THE UNIVERSITY OF CHICAGO

DEVELOPMENTAL MISPROGRAMMING OF METABOLISM BY ENDOCRINE-DISRUPTING CHEMICALS: A FOCUS ON THE GLUCOCORTICOID RECEPTOR-MODULATING FUNGICIDE TOLYLFLUANID

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ACTH	Adrenocorticotropic Hormone
AF	Activation Function
AhR	Aryl Hydrocarbon Receptor
ANOVA	Analysis of Variance
AP	Activator Protein
AR	Androgen Receptor
ARE	Androgen Response Element
AUC	Area Under the Curve
BDE	Brominated Diphenyl Ether
BMI	Body Mass Index
BPA	Bisphenol A
CBE	Communities for a Better Environment
CBPR	Community Based Participatory Research
CI	Confidence Intervals
CRH	Corticotropin-Releasing Hormone
DBD	DNA Binding Domain
DDE	*p,p'*- dichlorodiphenylethylene
DDT	*p,p'*- dichlorodiphenyltrichloroethane
DEHP	di(2-ethylhexyl) phthalate
DEX	Dexamethasone
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DOHaD	Developmental Origins of Health and Disease
E2	Estradiol
EDC	Endocrine Disrupting Chemical
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Environmental Protection Agency
ER	Estrogen Receptor
ERE	Estrogen Response Element
EYCEJ	East Yard Communities for Environmental Justice
FOX	Forkhead Box
G6PC	Glucose 6-phosphatase
CBG	Corticosterone-Binding Globulin
GC	Glucocorticoid
GH	Growth Hormone
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
GTT	Glucose Tolerance Test
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HMW	High Molecular Weight
HNF	Hepatocyte Nuclear Factor
HOMA-β	Homeostatic Model Assessment of β-cell function
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance

HPA	Hypothalamic-Pituitary-Adrenal
HR	Hazard Ratio
HSD	Hydroxysteroid Dehydrogenase
IDR	Incidence Density Ratio
IGF	Insulin-like Growth Factor
IQR	Interquartile Range
IP	Intraperitoneal
IRR	Incidence Rate Ratio
ITT	Insulin Tolerance Test
IUGR	Intra Uterine Growth Restriction
LBD	Ligand Binding Domain
LMW	Low Molecular Weight
LVEJO	Little Village Environmental Justice Organization
MR	Mineralocorticoid Receptor
NAFLD	Non-Alcoholic Fatty Liver Disease
NO_x	Nitrous Oxides
NHS	Nurses' Health Study
NHANES	National Health and Nutrition Examination Survey
OC	Organochlorine
OR	Odds Ratio
PAH	Polyaromatic Hydrocarbon
PBDE	Polybrominated Diphenyl Ether
PCB	Polychlorinated Biphenyl
PEPCK	Phosphoenolpyruvate carboxykinase
PI3K	Phosphatidylinositol 3 phosphate kinase
PIVUS	Prospective Investigation of the Vasculature in Uppsala Seniors
POMC	Proopiomelanocortin
POP	Persistent Organic Pollutants
PPARγ	Peroxisome Proliferator-Activated Receptor-γ
PVC	Polyvinyl Chloride
$PM_{2.5}$	Particulate Matter ≤ 2.5 μm
PM_{10}	Particulate Matter ≤ 10 μm
PTT	Pyruvate Tolerance Test
PW	Postnatal Week
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RR	Relative Risk
SEM	Standard Error of the Mean
SES	Socioeconomic Status
SRY	Sex-Determining Region Y
STAT	Signal Transducer and Activator of Transcription
T	Testosterone
T2D	Type 2 Diabetes
TF	Tolylfluanid
TSCA	Toxic Substances Control Act
TSS	Transcription Start Site

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Abstract

Type 2 diabetes disproportionately afflicts African-Americans, Latinx communities, and people with low income in the U.S. due to complex environmental influences. Despite the history of environmental inequality in the U.S., the potential contribution of endocrine-disrupting chemicals (EDCs) to racial/ethnic and socioeconomic differences in diabetes risk is not known. Evidence from recent decades gathered from the present comprehensive literature review showing uneven socioeconomic and racial/ethnic EDC exposures raises the possibility that EDCs are underappreciated contributors to diabetes disparities. Notably, EDC exposures during critical windows of development can program metabolic disease risk in a sex-specific way. Importantly, EDCs that modulate glucocorticoid receptor (GR) signaling are an understudied class of environmental pollutants of likely public health significance given that glucocorticoids regulate the development of tissues that control glucose homeostasis. The present studies examined the impact of perinatal exposure to the fungicide tolylfluanid (TF) on metabolic physiology in adult mouse offspring to understand how GR-disrupting EDCs can misprogram metabolism. C57BL/6J dams received standard rodent chow or the same diet containing 67 mg/kg TF. Female offspring exhibited reduced glucose tolerance, markedly enhanced systemic insulin sensitivity, reduced adiposity, and normal gluconeogenic capacity during adulthood. In contrast, male offspring exhibited impaired glucose tolerance with unchanged insulin sensitivity, no differences in adiposity, and increased gluconeogenic capacity. These findings indicating that perinatal TF exposure programs metabolism in a sex-specific manner imply that exposure to other GR-modulating EDCs may elicit similar effects, and suggest that unequal exposures to GR-modulating EDCs may program metabolic disease risk differently by sex in exposed populations.

Chapter 1

Disparities in Environmental Exposures to Endocrine-Disrupting Chemicals and Diabetes Risk in Vulnerable Populations

Sections of this chapter have been adapted verbatim from:

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Section 1.1: Introduction to Metabolic Disease Disparities: Focus on Development and the Environment

Section 1.1.1: The Global Diabetes Pandemic

Diabetes is a complex and devastating metabolic disease that arises from impairments in insulin production and/or action with consequential derangements in global energy metabolism. Currently afflicting nearly 10% of the U.S. population [1] and projected to impact 640 million individuals globally by 2040 [2], diabetes is the leading cause of adult blindness, kidney failure, and non-traumatic amputations; moreover, it is a central driver of cardiovascular disease, the leading cause of death among people with diabetes [3].

Critically, in the U.S. diabetes disproportionately afflicts African-Americans, Latinx communities, and people with low income. Compared to non-Hispanic Whites, the risk of developing diabetes is estimated to be 66% higher for Hispanics and 77% higher for African-Americans [4]. Indeed, 17.9% of African-Americans and 20.5% of Mexican-Americans have diabetes compared to only 9.1% of non-Hispanic Whites [5]. Furthermore, age-adjusted diabetes

mortality rates are significantly higher among Hispanics (28.8 per 100,000 population) and Non-Hispanic Blacks (43.6) than Non-Hispanic Whites (19.8) [6]. Importantly, recent analyses suggest that diabetes prevalence has increased for African-Americans and Mexican-Americans over the last decade while rates among non-Hispanic whites have remained constant [5]. Although physical inactivity and caloric excess are key drivers of the diabetes pandemic, mechanistically understanding the myriad of factors that promote diabetes risk is essential for developing strategies to mitigate the societal impact of diabetes that devastates the lives of millions, and to address the disproportionate burden of diabetes in vulnerable demographics.

Section 1.1.2: Developmental Origins of Metabolic Disease

While chronic exposure to metabolic disease risk factors throughout life can promote the onset of diabetes, metabolic disease risk can also be programmed during sensitive windows of development as proposed by the Developmental Origins of Health and Disease (DOHaD) hypothesis. Evidence for the DOHaD first came from studies of the Dutch Hunger Winter, a period in which Nazi Germany imposed a food blockade on the western Netherlands at the end of the Second World War. Studies of individuals who were exposed to the Dutch Famine *in utero* showed lower birth weights, and these individuals were more likely to develop type 2 diabetes and suffer from cardiovascular disease by age 50 compared with those individuals born either the year before or the year after the famine [7]. Based on numerous epidemiological and animal studies, we now know that environmental factors can disrupt the organization of key metabolic tissues during fetal development and impair their ability to maintain glucose homeostasis throughout later in life [8]. As such, exposure to numerous common environmental factors during pregnancy; including overnutrition, psychological stress, and diabetes have been linked to the onset of metabolic disease

in the offspring in animal and epidemiological studies [8]. Additionally, developmental exposures to environmental pollutants can also promote later-life metabolic disease risk. Importantly, developing fetuses are more sensitive to xenobiotic exposures due to their underdeveloped detoxifying mechanisms, higher anabolic rate, and higher toxicant-to-weight ratios [9]. Because of these attributes, *in utero* and early post-natal development are periods of enhanced susceptibility to malprogramming by numerous environmental factors that can predestine individuals to greater lifetime metabolic disease risk [10].

Section 1.1.3: Metabolism-Disrupting Chemicals

The mass production of structurally diverse synthetic chemicals has resulted in an unprecedented burden on the environment and public health worldwide. As endocrine and metabolic diseases continue to rise globally [11], nearly 800 compounds have been identified as putative endocrine-disrupting chemicals (EDCs) [11], while tens of thousands of compounds still lack basic toxicological screening [12]. As defined by the Endocrine Society, EDCs are exogenous chemicals, or mixtures of chemicals, that interfere with any aspect of hormone action [13]. Historical EDC research focused largely on endpoints related to sexual-development and reproduction following developmental and adult exposures to sex-hormone modulating agents given the prominent roles that androgens and estrogens play in these physiological processes. However, the metabolic systems that control energy homeostasis are established by numerous hormones during fetal development, and glucose homeostasis is maintained by diverse endocrine signals throughout the lifespan. For this reason, metabolism is a physiological endpoint susceptible to disruption by EDCs both during development and throughout postnatal life. Of note, the dramatic rise in U.S. diabetes rates correlates closely with synthetic chemical production [14], and

these associations are now supported by epidemiological, animal, and cellular data that demonstrate that EDCs can interfere with insulin secretion and action, as well as with other pathways that regulate glucose homeostasis. Thus, exposures to EDCs have the potential to disrupt metabolism. However, it is important to recognize that the burden of that risk is not uniformly borne across society given the unequal distribution to exposures in the U.S. that disproportionately affects demographics already at higher risk for developing metabolic diseases.

Section 1.2: Overview of Environmental Racism in the U.S.

Despite the history of inequitable distribution of environmental pollution affecting communities of color in the U.S. [15], the potential contribution of environmental toxicants to racial/ethnic and socioeconomic differences in diabetes risk is underappreciated. The issue of environmental injustice first entered widespread consciousness in 1982 when residents of the predominantly African American community of Warren County, North Carolina, made national news by laying themselves across a rural road to prevent encroaching trucks from dumping dirt laden with polychlorinated biphenyls (PCBs) in their community [16]. This media attention prompted empirical examination of the community's claim that toxic waste facilities were being disproportionately sited in low-income communities and communities of color. In 1987, the first national study on environmental discrimination documented the disproportionate siting of toxic waste facilities in African-American, Latinx, and poor neighborhoods throughout the U.S. [17]. Reevaluated 20 years later, these racial and socioeconomic disparities in toxic waste site proximity were even greater than previously reported [15]. Research on environmental inequality has grown substantially since the 1980s, with the majority of evidence showing racial and socioeconomic disparities in exposures to myriad of environmental hazards [16]. Moreover, the percentage of

African-Americans and Latinx living in “fenceline zones”, where people are least likely to escape a toxic chemical emergency, were 75% and 60% greater than the U.S. average, respectively [18]. In addition to higher exposure to industrial air pollution nationwide [19], unequal exposures amongst people of color are also rooted in patterns of occupation, housing conditions, and neighborhood infrastructure [20, 21].

Section 1.3: Unequal Environmental Exposures and Diabetes Risk

Section 1.3.1: Introduction

Diverse scientific evidence linking EDCs with the development of diabetes and other metabolic disorders continues to grow. Importantly, exposures to several toxicants have been prospectively linked to diabetes risk, including: PCBs, various chemical constituents of air pollution, bisphenol A (BPA), phthalates, and organochlorine (OC) pesticides (**Table 1.1**); moreover, exposure to these EDCs is higher among African-Americans, Latinx, and people with low income (**Table 1.2**). The wealth of evidence gathered from recent decades showing uneven socioeconomic and racial/ethnic exposures raises the possibility that these chemicals are underappreciated contributors to diabetes disparities. This section will review the state of the evidence linking ethnic, racial, and socioeconomic disparities in pollutant exposure in the U.S. to EDCs linked to diabetes.

Section 1.3.2: Polychlorinated Biphenyls (PCBs)

Introduced to the U.S. in the 1930s for a variety of industrial purposes, PCBs are a class of synthetic compounds in which various combinations of hydrogen atoms on the biphenyl ($C_{12}H_{10}$) structure have been substituted with chlorine, resulting in 209 congeners that are designated by a

unique number reflecting the extent and position of their chlorination (e.g. PCB 153). Although banned by the U.S. Environmental Protection Agency in 1977, PCBs remain detectable in human tissues due to their environmental and biological persistence [22]. Importantly, higher PCB exposures amongst African-Americans have been documented since the 1960s [23] (**Table 1.2**). Ongoing human exposure to PCBs is due to the legacy of contamination in food, including certain fish [24]; however, additional exposure sources include leaching from contaminated industrial sites and indoor construction materials [25, 26]. PCB waste is found in Superfund and toxic waste sites that are concentrated in neighborhoods of color [27]. Although catfish consumption has been suggested as the main contributor to increased PCB levels in African-Americans [28], the historical siting of PCB production and disposal sites in predominantly Black communities likely contributes to increased contamination of locally-sourced foods. One example of this phenomenon is Anniston, Alabama, a PCB-manufacturing city from 1929 to 1971. African-Americans not only lived closer to a former Monsanto PCB manufacturing plant but also had PCB levels three-times higher than Caucasians in Anniston [29]. Consumption of local fish and livestock were the strongest predictors of higher serum PCB levels among African-Americans [30], while consumption of local dairy products and dredging near another PCB-contaminated Superfund site also predicted higher cord blood PCB levels in infants [31].

Experimental studies suggest that exposure to various PCB congeners have the capacity to promote metabolic dysfunction *in vitro* and in animal models. Murine adipocytes treated with PCB-77 or PCB mixtures promoted insulin-desensitizing pro-inflammatory cytokine release, and decreased insulin-stimulated glucose uptake at sub-lethal concentrations [32, 33]. In animal models, exposure to coplanar PCB congeners (e.g. PCB-77) promoted glucose intolerance while antagonizing the metabolic benefits of weight loss, effects mediated by activation of the aryl

hydrocarbon receptor (AhR) signaling [34, 35]. Non-coplanar PCB congeners such as PCB-153, the most abundant congener found in humans, exhibit less affinity for the AhR; however, this PCB class has also been shown to promote metabolic dysfunction. In mice fed a high fat diet, PCB-153 synergistically increased non-alcoholic fatty liver disease (NAFLD), the liver component of the metabolic syndrome [36, 37]. At the cellular level, exposure to a PCB mixture down-regulated insulin signaling intermediates in skeletal muscle and liver in mice, resulting in hyperinsulinemia and systemic hyperglycemia [38]. Importantly, β-cell PCB treatment *in vitro* stimulated insulin secretion [39], suggesting that prolonged lifetime exposure can lead to β-cell exhaustion; however, additional studies are required to define the β-cell effects of PCB exposures. The effects of PCBs and their metabolites on energy metabolism are likely complex since they can disrupt the AhR, pregnane X-receptor, and constitutive androstane receptor, as well as sex steroid, glucocorticoid (GC), and thyroid hormone signaling [40-42], all of which regulate lipid and glucose homeostasis.

