samples were isolated. Tissues were isolated using Direct-zol RNA MiniPrep (Zymo Research #R2053). Tissues were homogenized with VWR Premium Micro-Homogenizer (#10032-328). Depending on the experiment, RNA from 4-5 randomly selected mice from each group were submitted to the University of California Davis Genome Center for 3' Tag-RNA-sequencing using an Illumina HiSeq 4000 platform. To ensure RNA quality and concentrations Nanodrop was used and gel electrophoresis was performed on RNA gels to assess purity of RNA. We obtained single-end reads (85nt) for each sample. Statistical evaluation of transcriptome variation was performed using the Galaxy Project platform (Galaxy version 23.0). FastQ files were processed using FastQC (Galaxy version 0.73). Indexing and alignment to the mouse genome (mm39) was done using STAR (Galaxy version 2.7.10b+galaxy3). FeatureCounts (Galaxy version 2.03+galaxy2) function was used to assign uniquely mapped RNA-seq reads to GRCm39 mouse reference genome count reads. DESeq2 (Galaxy version 2.11.40.8+galaxy0) function was used to determine differentially expressed genes between treatment and control groups in either respective experiment.

Gene ontology term analyses

Gene ontology (GO) term analyses were accomplished using differentially expressed genes (DEGs) generated from Galaxy sequencing pipeline. In Galaxy, using GOSeq (Galaxy version 1.5.0+galaxy0) the overrepresented gene categories were generated.

Statistical analyses

Statistical analyses for metabolic endpoints (body weight, plasma triglycerides, plasma metabolites, and glucose and insulin tolerance tests were performed using GraphPad Prism 10.0 (GraphPad Software, Inc.). Statistical tests and specific comparisons are indicated in each figure and their respective figure legend.

RESULTS:

Chronic nicotine exposure significantly decreases body weight in male mice

Female and male mice were exposed to nicotine, TBT or the vehicle control DMSO for sixteen weeks, then sacrificed at twenty-two weeks old. Body weights were measured weekly and compared to the control animals (DMSO-treated). Males exposed to nicotine exhibited significantly decreased body weights between weeks 14 and 21 of age (Figure 1). Females exposed to nicotine did not exhibit significantly decreased body weights when compared to control animals. Animals exposed to TBT did not exhibit any statistically significant alterations to weight gain while on treatments.

Chronic nicotine or TBT exposure of animals elicits impaired insulin tolerance and plasma metabolite levels in male mice

Chronic nicotine or TBT exposure did not elicit any significant alterations to blood glucose levels during glucose tolerance test for males or females (Figure 2). However, we observed alterations in insulin sensitivity during the insulin tolerance test. Blood glucose levels were significantly higher in TBT-treated and nicotine-treated male mice when compared to DMSO-treated males (Figure 3A). These results are also consistent with an increased area under the curve of TBT and nicotine treated males when compared to DMSO males (Figure 3C). This would indicate that those male mice belonging to either chemical treatment group have target tissues that are unable to efficiently uptake glucose from the blood when stimulated with a bolus of insulin, which is consistent with a insulin resistance phenotype, and a risk factor for T2D. Female mice of either treatment group did not exhibit any differences when compared to DMSO females during the insulin tolerance test (Figure 3B and 3D).

We measure plasma levels of metabolites involved in different metabolic processes such as glucose homeostasis (Figure 4). We found significant decreased plasma metabolite

levels C-peptide 2, GIP, and PYY in the nicotine group compared to the control group in males. There were no significant differences to amylin, GLP-1, glucagon, PP, secretin or resistin when comparing nicotine to control treated animals. Ghrelin was significantly increased in nicotine males when compared to DMSO males. Leptin plasma concentrations were significantly decreased in nicotine female and male mice when compared to their counterparts treated with DMSO (Figure 4). Increased ghrelin levels and decreased leptin levels in males exposed to nicotine indicate an imbalance in metabolite concentrations involved in activated appetite regulation and increased risk of weight gain and obesity. There were no differences in plasma metabolite levels when looking at TBT treated animals compared to control animals.

Chronic nicotine or TBT exposure of animals elicits transcriptomic alterations associated with cardiometabolic processes in liver tissue

Male and female mice that were chronically exposed to either nicotine or TBT exhibited transcriptomic alterations in the hepatic liver associated with cardiovascular processes (Figures 5 and 6). The liver is involved in the regulation of cardiometabolic processes such as blood glucose homeostasis or cholesterol levels. Alterations of such pathways can contribute to atherosclerosis and inflammation to cardiovascular tissues (Wiernsperger et al., 2013). Hepatic transcriptomics can provide insight into cardiometabolic processes as liver dysfunction can regulate lipid metabolism, coagulation proteins, and inflammatory responses in turn affecting cardiovascular function (Cao et al., 2022; Fang et al., 2024). Transcriptomic analyses of liver tissues revealed an enrichment of differentially expressed genes (DEGs) associated with the GO terms ‘metabolic process’, ‘muscle cell development”, and ‘circulatory system development’ in both males and females when comparing the nicotine and the control groups (Figures 5 and 6). TBT exposed females compared to control animals exhibited differential gene expression in GO terms enriched for biological processes associated with ‘lipid metabolic process,’ ‘small molecule metabolic

process,' and 'carboxylic acid metabolic process' (Figure 7). Males exposed to TBT compared to their controls revealed differential gene expression of GO terms enriched for similar biological processes to their female counterparts, including processes like 'lipid metabolic process,' 'small molecule metabolic process,' and 'monocarboxylic acid metabolic process' (Figure 8). To assess any overlap of differentially expressed genes among treated groups, nicotine or TBT, we looked for shared GO term categories (Figure 10). Investigation into the gene ontology terms of shared elements of differentially expressed genes among females on either nicotine or TBT treatment did not reveal any statistically significant GO terms. Though there were 37 shared differentially expressed genes among females on either nicotine or TBT treatment. However, when looking into shared differentially expressed genes among TBT-and nicotine-treated males, there were GO terms that overlapped and were enriched in biological processes represented in Figure 10. Interestingly, the biological processes that TBT and nicotine males share include 'muscle system development,' 'cardiac cell development,' 'muscle contraction,' and 'heart contraction' (Figure 10). Many of the overrepresented GO terms associated with differentially expressed genes in nicotine versus DMSO or TBT versus DMSO are involved in cardiovascular processes. These data reveal that nicotine or TBT can elicit hepatic transcriptomic alterations that are involved with cardiovascular processes in male mice

DISCUSSION

Cardiometabolic disease prevalence is a global health concern as rates of disease are increasing (Janssen, 2023). Typical factors associated with cardiometabolic disease prevalence, physical inactivity and hypercaloric diets cannot fully explain the global increases of cardiometabolic disease (Elizabeth et al., 2020; Wali et al., 2020). In the past several decades, investigation into EDC exposure and the adverse health outcomes that arise reveal that some chemicals, like plasticizers, can elicit metabolic disruption and disease (Ema et al., 1990; Field et al., 1993). Humans are exposed to a myriad of chemicals and factors in their

environments within their lifetime that can lead to adverse health outcomes. We previously demonstrated that ancestral exposure to the anti-fouling agent TBT can elicit metabolic disruption in future offspring in rodent models (Chamorro-Garcia et al., 2013). However, potential cardiometabolic alterations at the physiological and transcriptomic level upon direct TBT exposure has not been previously characterized. Another important chemical that can lead to adverse cardiometabolic outcomes is nicotine, the main addictive substance in tobacco products. Though the rates of tobacco use are on the decline, many individuals continue to use nicotine products. Nicotine exposure can elicit cardiometabolic alterations in humans and in animal models (Dani and Heinemann, 1996; Bergman et al., 2012). Here we further characterize the physiological and transcriptomic alterations that arise upon direct chronic nicotine exposure. Specifically, we show that there were significant alterations to the liver, an important cardiometabolic tissue that regulates metabolic process like lipid or cholesterol metabolism which in turn impacts blood pressure and cardiovascular health (Møller and Bernardi, 2013).

This study demonstrates that exposure to nicotine and TBT chemical exposures can lead to liver alterations that are consistent with cardiometabolic disease in mice. Chronic nicotine exposure elicited impaired insulin tolerance and altered plasma metabolite levels in male mice while decreasing their body weight. Liver transcriptomic analyses in nicotine male mice reveal differential gene expression of genes that were enriched for gene ontology (GO) terms associated with cardiovascular processes. Specifically, biological processes like cardiac cell development processes were altered in nicotine and TBT male mice when compared to control animals. These findings highlight that chronic exposure to nicotine or TBT while maintained on the total western diet elicits cardiometabolic disruption in both animals, with a more pronounced phenotype in male mice.

Chronic tributyltin (TBT) exposure while on a hypercaloric diet led to metabolic disruption phenotypes in male mice. Like the nicotine males, the TBT males also experienced

impaired insulin tolerance and altered plasma metabolite levels. Liver transcriptomics reveal differentially expressed genes that were enriched for GO terms associated with cardiovascular processes. Specifically, there were alterations in representation with GO terms like muscle contraction, heart contraction, and muscle system development.

Humans are exposed to a variety of factors in their environments daily: chemicals, pollution, diet, stress, etc. Global rates of metabolic disease prevalence are steadily increasing, and the two commonly attributed factors, poor diet and sedentary lifestyle, cannot be the only factors eliciting the rise in metabolic disruption (Wali et al., 2020; Baillie-Hamilton, 2002). This study demonstrated that chronic exposure to environmental chemicals like nicotine and our positive control TBT while maintained on a hypercaloric diet elicits cardiometabolic disruption at the molecular level in hepatic tissue. Chronic nicotine exposure impaired insulin tolerance and altered plasma metabolites in male mice when compared to DMSO males. Like nicotine, TBT elicited alterations to insulin tolerance and altered plasma metabolites in male mice when compared to DMSO males.

This study highlights that chronic exposure to nicotine or tributyltin while maintained on a hypercaloric diet can further exacerbate cardiometabolic disruption effects in mice. These studies provide foundational information for the direct cardiometabolic effects of tobacco-related chemicals like nicotine, in an animal model. When investigating future generations that were ancestrally exposed to toxicants, we have highlighted the direct cardiometabolic effects at the physiological and transcriptomic level in adult mice.

FIGURES

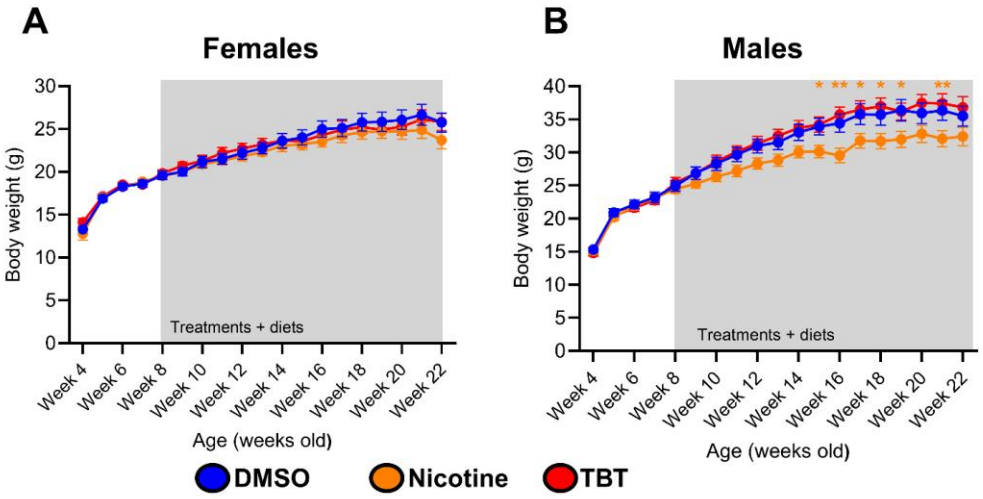

Figure 1. Chronic nicotine exposure leads to significantly decreased body weights in male mice. (A) Weekly body weights for female mice. (B) Weekly body weights for male mice. (Two-Way ANOVA, DF Column Factor = 2, Row Factor = 14, Dunnett's multiple comparison's test, *P<0.05, **P<0.01, n = 10; compared to DMSO-control).

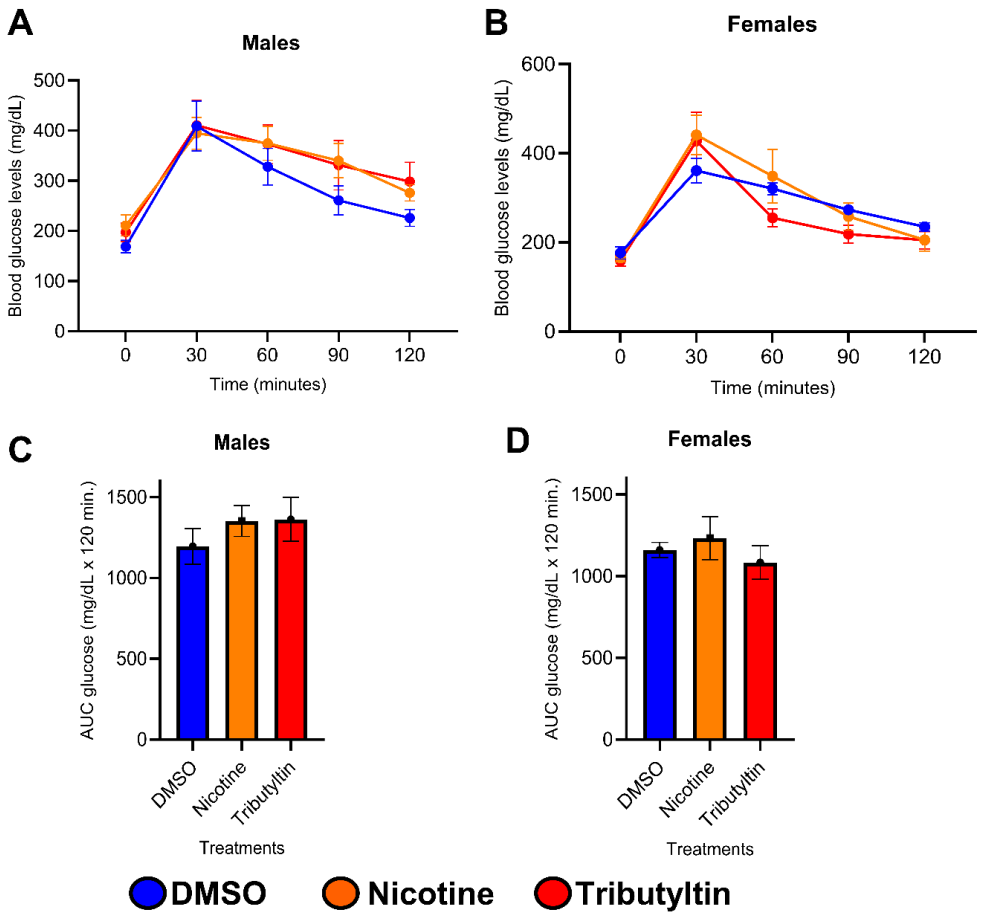

Figure 2. Chronic nicotine or tributyltin exposure does not lead to significant alterations in the glucose tolerance test in animals. (A) Glucose tolerance test curve for male mice. (B) Glucose tolerance test curve for female mice. (C) Area under the curve for glucose tolerance test for male mice. (D) Area under the curve for glucose tolerance test for female mice. (Two-Way ANOVA, DF Column Factor = 2, Row Factor = 4, Dunnett's multiple comparisons for GTT, and Kruskal-Wallis test for AUC, n = 5).