A large body of evidence, including prospective epidemiological studies, supports the hypothesis that PCBs are metabolic disease risk factors. Residential proximity to PCB-contaminated waste sites is associated with higher diabetes hospitalization rates [43]. Among female residents of Anniston, serum PCB levels were significantly associated with diabetes [44], while in a separate study with 25 years of follow-up, women with higher PCB levels exhibited increased diabetes incidence (Incidence Density Ratio: 2.33; 95% Confidence Interval [95%CI]: 1.25-4.34) [45]. Similarly, women exposed to PCB-laced rice bran oil during the Yucheng poisoning event in Taiwan also had an increased risk of developing diabetes (Odds Ratio [OR]: 2.1; 95%CI: 1.1-4.5), with markedly higher risk among those who developed chloracne, a cutaneous manifestation of dioxin-like PCB exposure (OR: 5.5; 95%CI: 2.1-13.4) [46]. A meta-analysis that pooled data from the Nurses' Health Study with six prospective studies showed that

total PCBs were associated with incident diabetes (OR: 1.70; 95%CI: 1.28-2.27) [47]. Further supporting these prospective links between PCB exposure and diabetes are data from cohort studies including the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) [48], as well as a group followed for nearly 20 years [49]. Finally, while not reaching statistical significance, a study of Swedish women suggested that higher levels of PCB 153 were similarly associated with increased rates of type 2 diabetes (T2D) diagnosed after more than 6 years of follow-up (OR: 1.6; 95%CI: 0.61-4.0) [50]. Collectively, these data suggest an association between PCBs and diabetes risk, especially among women; however, there are some discrepant findings in the literature. In a study of Great Lakes sport fish consumers, PCB 118 and total PCBs were not associated with diabetes [51], and in a Flemish study that adjusted for correlated exposures, PCBs showed a negative association with self-reported diabetes [52]. Despite these discrepancies, a meta-analysis of both cross-sectional and prospective studies published prior to March 2014 showed that, in aggregate, total PCBs were associated with increased diabetes risk (Relative Risk [RR]: 2.39; 95%CI: 1.86-3.08) [53]. Taken within the context of animal and cellular data demonstrating that PCBs alter metabolic function ((**Table 1.3**) and references therein), this evidence collectively suggests that differential exposure to PCBs could contribute to diabetes disparities.

Section 1.3.3: Traffic-Related Air Pollution and Particulate Matter

Traffic-related air pollution is composed of various chemical components, including nitric oxides (NO_x), ozone (O_3), and particulate matter (PM), which is a mixture of particles and liquids classified by their diameter (e.g., <10 μm [PM_{10}] or <2.5 μm [$PM_{2.5}$]). Nationwide studies show that African-Americans and Latinos are exposed to significantly more $PM_{2.5}$ [19, 54], and ethnic

and racial disparities in exposure to traffic-related air pollution exceed those between income groups [55] **(Table 1.2**). NO_2 levels correlate closely with $PM_{2.5}$, ultrafine particles, and black carbon and thus serve as a proxy for traffic-related air pollution [56]. Exposure to NO_2 was 38% higher for people of color than for non-Hispanic Whites and 10% higher for people below the poverty line [55]. Among non-White individuals living in poverty, children under the age of 5 were exposed to 23% higher NO_2 concentrations than the rest of the population [55]. Importantly, racial differences in NO_2 exposure were greater in large metropolitan centers compared to small-to-medium urban areas, likely reflecting racial and ethnic segregation around traffic corridors in major U.S. cities.

Animal studies suggest that exposure to $PM_{2.5}$ alters whole body energy homeostasis through the development of a chronic inflammatory state, endoplasmic reticulum stress in metabolic tissues, and autonomic nervous system dysfunction [57]. Chronic inhalational exposure to $PM_{2.5}$ aberrantly activates innate immunity, increasing serum inflammatory cytokines and recruiting pro-inflammatory macrophages to adipose tissue resulting in the development of insulin resistance [58]. In animal models, $PM_{2.5}$ exposure impairs insulin signaling in adipose tissue, liver, skeletal muscle, and the vascular endothelium, which increases blood glucose levels, and promote hepatic lipid accumulation [58, 59]. Similar effects were also shown with developmental exposure to $PM_{2.5}$ during early life [60]. Although these studies examined $PM_{2.5}$ concentrations that are higher than average ambient U.S. concentrations (15 $\mu g/m^3$), they are on par with levels observed in low- and middle-income countries that are experiencing dramatic increases in diabetes rates, e.g. China, India, and several Latin American countries [61].

Increasing epidemiological evidence implicates air pollution in glucose dysregulation, including insulin resistance [62] (**Table 1.1**). In a small but elegant study, residents of rural

Michigan exposed to urban air for only 4-5 hours daily for 5 consecutive days exhibited an increase in the homeostatic model assessment of insulin resistance (HOMA-IR) for each 10 $\mu g/m^3$ increase in $PM_{2.5}$ [63]. Similarly, in insulin resistant adults with the metabolic syndrome in the Beijing metropolitan area, variations in black carbon and $PM_{2.5}$ were associated with worsening insulin resistance [64]. In Germany, long-term exposure to PM_{10} and NO_2 was associated with greater insulin resistance in 10-year old children [65]. Additionally, several studies have linked poor air quality with progression to diabetes. In one study of subjects without diabetes followed for 5.1 years, each interquartile range (IQR) increase in total PM_{10} was associated with a 20% increased risk of developing T2D (RR: 1.20; 95% CI: 1.01-1.42) [66]. Living closer than 100 m (relative to >200 m) from a busy road was associated with a 37% increased risk of developing diabetes (RR: 1.37; 95% CI: 1.04-1.81). In this study, higher levels of $PM_{2.5}$, traffic-specific PM_{10}, and traffic-specific $PM_{2.5}$ were each also associated with increased diabetes risk; however, these measures failed to reach statistical significance. In a study of Black women living in Los Angeles, CA followed for 10 years, incident diabetes rates were increased for each IQR increase in NO_X (Incidence Rate Ratio [IRR]: 1.25; 95% CI: 1.07-1.46), while $PM_{2.5}$ was associated with a non-significant increase in incident diabetes (IRR: 1.63; 95%CI: 0.78-3.44) [67]. Among non-diabetic women from the Study of the Influence of Air Pollution on Lung, Inflammation, and Aging cohort followed for 16 years, incident diabetes increased by 15-42% per IQR of PM_{10} or traffic-related air pollution [68]. The data from prospective studies are not, however, uniform. In the Multi-Ethnic Study of Atherosclerosis, NO_X was associated with *prevalent* diabetes, and $PM_{2.5}$ trended toward an association (OR: 1.09; 95% CI: 1.00-1.17), but no air pollution measure was associated with *incident* diabetes over 9 years of follow-up [69]. In a long-term analysis of the Black Women's Health Study with adjustment for multiple metabolic stressors, NO_2 was not associated with

diabetes incidence [70]. Despite this heterogeneity, epidemiological studies linking various chemical constituents of air pollution to diabetes risk coupled with animal studies demonstrating that exposures to air pollutants such as $PM_{2.5}$ and polyaromatic hydrocarbons (PAHs) disrupt metabolism and promote inflammation (**Table 1.3**) collectively suggest that differential exposure to air pollution may augment diabetes risk in low-income communities of color.

In addition to effects on diabetes development *per se*, air pollutants may also promote adverse outcomes in those with the disease. For example, $PM_{2.5}$ levels modeled for home addresses were linked to diabetes on death certificates [71], while a prospective analysis of over 2 million adults revealed that a 10 $\mu g/m^3$ increase in $PM_{2.5}$ was associated with increased diabetes-related mortality [72]. These findings may be related to adverse vascular effects in those with diabetes. In 22 patients with T2D living in North Carolina, daily measures of flow-mediated vasodilatation were decreased in association with $PM_{2.5}$ levels [73]. The clinical significance of this may be reflected in data showing that each 10 $\mu g/m^3$ increase in $PM_{2.5}$ was associated with an 11% increased risk of ischemic stroke in those with diabetes [74].

Section 1.3.4: Bisphenol A (BPA)

BPA is a ubiquitous synthetic chemical used in the manufacturing of polycarbonate and other plastics commonly used in consumer products; moreover, BPA is a component of sales receipts and epoxy resins lining food and beverage cans as well as water pipes. BPA exposure in the U.S. population is nearly universal [75]. Although BPA is rapidly cleared from the body and single measurements may not reflect cumulative exposure [76], African-Americans and people with lower incomes have higher BPA levels than the population at large (**Table 1.2**). The reasons for these disparities are not clear; however, reduced access to fresh food and consequential

consumption of processed foods may partly explain these associations [77] since consuming foods packaged in plastic or cans increases BPA exposure [78]. Moreover, among individuals with low food security, BPA levels were higher if they received emergency food assistance, which includes canned foods [79]. For example, 6-11 year-old children receiving emergency food assistance had BPA levels that were 54% higher than age-matched children from more affluent families [79].

Several animal studies have shown that prenatal exposure to BPA at concentrations at or below the U.S. EPA's daily allowable intake level (50 μg/kg/day) resulted in increased serum insulin, as well as decreased glucose tolerance later in life [80, 81]. Importantly, these effects were not observed at much higher doses, but were significantly worsened with a high-fat diet exposure during development [80]. Perinatal exposure to BPA induced promoter hypermethylation of hepatic glucokinase with consequent downregulation of glucokinase expression during adulthood [82]. BPA exposure decreases levels of the insulin-sensitizing adipokine adiponectin and disrupts insulin signaling in various animal models [83]. BPA exposure also has rapid effects on β-cell insulin secretion. Low-dose exposure can stimulate insulin secretion, whereas chronic exposure increases β-cell insulin content and glucose-stimulated insulin secretion, leading to post-prandial hyperinsulinemia that is often observed before the development of insulin resistance [84].

Disparities in BPA exposure may contribute to metabolic disease burden since increasing evidence associates BPA with diabetes. Analyses exploring the association between urinary BPA levels and metabolic disease are complicated by BPA's rapid excretion [85]; moreover, while there is no definitive evidence that urinary excretion of BPA is influenced by race/ethnicity, lack of adjustments for renal function can complicate urinary assessments in population studies [86]. Despite these caveats, the National Toxicology Program concluded that BPA is suggested to exert effects on glucose homeostasis and insulin release based upon animal and *in vitro* studies [87].

While there is some heterogeneity across studies, the literature supporting this conclusion demonstrates myriad BPA-induced metabolic disruptions across multiple animal and cellular model systems, including alterations in body weight regulation, insulin action, and insulin secretion as well as specific disruptions in β-cell, α-cell, hepatocyte, and adipocyte function and development (**Table 1.3**). This conclusion is further supported by limited prospective human studies (**Table 1.1**). In data from the Nurses' Health Study (NHS), extremes of BPA quartiles were associated with incident diabetes after adjusting for BMI (OR: 2.08; 95%CI: 1.17-3.69) in NHS II, but not NHS [88]. This potentially suggests that age may modify BPA-associated diabetes risk as the mean age in NHS II was 45.6 years versus 65.6 years in NHS. Alternatively, these differences may have arisen from period-cohort effects in which the extent, diversity, or timing of exposures may have been greater or more deleterious in NHS II. Furthermore, the relationship between BPA and diabetes risk was modulated by a diabetes genetic risk score, suggesting that some populations may be more sensitive to the diabetogenic effects of BPA [89]. Interestingly, among those with diabetes BPA may exacerbate diabetes complications since high levels of BPA were associated with a markedly increased rate of developing chronic kidney disease (OR: 6.65; 95%CI: 1.47-30.04) [90]. In one meta-analysis aggregating cross-sectional and prospective studies, comparing the highest to lowest exposure groups demonstrated a positive association between BPA and diabetes (RR: 1.45; 95%CI: 1.13-1.87) [53], a finding similar to a second, more recent meta-analysis of prevalent diabetes in three cross-sectional studies (OR: 1.47; 95%CI: 1.21-1.80) [91]. Thus, based on reasonable evidence, differential BPA exposure can contribute to diabetes disparities.

Section 1.3.5: Phthalates

Phthalates are a diverse class of widely used synthetic compounds. High-molecular-weight (HMW) phthalates are mainly employed as plasticizers in food packaging, toys, and building materials such as polyvinyl chloride (PVC); low-molecular-weight (LMW) phthalates are used in pharmaceuticals, personal care products, and solvents. Phthalates are not covalently bound within products and thus can volatilize or leach out, thereby facilitating absorption via dermal contact, ingestion, and inhalation. Higher phthalate exposure amongst people of color and people with low income have been documented in various studies (**Table 1.2**), although the sources of these exposure differences are difficult to discern given the widespread commercial use of phthalates. Reduced access to fresh fruits and vegetables and increased consumption of fat-rich foods in low-income populations may augment exposure differences since certain high fat foods are a major source of HMW phthalates [92]. Weathering of older construction materials in low-income households may increase inhalational phthalate exposure [93]. Furthermore, purchasing inexpensive products likely contributes to disproportionate phthalate exposures based on an evaluation of products at "dollar stores" revealing that 32% of PVC-containing products exceeded phthalate limits established for children's products by the Consumer Product Safety Commission [94]. Importantly, personal care products and cosmetics also contribute to phthalate exposure [95], especially in women, who typically have the highest concentrations of phthalates [96]. Indeed, certain feminine hygiene products were found to be at least partially responsible for higher levels of monoethyl phthalate in African-American women [97]. These data provide provocative evidence of racial, ethnic, and socioeconomic disparities in phthalate exposure; however, additional studies are needed to further illuminate the sources of unequal phthalate exposures.

Numerous animal studies demonstrate that phthalates affect energy metabolism, most of which have studied exposure to the widely used di(2-ethylhexyl) phthalate (DEHP). DEHP

decreased insulin content and secretion, and induced apoptosis of INS-1 cells by activating endoplasmic reticulum stress and suppressing antioxidant protection [98]. Phthalate exposure has been associated with increased oxidative stress biomarkers in humans such as decreased serum bilirubin [99], which is important since β-cells are particularly vulnerable to oxidative stress due to their low levels of catalase and glutathione peroxidase. *In utero* DEHP exposure led to reduced β-cell mass and insulin content with disruptions in β-cell ultrastructure at weaning, whereas at adulthood, female offspring of exposed dams developed hyperglycemia with reduced insulin levels [100]. Although male offspring of exposed dams showed elevated insulin levels without glucose intolerance [100], *in utero* DEHP exposure exacerbated glucose intolerance in males at adulthood upon high-fat diet feeding [101]. DEHP along with its breakdown products and other phthalates can directly activate the peroxisome proliferator-activated receptor-γ (PPARγ) [102], and may influence diabetes pathogenesis by their obesogenic activity.

Several epidemiologic studies have linked higher phthalate exposure with diabetes (**Table 1.1).** In data from NHS II, total urinary phthalate metabolites were associated with diabetes [88]. In this analysis, metabolites of butyl phthalates and DEHP were associated with diabetes with ORs of 3.16 (95%CI: 1.68-5.95) and 1.91 (95%CI: 1.04-3.49), respectively. Similar to BPA, these associations may be age-related or a consequence of period-cohort effects as similar findings were not observed with the older, original NHS. In the Early Life Exposure in Mexico to Environmental Toxicants cohort, *in utero* levels of monoethyl phthalate were associated with reduced insulin secretion in pubertal boys [103]. In the meta-analysis of Song and colleagues, urinary concentrations of phthalates were nearly significantly associated with diabetes (RR: 1.48; 95%CI: 0.98-2.25). With supportive cellular and animal data demonstrating that various phthalates have the capacity to promote dysfunction in multiple metabolic tissues (**Table 1.1**), further prospective

studies are justified to define the relationship between phthalate exposures and diabetes risk, particularly among vulnerable populations.

Section 1.3.6: Organochlorine Pesticides

Organochlorine (OC) pesticides were extensively used in the U.S. until the 1970s when most were banned due to their environmental persistence and toxicity to humans and wildlife; however, several OC pesticides and their metabolites are still detectable in the U.S. population. Importantly, levels of these compounds are greater in Mexican-Americans and African-Americans compared to Caucasians (**Table 1.2**). The prolonged use of OC pesticides outside of the U.S. for agricultural purposes or vector control is believed to contribute to higher levels in Latinx populations [22, 104, 105]. Indeed, based on NHANES data, people born outside of the U.S. are more likely to be exposed to OC pesticides [106]. However, the overrepresentation of Mexican-Americans in U.S. agriculture may also play a role in exposure disparities [107]. Additionally, direct exposures to OC pesticides before their phase-out may have been passed down to offspring through breast milk and cord blood [108], likely resulting in higher body burdens at the start of life that then persist into adulthood.

Organochlorine pesticides are persistent organic pollutants (POPs) that can bioaccumulate in adipose tissue, where they disrupt adipocyte function and development and from which they can leach to affect other tissues [109, 110]. Animal and *in vitro* studies support these links; however, the mechanisms by which OC pesticides perturb energy homeostasis remain elusive. Both *p,p'*-dichlorodiphenyltrichloroethane (DDT) and *p,p'*- dichlorodiphenylethylene (DDE), a metabolite of DDT, can promote differentiation of mature murine adipocytes *in vitro* by mechanisms that are unclear [111, 112]. Male rats that were fed crude salmon oil naturally contaminated with POPs

including OC pesticides, developed insulin resistance and hepatosteatosis compared to rats fed refined salmon oil [33]. Moreover, this study demonstrated that OC pesticides present in farmed salmon oil were the most potent POP-inducers of insulin resistance in an *in vitro* adipocyte model. A study examining perinatal DDT exposure found that female offspring developed hyperglycemia and hyperinsulinemia, and exhibited reduced thermogenesis and energy utilization during adulthood, suggesting a novel mechanism by which OC pesticide exposure might promote metabolic dysfunction [113]. In addition, earlier studies demonstrated the capacity of DDT to inhibit ß-cell insulin secretion [114]. However, other studies have reported conflicting results on the effects of *p,p'*-DDE on fasting hyperglycemia and insulin resistance in mice, suggesting that these phenotypes are diet-dependent [115, 116]. Supporting these basic science studies, epidemiological data from different parts of the world have associated exposure to OC pesticides including *p,p'*-DDT and its major metabolite *p,p'*-DDE, ß-Hexachlorocyclohexane (HCH), oxychlordane, and Hexachlorobenzene (HCB) with diabetes and the metabolic syndrome [117]. Thus, exposure to OC pesticides may be a further contributor to metabolic disease disparities.

In concordance with animal and cellular data demonstrating the capacity of OC pesticides to disrupt multiple aspects of cellular and systemic glucose regulation (**Table 1.3**), epidemiological studies from various regions of the world have associated OC pesticide exposure with diabetes and the metabolic syndrome (**Table 1.1**). For example, plasma HCB was positively associated with incident T2D, an effect confirmed in an accompanying meta-analysis (OR: 2.00; 95%CI: 1.13-3.53) [47]. Among Great Lakes sport fish consumers, levels of DDE were associated with incident diabetes [51], while a study in Swedish women showed that being in the highest quartile of DDE levels relative to the lowest quartile was associated with incident diabetes (OR: 5.5; 95%CI: 1.2-25) [50]. In a nested case-control cohort of individuals followed for nearly 20 years, the OC

pesticides *trans*-nonachlor, oxychlordane, and mirex were nonlinearly associated with new onset diabetes [49]. While in the PIVUS study, *trans*-nonachlor and a summary index of 3 OC pesticides were also positively associated with diabetes at age 75 [48]. In a Flemish biomonitoring program, OC pesticides levels measured in 2004-5 were associated with self-reported diabetes in 2011; this included hexachlorobenzene as well as DDE in men [52]. Finally, in the Agricultural Health Study, a large prospective cohort of pesticide applicators and their spouses, the OC pesticide dieldrin was associated with incident diabetes (Hazard Ratio: 1.99; 95%CI: 1.12-3.54) [118]. Compiling data across studies, a meta-analysis comparing the highest to lowest exposure groups demonstrated a strong positive correlation between OC pesticide exposure and diabetes rates (RR: 2.30; 95%CI: 1.81-2.93) [53].

Section 1.3.7: Polybrominated diphenyl ethers (PBDEs)

PBDEs are synthetic chemicals that have been used as flame retardants since the 1970s. Because of their chemical stability and hydrophobicity, PBDEs have been found to bioaccumulate in animal fat, breast milk and placental tissue [119, 120]. United States residents have some of the highest levels of PBDEs in the world [119]. Within the U.S., the highest dust and serum levels have been reported in California [121], which may be due to California's strict furniture flammability standards.

Epidemiological evidence thus far suggests that in California, Mexican-Americans and people with low socioeconomic status (SES) may be disproportionately exposed to PBDEs (**Table 1.2**). House dust contaminated with PBDEs that have leached out of furniture is currently thought to be the most significant exposure route. Low-income households may, on average, replace damaged furniture less often and be more likely to own used furniture, which may contribute to

the higher dust levels documented. Residential zoning may further contribute to PBDE exposure by confining children indoors where exposure is highest. This concept is supported by associations between higher PBDE levels and the absence of safe places to play among low-income, Mexican-American children from Salinas, California [122]. Importantly, lower levels of PBDEs have been found in foreign-born Mexican-Americans relative to U.S.-born Mexican-Americans [121, 123, 124]. Thus, PBDE exposure may be another possible health risk factor affecting the already vulnerable Mexican immigrant population of California.

Although studies suggest that PBDE exposure disparities exist outside of California, the affected populations seem to vary between studies and geographical regions. One national study that examined the influence of SES on PBDE exposure found that individuals living in a household with an income of less than $20,000/year had significantly more PBDE exposure compared to those of higher income households (>$20,000/year) [121]. A study based in Durham, North Carolina also associated higher serum levels of some PBDEs with lower SES [125]. However, studies carried out in New York and Baltimore, MD, showed that educational attainment positively correlated with increased PBDE exposure [126, 127]. An analysis of the NHANES 2003-2008 data, which did not look at SES, found no significant differences of PBDE levels across ethnic groups [22]. Thus, more studies are required to understand regional drivers of PBDE exposure disparities by race/ethnicity and SES.

The majority of the experimental studies that have investigated the metabolism-disrupting activity of PBDEs have examined the effects of BDE-47, the most prevalent congener measured in human samples [128]. BDE-47 promotes differentiation of 3T3-L1 preadipocytes into mature adipocytes [129-131]. Interestingly, some BDE-47 metabolites are potent ligands for PPARγ [132], a master regulator of adipocyte differentiation. Animal models of *in vivo* PBDE exposure

have shown impaired lipolysis and glucose utilization in adipose tissue, markers of reduced insulin response [133], and alterations in hepatic gene expression that may compromise liver glucose and lipid metabolism [134-136]. Penta-BDEs such as BDE-47 have been phased out in several states in the U.S. since 2006 and replaced with organophosphate flame retardants, which can also induce adipocyte differentiation via PPARγ activation [137]. Although exposure data on this relatively new class of flame retardants is limited, their similar routes of exposure may result in similar exposure disparities.

While, emerging epidemiological evidence links PBDE exposure to metabolic disease, the available evidence to date is not as strong as it is for the previously discussed EDCs. In a prospective cohort of French women, PBDE exposure showed a non-linear association with T2D risk, where the second and fourth quintile groups of exposure showed significant hazards ratios [138]. Further, evidence from two case-control community-based studies in China showed a positive association between serum PBDE-47 and diabetes risk [139]. In a national study, serum PBDE-153 levels showed an inverted U-shaped association with diabetes and metabolic syndrome [140]. Interestingly, PCBs, which have a similar molecular structure to PBDEs, have also displayed diabetogenic non-monotonic dose responses such as the one in this study [141-143]. A study in the Great Lakes region did not identify significant associations with PBDE exposure and diabetes [144]. Thus, further studies may be needed to investigate the high California PBDE levels with obesity and diabetes. However, evidence from recent case-control studies from the U.S., Iran, and China have consistently associated higher maternal serum PBDE levels with gestational diabetes [145-148]. Considering the higher PBDE exposure in Mexican-Americans, early life PBDE exposure should be considered as a possible factor that may contribute to the deterioration of metabolic health. Importantly, gestational diabetes increases the risk of developing T2D in

mothers after giving birth, and also increases the risk of metabolic disease in exposed children [149]. Overall, emerging epidemiological evidence supported by growing animal and *in vitro* studies now link PBDE exposure to diabetes, and raise the question of whether disparate exposures to this class of EDCs can be contributing to the unequal risk of T2D in the U.S.

Section 1.4: Linking Environmental Exposures to Diabetes Risk in Vulnerable Populations

Most studies examining links between EDCs and diabetes have done so without consideration of race, ethnicity, or SES; however, recently some reports have begun to interrogate these important interactions. In a cross-sectional study investigating the associations between phthalates and insulin resistance, there was an interaction with race demonstrating that Mexican-American (P=0.001) and non-Hispanic Black adolescents (P=0.002) had significant increments in HOMA-IR with higher levels of HMW phthalates or DEHP that were not observed in non-Hispanic Whites ($P \geq 0.74$) [150]. Similarly, in stratified models, HMW phthalates and DEHP were more strongly associated with HOMA-IR in adolescents from households with lower income. In another cross-sectional study, phthalate levels were positively associated with fasting blood glucose, fasting insulin, or HOMA-IR; however, the dose-response relationship was stronger among African-Americans and Mexican-Americans than Whites [151]. In the meta-analysis of Song and colleagues, the impact of PCBs on diabetes risk was higher in non-White populations (RR: 2.91; 95%CI: 1.60-5.30) compared to their White counterparts (RR: 1.94; 95%CI: 1.42-2.62); similarly, associations between OC pesticides and diabetes were also stronger in non-Whites (RR: 2.64; 95%CI: 1.56-4.49) than Whites (RR: 1.95; 95%CI: 1.40-2.71) [53]. While these associations are likely partially attributable to higher EDC exposures, these findings may also suggest that Blacks and Latinxs may also be more sensitive to the diabetogenic effects of environmental

contaminants due to potential synergy with other diabetes risk factors, including behavioral factors such as diet and exercise.

The current literature provides evidence that six classes of environmental toxicants are linked to diabetes risk in humans, and for each there is evidence that vulnerable populations are disproportionately exposed. The strength of epidemiological evidence for these six classes varies, with the most consistent findings observed for the persistent pollutants (PCBs and OCs). This likely reflects the long biological half-lives of these compounds, the stability of their quantitation, and the duration of time they have been studied. Among all six classes, however, there is provocative evidence of diabetes-promoting effects as well as disparities in exposure. Thus, while further study is required, the unequal burden of environmental risk factors in ethnically/racially, and economically-segregated neighborhoods of color may contribute to inter-ethnic differences in metabolic health.

Section 1.5: Origins of Differential Environmental Exposures

Addressing disparities in environmental health necessitates understanding the sociological forces that shape society. Segregation profoundly influences individual socioeconomic status, reinforces unhealthy neighborhood environments, and modifies individual behaviors [152], all of which influence metabolic disease susceptibility. Reduced access to affordable healthy foods, as seen in many African-American and Latinx neighborhoods, promotes unhealthy eating habits [77], while lack of safety and reduced access to green space can limit physical activity [153]. Thus, the built environment in many communities of color potentiates two key drivers of diabetes risk, namely diet and exercise.

In addition, the historic economic and political racialization of residential areas and the labor force promoted today's racial segregation and the co-decline of environmental health in these neighborhoods [154]. Indeed, living in highly segregated metropolitan areas is associated with greater health risk from industrial air pollution, with African-Americans at enhanced risk relative to non-Hispanic Whites [155]. Importantly, despite improvements in air quality over time, African-Americans remain exposed to significantly more air pollution than non-Hispanic Whites [156]. Accounts of industrial division of labor by race in major U.S. cities document how people of color were restricted to low-wage, hazardous occupations while simultaneously being confined to low-income housing near these industries [154]. Similar labor divisions also occurred in agriculture [107]. Grandfathering clauses allow older industrial facilities, often located in America's metropolitan centers, to opt out of stricter environmental regulations required of newer facilities, thereby clustering industrial toxins within these urban cores [155]. Suburbanization was accompanied by expansion and clustering of highways near and through neighborhoods of color [157], leading to higher traffic-related air pollution exposure amongst African-Americans and Latinos [55]. Shifted focus to suburban economic development with consequential disinvestment of inner city neighborhoods have perpetuated a legacy of environmental inequality [154]. The cumulative effects of these cultural forces enhance exposure to environmental toxicants among African-American, Latinx, and low-income communities; addressing this history is essential for eliminating disparities in metabolic health.

Section 1.6: Unanswered Questions and Objective of Dissertation

While racial/ethnic and socioeconomic disparities in numerous metabolic disease risk factors, including EDC exposures, have been extensively reported in the U.S. for decades, the

extent to which these exposure differences are programming disease risk *in utero* and contributing to the current disparities in metabolic disease is unknown. Additionally, the sex-specific manifestations of these developmental exposure disparities are not understood. Further, the impact that glucocorticoid receptor (GR) modulating EDCs have on the programing of metabolic disease in animal models and humans is understudied. Finally, recent advances in the field of epigenetics have allowed for increasingly refined explanations for the transgenerational of inheritance of metabolic outcomes triggered by environmental factors including developmental EDC exposures [158, 159]. The extent to which the history of social and environmental inequality in the U.S. has contributed to the current racial and socioeconomic disparities in metabolic health and disease is unknown. Understanding how adverse health outcomes are programmed *in utero* in animal models will lead to a better understanding of how EDC exposure disparities are contributing to the current decline in metabolic health.