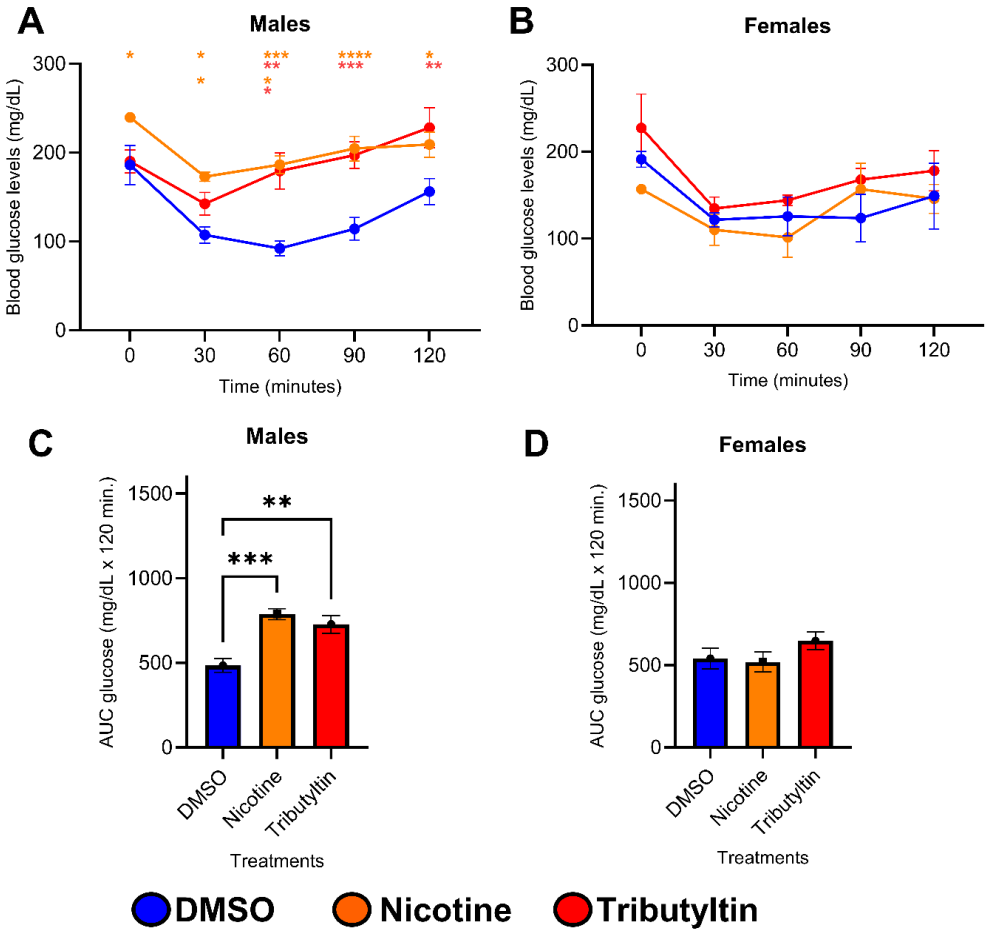

Figure 3. Chronic nicotine or tributyltin exposure led to significantly increased blood glucose levels during insulin tolerance test. (A) Insulin tolerance test curve for male mice. (B) Insulin tolerance test curve for female mice. (C) Area under the curve for insulin tolerance test for male mice (*** = nicotine compared to DMSO, ** = TBT compared to DMSO). (D) Area under the curve for insulin tolerance test for female mice. (Two-way ANOVA, DF Column factor = 2, Row factor = 4) Dunnett's multiple comparisons for ITT, and Kruskal-Wallis test for AUC; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001,n=5)

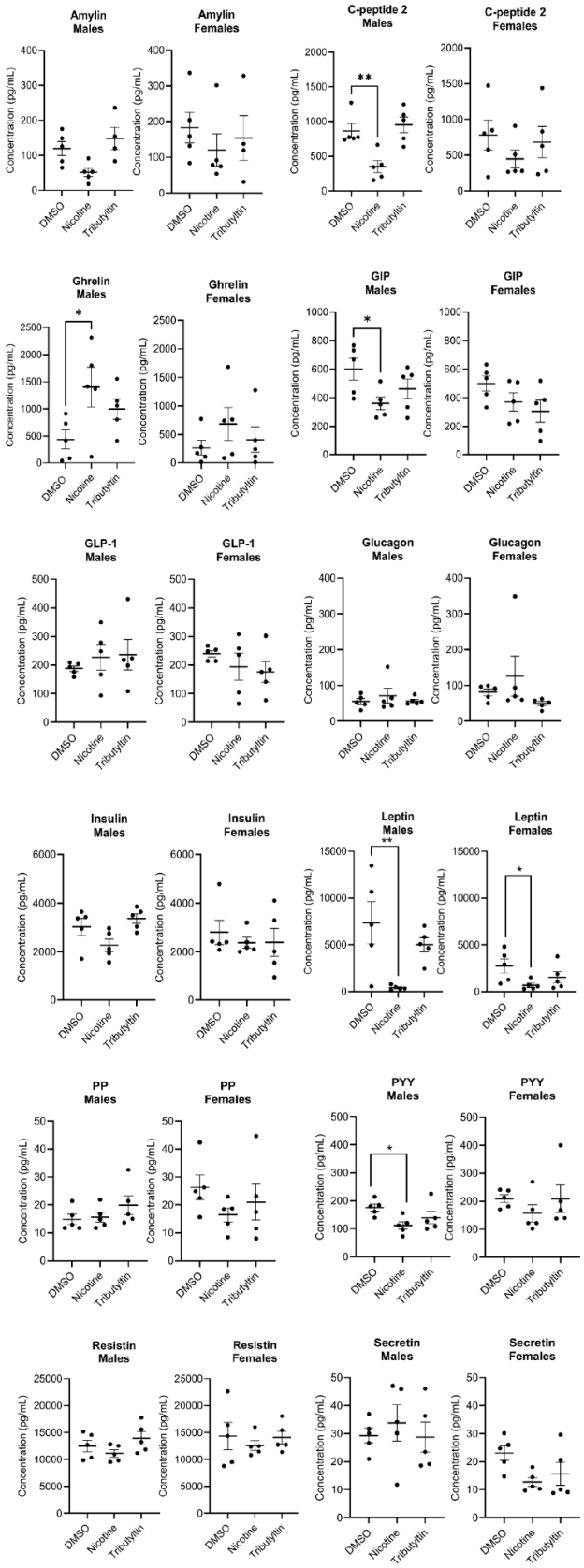
Amylin Males
Amylin Females
C-peptide 2 Males
C-peptide 2 Females
Ghrelin Males
Ghrelin Females
GIP Males
GIP Females
GLP-1 Males
GLP-1 Females
Glucagon Males
Glucagon Females
Insulin Males
Insulin Females
Leptin Males
Leptin Females
PP Males
PP Females
PYY Males
PYY Females
Resistin Males
Resistin Females
Secretin Males
Secretin Females
Concentration (pg/mL)
DMSO
Nicotine
Tributyltin

Figure 4. Chronic nicotine exposure led to significant alterations in plasma metabolite levels in male mice. Plasma metabolite concentrations in twelve metabolites. (One-WAY ANOVA, DF treatment between columns = 2 Dunnett's multiple comparisons for; *P<0.05, **P<0.01,n=5)

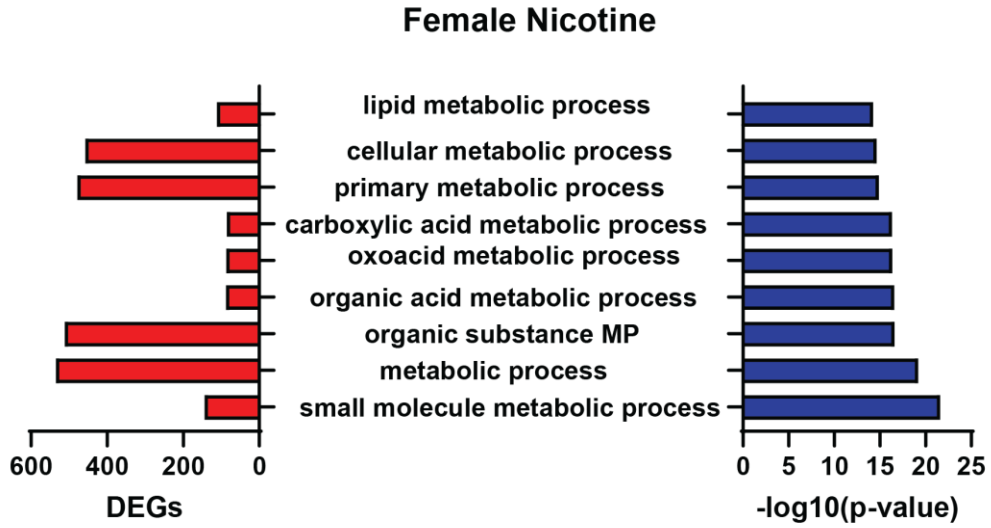

Figure 5. Chronic nicotine exposure led to alterations of differentially expressed genes that were enriched for these biological processes in hepatic tissue of female mice. Differentially expressed genes (DEGs) in nicotine-treated females on the total western diet (TWD) compared to control females on TWD. DEGs were enriched for gene ontology terms associated with biological processes like small molecule metabolic process, organic substance metabolic process, and primary metabolic process. Gene ontology (GO) terms were generated using Galaxy and the top ten GO terms were selected based upon significance of a p-value < 0.05. (MP = metabolic process)

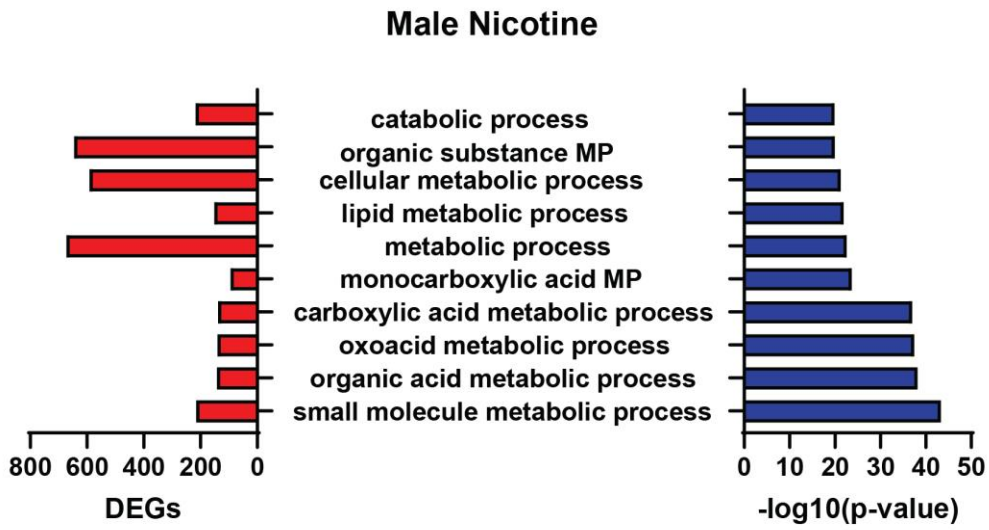

Figure 6. Chronic nicotine exposure led to alterations of differentially expressed genes that were enriched for these biological processes in hepatic tissue of male mice. Differentially expressed genes (DEGs) in nicotine-treated males on the total western diet (TWD) compared to control males on TWD. Differentially expressed genes were enriched for gene ontology (GO) terms associated with biological processes like small molecule metabolic process, lipid metabolic process, and catabolic process. . GO terms were generated using Galaxy and the top ten GO terms were selected based upon significance of a p-value < 0.05. (MP = metabolic process)

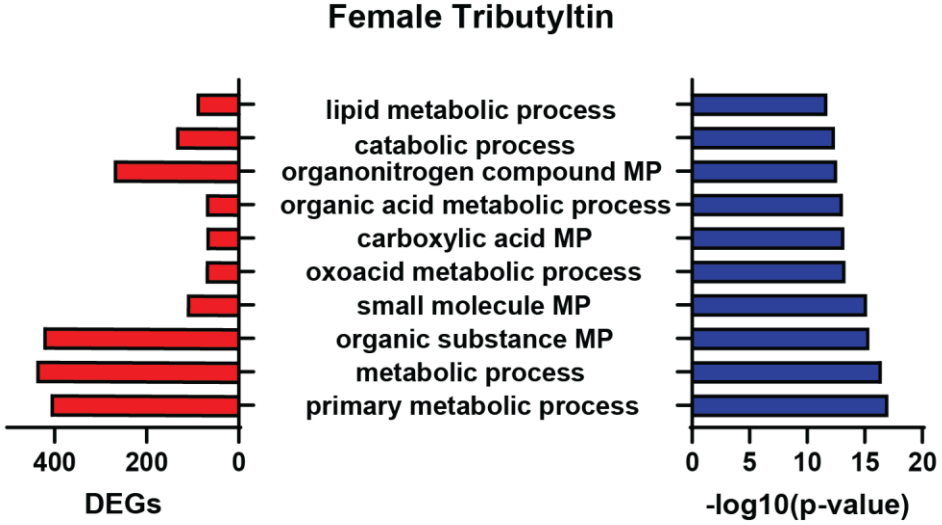

Figure 7. Chronic tributyltin exposure led to alterations of differentially expressed genes that were enriched for these biological processes in hepatic tissue of female mice. Differentially expressed genes (DEGs) in TBT-treated females on the total western diet (TWD) compared to control females on TWD. DEGs were enriched for gene ontology (GO) terms associated with biological processes like primary metabolic process, organic substance metabolic process, and lipid metabolic process. GO terms were generated using Galaxy and the top ten GO terms were selected based upon significance of a p-value < 0.05. (MP = metabolic process)

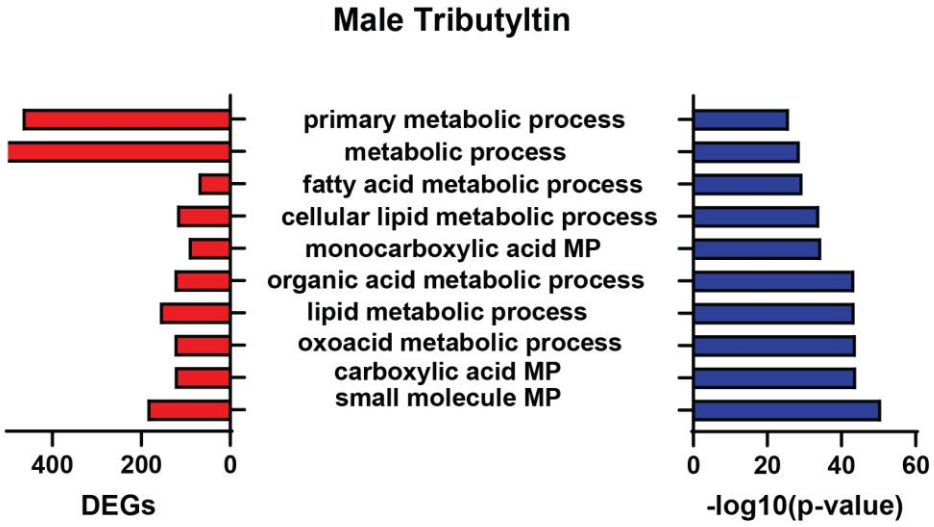

Figure 8. Chronic tributyltin exposure led to alterations of differentially expressed genes that were enriched for these biological processes in hepatic tissue of male mice. Differentially expressed genes (DEGs) in TBT-treated males on the total western diet (TWD) compared to control males on TWD. DEGs were enriched for gene ontology terms associated with biological processes like lipid metabolic process, small molecule metabolic process, and primary metabolic process. GO terms were generated using Galaxy and the top ten GO terms were selected based upon significance of a p-value < 0.05. (MP = metabolic process)

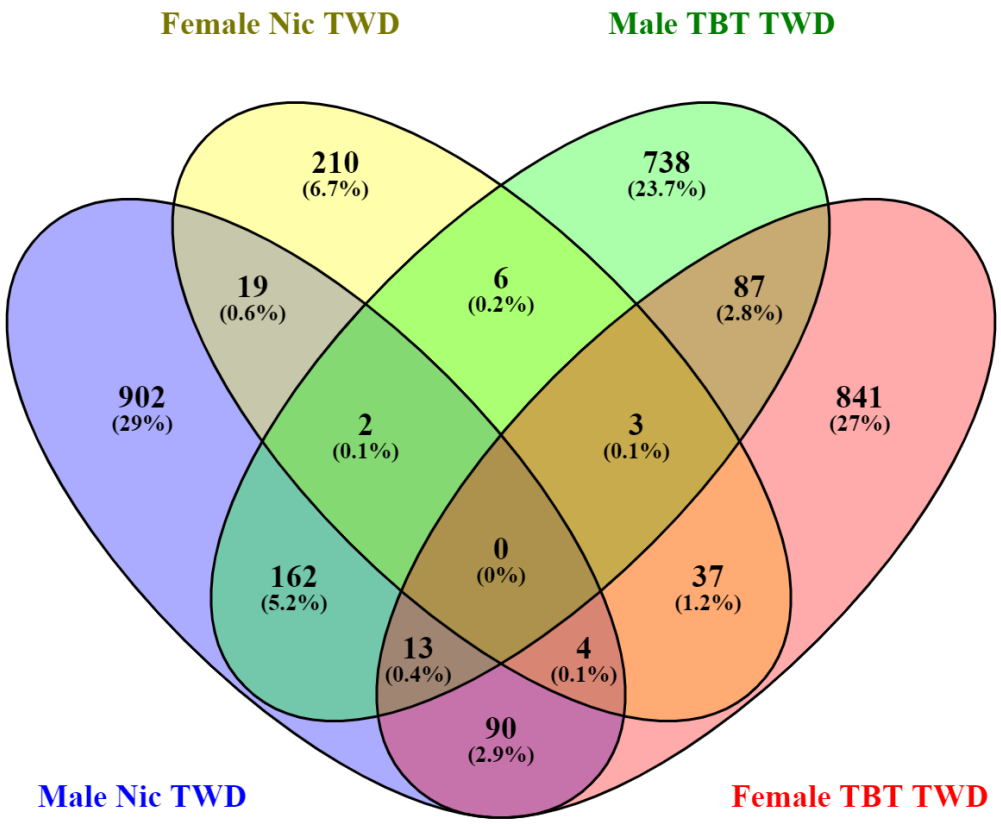

Figure 9. Venn diagram of differentially expressed genes of either nicotine or tributyltin versus control (DMSO). Differentially expressed genes in treated versus control animals on TWD, separated by sex and treatment. Differentially expressed genes that were considered statistically significant (p-value < 0.05) are listed under each category.

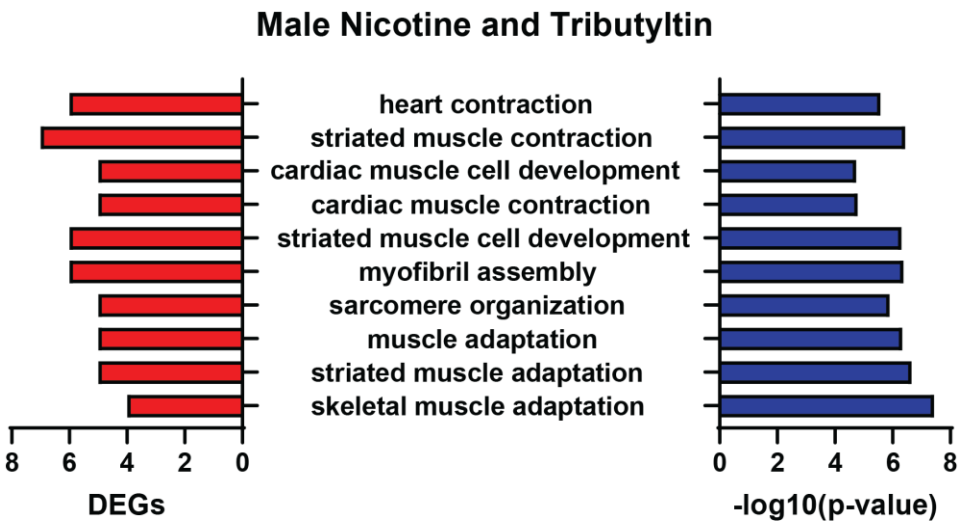

Figure 10. Similar differentially expressed genes among TBT- and nicotine-treated males enriched for these biological processes in hepatic tissue of male mice on the total western diet. Differentially expressed genes (DEGs) that were similarly shared among TBT- and nicotine-treated males when compared to control males. These shared DEGs were enriched for gene ontology (GO) terms to biological processes associated with striated muscle cell development, cardiac muscle development, and heart contraction. GO terms were generated using Galaxy and the top ten GO terms were selected based upon significance of a p-value < 0.05.)

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CHAPTER 3

PATERNAL EXPOSURE TO NICOTINE LEADS TO LIVER TRANSCRIPTOMIC ALTERATIONS ASSOCIATED WITH METABOLIC FUNCTION IN OFFSPRING THAT ARE EXACERBATED BY A HYPERCALORIC DIET

ABSTRACT

Metabolic syndrome prevalence in the United States is on the incline and projected to affect half of the population by 2050. Factors often attributed with incidence of metabolic syndrome and its associated disorders include genetics, caloric surplus and an inactive lifestyle. Smoking tobacco, one of the leading preventable causes of adverse health issues, can elicit metabolic disruption and incidence of disease. Nicotine, the main psychoactive ingredient in tobacco, is metabolized by the liver and exposure to it has been associated with precursor factors that lead to metabolic disruption. In rodents, paternal nicotine exposure elicits metabolic disruption effects at least in male offspring. We previously demonstrated that ancestral exposure to the endocrine-disrupting chemical tributyltin leads to metabolic disruption in the next generation, which was further exacerbated by diet intervention. Here we build upon previous findings by investigating the offspring effect of paternal preconception exposure to nicotine. We exposed the offspring to a hypercaloric diet to determine the interaction between ancestral exposure to nicotine and F1 diet to mimic human habits that might explain the current metabolic disease trends. Physiological outcomes such as body weight, glucose and insulin tolerance alterations were mild among nicotine-sired offspring. However, hepatic transcriptomic findings reveal significant alterations to the transcriptome of animals whose fathers were exposed to nicotine and regardless of diet in a sexually dimorphic manner that was consistent with changes to plasma metabolites. These findings highlight that paternal chemical exposures can predispose the next generation to metabolic disruption that can further be exacerbated by dietary challenge.

INTRODUCTION

Metabolic syndrome is a cluster of physiological abnormalities that are associated with the development of both cardiovascular and metabolic diseases and include insulin resistance, hypertension, abdominal obesity, and hyperlipidemia (Saklayen 2018). In the United States, the prevalence of metabolic syndrome was around 34% of the adult population in 2016 (Hirode and Wong 2020). One disease associated with metabolic syndrome, type 2 diabetes, has a prevalence in the US of 11.3% in the adult population and those numbers are projected to increase to 12.2% by 2045 (Fang et al. 2022). Metabolic diseases account for increased risk of life-threatening conditions like cardiovascular issues, high blood sugar, and abnormal cholesterol levels. Health care expenses are costly for individuals that have metabolic diseases, like type 2 diabetes, obesity, and/or metabolic syndrome (Bolnick et al. 2020). Metabolic alterations affect sexes differently. Specifically, there are factors like body composition, fat storage, and hormone signaling that dictate physiological differences between males and females, which explain, at least in part, the increased prevalence of cardiometabolic diseases in females than in males (Blaak 2001).

Factors that have been attributed with metabolic disease include poor diet, low energy expenditure and, more recently, exposure to environmental toxicants (Heindel et al. 2017). In the United States, 50% of the population follows a Western diet, which includes processed and refined hypercaloric foods that are high in fat, sugar and sodium (Clemente-Suárez et al. 2023). However, the increasing incidence of metabolic syndrome in infants and children cannot be explained solely by caloric surplus and a sedentary lifestyle (Fock and Khoo 2013). In the last 20 years, a growing body of evidence using rodents showed that exposure to environmental toxicants can lead to metabolic disruption, not only in the individuals directly exposed to them, but also in future unexposed generations (Chamorro-García et al. 2013; Chamorro-Garcia et al. 2017; Chamorro-García et al. 2021; King et al. 2019b, 2019a; Nilsson et al. 2023). Among those environmental exposures, smoking has

been shown to increase susceptibility to metabolic alterations such as obesity, type 2 diabetes and cardiovascular disease (Akbartabartoori et al. 2006) but little is known about the effect chemicals found in tobacco have on these processes.

Smoking tobacco products is one of the leading preventable causes for adverse health effects, like respiratory diseases, increased risk of developing type 2 diabetes, and high blood pressure in adults in the United States (Cornelius et al. 2023). Although cigarette smoking has decreased in recent decades (The Health Consequences of Smoking—50 Years of Progress), the advent of electronic cigarettes (e-cigarettes) has led a new generation of young adults to use nicotine products (Erhabor et al. 2023), with a tendency of higher rates of male-smokers than female-smokers. In 2020, the global prevalence of tobacco use among men was 36.7% and among women was 7.8% (Siddiqi et al. 2020). Human studies showed that there is a positive dose-response relationship between the number of cigarettes smoked daily and increased risk of metabolic syndrome (Park et al. 2003; Balhara 2012). Through smoking tobacco, individuals can be directly exposed to a myriad of chemicals, such as nicotine, arsenic, benzene or cadmium, and other factors, such as products derived from combustion of organic materials in tobacco (CDC 2010). Nicotine, the main psychoactive ingredient in tobacco and a known endocrine disrupting chemical, is metabolized by the liver and has been associated with various adverse effects including increased blood pressure and free fatty acids in plasma, and decreased mobilization of blood glucose, which are risk factors for metabolic disruption (Kassotis and Stapleton 2019; Waldum et al. 1996).

Investigation into paternal contributions to next generation's health reveal that diet modifications can lead to metabolic alterations in progeny in rodents (Carone et al. 2010; Mima et al. 2018; Wu et al. 2015; Chen et al., 2016). It has been previously shown that paternal environmental exposures to an unhealthy diet, alcohol, and cocaine leads to neurological, behavioral, and physiological alterations in the next generation (Goldberg and Gould 2019; Holloway et al. 2007; McCarthy et al. 2018; Vassoler et al. 2014). In rodents,

paternal nicotine exposure is known to elicit heritable phenotypes in unexposed generations, including alterations to hepatic lipid, fatty acid, and xenobiotic metabolism expression in the liver transcriptome in a mouse model (Vallaster et al. 2017). In humans, paternal nicotine exposure can lead to cognitive deficits and behavioral alterations in the next generation (Maurer et al. 2022). The mechanisms underlying these heritable alterations upon paternal nicotine exposure are still being elucidated, but a well-supported hypothesis is that paternal nicotine exposure alters sperm small ncRNAs leading to alterations in the next generation (Zeid and Gould, 2023).

We showed that ancestral exposure to the endocrine-disrupting chemical tributyltin (TBT) leads to multigenerational metabolic disruption, which is exacerbated in unexposed descendants when they feed on a diet with a slightly higher fat content than the previous generations (Chamorro-Garcia et al. 2017; Chamorro-García et al. 2021; Diaz-Castillo et al. 2019). Building upon those findings, here, we investigate the offspring metabolic effect of the interaction between paternal exposure to nicotine and diet. We exposed male mice to nicotine, via drinking water, and mated them to untreated females. The resulting offspring were placed on either a control diet (CD) or total western diet (TWD), a hypercaloric rodent diet equivalent to the Western diet with a higher sugar and carbohydrate content. We analyzed metabolic endpoints including body weight, plasma metabolites, and the transcriptome data from liver. We found that effects on body weight changes and glucose and insulin tolerance were mild. However, transcriptomic analyses of the liver showed significant alterations of the transcriptome in animals whose fathers were exposed to nicotine in a sexually dimorphic manner that were consistent with changes in plasma metabolites. This research gives insight into how predisposition to metabolic disease due to paternal exposures can be further exacerbated by other factors such as diet. Since Western diet represents a widespread diet pattern in the U.S. and there is a prevalence of 17.9% of paternal smoking

(King et al. 2009), our approach models habits of a significant percentage of the US population.