The aim of this dissertation is to advance the current understanding of how gestational exposure to GR-modulating EDCs affect the metabolic health of exposed offspring. Discussion of metabolic outcomes following gestational pharmacological GC overexposures will put in context how exposures to GR-modulating EDCs can affect the metabolic health of exposed offspring. Sex-specific outcomes and potential mechanisms mediating these differences will be emphasized. Current knowledge about GR-modulating EDCs will be examined. Finally, the metabolic outcomes of perinatal exposure to the GR-modulating EDC tolylfluanid (TF) on exposed offspring will be discussed.

Chapter 2

Metabolic Misprogramming by Developmental Glucocorticoid Disruption

Section 2.1: General Overview of Glucocorticoids: Physiology, Receptor Biochemistry, and Gene Regulation

Section 2.1.1: Introduction to Glucocorticoid Physiology

EDCs that modulate GR signaling are an understudied class of environmental pollutants of likely public health significance given the numerous physiological outcomes that GCs regulate during development and throughout life, including the control of glucose homeostasis. Developmental GC overexposure results in sex differences in outcomes that lack fundamental mechanistic understanding; moreover, these differences have been understudied in EDC studies. This chapter will discuss the current understanding of GC action, the role that GCs and sex hormones play during fetal development, how pharmacological overexposures to GCs promote the development of metabolic disease with a focus on sex differences in outcomes, and the state of knowledge regarding GR-modulating EDCs. Finally, this chapter will discuss potential mechanisms by which developmental GR disruption results in sex specific outcomes.

GCs are life-sustaining steroid hormones that regulate physiological processes ranging from fetal development, metabolism, reproduction, circadian rhythmicity, inflammation, and behavior. GCs are synthesized in the zona fasciculata of the adrenal cortex under control of the Hypothalamic-Pituitary-Adrenal (HPA) axis (**Figure 2.1**). In response to HPA activators such as stress, the paraventricular nucleus of the hypothalamus releases Corticotropin-Releasing Hormone (CRH), which signals corticotropic cells in the anterior pituitary that produce Adrenocorticotropic hormone (ACTH) from pro-opiomelanocortin (POMC) to release ACTH. ACTH promotes the

synthesis of GCs by the adrenal cortex, namely cortisol in humans and corticosterone in rodents, which negatively feedback on the HPA axis.

GC bioavailability is regulated by several mechanisms, which directly influence GR binding and transcriptional activity. Circulating GC levels follow diurnal rhythms characterized by peak morning levels and low nighttime levels in humans, whereas rodents exhibit opposite patterns [160]. Most circulating GCs are bound to corticosterone-binding globulin (CBG) and albumin, which reduce circulating GC bioavailability. At the cellular level, GC availability is regulated by 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes. 11β-HSD1 favors the conversion of inactive GCs such as cortisone and 11-dehydrocorticosterone into active cortisol and corticosterone, whereas 11β-HSD2 favors the conversion of active GCs into their inactive forms [160]. Because GCs have high affinity to the mineralocorticoid receptor (MR), 11β-HSD2 is abundant in tissues where endogenous GCs would aberrantly induce MR action, such as the kidney.

GCs were named based on initial observations of their role in regulating blood glucose levels. As such, GCs are counterregulatory hormones that increase blood glucose levels in times of fasting or psychological stress by reducing peripheral insulin sensitivity, promoting lipid and amino acid mobilization, and inducing hepatic glucose output. Increased GC exposure during adult life through either pharmacological treatment, endogenous hypercortisolemia due to Cushing's Syndrome, and to a lesser extent, chronic psychological stress promotes the development of metabolic derangements characterized by hyperglycemia, hyperlipidemia, and visceral adiposity [161]. Increased GC exposure during fetal development has also been linked to adverse metabolic phenotypes later in life [162]. Thus, GCs are essential metabolic hormones that program and

maintain glucose homeostasis throughout the lifespan, making GR action a susceptible target for disruption by EDCs that could result in metabolic derangements.

Section 2.1.2: Glucocorticoid Receptor (GR) Biochemistry

GCs elicit their actions by binding to GR, a member of the nuclear receptor superfamily that is encoded by the NR3C1 gene. GR possesses an N-terminal domain, a central DNA binding domain (DBD), C-terminal ligand binding domain (LBD) and a flexible hinge region connecting the DBD and LBD that contributes to conformational changes upon ligand binding. GR activity is open to regulation by different posttranslational modifications including acetylation, phosphorylation, SUMOylation, and ubiquitination [163]. The N-terminal domain possesses the activated function (AF-1) transactivation domain which is needed for maximal transcriptional activity. The AF-1 domain from human GR contains 3 serine residues that can be phosphorylated (Ser-203, Ser-211, Ser-226); of these, pSer-211 is dependent on agonist activation, and promotes GR-transcriptional activation [164]. Further, human GR has been found to harbor at least three SUMOylation sites: K277 and K293 in the N-terminal domain, which promote gene repression, as well as K703 in the C-terminal ligand binding domain, which promotes GR activity [165, 166]. The LBD also possesses sequences that allow the receptor to dimerize and translocate to the nucleus, as well as a second transactivation AF-2 domain, which mediates interactions with coregulators after ligand binding [167].

The NR3C1 gene is comprised of nine exons that via alternative splicing give rise to several transcriptional isoforms: GRα, GRβ, GRγ, GR-A, and GR-P. Alternative translation initiation of each GR transcriptional isoform results in up to 40 potential GR isoforms with differences in tissue distribution and transcriptional activity [160]. GRα is the most highly studied isoform and will be

the focus of this chapter. GR is localized in the cytosol bound to several proteins including chaperones such as heat shock protein 90 (Hsp90), Hsp70, and immunophilins such as FKBP51 and FKBP52, which help maintain GR in a conformation with the highest ligand affinity [160]. Upon ligand binding, cytosolic GR changes conformation, dissociates from its chaperones, and translocates into the nucleus where it regulates gene expression. Overall, the structure and processing of GR is receptive to diverse regulatory signals that allow for the dynamic control of transcriptional activity leading to tissue-specific actions throughout development and life.

Section 2.1.3: Gene Regulation by the Glucocorticoid Receptor

Once bound to DNA, GR can promote the expression of extensive gene networks by consolidating the nucleation of coregulators and the general transcription machinery. GR binds to palindromic DNA sequences as homodimers, but may also bind as homotetramers [168]. GR monomers bind to half-site motifs in the genome at a ratio of around 5:1 of homodimer binding and can drive transcription, but the relative role of monomeric GR in gene expression is still being explored given the preference of pharmacological GCs to induce GR homodimerization [169]. GR-DNA binding depends on chromatin accessibility to glucocorticoid response elements (GREs) [170-172]. Available data indicate that GR binding patterns overlap less than 5% across different cell types [170, 173] suggesting that cell-specific differences in the chromatin landscape dictate differential gene expression by GR. While cell-specific DNA accessibility is established during cell differentiation, differences in the expression of chromatin remodeling factors that could be signaled to open or close genomic regions harboring GREs such as Activator Protein 1 (AP-1) [174] and Forkhead Box A1 (FoxA1) [175] can also contribute to tissue-specific differences in transcriptional regulation by GR.

Tissue-specific differences in the expression of other transcription factors and coregulators can also contribute to differential GR gene regulation. Through a mechanism termed "assisted loading," DNA-bound GR can increase accessibility to other transcription factors and promote gene transcription [176]. Likewise, other DNA-bound transcription factors can recruit GR through assisted loading [177]. GR has the ability to regulate genes that do not possess GREs through a mechanism called "tethering", where GR forms protein-protein interactions with DNA-bound transcription factors and contributes to the transcriptional complex [178]. DNA-bound GR can also tether other transcription factors in regions that don't harbor their respective DNA-binding regions and involve them in the transcriptional complex [177]. Notably, various transcription factors which have tissue-specific differences in relative expression and which show sex differences in activity, such as signal transducer and activator of transcription 5 (STAT5), can engage in tethering with GR and mediate gene expression [177]. Further, GR can cooperatively bind to DNA with other DNA-bound transcription factors and promote gene expression [177]. Tissue-specific expression and modulation of coregulators may also contribute to cell-specific differences in GR action [179]. GR-mediated gene repression is thought to happen by upregulation of gene products that antagonize other gene endpoints, by binding or tethering to other transcription factors and inhibiting their activity, or by GR binding to negative glucocorticoid response elements [180-182]. The regulation of gene expression by GR depends on numerous tissue-specific elements that can be modulated by factors including developmental stage, hormones, and other environmental influences. Comprehensively understanding how GR activity is modulated in a tissue-specific manner by EDCs during fetal development can clarify the nature behind tissue-specific phenotypes that are sex-dependent, which still lack a mechanistic understanding.

Section 2.2: Estrogens, Androgens, and Glucocorticoids During Fetal Development

Section 2.2.1: Conceptualizing Developmental Misprogramming as a Multi-Hormone Issue

Numerous hormones including estrogens, androgens, and glucocorticoids control fetal development; however, the ways in which these three hormone classes cooperate to promote development are not completely understood. Importantly, these three hormone classes are known to crosstalk during adulthood **(Section 2.4.2-2.4.3),** suggesting that hormone crosstalk may also happen during fetal development. Differences in the levels of testosterone (T) between male and female fetuses promote sex-differentiation and masculinization of reproductive organs [183], and recent evidence suggests that sex-differences in the development of key metabolic tissues may also happen during early development [184, 185]. Whether disrupting GC signaling during the establishment of sex-differences of key metabolic tissues results in sex-specific outcomes remains to be studied. In support of this idea, developmental GC overexposure leads to sex differences in later-life outcomes (**Table 2.1**), suggesting that aberrant GR activation during development can interact with AR and/or ER signaling and result in sex-specific organizational differences leading to adverse outcomes later-in-life. This section will introduce the developmental roles and dynamics of estrogens, androgens, and glucocorticoids to better outline susceptible windows of malprogramming during fetal development and better understand how disruption of these hormone pathways can potentially lead to sex-specific outcomes.

Section 2.2.2: Estrogens During Fetal Development

Early in pregnancy estrogens are synthesized by the corpus luteum in the maternal ovaries, but by the eighth week of gestation (in primates), the placenta becomes the primary source of estrogens. The rodent placenta has minimal steroidogenic activity and depends on ovarian estrogen

synthesis throughout gestation [186]. In primates, the placenta cannot synthesize C19 steroids from pregnenolone or progesterone since it does not express 17α-hydroxylase/17:20-lyase (P450c17), and thus depends on the developing fetal adrenal gland for androgen precursors such as dehydroepiandrosterone (DHEA) and DHEA-S for placental estrogen synthesis [187]. This increased estrogen during gestation regulates fetal exposure to maternal GCs by upregulating placental 11-βHSD2, which converts active cortisol and corticosterone into inactive cortisone and 11-dehydrocorticosterone [188]. By limiting the elevated maternal GCs from entering fetal circulation, which would otherwise inhibit ACTH release by the fetal pituitary, fetal ACTH remains high and stimulates DHEA secretion from the fetal adrenal gland to continue providing substrate to drive increasing estrogen production in primates [187]. This estrogen-mediated increase in fetal ACTH levels promotes the maturation of the transitional zone of the fetal adrenal gland, which becomes a key source of fetal GCs later in gestation [187]. As fetal glucocorticoids rise, they stimulate placental CRH secretion (as opposed to their inhibitory effects on the hypothalamus), resulting in a positive feedback loop that further promotes GC secretion from the fetal adrenal gland towards the end of gestation [187].

The described increase in circulating estrogens in the mother ensure a healthy pregnancy and postpartum period through direct actions on maternal physiology and by regulating other hormones [189, 190]. Estrogens also elicit a variety of actions on the fetus that includes sexual differentiation and the development of reproductive tissues and the brain [191-193], but also likely regulate the development of non-reproductive organs important for maintaining glucose homeostasis [184, 194, 195]. Importantly, while T levels are markedly higher in developing male fetuses, tissue-specific aromatization of androgens to estrogens is common during development, and known to contribute to sex-specific differences in developmental outcomes. For example,

levels of estradiol (E2), estrogen receptors, and aromatase activity are at their highest in the brain prenatally and in the first postnatal days before declining to adult levels [191]. Whether such tissue-specific conversion happens in developing key metabolic tissues is possible, but needs further study. Interestingly, inhibition of ER activity during fetal development by inhibiting aromatase in baboons results in insulin resistance later in life [196, 197], while prenatal exposure to ER disrupting EDCs leads to adverse metabolic outcomes in mice [198]. Whether indirect disruption of ER signaling through GR disruption contributes to adverse metabolic programming is possible and should be formally tested.

Section 2.2.3: Androgens During Fetal Development

Fetal androgens are best known for promoting sexual differentiation and masculinization of reproductive organs; however, androgens likely mediate sex differences in the development of key metabolic tissues as well [184]. Fetal T levels in humans and rats are regulated differently and follow somewhat different circulating patterns during pregnancy [183]. In humans, male fetal Leydig cells start to produce T at about 8 weeks of gestation [199], and levels peak during the second trimester, from 11-14 weeks of gestation [200-202] as measured in fetal blood and the testis. Notably, male serum T levels are between 3- to 8-fold higher than in female fetuses between gestation week 12-20 [203, 204]. In the fetal male rat, testicular testosterone production begins at around gestational day 14.5-15.5 [205, 206]. T levels in the rat peak near term at around gestation day 16.5-17.5 if measured in the testis and blood, [207, 208] or gestation day 18.5-19.5 when measuring whole body T levels [209]. Further, whole body T levels in rats are higher in males in comparison to female levels, but only on gestational days 18.5 and 19.5 [209].

Notably, prenatal overexposure to androgens leads to metabolic perturbations in female offspring characterized by increased adiposity, impairments in insulin secretion, and/or insulin resistance in mice, rats, sheep, and monkeys [210-214]. Whether these perturbations result from direct organizational programming of key metabolic tissues through AR signaling, or indirectly, by altering the development of reproductive, or neuronal endpoints that increase susceptibility to metabolic disease by altering sex hormone levels during adulthood or eating behavior need further study. Nonetheless, aberrant androgen action during fetal development is implicated in the misprogramming of metabolism, that may be sex specific. Critically, overexposure to GCs during pregnancy leads to outcomes indicative of alterations in fetal androgen levels and action. For example, dexamethasone (DEX) treatment during the last week of gestation in rats lead to altered anogenital distance [215]. Further, maternal corticosterone use during pregnancy was associated with increased risk of hypospadias [216]. Whether aberrant GR signaling during fetal development promotes the misprogramming of key metabolic tissues by directly altering AR signaling or T levels is a possibility that should be further studied. Further, the extent by which T overexposure leads to metabolic misprogramming by disrupting GR signaling during development remains unknown.