METHODS

Chemicals and Reagents

(-)-Nicotine (#N3876), D-(+)-glucose (#G8270), and human recombinant insulin (dry powder, #91077C) were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific, LLC. Nicotine was stored out of light and in a desiccator. Glucose and insulin stocks for glucose and insulin tolerance tests were prepared fresh the day of metabolic testing.

Animal Maintenance and Exposure

Mice were purchased at Jackson Laboratory (Sacramento, CA). Animals were housed in micro-isolator cages in a temperature-controlled room (21-22°C) with a 12 h light/dark cycle and provided food (Envigo; Teklad Global Soy Protein-Free Extruded Rodent Diet, irradiated #2920X) and water *ad libitum* unless otherwise indicated. Animals were treated humanely and with regard for alleviation of suffering. All procedures conducted in this study were approved by the Institutional Animal Care and Use Committee of the University of California, Santa Cruz.

Three-week-old C57BL/6J male mice (n=30) were purchased from Jackson Laboratory (Sacramento, CA), and randomly assigned to two treatment groups (15 animals per treatment) receiving 200 µg/mL nicotine or 0.1% dimethyl sulfoxide (DMSO; vehicle control) in drinking water. Every 3-4 days, we measured and discarded the remaining water in the bottles, and water bottles were refilled with fresh water and the corresponding treatment. The treatment continued for six weeks, to encompass the entirety of spermatogenesis process to ensure that sperm at all stages were being exposed to oral consumption of nicotine (Oakberg 1956). This concentration of nicotine achieves the average levels of cotinine an average

smoker has in blood (Klein et al., 2004; Collins et al., 2012; Supplemental Figure S1). After the sixth week of water treatments, male mice were mated with age-matched (1 male:1 female) unexposed female C57BL/6J mice purchased from Jackson Laboratory.

Once gestational plugs were confirmed in the F0 females, the F0 males were sacrificed *via* isoflurane overdose and cervical dislocation. F0 females were weighed to ensure pregnancy 10 days after plug detection. No statistically significant differences were observed in terms of number of pups and sex bias among treatments (Supplemental Table S1). Since litter size can affect growth trajectories of the pups, we only considered litters that had between 6-8 pups (average litter size in our cohort = 7), and litters with less than 2 members of each sex were excluded. We considered both male and female offspring separately in our analysis.

Fifteen F1 animals per paternal treatment and per sex were weaned from dams at 3 weeks old and placed on either a Control Diet (CD, 93G, TD.140148) or Total Western Diet (TWD, New Total Western Diet VI, TD.110919) for five weeks. Diets were supplemented with fresh pellets every week (120 g of food per cage). Weekly body weight measurements were recorded between weeks 2-8. At 8 weeks old, animals were euthanized via isoflurane overdose. Blood was drawn from direct heart puncture into an EDTA-treated syringe and placed in a clean tube containing protease inhibitors (Protease Inhibitor Cocktail, EDTA-free, Sigma-Aldrich #S8830). Blood was centrifuged for 10 min at 5,000 rpm at 4ºC. Plasma was transferred to a clean tube, snap-frozen in liquid nitrogen and preserved at -80ºC. Samples were shipped to Eve Technologies Corporation (Calgary, AB) for analysis of a panel of plasma metabolites (Mouse/Rat Metabolic Hormone Discovery Assay® 11-Plex, MRDMET). Liver samples were collected and weighed from animals. All tissue harvesting was performed with the dissector blinded to which groups the animals belonged. At the moment of euthanasia, each mouse was assigned a code, known only to the lab member not involved in dissections. Both tissues were snap frozen and stored at -80ºC for RNA sequencing analyses.

Glucose and insulin tolerance tests

On weeks six and seven, the same five animals per group were subjected to glucose and insulin tolerance tests (GTT and ITT), respectively. Glucose and insulin stocks were prepared fresh the day of the assay in 0.9% saline. Animals were given 2 g of glucose/kg body weight (b.w.) or 0.75 IU of insulin/kg b.w. via intraperitoneal injection after 4H of fasting. Blood glucose levels were measured with Contour® blood glucose meter (BAYER) and Contour® blood glucose strips (BAYER) every 30 minutes for 120 minutes after injection of glucose or insulin. After tests were completed, animals were given their respective diets.

Plasma triglyceride levels

Triglyceride levels were measured in plasma samples collected from sacrificed F1 animals with the Promega Triglyceride-Glo Assay® which measures luminescence of glycerol in each sample. Glycerol is a byproduct of triglycerides that enzymatically interacts with added lipases, and the presence of glycerol is then measured. Glycerol is measured in a coupled reaction scheme that links production of NADH to the activation of pro-luciferin that produces light with luciferase. Triglyceride levels are determined from the difference of glycerol measured in the absence (free glycerol) and presence (total glycerol) of lipase. Samples were aliquoted into a 96-well plate and read by a spectrophotometer. Glycerol concentrations were measured in samples based off the slope of the standards curve.

RNA Isolation and sequencing

RNA from F1 liver was isolated using Direct-zol RNA MiniPrep (Zymo Research #R2053). Tissues were homogenized with VWR Premium Micro-Homogenizer (#10032-328). RNA from five randomly selected non-sibling mice from each group were submitted to the University of California Davis DNA Technologies & Expression Analysis Core Laboratory for 3’ Tag-RNA-sequencing using an Illumina HiSeq 4000 instrument. We obtained single-end reads (length=85 nt) for each sample. Statistical evaluation of transcriptome variation was

performed using Galaxy Project platform (Galaxy version 23.0) (Afgan et al. 2022). FastQ files were processed using FastQC (Galaxy version 0.73). Indexing and alignment to the mouse genome (mm39) was done using STAR (Galaxy version 2.7.10b+galaxy3). FeatureCounts (Galaxy version 2.03+galaxy2) function was used to assign uniquely mapped RNA-seq reads to GRCm39 mouse reference genome count reads. DESeq2 (Galaxy version 2.11.40.8+galaxy0) function was used to determine differentially expressed genes between the nicotine group and control.

Gene Ontology (GO) term analyses

We carried out functional enrichment analyses of differentially expressed genes using Galaxy sequencing pipeline (Goseq function version 1.5.0+galaxy0). Supplementary files were generated for paternal nicotine animals versus paternal DMSO animals and separated by sex, diet, and tissue. Venn diagrams were generated using Venny 2.1.0 (Oliveros 2007) to determine shared elements among differentially expressed genes from various treatment groups.

Statistical analyses

Statistical analyses for metabolic endpoints (body weight, plasma triglycerides, plasma metabolites, and glucose and insulin tolerance tests were performed using GraphPad Prism 10.0 (GraphPad Software, Inc.). Statistical tests and specific comparisons are indicated in each figure and their respective figure legend.

RESULTS

F0 Males exposed to nicotine had significantly decreased body weights

Male mice were exposed to 200 µg/mL nicotine in their drinking water for six weeks to ensure exposure was present throughout most of the cycle of spermatogenesis (Oakberg, 1957). The control group was exposed to 0.1% DMSO as DMSO is the solvent used for nicotine in

this study. The amount of nicotine used rendered between 200-500 µg of cotinine in blood, which is equivalent to cotinine levels found in an average smoker (Sharma et al. 2019) (Supplemental Figure S1A). Male mice that were exposed to nicotine had significantly decreased weekly body weights when compared to vehicle control animals during the last two weeks of treatment (Supplemental Figure S1B), which is consistent with previously published data (Mangubat et al. 2012). We observed that nicotine-treated males tended to drink less water than DMSO-treated males. Males were mated to age-matched unexposed female mice. We did not encounter any biases in animal numbers of the F1 generation (Supplemental Table S1).

Paternal exposure to nicotine leads mild metabolic alterations in the offspring

At three weeks of age, F1 male and female offspring were separated in two groups that were fed either a Total Western Diet (TWD) or the corresponding Control Diet (CD). To assess whether male preconception exposure to nicotine lead to metabolic disruption in their offspring, we performed a longitudinal study of body weight, dynamic analyses of glucose metabolism, determine plasma levels of relevant metabolism regulators, and transcriptomic analyses of the liver. Male mice from the nicotine group on the TWD (Nic/TWD) showed a significant increase in body weight in weeks 4 and 5 when compared to animals from the DMOS group on the CD (DMSO/CD), while no differences were observed in any other treatment group comparisons (Figure 1A), suggesting that paternal exposure to nicotine increased predisposition to body weight gain when animals are exposed to a secondary metabolic challenge such as a hypercaloric diet. In females, we did not observe any differences in body weight (Figure 1B).

To test whether paternal exposure to nicotine affects glucose metabolism in their offspring, we performed a glucose tolerance test (GTT) in F1 females and males at six weeks of age and insulin tolerance test (ITT) at seven weeks of age (Figure 1). We did not observe significant changes in glucose or insulin sensitivity in males. In females, we found that fasting

glucose in the Nic/CD group was significantly decreased compared to the DMSO/CD group in the GTT although no differences were observed in fasting glucose the day of the ITT, suggesting that glucose levels at fasting are variable.

Upon euthanasia, we further analyzed plasma levels of 12 metabolites involved in metabolic processes. In males, we found that plasma levels of glucagon and insulin in the Nic/TWD group were significantly lower compared to the DMSO/CD group (Figure 2). Insulin levels were also reduced in males from the DMSO/TWD group compared to DMSO/CD, with no statistically different changes between DMSO/TWD and Nic/TWD, suggesting that the effect is driven by the diet and not the ancestral exposure to nicotine. In contrast, the reduction of glucagon levels seems to be driven by paternal exposure to nicotine, since its levels in the nicotine group are significantly reduced compared to the control group on their corresponding diet. Also in males, resistin levels were increased in the Nic/TWD group compared to the DMSO/CD group. We found a significant decrease in amylin and pancreatic polypeptide (PP) levels in Nic/TWD group compared to animals from the DMSO/TWD group, but no significant differences were found with all other comparisons among groups.

In F1 females, we found that animals from the Nic/TWD group had increased ghrelin levels in plasma compared to animals from DMSO/CD. Females from DMSO/TWD group also showed increased plasma levels of ghrelin compared to their counterparts fed CD (Figure 2). Taken together, these data suggest that the increase in ghrelin levels is led by the TWD but not to paternal exposure to nicotine. Principle component analyses were performed to assess separation of each sample regarding paternal treatment and F1 diet as well as measurements of the twelve plasma metabolites (Figures 3). PCA helps uncover differences in metabolite profiles between treated and untreated samples by identifying directions, or principle components, where variance is greatest. If treated and untreated samples cluster separately in the PCA plot this would indicate a strong effect of treatment on metabolic profile.

Paternal exposure to nicotine leads to transcriptomic alterations in the liver associated to metabolic processes

We analyzed changes in transcript abundance in the offspring of males exposed to nicotine or DMSO fed with either diet to further characterize alterations of paternal nicotine exposure at the expression level in the liver. Livers were collected from F1 animals at the time of euthanasia at 8-weeks of age. RNA was isolated and prepared for 3'Tag Sequencing to assess differential gene expression between groups.

In males, we found 1,357 differentially expressed genes (DEGs) in Nic/CD animals compared to the DMSO/CD animals based on p-adjusted value < 0.05. Since TWD can lead to further changes in the expression, we looked for overlapping genes that were differentially expressed in the same direction in both comparisons, which would be indicative of changes due to ancestral exposure to nicotine independent of the diet. We found 1,033 DEGs comparing animals from DMSO/CD group with the Nic/TWD (Supplemental File 9). Of those, 336 DEGs were shared between the two groups, but only 5 and 2 were underexpressed and overexpressed, respectively, in both datasets following a cut-off of p-adjusted value < 0.05 and log 2 fold change of 2 (Figure 4A). The 5 underexpressed genes are Bhlhe40, Kank1, Rnf125, Acaa1b, Dlc1 and the overexpressed genes were Klf6, Gstm3. Of note, underexpression of Rnf125 has been associated with type 2 diabetes and Acaa1b is an enzyme involved in cholesterol biosynthesis in the liver (Mao et al. 2011). Rnf125 gene encodes for the protein E3 ubiquitin-protein ligase and this protein is involved in various processes, and may influence insulin signaling by indirectly modulating pathways related to inflammation and/or stress (Mao et al., 2011; Hu et al., 2023). Gene ontology (GO) enrichment of DEGs comparing DMSO/CD with Nic/CD showed 83 significant gene ontology terms (p-adjusted<0.05) including "lipid metabolism' and several cholesterol synthesis related categories in the top 10 with lowest p value (Figure 4B and Supplemental Files 3 and 4). GO enrichment of DEGs comparing DMSO/CD with Nic/TWD showed 602 significant categories

(p-adjusted<0.05) that included “metabolic process” and “lipid metabolic process” in the top ten with lowest p value (Figure 4C and Supplemental File 10). When comparing DMSO/CD and Nic/TWD, we found enrichment of the GO terms “metabolic process” and “lipid metabolic process” significantly enriched in the top 10 GO terms with lower p values. Additionally, we found GO terms associated with glucagon metabolism, glycogenolysis and insulin signaling that were significantly enriched (Supplemental File 10). We found that the p-values of the GO term analyses are significantly lower in the DMSO/CD-Nic/TWD comparison in male and female datasets (Supplemental Files 10 and 12), suggesting that TWD further exacerbates the metabolic response to paternal exposure to nicotine observed in the Nic/CD group compared to the DMSO/CD. Taken together, these data suggest that ancestral paternal exposure to nicotine leads to alterations of the transcriptome in the liver that are consistent with alterations in the metabolic function of the organisms and that those alterations are exacerbated by the consumption of a hypercaloric diet.

In females, we identified 1,046 DEGs in the Nic/CD group compared to the DMSO/CD group. Comparisons of Nic/TWD and DMSO/CD showed 915 DEGs (Supplemental File 11). We found 261 overlapping DEGs, of which 29 and 15 were under expressed and overexpressed, respectively, in both data sets, suggesting that the changes occurred because of the paternal exposure to nicotine (Figure 5A and Supplemental File 11). Of note, shared under expressed genes included Fbp1 whose deficiency has been associated with hypoglycemia and acute liver failure, and Dbi, which has been associated with anorexa nerviosa (Joseph et al. 2020). Fasting glucose levels of females were significantly lower in the Nic/CD compared to the DMSO/CD which is consistent with the decreased levels of Fbp1. Shared overexpressed genes include Nr1i3, Lpin2 and Lipg, which are involved in cholesterol metabolism, and PGC-1a and AldoC that are both involved in liver gluconeogenesis at different stages of the pathways. GO enrichment analyses of DEGs (p<0.05) comparing DMSO/CD with Nic/CD showed 391 significant gene ontology terms (p-adjusted<0.05) including “metabolic

processes” and “lipid metabolic processes” in the top ten categories with lowest p-adjusted values, with 233 and 59 DEGs, respectively (Figure 5B and Supplemental File 2). Gene ontology enrichment of DEGs comparing DMSO/CD with Nic/TWD showed 808 significant terms (p-adjusted<0.05) that also included “metabolic processes” and “lipid metabolic processes” in the top ten with lowest p-adjusted value, with 733 and 210 DEGs, respectively (Figure 5C and Supplemental File 12).

Sexually dimorphic response

The physiological data show that paternal exposure to nicotine leads to metabolic alterations in both male and female offspring. Although the differences are mild, the response to paternal exposure to nicotine is different in each offspring sex. We first analyzed the basal transcriptomic differences in the offspring by comparing liver DMSO/CD data sets of males and females. We identified 2,178 DEGs (p<0.05) (Supplemental Files 1 and 3). Gene ontology analyses rendered 1,056 significant categories (p adjusted<0.05), with “lipid metabolism” and “metabolic processes” being in the top 10 with lower p value, suggesting that processes involved in overall metabolic function and lipid function is significantly different between males and females (Supplemental Files 2 and 4). To identify the genes that were differentially expressed due to paternal exposure to nicotine, we compared liver Nic/CD datasets between males and females. We found 2,214 DEGs (p<0.05) with a GO enrichment of 778 categories (Supplemental Files 2 and 4). The category with lowest p adjusted is “metabolic processes”, and “lipid metabolic process” and “fatty acid metabolic process” being in the top twenty with lowest p value.

We compared the DEGs between males and females on the DMSO/CD group with the Nic/CD group and identified 829 shared genes, with 429 under expressed and 380 overexpressed in males vs females. Gene ontology enrichment analyses rendered categories such as “lipid metabolism” and “metabolic process” in the top ten with lowest p values,

suggesting that paternal exposure to nicotine further exacerbates the metabolic differences between males and females (Supplemental Files 1 and 3).

DISCUSSION

Metabolic conditions such as obesity and type 2 diabetes are a current concern due to their increasing prevalence worldwide. These conditions can originate by multiple factors, including genetics, lifestyle choices such as diet or exercise, and exposure to environmental factors. One current limitation in our understanding of the contributing factors to metabolic conditions relates to the lack of information about how ancestral exposure effects are modulated by the current environment. Here, we use a paternal exposure paradigm to determine how hypercaloric diets exacerbate the metabolic effect in the offspring of male mice exposed to nicotine.

It is currently accepted that exposure to nicotine can increase the risk of certain diseases such as cardiovascular disease and cognitive function and if exposed during pregnancy, similar effects can be observed in the offspring. It was previously shown that paternal exposure to nicotine leads to metabolic alterations in their offspring (Vallaster et al. 2017), but little was known about how nicotine exposure could interact with other factors such as diets to further exacerbate metabolic conditions and the mechanism through which paternal exposure leads to such phenotype. Given that fathers contribute to the next generation exclusively via the germline, the only elements that might be contributing to altering the development of the offspring must be carried by the sperm, as opposed to what occurs with maternal preconception exposure in which the oocyte and the maternal milieu can be affected and contribute to disease in their offspring.

We exposed male mice to nicotine via drinking water for five weeks, which spans the window of spermatogenesis. Female and male offspring from nicotine (Nic) and control (DMSO) groups were separated in two subgroups that were fed a control diet (CD) or a

hypercaloric diet known as Total Western Diet (TWD). In the female offspring, we did not find significant changes in body weight or in glucose and insulin tolerance tests. We observed a significant increase of ghrelin levels in plasma in both TWD groups compared to DMSO/CD group, but no difference between Nic/TWD and DMSO/TWD, suggesting that the factor driving the phenotype is TWD and not nicotine. Ghrelin, also known as the “hunger hormone”, is secreted by the intestine to stimulate food intake. Ghrelin secretion from the gut can inhibit insulin secretion and regulate hepatic gluconeogenesis (Pradhan et al. 2013). Increased ghrelin levels can suppress insulin secretion from the pancreas and insulin-mediated glucose uptake (Pradhan et al., 2013). Although we did not identify significant changes in insulin levels in plasma or insulin sensitivity through ITT with any of the interventions, transcriptomic analyses in the liver revealed that two genes involved in gluconeogenesis, PGC-1a and AldoC, were overexpressed in the nicotine group regardless of the diet. In an *in vivo* mouse models knockdown of PGC-1a has been associated with decreased dopamine neurons, and increased neuropathy that is heightened in diabetic mice (Choi et al., 2014). Another mouse model with a knockout of AldoC was associated with decreased plasma total cholesterol and triglycerides but with no significant changes to liver levels of these molecules (Votava et al., 2024). Gluconeogenesis is the process of glucose production from non-carbohydrate sources, including glycerol, amino acids, lactate and pyruvate during long periods of fasting. The apparently inconsistent result of not seeing significant effects in GTT and ITT but seeing changes in gene expression levels of genes involved gluconeogenesis in the liver may be explained by the fact that GTT and ITT were performed after a short fasting period (4 hours) while the fasting before euthanasia and isolation of liver tissue occurred overnight. As such, the pathways stimulated for glucose production in both assays were different, glycogenolysis for the short fasting and gluconeogenesis for the long fasting.

Nic/TWD male offspring showed significantly increased body weights at 4 and 5 weeks of age compared to the DMSO/CD counterparts, while Nic/CD males did not show

significant changes at any timepoint analyzed, suggesting that the differences observed between the Nic/TWD group and the DMSO/CD group were due to the TWD. Although we did not observe any significant changes in GTT and ITT in males, we found significant differences in plasma levels of metabolic analytes involved in the regulation of metabolic pathways. We found that paternal exposure to nicotine leads to a reduction of glucagon levels in plasma of males on either diet compared to the DMSO groups in their same diet. Glucagon is secreted by the pancreas when levels of glucose are low to stimulate glycogenolysis in liver and promote glucose release in the bloodstream and it is inhibited by free fatty acids and keto acids during long periods of fasting (Jiang and Zhang 2003). In this study, animals on TWD from either exposure group had significantly lower levels of insulin than the DMSO/CD group. Low insulin levels are associated with increased levels of glucagon, which is opposite to what we observed. The fact that both treatment groups had lower levels of insulin on the TWD suggest that the driving contributing factor to the low levels of insulin is the diet and not the nicotine treatment which would suggest that the effects on glucagon and insulin levels are somewhat independent. When performing analyses of liver function, we found GO term enrichments of categories associated to glycogenolysis (glucose production during short periods of fasting), glucagon and insulin signaling, which would be consistent with altered plasma levels of glucagon and insulin levels in the Nic/TWD group compared to the DMSO/CD. Specifically, there was underexpression of genes like Rnf125 and Acaa1b, which are involved in metabolic processes such as glycogenolysis in the liver. Investigation into knockdowns of either gene reveal that Rnf125 knockdown activates inflammatory processes in a mouse model (Hu et al., 2023). There was little information on manipulation of the gene Acaa1b in a mouse model.

Analyses of transcript abundance in liver samples showed significant changes in genes involved in metabolic processes and lipid metabolism. Interestingly, we found that in males and females there were DEGs involved in different pathways regarding glucose

metabolism. In males, we found that genes involved in glycogenolyses were differentially expressed in the same direction in animals ancestrally exposed to nicotine independent of the diet they were exposed to. In females, genes involved in the gluconeogenic pathways were differentially expressed in the same direction regardless of the diet, suggesting that the alterations were due to paternal nicotine exposure. In humans, the prevalence of metabolic diseases such as metabolic syndrome, type 2 diabetes and hypertension has been shown to increase with aging (Ford et al. 2002; Hirode and Wong 2020). As such, although the physiological phenotypes in this study were mild and we did not observe robust alterations in glucose and insulin sensitivity, we hypothesize that larger metabolic effects would be identified if animals had been allowed to age.

Nicotine has been present in our society for multiple centuries. Early studies on the effects of exposure to nicotine were mostly focused on addiction and cognitive function. More recently, it has been demonstrated that paternal exposure to nicotine can lead to metabolic alterations in the offspring associated to metabolism of xenobiotics (Vallaster et al. 2017). However, little was known about the interaction between ancestral exposure to nicotine and other exposure elements such as hypercaloric diets, which are two prevalent elements of the exposome in Western societies. Our study demonstrates that paternal exposure to nicotine predisposes the offspring to alterations in glucose metabolism that are further exacerbated by hypercaloric diets that can potentially worsen with aging.

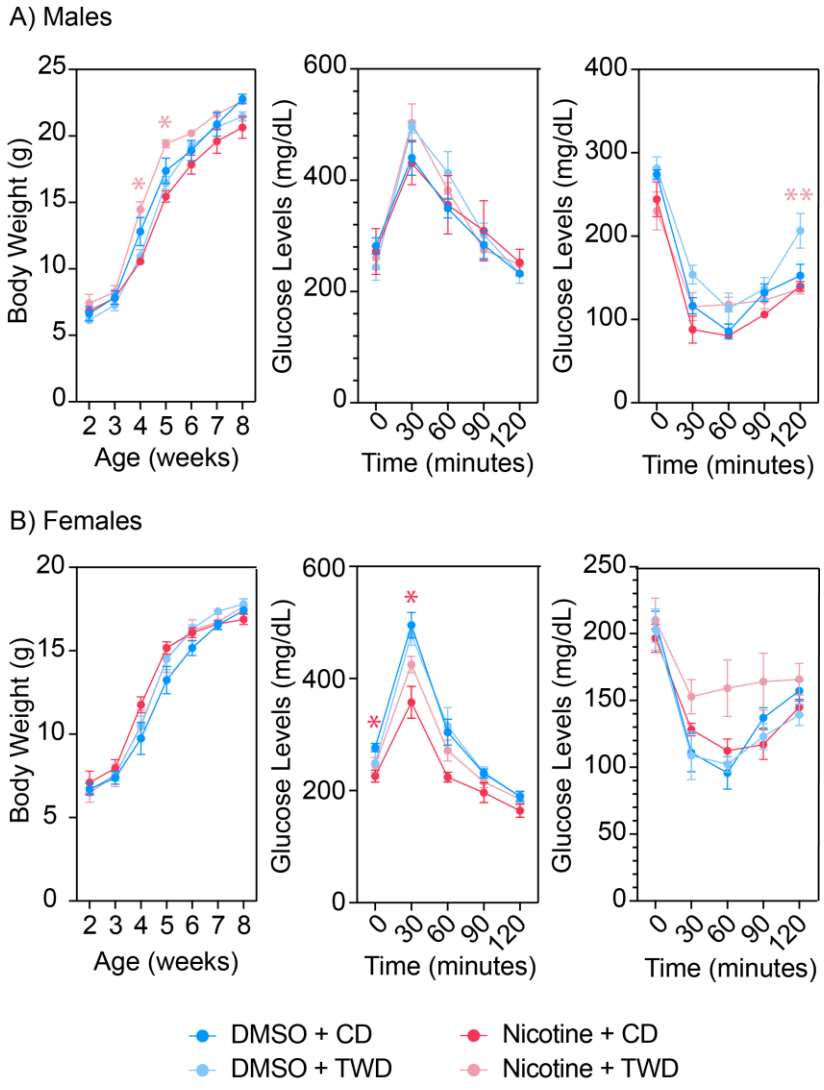

Figure 1. Paternal nicotine exposure elicits mild and sexually-dimorphic metabolic outcomes when analyzing weekly body weights, glucose and insulin tolerance tests in the F1 generation. (A) F1 males weekly body weight measurements, glucose and insulin tolerance tests. (B) F1 females weekly body weight measurements, glucose and insulin tolerance tests. F1 animals were measured for body weights weekly. Animals were fasted for four hours before either glucose or insulin tolerance test and blood glucose levels were measured every thirty minutes for two hours. (Body weight statistical tests: Two-Way ANOVA, DF Time x Column Factor = 8, Šídák's multiple comparisons test, *P<0.05, n=15; glucose/insulin tolerance test statistical tests: Two-Way ANOVA, DF Time x Column Factor = 4, Šídák's multiple comparisons test, *P<0.05, **P<0.01, n=5).