Section 2.2.4: Glucocorticoids During Fetal Development

Systemic GC production in humans and rodents is enhanced during pregnancy [217]. Circulating maternal CRH levels increase up to one thousand times their nonpregnant concentrations [218, 219] due to increased CRH production from the placenta, decidua, and fetal membranes [220-222]. While CRH regulates several physiological functions during pregnancy, including fetal adrenal steroidogenesis, maintenance of fetoplacental circulation, and onset of

parturition [223], CRH also increases maternal ACTH and results in a two- to three-fold increase in total maternal circulating cortisol in humans that can reach levels observed during Cushing's Syndrome [224]. Estrogens upregulate hepatic CBG production in the mother [225], which bind and reduce GC bioavailability, but nonetheless, free GC levels increase throughout pregnancy [226]. Interestingly, GC sensitivity decreases in certain maternal tissues, such as the liver, potentially to protect against adverse metabolic effects that would otherwise result from the Cushing-like state of pregnancy. Restoration of hepatic GC sensitivity in the pregnant mouse impairs pregnancy-induced hepatomegaly and ultimately leads to IUGR, suggesting that tissue-specific changes in maternal GCs sensitivity is essential for healthy fetal development [227]. Proposed mechanisms explaining the lack of negative feedback by the higher GC levels on the HPA axis are enhanced pituitary response to CRH and decreased pituitary sensitivity to GC negative feedback [223].

GCs are important regulators of fetal development that optimize offspring fitness [228]. Throughout gestation fetal GCs serve as signals of resource availability that support fetal survival in response to drops of essential nutritional substrates during development such as glucose and oxygen, and prioritize substrate availability for key tissues at the expense of others [229]. The rise in GCs towards the end of gestation promotes the developmental transition from tissue accretion to differentiation in multiple tissues [162]. These GC-mediated physiological changes are driven by the upregulation of enzymes in multiple tissues that sustain life, including the lungs, liver, adipose tissue, and the gastrointestinal tract [162]. For example, GCs upregulate genes needed to produce glycogen, as well as genes necessary for gluconeogenesis, including phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pc) [162]. Thus, throughout pregnancy, carefully regulated GC levels and their tissue specific actions in the mother and fetus are essential

for controlling a healthy developmental trajectory that will optimize offspring fitness after birth. Disrupting GC action during fetal development can therefore have life-lasting physiological impacts on the offspring that can increase disease risk.

Section 2.3: Metabolic Outcomes Following Developmental Overexposure to Glucocorticoids

Section 2.3.1: Introduction

Gestational exposure to excess GCs leads to adverse outcomes in the offspring later-in-life, including alterations in lipid and glucose homeostasis. Prenatal treatment with pharmacological GCs administered to augment lung development in preterm births decreases birth weight, a known cardiometabolic disease risk factor [230], and can increase the individual's risk of insulin resistance during adulthood [231]. Moreover, animal models of *in utero* GC excess, such as pharmacological inhibitors of placental 11β-HSD2 and chronic maternal psychological stress, as well as other indirect models such as maternal protein restriction and hypoxia have all shown metabolic defects in the offspring [232-236]. This section will focus on studies that have treated dams with known doses of pharmacological GCs such as betamethasone or DEX (**Table 2.1**), which bypass 11β-HSD2 deactivation and readily cross the placenta and expose the developing fetus.

Section 2.3.2: Adverse Metabolic Outcomes Following *In Utero* Pharmacological Glucocorticoid Exposure

While prenatal GC exposures lead to varying effects on glucose homeostasis in offspring, most studies have shown adverse metabolic outcomes characterized by either impaired glucose tolerance or decreased insulin sensitivity (**Table 2.1**). Interestingly, the most commonly affected

organ from these studies is the liver, which exhibits related metabolic derangements such as lipid accumulation, reduced sensitivity to insulin, and upregulated expression of gluconeogenic genes such as PEPCK with resulting increased hepatic glucose output. Other important metabolic tissues such as adipose and the pancreatic β-cells have also shown functional and structural abnormalities following prenatal DEX exposure (**Table 2.1**). Pancreatic β-cells are reduced following prenatal GC overexposure, with some studies reporting decreased glucose-stimulated insulin secretion during glucose tolerance test [237-239]. Glucocorticoid misprogramming of β-cell development and function can increase diabetes risk later in life by decreasing the β-cell pool that the body will depend on to stay at euglycemia during adulthood, especially since most of the lifelong β-cell population from which adult β-cells will replicate from is established early in life [240, 241]. Gestational DEX exposure reduced epididymal and/or total visceral adiposity in several studies [242-244]. Two studies found no differences in adiposity, likely because the choice of adipose depot (retroperitoneal) [245], or because adiposity was assessed at weaning [238] when fat mass is minimal. Critically, the reduction or no change in adiposity from these studies suggest that developmental exposures can result in adiposity phenotypes different from those observed in adults with glucose intolerance and insulin resistance, which are usually characterized by increased adiposity. Gestational GC overexposure could potentially inhibit adipose development and consequent expansion during adulthood, which can promote increased hepatic lipid storage and decrease liver insulin sensitivity, as has been observed in several studies (**Table 2.1**). In support of this point, nutrient excess during adulthood leads to worse liver steatosis in rats prenatally exposed to DEX [245-248]. Finally, several studies have found that DEX increases circulating GC levels [249, 250], and can upregulate GR levels in adipose and liver [242, 251], all of which promote hepatic glucose output. Overall, glucose intolerance and insulin resistance are common

metabolic endpoints resulting from developmental GC overexposure in animal models. While the mechanisms leading to these adverse outcomes warrant more work, evidence from current studies show that metabolic misprogramming results from structural and functional defects at multiple tissues.

Section 2.3.3: Potential Mechanisms by Which Developmental Glucocorticoid Overexposure Results in Adverse Metabolic Outcomes

We still lack a mechanistic understanding of how early life overexposures to GC result in adverse metabolic outcomes later in life such as glucose intolerance and insulin resistance, but evidence from available studies suggest that these effects are mediated through multiple tissues and may be programmed epigenetically [252]. Maternal and fetal GCs are critical for the development of key metabolic tissues, including pancreatic β-cells [253], adipose tissue [254], and the liver [255]. All three of these tissues have been shown to be adversely affected by prenatal DEX exposures (**Table 2.1);** however, the mechanisms by which GCs misprogram these tissues are likely tissue-specific and dependent on the developmental period of exposure. For example, GCs suppress β-cell development and promote acinar cell development [253]; thus prenatal GC excess can lead to the observed decreased pancreatic β-cell content by suppressing β-cell development. Fetal overexposure to GCs results in a lifelong increase in hepatic expression of the rate-limiting enzyme of gluconeogenesis, PEPCK (**Table 2.1**), which is implicated in the pathogenesis of type 2 diabetes and its complications. PEPCK can also be upregulated by multiple additional factors that are similarly altered following prenatal GC overexposure, such as hepatocyte nuclear factor 4 (HNF4) [256], GR [251], and reduced insulin sensitivity, which is commonly observed after gestational DEX exposure. Whether PEPCK, GR and/or HNF4 are

epigenetically programmed after gestational GC overexposure remain to be tested. Evidence for epigenetic malprogramming some of these factors exists in other contexts. For example, low maternal grooming in rats resulted in a higher stress response in the offspring, which was attributed to lower hippocampal GR expression driven by lower GR promoter accessibility, which blunted HPA-axis negative feedback [257]. Further, paternal stress resulted in increased hepatic PEPCK expression in the offspring, which was associated with the epigenetic silencing of a micro-RNA that negatively regulates PEPCK [258]. Prenatal exposure to excess pharmacological GCs may alter the hepatic chromatin architecture during fetal development in such a way that directly promotes the increased expression of PEPCK, or modulates the expression of PEPCK-regulating genes. Unfortunately, epigenetic programming of metabolic phenotypes following prenatal GC overexposure through pharmacological exposures or EDC exposures remains relatively understudied. Future studies are warranted to test at how GC overexposure affects fetal organ development to better understand the mechanisms by which GC program metabolic disease risk later in life.

Section 2.3.4: Sex Differences in Metabolic Outcomes Following Developmental Glucocorticoid Overexposure

Sex differences in HPA axis function, behavior, and neurological endpoints following gestational GC overexposure are established [259-261]; however, few studies have reported studying how prenatal pharmacological GC overexposure affects the metabolic health of both male and female offspring. Out of the 15 studies included in **Table 2.1** that have studied metabolic outcomes following pharmacological GC exposure during gestation, only four studies have presented results from male and female offspring. Importantly, every one of these studies found

sex differences in metabolic outcomes. Interestingly, the phenotypes and sex differences observed varied by study, and ranged from changes in hepatic PEPCK expression, steatosis, adiposity, insulin sensitivity, glucose tolerance, and circulating corticosterone. Sex differences in these studies were characterized by only one sex having an adverse metabolic phenotype, whereas the other sex was unaffected, even though both males and females were exposed to DEX during gestation. There was no clear trend suggesting that either females or males were more susceptible to metabolic misprogramming by gestational GC overexposure, since depending on the study, both sexes experienced adverse metabolic outcomes. While the mechanisms behind the observed sex differences were not elucidated, one study attributed suppressed growth hormone (GH) axis as a potential mediator of liver steatosis in DEX-exposed female offspring that was not observed in male offspring [246]. Thus, a thorough understanding of potential mediators of sex differences in metabolic outcomes following gestational GC overexposures is warranted to truly understand how environmental factors that disrupt GC signaling during development are implicated in today's metabolic disease pandemic.

Section 2.4: Potential Mechanisms Explaining Sex Differences in Metabolic Outcomes

Section 2.4.1: Placental Mediated Sex Differences

The placenta is a transient organ that sustains pregnancy and promotes fetal growth by acting as the fetal-maternal interface mediating the exchange of metabolic substrates, waste, and hormones, while also synthesizing hormones necessary for development and parturition, and serving as a barrier limiting environmental contaminants from entering fetal circulation [262]. The placenta originates from the embryo, resulting in sex differences in numerous placental aspects including formation, function, and response to environmental factors [263]. As such, sex-specific

outcomes in offspring prenatally subjected to nutritional, pharmacological, hormonal, and chemical insults have been attributed to the placenta by mechanisms that remain unclear [262, 264].

Importantly, sex differences in placental outcomes following prenatal GC overexposures have been extensively reported in humans and mice [264] despite known differences in placental morphology and function across these species [186], emphasizing the pervasiveness of sex differences in placental GC response. For example, a single dose of DEX in spiny mice led to latent sex-specific differences in structure and gene expression in the placenta, which included differential regulation of the primary placental glucose transporter SLC2A1 [265-267]. Critically, differential expression of nutrient transporters by GC overexposure can potentially result in adverse metabolic outcomes by limiting essential nutrient availability in a sex-specific manner, however, this hypothesis needs to be tested. These different outcomes in glucose transporter differences were not attributed to differences in placental GR expression by sex [265]; however, different GR isoforms were not assessed. Notably, human placentas express several GR isoforms that are differentially expressed according to sex [268, 269], which may contribute to differences in placental and fetal response to GCs during development. As such, prenatal DEX exposure in mice altered placental GR isoform expression in a sex-dependent manner [270]. Interestingly, pharmacological GC exposure during gestation has led to increased placental 11β-HSD2 levels in female placentas in both humans and mice [271, 272], suggesting that differential GC-induced changes in placental GC metabolism might contribute to sex-specific outcomes. Further, placental response to GC treatment in asthmatic mothers have been reported to exhibit sex differences [273-275]. Thus, while mechanisms are lacking, evidence showing distinct placental responses to GCs

suggests that the placenta can also respond in a sex-specific manner to GR-modulating EDCs, and may result in different metabolic outcomes between male female offspring.

Section 2.4.2: Glucocorticoid Receptor and Estrogen Receptor Crosstalk

Crosstalk between GR and ERα has been established in numerous cell types from different species including humans, rats, and mice; this underscores the physiological importance of this hormonal interplay. ER and GR have been shown to affect each other's action both by affecting receptor and ligand availability, and by altering genomic transcriptional binding and activity. The bulk of the evidence describing the nature of GR/ER crosstalk described here is based on breast cancer cell models, uterine tissue, and the liver; however, GR/ER crosstalk in different brain regions has also been reported [276, 277]. This section will describe what is known about GR and ER crosstalk to place into context how a common hormone transcriptional mechanism of communication that is currently not studied during fetal development can lead to a better understanding of developmental misprogramming by EDCs that alter either GR or ER signaling.

The significance of GR and ER crosstalk is evident in breast cancer given that GR expression in ERα-positive tumors is associated with better clinical outcomes, and worse outcomes in triple negative breast cancer [278, 279]. In the ERα positive breast cancer cell line MCF-7, along with mouse livers, and human hepatocytes, GCs can inhibit E2 from binding to ER by upregulating estrogen sulfotransferase and inducing estrogen sulfation [280]. Likewise, E2 promotes the proteasomal degradation of GR in MCF-7 cells by upregulating p53 and the E3 ubiquitin ligase Mdm2 [281]. E2 can also reduce GR activity by reducing GR Ser-211 phosphorylation by upregulating protein phosphatase 5 (PP5) [282]. Apart from crosstalk at the ligand and receptor level of action, GR and ER influence each other's binding to chromatin and

consequent control of gene expression. As such, a large overlap of DNA binding sites for ER, AR, and GR have been identified in male breast tumors [283]. DEX can inhibit E2-mediated proliferation and downregulated ERα target gene expression by promoting GR recruitment to ERα-binding regions, causing the destabilization of ERα transcriptional complex [284]. The observed direct interaction between GR and ERα was mediated through the GR DBD, and the binding of GR to estrogen binding sites was mediated by AP-1 and the pioneer factor FoxA1 [284], suggesting that GR binding to estrogen binding sites can happen in numerous tissues given widespread expression of FoxA1 and AP-1. Further, ligand-bound SUMOylated GR can repress ER-activated genes by inhibiting the recruitment of the mega transcription factor complex (MegaTrans) to ER-alpha-bound enhancers [285].

DEX has the ability to both inhibit and synergize ERα target gene expression, indicating that the transcriptional endpoint of GR-ERα crosstalk is gene-dependent [286]. Further, in this model, the co-regulator interaction domain of the ERα LBD was necessary for the co-recruitment of GR to the estrogen response element (ERE)-rich array, suggesting that coregulator proteins also contribute to GR/ER crosstalk [286]. Interestingly, the extent of ER and GR crosstalk goes beyond altering known genes regulated by each hormone receptor. For example, DEX and E2 co-treatment resulted in the abrogation of GR and ER genomic binding at a small number of genomic sites, but gave rise to more binding sites previously not measured in the absence of the co-treatment in mouse mammary epithelial cell lines. This induction of new chromatin binding resulted from ER and GR increasing chromatin accessibility for each other at assisted binding sites, and interestingly, ER-assisted loading of GR happened in the absence of EREs in these genomic sites, and were actually dependent on “assisted loading” by AP-1 [287]. Another study found similar results in in MCF7

cells, where ER and GR co-activation promoted GR chromatin association with ER and AP-1 response elements, as well as with FoxO response elements [288].