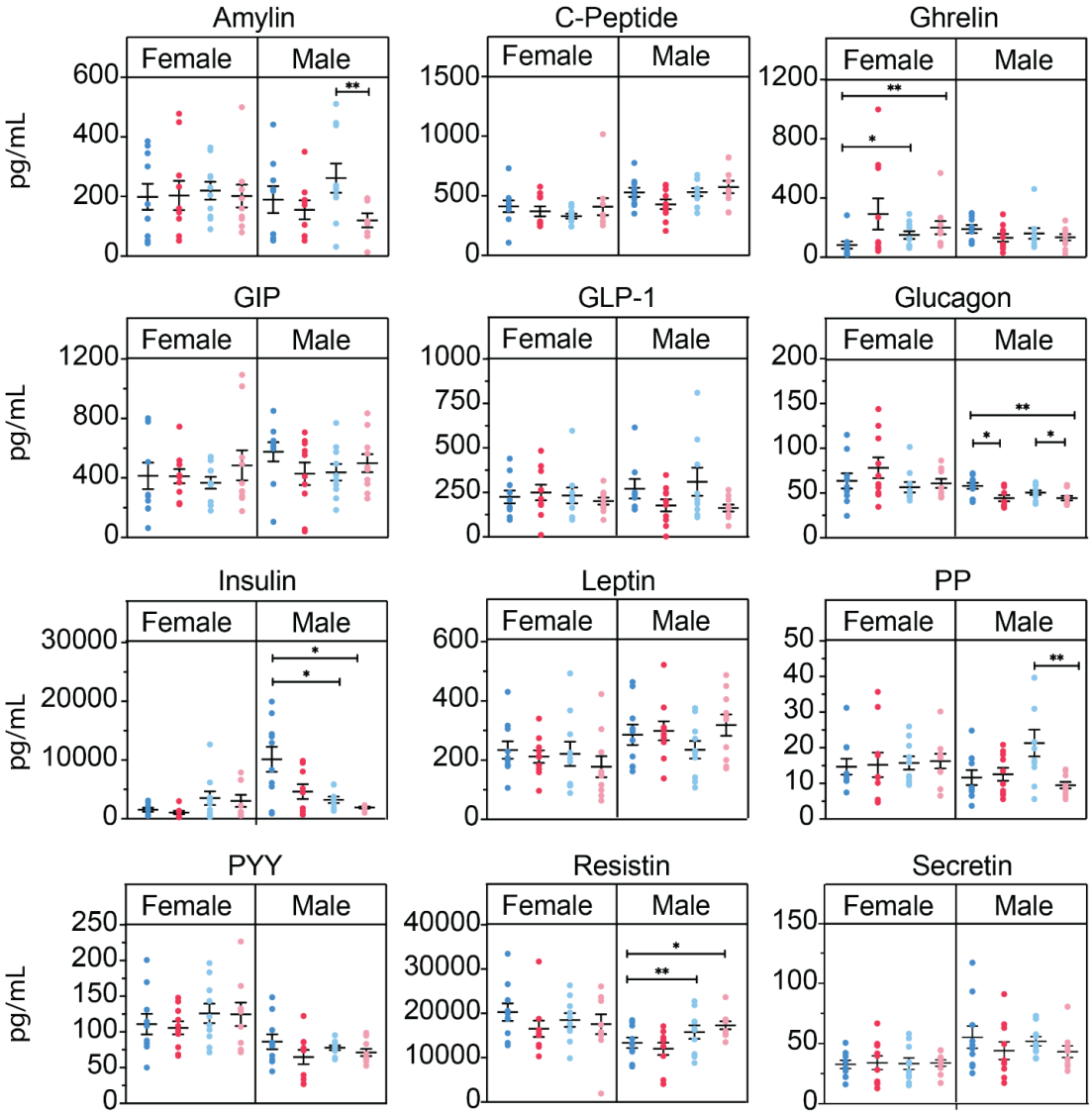

Figure 2. Several plasma metabolite levels were significantly altered in F1 animals upon paternal nicotine exposure and/or TWD; amylin, ghrelin, glucagon, insulin, PP, and resistin. KEY: Dark blue = DMSO+CD, light blue = DMSO+TWD, dark red = Nicotine+CD, light red = Nicotine+TWD. Plasma metabolite levels were measured across twelve different metabolites in pg/mL. Plasma was isolated from blood collected at the time of sacrifice (Mann-Whitney test, *P<0.05, **P<0.01, n=10).

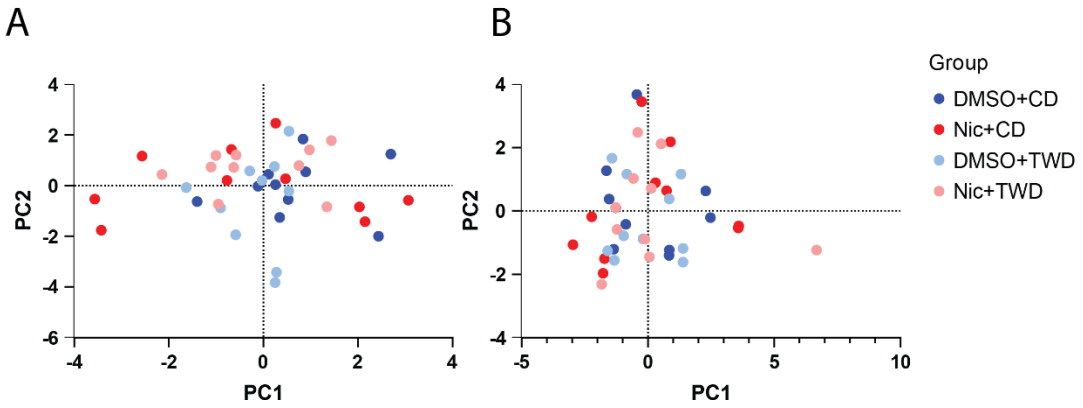

Figure 3. Principle component analysis (PCA) of plasma metabolites in F1 males (A) and females (B) reveals treatment effect on all twelve metabolites. Principle component (PC) scores of twelve plasma metabolites for each treatment group and their respective diets.

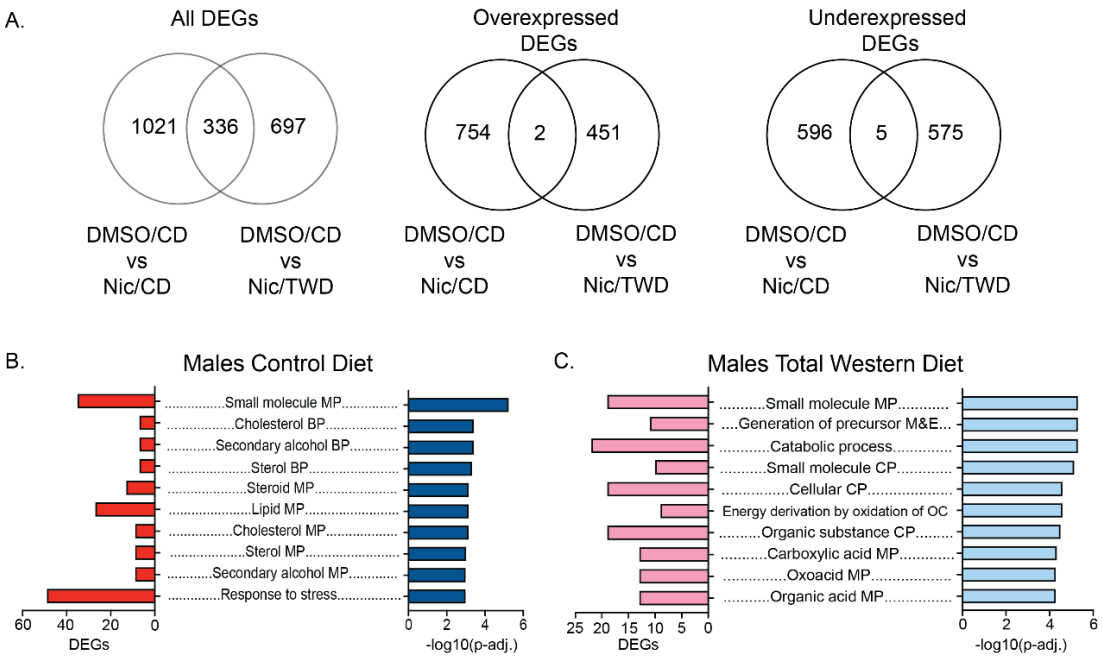

Figure 4. Differential gene expression analysis of paternal nicotine exposed compared to control males reveals overrepresented GO terms involved with small molecule metabolic process, lipid metabolic process and cholesterol metabolic process. A) Venn diagrams representing the overlapping differentially expressed genes (DEGs) in DMSO/CD vs Nic/CD and DMSO/CD vs Nic/TWD comparisons using Venny 2.1 (Oliveros 2007). Left panel: all DEGs, middle panel: overexpressed DEGs, right panel: underexpressed DEGs. **B)** Gene ontology terms associated with the differentially expressed genes are highlighted in the middle of each figure. The top ten gene ontology terms that were enriched in biological processes are provided. Differentially expressed genes were considered statistically significant if p-value < 0.05. MP: Metabolic Processes, BP: Biosynthetic Process, M&E: Metabolites and Energy, CP: Catabolic Process, OC: Organic compounds.

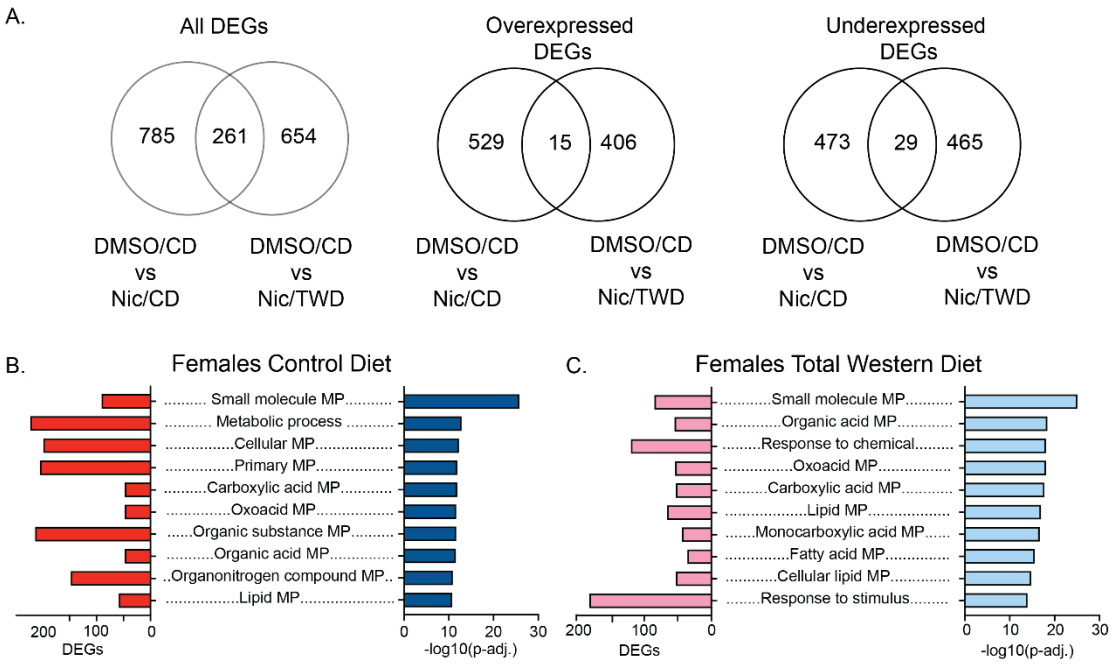

Figure 5. Differential gene expression analysis of paternal nicotine exposed compared to control females reveal overrepresented GO terms involved with biological processes such as small molecule metabolic process, lipid metabolic process, and primary metabolic process. A) Venn diagrams representing the overlapping differentially expressed genes (DEGs) in DMSO/CD vs Nic/CD and DMSO/CD vs Nic/TWD comparisons using Venny 2.1 (Oliveros 2007). Left panel: all DEGs, middle panel: overexpressed DEGs, right panel: underexpressed DEGs. **B)** Gene ontology terms associated with the differentially expressed genes are highlighted in the middle of each figure. The top ten gene ontology terms that were enriched in biological processes are provided. Differentially expressed genes were considered statistically significant if p-value < 0.05. MP: Metabolic Processes.

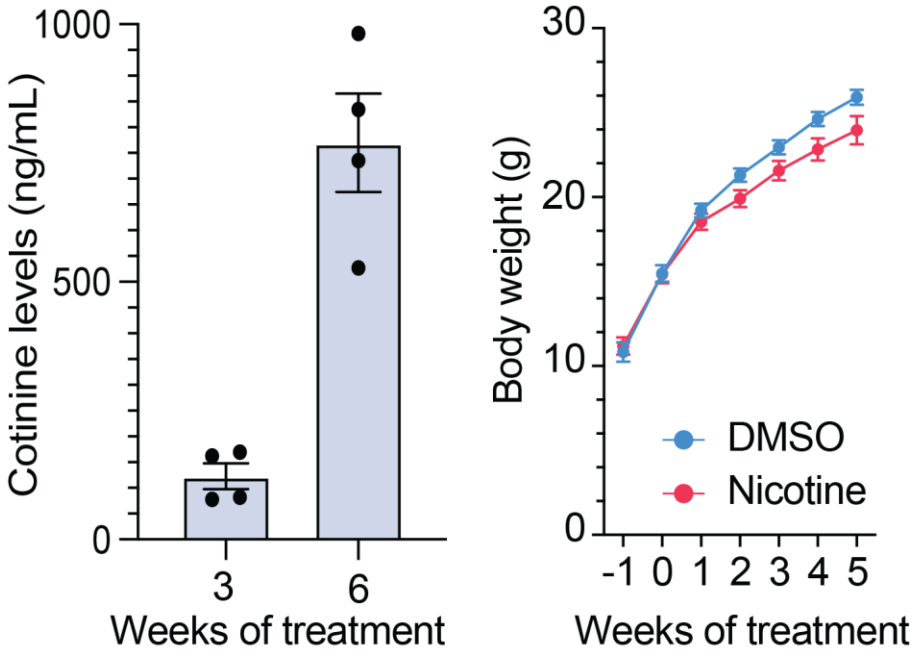

Figure S1. F0 male cotinine levels and weekly body weights after nicotine exposure. Male mice were exposed to nicotine via drinking water for six weeks. Cotinine levels were measured at the time of sacrifice after either three or six weeks of nicotine exposure (n=4). Weekly body weights were measured during the duration of the treatment (Two-Way ANOVA, Šídák's multiple comparisons test, n=10). There were no significant differences between body weights among nicotine-treated and control males.

Table S1.

	Females bred		Pregnant females		Pups born		Pups weaned		Females weaned		Males weaned	
Generation	DMSO	Nicotine	DMSO	Nicotine	DMSO	Nicotine	DMSO	Nicotine	DMSO	Nicotine	DMSO	Nicotine
F1	19	19	7	11	71	99	68	99	35	42	33	57

Table S1. F1 litter size and sex demographics. F0 Females were bred with either nicotine or DMSO treated age-matched males.

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CHAPTER 4

CONCLUSIONS

Cardiometabolic disease global prevalence is rapidly increasing with individual risk factor diseases, like cardiovascular disease, obesity, and/or type 2 diabetes projected to affect about 60% of the world's population by 2050 (Chong et al., 2024; Shi et al., 2023). Risk factors for cardiometabolic diseases have often been attributed to sedentary or inactive lifestyle paired with diets high in fat. Recent studies demonstrated that exposure to environmental factors, such as chemical substances like pesticides, can elicit adverse health outcomes in the form of metabolic disease (Lamat et al., 2022; Rosenbaum et al., 2017). Tobacco-related chemicals, like the main addictive ingredient nicotine, have been shown to elicit increased incidence of cardiometabolic disruption and disease (Balhara, 2012; Rehman et al., 2021). Though global tobacco use continues to decrease, men represent a high percentage of the communities that continue to smoke (Reitsma et al., 2021). Investigation into paternal contributions to the next generations' health are starting to be elucidated; however further characterization of paternal nicotine exposure and a dietary challenge in the next generation have not been explored. This dissertation highlights the gaps in knowledge of paternal nicotine exposure paired with a hypercaloric dietary challenge in the next generation and the sexually dimorphic metabolic phenotypes that arise. This dissertation also demonstrates the gaps in knowledge of direct exposures to EDCs like nicotine or TBT and associated metabolic outcomes at the physiological and transcriptome levels.

This dissertation aims to highlight that nicotine exposure paired with a secondary factor like a hypercaloric diet can lead to further detrimental metabolic effects in a mouse model. Both data chapters highlight two different exposure paradigms to nicotine, with Chapter 2 focusing on chronic direct exposure to nicotine and Chapter 3 focusing on the metabolic effects of paternal nicotine exposure and F1 TWD dietary challenge. I have shown in both data chapters that nicotine exposure in males can elicit metabolic disruption outcomes

and increase susceptibility to metabolic disease. I have also shown that the introduction of a secondary factor, the hypercaloric TWD, can further intensify these metabolic disruption outcomes and even produce metabolic effects at the transcriptomic level. Investigation into a potential epigenetic mechanism underlying the phenotypes observed upon paternal nicotine exposure were not explored in this dissertation. However, we ultimately hypothesize that paternal nicotine exposure leads to alterations in sperm small ncRNAs, which are transferred to the developing zygote upon fertilization. These introduced small ncRNAs can contribute to alterations of gene expression during early embryogenesis that can lead to further gene expression alterations later in life. For those changes to be sustained during differentiation, mitosis and epigenetic reprogramming, we hypothesize that there are changes that occur at the level of expression of genes that participate in nuclear genome organization at the very early stages of development. The embryonic cells with altered nuclear genome organization lead to phenotypes observed in the F1 generation of paternal nicotine-exposed animals. The studies presented in this dissertation shed new light on the effects of dual exposure to environmental insults like chemicals (nicotine) and diet (TWD) in a mouse model a propose a new epigenetic mechanism by which paternal exposure to nicotine might be contributing to metabolic alterations in the next generation. The data in this dissertation should further corroborate previous findings that demonstrated the hepatic alterations upon paternal nicotine exposure (Vallaster et al., 2017).

In Chapter 1 of this dissertation, background information on metabolic disease prevalence and types of disorders associated with metabolic disruption was thoroughly detailed. Global metabolic disease prevalence is projected to keep increasing by 2050, with obesity leading to the largest number of deaths (Chong et al., 2023). Weight gain and/or obesity are not the only risk factors for metabolic disease. Other conditions associated with metabolic disease include hyperlipidemia, type 2 diabetes, and hypertension (Swarup et al., 2024). This chapter also highlights the factors that contribute to incidence of metabolic

disease, such as sedentary or inactive lifestyles, poor diets high in fat, and recently determined environmental chemical exposures (Kim et al., 2021; Okube et al., 2020; Khalil et al., 2023). The exposome, or the health outcomes that arise from all environmental exposures an individual is exposed to within their lifetime, is susceptible to chemical exposures that can lead to adverse metabolic disruption and disease (Wild, 2005; Yilmaz et al., 2020). Specifically, exposure to endocrine-disrupting chemicals, or EDCs, that can regulate hormone action directly and increase likelihood of metabolic disease (Heindel et al., 2022). Exposure to EDCs found in tobacco products, like nicotine, can elicit metabolic disruption (Tweed et al., 2012). Chemical exposures are also suggested to interfere with multigenerational disease (Xin et al., 2015). Prenatal nicotine exposure has been shown to lead to adverse health effects in the developing fetus including decreased birth weights which may lead to increased susceptibility to metabolic disease later in life (Wells and Lotfipour, 2023). Though global tobacco use is on the decline, there are still some affected communities that continue to smoke, like men. Many male smokers continue to smoke while trying to conceive children and thus their sperm is altered and leads to adverse effects in the resulting offspring (Dai et al., 2015; Barbagallo et al., 2024). There has been limited investigation into paternal nicotine exposure and metabolic disruption outcomes in the next generation (Vallaster et al., 2017), but it is hypothesized that paternal nicotine exposure alters certain epigenetic marks in sperm, like expression of small non-coding RNAs (ncRNAs), that are delivered to the zygote upon fertilization and lead to alterations in gene expression in the developing offspring. There is also limited investigation into predisposition to paternal nicotine exposure and a secondary factor challenge, like a high-fat diet, in the next generation and adverse metabolic outcomes. In our studies we introduced a high-fat diet known as the total western diet (TWD) to rodents as a positive control in Chapter 2 of this dissertation.

In Chapter 2 of this dissertation, we first determine metabolic outcomes upon direct exposure to nicotine in adult male and female mice. We also used a known EDC, tributyltin,

as our positive control group. Animals were exposed to either chemical, or the vehicle control dimethyl sulfoxide (DMSO), for sixteen weeks. Metabolic outcomes that were assessed included measured weekly body weights, performed glucose and insulin tolerance tests, and analyzed plasma metabolites and hepatic transcriptomics at the time of sacrifice. Chronic nicotine and TBT exposure induced impaired insulin tolerance and altered plasma metabolite levels, as well as decreased weekly body weights in male mice. Differential gene expression analysis of hepatic transcriptomics revealed that males exposed to nicotine when compared to control males on DMSO had different genes that were enriched for gene ontology (GO) terms associated with cardiovascular processes, specifically cardiac cell function. TBT and nicotine male mice compared to control DMSO animals had shared elements of GO terms associated with cardiovascular processes, specifically cardiac cell development. Female mice exposed to nicotine or TBT did not exhibit significant differences when compared to DMSO control animals. The findings from this study reveal a sexually-dimorphic phenotype when chronically exposed to nicotine and TBT. Specifically, nicotine males exhibited both physiological metabolic alterations in the form of impaired insulin tolerance, decreased weekly body weights, and altered plasma metabolite levels, and transcriptomic cardiometabolic alterations with certain cardiovascular processes enriched for differentially expressed genes when compared to DMSO control animals. TBT males also seemed to elicit similar physiological and transcriptomic cardiometabolic alterations when compared to DMSO control animals. The findings in this study demonstrate that toxicant exposure on hypercaloric diet can further exacerbate cardiometabolic outcomes in adult mice. This study is important to determine the basal effects of chronic nicotine exposure while on a total western diet to determine the metabolic outcomes that arise.

In Chapter 3 of this dissertation, I investigate the effect of paternal preconception nicotine exposure of the offspring in the presence or absence of a secondary dietary challenge and metabolic outcomes. Although it was previously shown that paternal exposure

to nicotine leads to transcriptomic alterations in the liver, some observations that were previously overlooked include 1) the sexually dimorphic response to ancestral exposure to nicotine and 2) the interaction between paternal exposure to nicotine and diets. In this study, F1 animals that were sired from nicotine-treated or DMSO-treated males were placed on either a control diet (CD) or a hypercaloric rodent TWD for eight weeks. Metabolic outcomes assessed in the F1 generation include measured weekly body weights, performed glucose and insulin tolerance tests, and analyzed plasma metabolites and hepatic transcriptomics. Interestingly, in this study we found that nicotine-sired F1 males on either diet exhibited significantly altered plasma metabolites involved in regulating glucose homeostasis. F1 nicotine/CD or TWD males also demonstrated differential gene expression analysis in hepatic tissue that revealed upregulation of biological processes involved with metabolic processes, such as small molecule metabolic process and xenobiotic metabolism. Similarly, there was shared downregulation of biological processes involved with regulation of cholesterol metabolic process and regulation of lipid transport. This study revealed a sexually dimorphic phenotype among F1 males and females sired from nicotine-treated fathers, and the addition of the TWD further exacerbated metabolic outcomes arose from predisposition to paternal nicotine exposure. Investigation into a potential epigenetic mechanism to explain the changes observed upon paternal nicotine exposure reveal that there is different chromatin organization within F1 animals based on paternal treatment group and sex. We ultimately hypothesize that paternal nicotine exposure alters sperm small non-coding RNAs that alter nuclear genome organization in the developing embryo, which leads to modulation of gene expression and altered phenotypes and outcomes. Specifically, these altered sperm small ncRNAs modulate gene expression in the next generation to elicit metabolic outcomes observed. These data further highlight the sexual dimorphism upon paternal nicotine exposure that gives rise to different phenotypes across the sexes in the F1 generation. In future ongoing directions in the laboratory we ultimately hypothesize that paternal nicotine exposure elicits alterations in sperm small ncRNAs that lead to modifications in the developing offspring that elicit metabolic

disruption later in life. Specifically, we hypothesize that there are increased loads and mixture of small ncRNAs introduced by sperm into the fertilized oocyte which alters nuclear genome organization which leads to alterations in gene expression and subsequently adverse health outcomes in the unexposed offspring (Figure 1).

This dissertation contributes to a better understanding of how exposure to environmental factors can lead to adverse metabolic outcomes. Humans are continuously exposed to multiple factors present in their environment and it is important to determine the interplay between these factors and the adverse health outcomes that arise. This dissertation highlights the importance of paternal preconception exposure to nicotine and the introduction of a dietary challenge in the next generation on metabolic health outcomes. Future studies in the laboratory will investigate how these alterations originate in the paternal sperm and how they are propagated to the next generation.

FIGURES

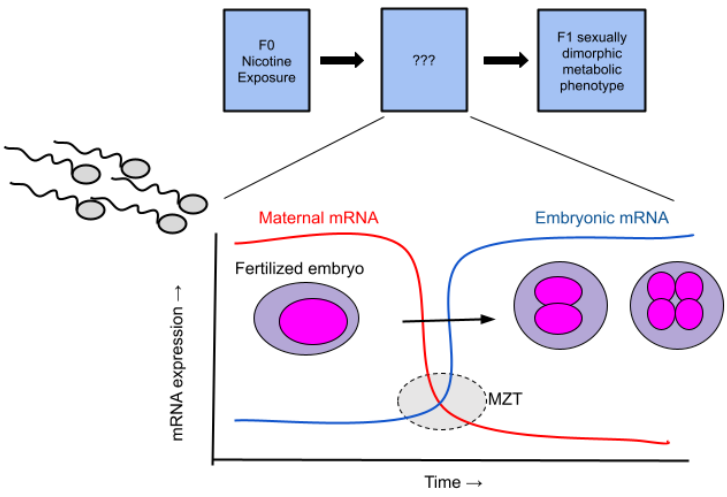

Figure 1. Paternal nicotine exposure alters sperm small ncRNAs that are delivered to the oocyte upon fertilization and lead to modifications of nuclear genome organization that lead to altered gene expression in the developing embryo schematic. Our

hypothesis postulates that these small ncRNA species/loads alter nuclear genome organization and thus gene expression and escape epigenetic reprogramming during early embryonic events such as the maternal-to-zygotic transition (MZT). Alterations to the nuclear genome of the developing embryo that can evade the MZT will have lasting impacts on gene expression in the offspring and may lead to adverse health outcomes previously observed.

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Appendix

Appendix Material

Appendix Section 1. Chronic arsenic and total western diet exposure experiment rationale.

Appendix Figure S1. Chronic total western diet does not significantly alter weekly body weights but alters glucose tolerance in female mice.

Appendix Figure S2. Chronic total western diet and arsenic exposure does not significantly alter weekly body weights but alters glucose tolerance in male mice.

Appendix Figure S3. Over-represented gene ontology (GO) terms in gonadal white adipose tissue (gWAT) transcriptomics of arsenic-treated females.

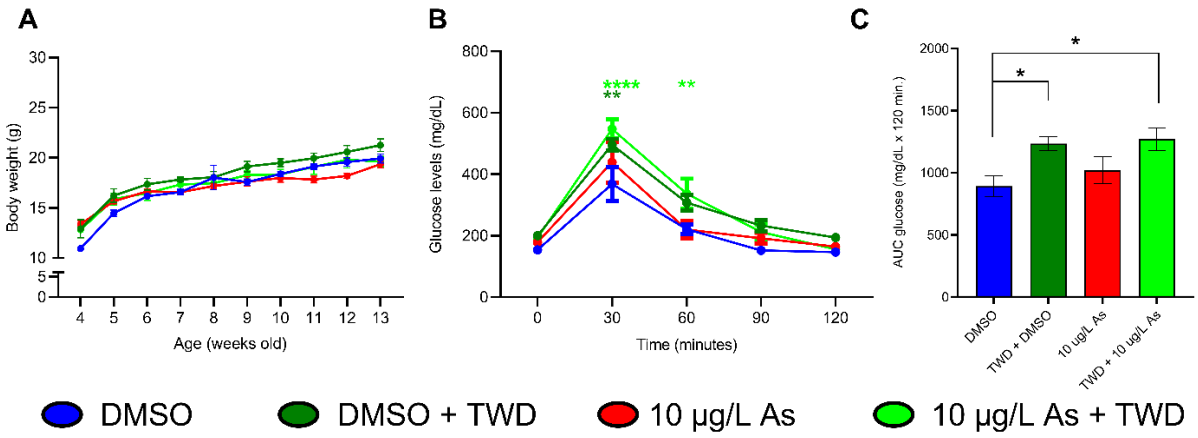
Appendix Figure S4. Over-represented gene ontology (GO) terms in gonadal white adipose tissue (gWAT) transcriptomics of arsenic-treated males.

Appendix Section 2. Chronic arsenic and total western diet exposure experimental findings and discussion.

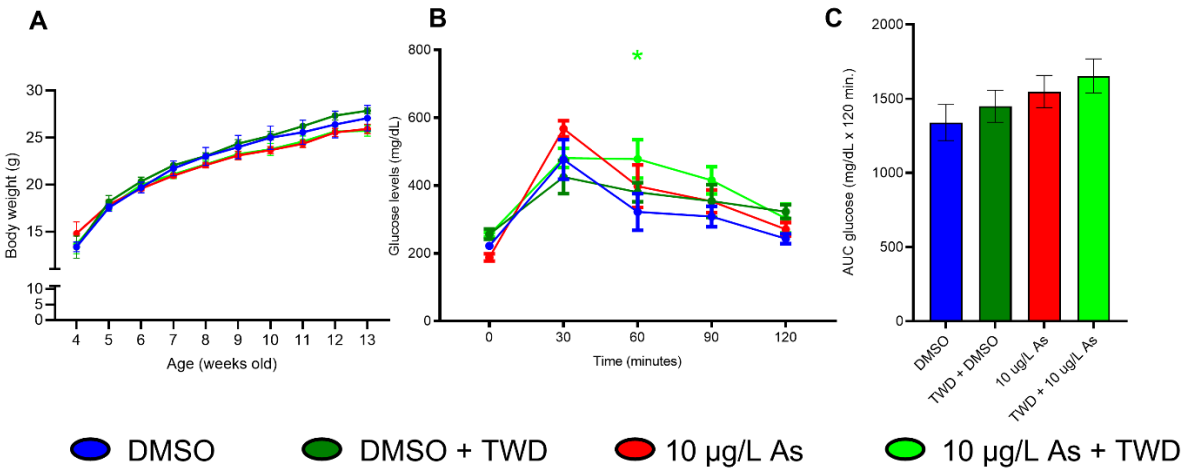
Appendix Section 1. Chronic arsenic and total western diet exposure experiment rationale.