In addition to crosstalk in breast cancer, GCs are known to antagonize uterotrophic estrogen action [289-291]. DEX decreases estrogen-stimulated insulin-like growth factor 1 (IGF-1) gene expression [292] and inhibits the proinflammatory and bactericidal activity of E2 in the rat uterus [293, 294]. E2 can prevent GR from binding gene promoters and consequently inhibit gene expression by promoting ER binding to GREs and decreasing polymerase 2 occupancy [295] as well as reducing recruitment of pioneer factors FoxA1/2 to GREs [296]. In single human uterine leiomyoma and myometrium cell types, about 97% of the examined genes that were simultaneously regulated by DEX and E2 had similar expression patterns, while few genes were identified as antagonistically regulated by DEX and E2 [297]. Likewise, co-treatment of DEX and E2 in the human uterine endometrial cancer cell line ECC1 resulted in only 5.2% of the co-regulated genes antagonistically regulated [298]. For these antagonistically regulated genes, GREs were shown to be present in 80% of the promoters, while EREs were only present in 45% of the antagonistically regulated promoters, suggesting that GR binding to GREs was a more common way of estrogen antagonism. In the human endometrial adenocarcinoma Ishikawa cell line, DEX and E2 co-treatment resulted in a transcriptional profile that was most similar to that of E2 [299]. Co-treatment of DEX and E2 dramatically increased shared GR and ER genomic binding sites to 46.5%, with most of these sites being new binding sites for GR, but not for ER, suggesting that ER enables GR to bind new genomic loci [299]. Contrary to this study, simultaneous activation of GR and ER resulted in the most differentially expressed genes and were unique to the DEX and E2 co-exposure condition [298] as observed in mammary epithelial cells [287], suggesting that the unique transcriptional profiles resulting from simultaneous GR and ER activation may be a method

of controlling cell function common to other tissues. Thus, depending on the uterine cell line, E2 and DEX co-treatments can lead to similar genomic binding patterns and consequent similar regulation of gene expression, or can result in unique regulation of gene expression. The implications of this cell type specific ER and GR crosstalk for fetal development remains to be studied.

Section 2.4.3: Glucocorticoid Receptor and Androgen Receptor Crosstalk

The DBD of the androgen receptor (AR) and GR have a high degree of amino acid sequence similarity, including a conserved P Box, which allow them to bind similar and even identical hormone response elements [300-302], but GR is unable to bind selective androgen response elements (AREs) [301, 303]. The functional overlap between AR and GR is evident in castration resistant prostate cancer, where GR activity regulates a different yet considerably overlapping transcriptome that renders AR inhibition therapy ineffective [304]. As such, about one half of the AR binding regions overlap GR binding regions in antiandrogen-resistant xenograft tumors and GR-expressing LNCaP-1F5 cells [304, 305]. The presence of ligand-bound AR also influences genomic GR binding activity; liganded GR can antagonize AR transcription in the presence of androgens, but GR can promote AR transcriptional endpoints in the absence of androgens [302, 305].

Depending on the DNA binding sequence, both AR and GR can either promote transcription, or interfere with transcriptional activity [306, 307]. The ability of GR to inhibit AR transcription is probably not due to competition of DNA binding, since DEX and DHT co-treatment actually results in increased AR chromatin binding, likely through assisted loading [305]. One possible way by which AR and GR inhibit each other's transcriptional activity at certain

genes is by forming heterodimers at GREs [308]. Further, SRC-1, the coactivator for several steroid hormone receptors including GR can actually inhibit AR transactivation, suggesting that coactivators for each hormone receptor can also play a role in crosstalk between GR and AR [309]. Additionally, the weak androgen DHEA can upregulate and preferentially direct splicing of GR mRNA towards the β isoform, which is known to inhibit the expression of some GRα-regulated genes, providing another possible mechanism of AR mediated GR antagonism [310]. Apart from genomic crosstalk, AR signaling suppresses GR gene expression in prostate cancer [311], however, whether AR suppresses GR expression in other tissues needs to be tested. The anabolic steroid oxandrolone antagonized GR transactivation in an *in vitro* monkey kidney CV-1 cell luciferase model without affecting cortisol binding to GR [312]. This effect was AR-dependent, and interestingly, the DDT metabolite DDE, which is a known anti-androgenic EDC, also suppressed GR transactivation, suggesting AR modulating EDCs can affect GR endpoints as well.

Recent evidence shows that crosstalk between AR and GR also happens in metabolic tissues such as the pancreatic β-cells, adipose tissue, and the liver. One study suggests that AR can decrease DEX-induced β-cell apoptosis in an INS-1 model [313]. GR was shown to upregulate AR expression and promote nuclear AR translocation during adipogenesis in human pre-adipocytes, while concurrently decreasing AR transcriptional activity [185]. While the purpose for upregulating AR while suppressing its activity remains unclear, it is possible that GR upregulates AR to promote GR chromatin binding through assisted loading, however, this hypothesis needs to be formally tested. GCs have been shown to deactivate androgens during adipogenesis in human preadipocytes [314]. Likewise, T can upregulate 11β-HSD1 in omental adipose tissue in children [315], suggesting that AR crosstalk with GR can also be mediated by altering tissue availability to GCs. Finally, DHT and corticosterone co-treatment in white and brown adipose resulted in

synergistic upregulation of GR-dependent genes that were not upregulated with DHT alone, while AR antagonism decreased GR transcriptional activity in adipose and the liver [316]. Overall, crosstalk between GR and AR mediates several physiological outcomes during adulthood including the regulation of tissues important for regulating glucose and lipid homeostasis in humans and rodents. Given that the basis of AR/GR crosstalk is largely dependent on similarities in DBDs, and T levels during development are higher in male fetuses, it is likely that GR/AR crosstalk during fetal development may contribute to sex differences in the development of key metabolic tissues. Thus, disruption of either GR or AR by EDC exposures could potentially lead to sex-differences in metabolic misprogramming.

Section 2.5: Glucocorticoid Receptor Signaling Modulating EDCs

An increasing body of evidence suggests that a wide variety of synthetic and plant-made chemicals have the ability to disrupt GR signaling. Some of the most prevalent of these EDCs include genistein [317] which is consumed from soy, arsenic [318], which is found in groundwater, rice, and industrial pollution, and methylsulfonyl PCBs [40], which are metabolites of legacy contaminants found throughout the environment. Recent high throughput studies using human GR reporter assays have identified numerous pesticides [319], organophosphate flame retardants [320], and metals [321] that antagonized GR in their cell models. Several of the chemicals tested in these studies had previously shown to disrupt other hormone receptors, emphasizing the complexity of endocrine disruption by synthetic chemicals and the need to test EDC mixtures for additive, synergistic, and opposing activity in cellular and physiological contexts. One study found that POP mixtures did not affect GR activity in their cell based reporter assay, but found GR antagonism by the pesticide DDE, and enhancement of cortisol-induced transcriptional activity by

the flame retardant BDE-47 [322]. Notably, exposure to both of these EDCs have been shown to be consistently higher in Mexican-Americans, African-Americans and people with lower income [323, 324]. In addition to single chemical screens, ecotoxicology studies have found widespread GC activity from water samples from numerous U.S. states [325, 326]. The chemical constituents in these water samples responsible for inducing GR activity were not characterized; however, over-the-counter pharmacological GCs and GR-modulating EDCs are likely contributors. Further, a handful of studies have also shown that several synthetic chemicals have the ability to disrupt GC synthesis in human adrenocortical carcinoma cells [327, 328], suggesting that GC action can also be affected by disruption of GC synthesis in addition to disrupting GR signaling. Out of the few studies that have tested GR modulating EDCs for adverse metabolic health outcomes, TF has been extensively studied in adipocytes and adult mice, and was shown to promote cellular insulin resistance [329], as well as systemic metabolic dysfunction characterized by glucose intolerance and insulin resistance [330]. However, no study had previously tested whether a well characterized GR-modulating EDC known to elicit metabolic defects in cells and live animals could affect the metabolic health of animals exposed throughout fetal development and lactation. Given the widespread prevalence of GR-modulating EDCs, studies are warranted to examine how exposure to this class of EDCs misprogram metabolism in animal models to better understand their effects on people.

Chapter 3

Developmental Exposure to the Endocrine Disruptor Tolylfluanid Induces Sex-Specific Later-Life Metabolic Dysfunction

Sections from this chapter have been adapted verbatim from:

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Section 3.1: Introduction

Sex differences in metabolic disease prevalence are characterized by higher diabetes rates in males and higher obesity rates in females that are attributed to differences in physiology and environmental interactions [331, 332]. An expanding body of epidemiological and animal studies suggests that developmental exposures to endocrine disrupting chemicals (EDCs) can lead to sex-specific, adverse effects on metabolic physiology [10, 333]. Sex-specific differences in metabolic outcomes are known to occur after developmental exposure to other environmental stressors, including both overnutrition and undernutrition [331]; however, the mechanisms underlining these sex-specific outcomes remain poorly understood. Assessing alterations in whole-body glucose-regulating physiological parameters is essential to subsequently mechanistically delineate how EDC exposures misprogram metabolism and increase disease risk in a sex-specific manner. The urgency to address this data gap is heightened by the fact that nearly 10% of the U.S. population has diabetes [1], and an estimated 629 million individuals across the globe are projected to have the disease by 2045 [334].

While tens of thousands of chemicals lack basic endocrine toxicological screening [12], 800-1000 compounds have already been identified as putative EDCs [11]. Among these EDCs that modulate glucocorticoid receptor (GR) signaling remain understudied, and little is known about the long-term consequences of early-life exposure to GR-modulating EDCs despite the critical role that maternal and fetal glucocorticoids play in the development of key metabolic tissues, including pancreatic β-cells [253], adipose tissue [254], and liver [255]. In humans, prenatal treatment with pharmacological glucocorticoids administered to augment lung development during preterm births has been shown to decrease birth weight [230] and may lower insulin sensitivity during adulthood, with some evidence of more pronounced effects in women [231]. Multiple animal studies have shown that dexamethasone (DEX) treatment during the last week of gestation promotes later-life metabolic defects, including insulin resistance and the upregulation of the hepatic gluconeogenic machinery [238, 249, 251]. While the prenatal programming of metabolic health by pharmacological glucocorticoids has been extensively studied in a variety of animals [162, 335], large data gaps exist regarding the later-life metabolic consequences of exposure to GR-active chemicals with lower GR affinity. Given that at least 34 putative human GR-modulating pesticides have been identified [319] and that relatively high glucocorticoid activity has been detected in U.S. surface waters [326], it is essential to understand the impacts of developmental exposure to GR-modulating EDCs on metabolic physiology and long-term metabolic disease risk.

Tolylfluanid (TF) is a phenylsulfamide fungicide used in agriculture and as a booster biocide in marine paints [330]. TF has been found on agricultural goods in Europe [336-338], where it has also been detected in groundwater in agricultural regions [339]. While not approved for use in the United States, TF is permitted on foods imported into the U.S. Previous studies have shown that TF activates GR signaling in adipocytes, with consequential induction of cellular

insulin resistance [329, 340, 341]. Adult mice exposed to TF near the maximum U.S. tolerance limit for imported foods exhibit weight gain, glucose intolerance, insulin resistance, and disrupted circadian rhythms [330]. While the impact of dietary TF on energy homeostasis remains controversial [342], data suggest that the precise physiological effects may be nutrient-dependent [343, 344]. The present study sought to expand upon these data to ascertain the sex-specific physiological effects of perinatal exposure to TF on later-life metabolic health.

Section 3.2: Materials and Methods

Section 3.2.1: Animals, TF exposure, and tissue processing

Eight-week old C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed at 22.2 ± 1.1°C under a 12:12-hour light-dark cycle. Mating cages were arranged with one male and two females per cage. Control mating cages received ad libitum access to a standard rodent chow (Teklad Global Diet 2918, Harlan Laboratories, Madison, WI), while TF cages received the identical diet supplemented with 100 mg/kg TF added at the time of manufacturing (Harlan Laboratories, Madison, WI); diet preparation led to a final TF concentration of 67 mg/kg. This dose was shown to increase adiposity and lower insulin sensitivity in adult male mice in previous studies [330]. Upon pregnancy confirmation by vaginal plug formation, dams were housed singly and continued on their respective diet throughout gestation and lactation until postnatal day (PD) 21. Initial breeding was performed with a 1:1.5 control:TF ratio to account for potential unknown effects of TF on fertility. The offspring analyzed in this study came from four distinct cohorts that included 29 control pregnancies and 42 TF pregnancies. Litter size and litter sex ratios were assessed at weaning. None of the litters were culled, and metabolic phenotyping to assess glucose tolerance or insulin sensitivity was performed in a

randomly selected subset of the offspring. Offspring were housed by treatment and sex in groups of 2-3 per cage. Offspring were handled and weighed weekly after weaning, and food was weighed and replaced weekly. All animals were treated humanely in accordance with protocols approved by the Institutional Animal Care and Use Committees at the University of Chicago and the University of Illinois at Chicago. Dams were euthanized after weaning by CO_2 asphyxiation followed by cervical dislocation. Offspring were euthanized by isoflurane intoxication followed by exsanguination via cardiac puncture. Relevant metabolic tissues were dissected, weighed, flash frozen in liquid nitrogen, and stored at -80°C prior to processing. The study timeline is summarized in **Figure 3.1**.

Section 3.2.2: Intraperitoneal glucose tolerance test (IP-GTT)

At postnatal week (PW) 10, mice were fasted for 6 hours beginning at 7:30 am. Fasting blood glucose was measured using a Freestyle Lite glucometer (Abbott Laboratories, Abbott Park, IL) by tail vein sampling. Mice then received an intraperitoneal (IP) injection of dextrose (2 g/kg body weight), and blood glucose concentrations were measured serially for 120 minutes. Blood samples for insulin levels were obtained using heparinized tubes at 0, 10, 30, and 60 minutes post IP injection, placed immediately on ice, and centrifuged at 1500 *g* for 15 minutes at 4°C. Plasma insulin concentrations were determined using the ALPCO mouse insulin ELISA kit per the manufacturer's instructions (ALPCO, Salem, NH). The sensitivity of the insulin ELISA was determined by the manufacturer to be 0.115 ng/mL for the 5 μL sample size, and the intra-assay coefficient of variation was determined to be between 4.53-9.30%. Homeostatic model assessment of insulin resistance (IR) and β-cell function (HOMA-IR and HOMA-β, respectively) were calculated using fasting blood glucose and fasting plasma insulin levels as previously described

[345].