Arsenic is a naturally occurring element that can leach into the groundwater from soil and contaminate drinking water. There's been little investigation into lower concentrations, 10 µg/L and less, arsenic exposure and potential adverse health effects in epidemiological or animal studies, specifically co-exposure to a total western diet.

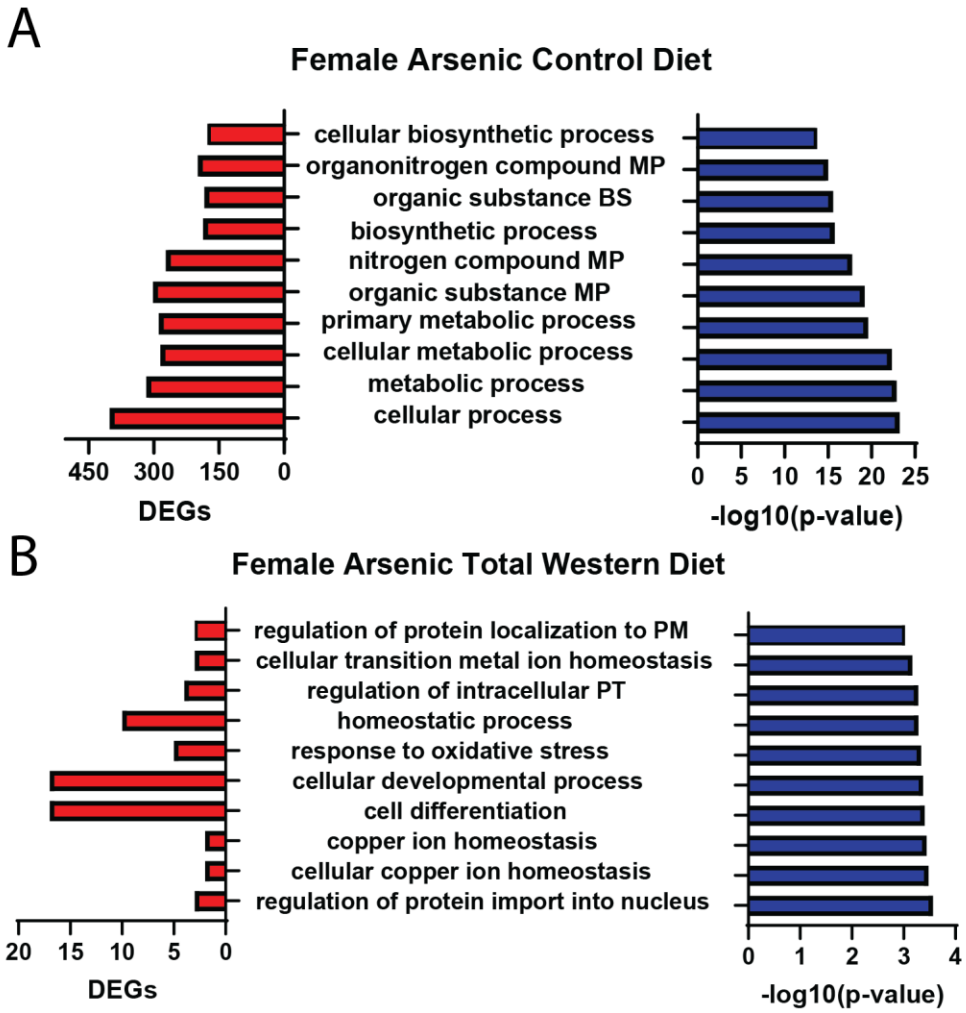
As a pilot study for environmental toxicant exposure paired with total western diet, we used low-level concentrations of inorganic arsenic or the vehicle control dimethyl sulfoxide (DMSO) in drinking water of adult mice for ten weeks. Weekly body weights were measured, and data are highlighted in Supplemental Figures S1 and S2. Before the time of sacrifice, animals were subjected to a glucose tolerance test (GTT). At the time of sacrifice, thirteen-week-old C57BL/6J mice had gonadal white adipose tissue (gWAT) collected for transcriptomic analyses. Differential gene expression analysis of arsenic-treated versus control gWAT revealed gene ontology (GO) terms that were overrepresented in biological processes listed in Supplmental Figure S3 and S4.



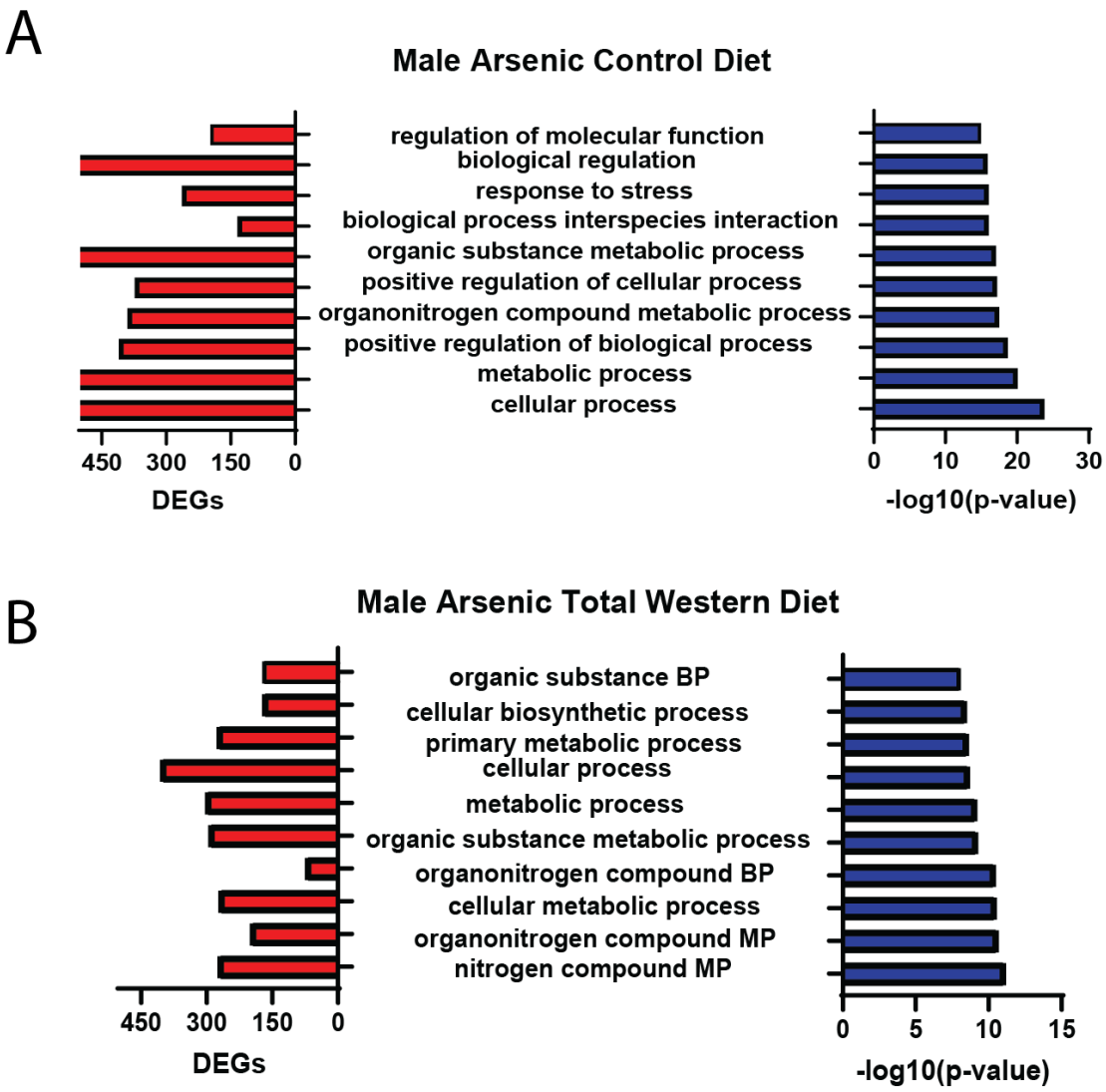
Appendix Figure S1. Chronic total western diet (TWD) does not significantly alter weekly body weights but alters glucose tolerance in female mice. (A) Weekly body weights were measured. No statistically significant differences when compared to control group (DMSO)(Two-way ANOVA; Dunnett's multiple comparisons, n=15). **(B)** Glucose tolerance test on 11-week-old female mice. Animals were fasted for 4 hours, then given an intraperitoneal (IP) injection of a bolus of glucose (2g/kg of body weight). Blood glucose levels were measured before IP injection of glucose (Time 0 min.) and at 30-minute intervals after glucose bolus for two hours. TWD-fed animals exhibited statistically significant blood glucose levels at time 30 minutes and 60 minutes when compared to vehicle control (DMSO). **(C)** Area under the curve (AUC) of the GTT in panel (B), highlighting statistically significant differences of TWD-fed animals when compared to the vehicle control (DMSO)(Two-way ANOVA; Dunnett's multiple comparisons for GTT, and Shapiro-Wilk test for AUC,*P<0.05, **P<0.01, ****P<0.0001, n=4).



Appendix Figure S2. Chronic total western diet (TWD) and arsenic (iAs) exposure does not significantly alter weekly body weights but alters glucose tolerance in male mice. **(A)** Weekly body weights were measured. No statistically significant differences when compared to control group (DMSO)(Two-way ANOVA; Dunnett's multiple comparisons, n=15). **(B)** Glucose tolerance test on 11-week-old male mice. Animals were fasted for 4 hours, then given an intraperitoneal (IP) injection of a bolus of glucose (2g/kg of body weight). Blood glucose levels were measured before IP injection of glucose (Time 0 min.) and at 30-minute intervals after glucose bolus for two hours. TWD-fed and iAs-treated animals exhibited statistically significant blood glucose levels at time 60 minutes post glucose bolus when compared to vehicle control (DMSO). **(C)** However, the area under the curve (AUC) of the GTT in panel (B), does not highlight statistically significant differences of TWD-fed iAs-treated animals when compared to the vehicle control (DMSO)(Two-way ANOVA; Dunnett's multiple comparisons for GTT, and Shapiro-Wilk test for AUC,*P<0.05, n=4).



Appendix Figure S3. Over-represented gene ontology (GO) terms in gonadal white adipose tissue transcriptomics of Arsenic-treated females. (A) Top ten over-represented categories in biological processes (BP) in Arsenic-treated females on the control diet. **(B)** Top ten over-represented categories in BP in Arsenic-treated females on the total western diet. (GOseq analysis in GalaxySeq, Wallenius method; n=4, $p<0.05$). (MP = metabolic process, BS = biosynthetic process, PM = plasma membrane, PT = protein transport)



Appendix Figure S4. Over-represented gene ontology (GO) terms in gonadal white adipose tissue transcriptomics of Arsenic-treated males. (A) Top ten over-represented categories in biological processes (BP) in Arsenic-treated males on the control diet. **(B)** Top ten over-represented categories in BP in Arsenic-treated males on the total western diet. (GOseq analysis in GalaxySeq, Wallenius method; n=4, $p<0.05$).(BP = biosynthetic process, MP = metabolic process)

Appendix Section 2. Chronic arsenic and total western diet exposure experimental findings and discussion.

Chronic total western diet exposure elicits impaired glucose tolerance in female mice

We wanted to determine the metabolic effects of chronic arsenic exposure paired with total western diet. We found that chronic arsenic exposure did not elicit significant differences in body weight or glucose tolerance tests, two important physiological indicators of metabolic disruption (Supplementary Figure S1 & S2). Total western diet exposure, however, elicit statistically significant alterations in blood glucose levels during the glucose tolerance test in female mice (Supplementary Figure S1B,C). Both groups on the total western diet, whether treated with arsenic or the vehicle, exhibited increased blood glucose levels at time 30 post-injection of glucose bolus (Supplementary Figure S1B). The area under the curve (Supplementary Figure S1C) also demonstrates that female mice on the total western diet had significantly increased blood glucose levels when compared to the vehicle control group on the control diet. In male mice, total western diet did not significantly alter blood glucose levels when compared to the vehicle control group on control diet (Supplementary Figure S2). Through these physiological measurements we determined that chronic arsenic exposure does not elicit metabolic alterations in either male or female mice.

Chronic arsenic exposure elicits alterations in gene expression in adipose tissue transcriptomics

Gonadal white adipose tissue (gWAT) was isolated, and RNA was extracted to perform differential gene expression analyses via 3'Tag Sequencing. Differential gene expression analyses were performed to assess alterations in gene expression between chronic arsenic exposed animals versus control animals separated by animal diet. Arsenic-treated female mice on the control diet exhibited differential gene expression in overrepresented gene ontology (GO) terms involved in molecular metabolic processes, like: 'organic substance metabolic process,' 'primary metabolic process,' and 'cellular metabolic process.' Arsenic-treated female mice on the total western diet also displayed differential gene expression in overrepresented GO terms involved in cellular processes, including: 'cell differentiation,' 'copper ion homeostasis,' and 'response to stress.' These data demonstrate that arsenic-treated versus control-treated female mice have differing overrepresented GO terms involved in different biological processes.

Adipose tissue transcriptomics in male mice exposed to arsenic on the control diet or the total western diet demonstrated similar differential gene expression in overrepresented GO terms involved in cellular processes, such as: 'metabolic process,' 'nitrogen compound metabolic process,' 'organic substance metabolic process,' and 'response to stress.' These data demonstrate that arsenic exposure in male mice, regardless of diet, led to alterations in gene expression in processes that are important for molecular metabolic processes. At the expression level, chronic arsenic exposure elicits alterations that might suggest metabolic disruption in adipose tissue. Future studies might explore hepatic transcriptomics after chronic arsenic exposure paired with total western diet, as liver tissue is another important metabolically relevant tissue.