Section 3.2.3: Intraperitoneal insulin tolerance test (IP-ITT)

At Postnatal Week (PW) 16 (PW16), mice were fasted at 9:00 am for 3 hours. Fasting blood glucose was measured, followed by IP injection of Humalog insulin (Eli Lilly, Indianapolis, IN) (0.4 U/kg body weight for females, 0.5 U/kg body weight for males). Mice with blood glucose readings that dropped below the limits of detection for the glucometer (20 mg/dL per the manufacturer) were confirmed on repeat testing, and if confirmed, hypoglycemic mice immediately received a rescue IP injection of dextrose. Post-hypoglycemia data points were excluded from analysis.

Section 3.2.4: Intraperitoneal pyruvate tolerance test (IP-PTT)

At PW 20-22, mice were fasted for 16 hours overnight from 5:00 pm to 9:00 am. Fasting blood glucose was measured, followed by IP injection of sodium pyruvate (Sigma, St. Louis, MO) (1 g/kg body weight), and blood glucose was measured serially by tail vein sampling using a Freestyle Lite glucometer.

Section 3.2.5: Quantitative polymerase chain reaction

Total RNA was extracted from perigonadal adipose tissue and liver from mice fasted overnight using the E.Z.N.A. Total RNA Kit II (Omega Bio-tek Inc., Norcross, GA) and a BeadBug microtube homogenizer (Benchmark Scientific Inc., Edison, NJ) according to the manufacturers' instructions. Total RNA was quantified by UV spectrophotometry using a Nanodrop One (Thermo Fisher Scientific, Waltham, MA). RNA purity was verified by 260/280

and 260/230 ratios of ~2.0. Primers were generated by Primer-BLAST (National Center for Biotechnology Information, Bethesda, MD) and confirmed to have amplification efficiency of 90%–110%. Primer sequences (Integrated DNA Technologies, Coralville, IA) can be found in **Table 3.1**. Gene expression levels were evaluated by the ΔΔ-Ct method [346] with GAPDH used to control for total mRNA recovery; control values normalized to a group mean of 1.0.

Section 3.2.6: Serum collection and analysis

At the time of terminal sacrifice, blood was collected by cardiac puncture. Whole blood was collected in microfuge tubes, allowed to clot at room temperature for 45 minutes, and then centrifuged at 1500 *g* for 15 minutes at 4°C to collect serum. Serum was aliquoted in separate tubes to minimize repeated freezing-thawing during later analyses; samples were stored at -80°C.

Section 3.2.7: Adipose tissue insulin signaling western blotting

At the time of tissue harvest following a 3-hour fast, perigonadal fat was assessed for insulin sensitivity by quantifying the ratio of phosphorylated-to-total Akt at the serine 473 site (S473) as previously described [340]. Briefly, perigonadal fat was minced in Krebs-Ringer bicarbonate HEPES (KRBH) buffer pre-warmed to 37ºC, equally distributed into four Eppendorf tubes with different insulin concentrations (0, 1, 5, and 10 nM) and incubated at 37ºC for 10 minutes. After incubation, samples were put on ice, insulin-containing media was removed, and samples were washed with ice-cold KRBH. Homogenization buffer was added, samples were lysed by sonication, and centrifuged at 10,000 *g* at 4ºC for 10 minutes. Infranatant was used for immunoblotting. Adipose tissue lysate was separated by SDS-PAGE and transferred onto PVDF membrane. Membranes were blocked in 5% non-fat milk in Tris-buffered saline-Tween (TBS-T)

(0.1%) for one hour at room temperature and incubated overnight at 4°C with primary antibodies. Mouse monoclonal anti-total Akt (40D4) at 1:750 dilution and rabbit anti-phospho-S473 Akt (D9E) at 1:500 dilution (Cell Signaling Technology, Danvers, MA) were used as primary antibodies. Goat anti-rabbit IRDye® 680RD and goat anti-mouse IRDye® 800CW (LI-COR, Inc., Lincoln, NE) were used as secondary antibodies to simultaneously image Akt and phospho-Akt. Densitometry was performed using ImageStudioLite version 5.2.5 (LI-COR, Inc., Lincoln, NE). Insulin sensitivity was assessed as the ratio of the area of bands corresponding to phosphorylated Akt (S473) divided by total Akt, normalized within each animal to the basal condition (0 nM insulin) to specifically assess relative insulin responsiveness.

Section 3.2.8: Pancreatic histology and immunohistochemistry

At the time of tissue harvest, the pancreas was dissected, weighed, and fixed in 4% paraformaldehyde overnight followed by paraffin embedding. Pancreas sections (5 μm in thickness) were immunostained with the following primary antibodies at 1:500 dilution: polyclonal guinea pig anti-porcine insulin (DAKO, Carpinteria, CA), mouse monoclonal anti-human glucagon (Sigma-Aldrich), polyclonal goat anti-somatostatin (Santa Cruz Biotechnology, Santa Cruz, CA), and DAPI (Invitrogen, Carlsbad, CA). The primary antibodies were detected using a combination of DyLight 488-, 549-, and 649-conjugated secondary antibodies (1:200, Jackson Immuno Research Laboratory, West Grove, PA). Antibodies used in this study have been previously validated [347].

Section 3.2.9: Image capture and endocrine cell quantification

As previously described [347], microscopic images of pancreatic sections were taken with an Olympus IX8 DSU spinning disk confocal microscope (Melville, NY) with Stereo Investigator imaging software (SI; Micro Bright Field, Williston, VT). A modified method of "virtual slice capture" was used. Quantification of cellular composition (i.e., each area of β-, α-, and δ-cell populations, or sum of endocrine cell populations per islet area) was carried out using custom-written scripts for Fiji/ImageJ (https://rsbweb.nih.gov/ij/). MATLAB (MathWorks, Natick, MA) was used for mathematical analyses.

Section 3.2.10: Acute Restraint Test

At PW5, mice subjected to an acute restraint test. Blood was collected for baseline analyte measurements. Mice were then placed in small mouse restraint devices for 30 minutes, then released back to their respective cages. Blood samples were obtained using heparinized tubes at the end of the 30-minute restraint, then at 60 and 90 minutes. Blood was placed immediately on ice and centrifuged at 1500 *g* for 15 minutes at 4°C. Plasma corticosterone concentrations were determined using the Corticosterone ELISA kit (Abcam, MA). Plasma epinephrine concentrations were determine using the Adrenaline (Epinephrine) High Sensitive ELISA Assay Kit (Eagle Biosciences Inc., NH). Blood glucose levels were measured using a Freestyle Lite glucometer (Abbott Laboratories, Abbott Park, IL).

Section 3.2.11: Statistics

Relative to control mice, perinatal TF exposure consistently decreased weaning weight, our primary outcome measure, with no differences in the magnitude of decrease across the cohorts (data not shown); therefore, data were pooled from all studies. In collaboration with the Statistical

Laboratory at the University of Illinois at Chicago, Analysis of Response Profile, which does not make a parametric assumption on the form of the mean trajectory, was used to analyze effects on glucose tolerance, insulin sensitivity, and pyruvate tolerance using R (R Foundation for Statistical Computing, Vienna, Austria). The main goal in the Analysis of Response Profiles is to characterize the patterns of change in the mean response over time in the two groups and to determine whether the shapes of the mean response profiles do or do not differ for the two groups. The model used for these analyses used time and treatment as main effects, and treatment-by-time interaction effects. Post-hoc unpaired *t* tests without assumption of consistent standard deviations were performed on datasets with different Analysis of Response Profiles to ascertain time points at which blood glucose levels were significantly different. For non-time-dependent outcomes, control and TF treatment groups were compared by F-testing to determine differences in variance; for $F < 0.05$, *t* tests were performed with Welch's correction, whereas when $F > 0.05$, standard Student's *t* tests were performed. Log-Rank test was used to compare survival curves. Data are presented as mean ± standard error of the mean (SEM). A value of $P < 0.05$ was considered statistically significant. GraphPad Prism version 7.0 (La Jolla, CA) was used for all other comparisons.

Section 3.3: Theory

Glucocorticoids play a central role in metabolic programming, and pharmacologic treatment with glucocorticoids during development alters metabolic outcomes. This study was designed to test the hypothesis that the environmental GR-modulating EDC TF disrupts later-life metabolic homeostasis.

Section 3.4: Results

Section 3.4.1: Perinatal TF exposure does not alter litter size or sex-ratio

Litter size and sex ratio were assessed at weaning as a crude measure for developmental toxicity. Mean litter size (Control = 6.2, TF = 5.6) was not significantly different between groups ($p = 0.31$) (**Figure 3.2A**). Similarly, litter male-to-female sex ratio was also not significantly different at weaning between groups (Control = 1.7 versus TF = 1.2; $p = 0.20$) (**Figure 3.2B**). These data suggest that developmental exposure to TF did not result in significant gestational toxicity.

Section 3.4.2: Perinatal TF exposure reduces birth weight, weaning weight, and long-term body weights in offspring

Offspring body weight was measured at birth, at weaning on PD21, and once weekly post-weaning. TF exposure lowered birthweights by 3.7%, and lowered weaning weights in females and males by 13.4% and 6.8%, respectively (**Figures 3.2C, 3.2D, 3.2E**). Female offspring from TF exposed dams had significantly lower body weight throughout the entirety of the study (**Figure 3.2F**), while male offspring from exposed dams achieved comparable weight to that of control males by 17 weeks of age (**Figure 3.2G**).

Section 3.4.3: Perinatal TF exposure results in sex-specific patterns of mild glucose intolerance during acute glucose challenge

To assess whether perinatal TF exposure altered glucose homeostasis later in life, offspring underwent an IP-GTT at PW10. Perinatal TF exposure resulted in mildly impaired glucose tolerance in the offspring (**Figure 3.3**). Female offspring exposed to TF had higher blood glucose

levels early after glucose challenge (i.e., 12.4% higher at 10 minutes and 12.7% higher at 20 minutes post-injection) (**Figure 3.3A**). In addition, insulin levels were 22.4% higher at 30 minutes post-glucose load compared to controls (**Figure 3.3B**). Male offspring exposed to TF had higher blood glucose levels compared to controls later during the course of the GTT (i.e. 20% higher at 40 minutes, 17.9% higher at 90 minutes, and 16.2% higher at 120 minutes post-glucose injection) (**Figure 3.3C**), without significant differences in circulating insulin levels over the first 60 minutes of the IP-GTT (**Figure 3.3D**).

Section 3.4.4: Perinatal TF exposure increases whole-body insulin sensitivity in female offspring but not male offspring

Global insulin sensitivity was assessed to determine whether the observed impairments in glucose tolerance in TF-exposed offspring were attributable to impairments in insulin action. Unexpectedly, TF-exposed female offspring showed evidence of markedly enhanced insulin sensitivity (**Figure 3.4**). At PW10, assessment of steady-state glucose-insulin homeostasis demonstrated that TF-exposed female offspring had 29.7% lower HOMA-IR (**Figure 3.4A**) without significant differences in β-cell function as assessed by HOMA-β (**Figure 3.4B**). At PW16 TF-exposed female offspring exhibited a markedly enhanced response to insulin during an IP-ITT with significantly lower blood glucose levels at every time point post insulin injection (**Figure 3.4C**). Among TF-exposed female offspring, 38% experienced severe hypoglycemia (<20 mg/dL) during the IP-ITT requiring rescue with dextrose, while no control females exhibited this trait (Control n=0; TF n=7 with 4 at 45 minutes, 3 at 60 minutes, 1 at 90 minutes) (**Figure 3.4D**). Among male offspring, there were no significant differences in HOMA-IR and HOMA-β at PW10 (**Figures 3.4E** and **3.4F**), nor was there a difference in response to insulin during the IP-ITT at

PW16 (**Figure 3.4G**). Only one exposed male offspring became hypoglycemic during the course of the IP-ITT (Control n=0; TF n=1 at 45 minutes) (**Figure 3.4H**).

Section 3.4.5: Perinatal TF decreases adiposity and increases adipose tissue insulin sensitivity in female offspring but not male offspring

Adiposity and adipose tissue insulin sensitivity were assessed at the time of sacrifice in the offspring. Adiposity was defined as adipose tissue mass relative to total body weight for the perigonadal (epididymal or periuterine), perirenal, mesenteric, or total visceral (sum of perigonadal, perirenal, and mesenteric) depots. TF-exposed female offspring had 12.9% lower perigonadal adiposity and 26.4% lower perirenal adiposity; there were no differences in mesenteric adiposity (**Figure 3.5A**). Across all adipose depots analyzed, there was a trend toward lower total visceral adiposity at week 19 (p=0.052) (**Figure 3.5A**). Adipose insulin sensitivity was assessed *ex vivo* in perigonadal adipose tissue. Among TF-exposed female offspring, adipose tissue insulin sensitivity was enhanced relative to control mice with a 88.7% and 53.7% increase in Akt phosphorylation at 5 nM and 10 nM insulin, respectively (**Figure 3.5C**). Gene expression of insulin signaling intermediates upstream of Akt was quantified to ascertain the molecular mechanism responsible for the observed enhancement in adipose insulin sensitivity, but no differences were observed (**Figure 3.6**). Among male offspring, there were no differences in either depot-specific or total adiposity between control and TF-exposed mice (**Figure 3.5B**). Relative insulin-stimulated Akt phosphorylation was not significantly different between control and TF-exposed male offspring (**Figure 3.5D**).

Section 3.4.6: Perinatal TF does not affect pancreatic endocrine cell area in exposed offspring

To ascertain whether alterations in glucose homeostasis, insulin sensitivity, and adiposity were attributable to developmental disruption of the endocrine pancreas, pancreatic α-cell, β-cell, and δ-cell areas were quantified using immunohistochemistry. There were no differences in α-cell, β-cell, δ-cell, or total islet area relative to the total pancreatic area analyzed between control and TF-exposed offspring (**Table 3.2**).

Section 3.4.7: Perinatal TF exposure does not increase HPA reactivity, but increases blood glucose levels in male offspring only.

Circulating corticosterone after an overnight fast was measured at PW4, but no differences were observed between controls and TF-exposed offspring (**Figure 3.7**). On PW5, offspring were subjected to an acute restraint test to assess differences in HPA axis reactivity to psychological stress. No differences in circulating corticosterone were observed during the acute restraint test between control and TF-exposed offspring (**Figure 3.8A and B**); however, male offspring exposed to TF had increased circulating glucose immediately after the acute restraint (**Figure 3.8D**). Circulating epinephrine levels were measured to test whether increased epinephrine could potentially be responsible for the observed hyperglycemia in the TF-exposed male offspring. Circulating epinephrine concentrations were lower in the male offspring perinatally exposed to TF at 30 minutes during the acute restraint test (**Figure 3.8E**).

Section 3.4.8: Perinatal TF exposure results in sex-specific changes in hepatic gluconeogenic capacity in exposed offspring

On PW4 offspring were fasted overnight and hepatic expression of genes regulating gluconeogenesis was measured. Compared to control male offspring, male offspring perinatally

exposed to TF had 30.7% higher hepatic phosphoenolpyruvate carboxykinase (PEPCK) gene expression following overnight fasting with a trend toward higher expression of glucose-6-phosphatase (G6PC) (p=0.08) (**Figure 3.9B**). There were no differences in PEPCK or G6PC gene expression between female offspring (**Figure 3.9A**). Fasting circulating corticosterone, a key gluconeogenesis-stimulating hormone, was not different between groups in either sex (data not shown). Dynamic differences in gluconeogenic capacity among offspring were assessed by IP-PTT at PW20-22 to ascertain whether the observed differences in gene expression had lasting physiological effects. Male offspring perinatally exposed to TF showed higher blood glucose levels during the pyruvate challenge (p=0.047), (**Figure 3.9D**), while there were no differences in circulating glucose in female offspring during the pyruvate challenge (**Figure 3.9C**).

Section 3.5: Discussion

The current study adds to the growing body of research demonstrating sex-specific and long-term alterations in metabolic physiology caused by early-life exposures to EDCs [333]. Specifically, this study shows that perinatal TF exposure reduces birth and weaning weight, while leading to mild impairments in glucose tolerance later-in-life without disruptions in insulin secretion or endocrine pancreas morphology. Intriguingly, perinatal TF exposure decreased adiposity and markedly enhanced global insulin sensitivity in female offspring, but did not alter these parameters in male offspring. Finally, gluconeogenic capacity was enhanced in TF-exposed male offspring but unaffected in exposed females. These findings underscore the importance of *in vivo* studies to identify sex differences in metabolic fate arising from developmental EDC exposures. Moreover, these studies support the hypothesis that early-life exposure to metabolism-

disrupting EDCs may contribute to later-life metabolic disease risk in humans in a sex-dependent manner.

Previous work showed that dietary exposure to TF increased adiposity and decreased systemic and adipose-specific insulin sensitivity in adult male mice [330]. This motivated the present studies examining whether similar exposures during development elicited comparable metabolic derangements in exposed offspring. Importantly, the TF dose used for this study did not alter litter size or sex ratio at the time of weaning, indicating that this dose was not overtly toxic to developing fetuses. Perinatal exposure to TF resulted in lower birthweight, and reduced weaning weight in both female and male offspring, indicating that exposed offspring did not catch-up in growth by the time of weaning. Reduced birth weight results from impaired fetal growth that reflects an adverse intrauterine environment; it is a known risk factor for later-life cardiometabolic disease [262]. Furthermore, TF-exposed female offspring weighed less than control female offspring through PW19, while exposed male offspring achieved comparable weights to control males by PW17, showing long-term impacts of TF on growth and development.

Perinatal TF exposure resulted in modest, sex-specific impairments in whole-body glucose clearance during an acute glucose challenge. Despite having relatively higher insulin sensitivity at steady-state (as assessed by HOMA-IR), exposed female offspring exhibited elevated blood glucose levels relative to controls during the early phase of the glucose tolerance test. Importantly, the observed glucose intolerance among TF-exposed females was not due to impairments in insulin action as TF-exposed females were markedly more insulin sensitive than control mice. In evaluating insulin-glucose dynamics in this study, the modest blood glucose elevation in females was noted to normalize after 30 minutes when circulating insulin was significantly higher for exposed females. This suggests that hyperglycemia may have arisen from relative reductions or

delays in insulin release from pancreatic β-cells. Of note, however, TF exposure did not result in morphological changes in the endocrine pancreas, suggesting overt β-cell toxicity was not responsible for the observed findings.

In contrast to females, exposed male offspring experienced a more pronounced impairment in glucose clearance that was notable at later time points post-glucose load. Importantly, this hyperglycemia was not accompanied by compensatory β-cell insulin hypersecretion as seen in TF-exposed female offspring. Furthermore, the observed impairment in glucose clearance was not due to diminished systemic insulin action. Thus, we hypothesized that increased or sustained hepatic glucose output may have contributed to hyperglycemia in TF-exposed male offspring. Indeed, male TF-exposed offspring exhibited enhanced hepatic PEPCK gene expression at PW4, suggesting an upregulation of the gluconeogenic machinery that was not observed in female offspring. Indeed, with provocative testing TF-exposed male offspring exhibited enhanced gluconeogenic capacity as evidenced by higher hepatic glucose production following pyruvate challenge. Importantly, this was a sex-dependent effect as exposed female offspring did not exhibit this effect. Likewise, only male offspring perinatally exposed to TF exhibited elevated glucose levels during the acute restraint test. Taken together, these data indicate that the path to glucose intolerance among mice exposed perinatally to TF is sexually dimorphic.

The observed effects of developmental TF exposure on insulin sensitivity and adiposity in female offspring coincide with results from previous *in utero* growth restriction studies demonstrating that prenatal growth restriction that is not followed by catch-up growth is accompanied by increased insulin sensitivity and decreased adiposity [348-357]. Studies in sheep showed that increased insulin sensitivity resulting from *in utero* growth restriction originates during fetal development [358-362] and carries on into early postnatal life [362]. It has been

proposed that increased global insulin sensitivity arising from an adverse *in utero* environment is a compensatory mechanism to support anabolic metabolism and promote offspring survival [360]; however, the mechanisms by which insulin sensitivity is enhanced under these circumstances remains unclear. The results reported herein suggest that adipocyte insulin sensitivity is enhanced upstream of Akt; however, our studies were unable to identify changes in insulin signaling intermediates that could explain this phenomenon. One possibility is that insulin sensitivity is tuned to be more responsive through the differential phosphorylation of insulin receptor substrates [363]; however, further molecular analyses will be required to test this hypothesis and ascertain the precise mechanism by which insulin signaling is potentiated in exposed female offspring. Interestingly, evidence from rat studies suggest that compensatory increases in insulin sensitivity following developmental growth-inhibiting insults are followed by later-life onset of insulin resistance and a decline in metabolic health [236, 351, 364, 365]. For example, in rat models of uteroplacental insufficiency, female offspring exhibited enhanced insulin sensitivity at up to 12 months of age [352]; however, age-related deterioration in metabolic health and the development of insulin resistance was observed at 21 months of age [236]. The current study followed the exposed offspring for up to 22 weeks of age, which is within the timeframe during which other rodent models of growth restriction have observed enhanced insulin sensitivity [348-353]. Whether mice developmentally exposed to TF will ultimately go on to develop insulin resistance and worsened glucose intolerance with aging requires additional study.

Importantly, the current study adds to the growing body of evidence implicating hepatic gluconeogenesis as a sensitive metabolic endpoint for developmental misprogramming [238, 243, 249, 251, 366]. The upregulation of gluconeogenesis with attendant increased PEPCK expression in TF-exposed male offspring is consistent with several studies in rats demonstrating that

gluconeogenesis in male offspring is disrupted by either glucocorticoid exposure or protein restriction during development [238, 243, 249, 251, 366]. Importantly, in the present studies PEPCK upregulation was not due to enhanced corticosterone secretion, suggesting that another mechanism is responsible, including potential epigenetic alterations; however, this hypothesis requires further study. Interestingly, our study suggests that gluconeogenic capacity in exposed female offspring trends lower and that these mice are more prone to hypoglycemia with insulin challenge. The reasons behind this sex-specific defect in counterregulation needs further study.

Critically, the sex-specific effects on growth, insulin sensitivity, glucose tolerance, adiposity, and hepatic glucose output resulting from perinatal TF exposure highlights the importance of studying males and females in developmental studies that assess GR signaling modulation, especially since most of the prenatal studies published to date have focused on male offspring [238, 243, 249, 251, 366]. Taken together, animal studies have consistently shown that later-life metabolic physiology can be altered by *in utero* disruption of GR signaling by dexamethasone (DEX) treatment despite variations in the specific outcomes measured. The current study adds to this area of study by demonstrating that perinatal exposure to a fungicide that alters GR signaling also has lasting effects on growth and metabolism. The differences in metabolic outcomes between the current study and previous developmental DEX studies may be attributable to differences in species (rats vs. mice), timing of assessments, potency of GR-signaling modulation (DEX>TF), the timing and length of exposure, or non-GR-effects of TF. As evidence continues to emerge suggesting that a subset of EDCs modulate GR signaling, the current studies provide a useful framework for interrogated the long-term metabolic consequences of developmental exposure to these agents.

Despite the strengths of the current study in examining sex-specific differences in metabolic physiology after developmental exposure to TF, work presented herein has several limitations. The current study only followed offspring for 20-22 weeks after birth; thus, it did not determine whether further aging would unmask metabolic deterioration in exposed mice as seen in other growth restriction models. While this work found enhanced adipose-specific insulin sensitivity that could partly explain the increase in whole body insulin sensitivity in the exposed female offspring, additional mechanistic studies are needed to understand the molecular bases for the physiological alterations identified herein. It is also possible that the observed effects of TF could be mediated by alterations in maternal metabolism. Based on data that exposure of adult male mice to TF did not alter adiposity, glucose tolerance, or insulin sensitivity until 8 weeks of exposure [330], it does not seem likely that this was a major driver of the observed effects; however, this needs to be formally assessed. Despite these limitations the observed phenotypic disruptions in body weight, adiposity, insulin sensitivity, and gluconeogenesis induced by TF reflect growth and metabolic changes observed in other developmental stress models including prenatal DEX exposure, *in utero* protein restriction, or uterine artery ligation studies [348-353]. Future studies are warranted to elucidate the common molecular mechanisms by which these diverse stressors impact long-term metabolic health.

Chapter 4

Future Directions for Characterizing Mechanisms Driving Metabolic Outcomes of Developmental GR-Modulating EDCs and Preventing Exposures

Section 4.1: Comprehensive Assessment of Hepatic Transcriptomic Alterations During Adulthood After Perinatal TF Exposure

Evidence that perinatal TF exposure upregulates hepatic gluconeogenesis in the male offspring reflects outcomes observed from previous developmental GC overexposure animal models that have consistently shown altered gluconeogenesis in the offspring. Future work aimed at understanding the underlying mechanisms explaining impaired glucose tolerance resulting from perinatal TF exposure should thus begin with interrogating hepatic gluconeogenesis during adulthood. Unbiased transcriptomic approaches such as RNA-Seq would allow a genome-wide assessment of changes in gene expression that could identify causal genes responsible for the observed impairment in glucose tolerance and aberrant hepatic glucose output. Data gathered from such assessment could be analyzed by gene set enrichment analysis to test whether genes that compose or regulate pathways including gluconeogenesis, glycogenolysis, insulin sensitivity, or GR signaling are significantly altered. Examining the hepatic transcriptomic signature resulting from perinatal TF exposure could delineate whether the cause for upregulated gluconeogenesis is due to the upregulated gluconeogenic pathway itself, or pathways that regulate gluconeogenesis (such as glucocorticoid or glucagon signaling, for example). Upregulation of a regulatory pathway, such as GR signaling, could suggest that the HPA axis is altered, or hepatic sensitivity to GCs is altered, and could thus lead to a more comprehensive understanding of the observed phenotype. Further, unexpected regulatory factors such as microRNAs, or unanticipated pathways indirectly

regulating gluconeogenesis could be illuminated from an unbiased assessment that could broaden the current understanding of developmental programming of metabolism and basic regulatory mechanisms of hepatic glucose output. Finally, targeted gene expression analysis with qRT-PCR and western blotting could be performed at an earlier time point, such as after weaning, on the genes with the largest and most significant changes to test for the persistence of differential expression throughout the lifespan that would suggest epigenetic mis programming.

Section 4.2: Comprehensive Assessments of Chromatin Alterations Leading to Altered Gene Expression

Expression of genes persistently altered by perinatal TF treatment would warrant an extensive assessment of the epigenetic mechanisms leading to lifelong altered expression. Analysis of whole genome bisulfite sequencing targeted for the most significantly and persistently altered genes would give an assessment of differences in DNA methylation at gene regulatory sites that would suggest differences in DNA accessibility for the transcriptional machinery. Assessing the distribution of covalent histone modifications indicative of active (i.e. H3K4me3, H3K27ac) and repressive (i.e. H3K27me3) chromosomal regions using ChIP-Seq would further explain how changes in chromatin landscape at gene regulatory sites contribute to differential gene expression caused by perinatal TF exposure. Consistent observations of concordant changes in DNA methylation and histone modifications that explain upregulated or downregulated genes would justify further assessments targeted at understanding the developmental origins of observed chromatin alterations.

Comprehensive assessments of hepatic chromatin architecture between treatment groups before and after liver maturation has occurred would help determine alterations in the chromatin

landscape leading to abnormal gene expression later in life. Comparing differences in chromatin accessibility in unexposed offspring before and after liver maturation would provide a reference for chromatin organization during normal liver development. Abnormalities in accessibility of chromosomal regions relative to this standard could then be identified in the livers of TF-exposed offspring. Proposed assessments would include bisulfite sequencing to assess DNA-methylation differences, ChIP-Seq for active and repressive histone distribution, ATAC-Seq to assess DNA-accessibility patterns, and Hi-C to measure global chromatin interactions. Isolation of hepatocytes would be performed before liver maturation and at birth, when substantial liver maturation has happened. Results from these experiments would be compared between male offspring from untreated dams, dams treated with TF, and dams given the GR antagonist RU486, which would prevent endogenous GCs from inducing liver maturation. Comparing differences in chromatin architecture as the liver matures between control mice, TF-treated mice, and mice deprived of liver maturation via RU486 treatment would outline the establishment of aberrant chromatin modifications belonging to regulatory elements of differentially programed genes implicated in adverse metabolic outcomes later in life. For example, the epigenetic programming of a gene that is upregulated might be due to increased accessibility and activity of an enhancer rather than a promoter [367]. The proposed experiments could show this by measuring decreased enhancer methylation, increased DNA-accessibility and positioning of histones denoting active enhancers, as well as increased interactions between this hypothetical enhancer and the promoter regulating the gene of interest. Such comprehensive assessments would be possible at a genome-wide scale that would be more informative than targeted analysis on specific gene regulatory regions. Further, these unbiased tests could uncover underlying mechanisms responsible for the regulation of multiple genes. For example, the increased activity of an enhancer could result in the upregulation

of several genes [368]. Finally, identifying gene regulatory regions responsible for altered gene expression could help pinpoint proteins responsible for the alteration of the epigenetic makeup of such regulatory regions, such as pioneer factors, transcription factors, or other factors responsible for altering chromatin architecture such as CTCF. Overall, comprehensively assessing the chromatin landscape during fetal development would help resolve unanswered questions in the field of DOHaD, such as how altered gene expression is programmed during development, and what protein factors mediate such changes.

Section 4.3: Evaluating Potential Sources of Sex-Specific Hepatic Outcomes

Section 4.3.1: Assessing Potential Placental Contributions to Sex Differences in TF Outcomes

Sex-specific differences in placental function and response to environmental factors have been extensively reported, yet the role that the placenta plays in mediating distinct metabolic outcomes between male and female offspring following GC overexposure remains unclear. Testing for differential placental response to TF would be critical in resolving whether the placenta mediates the sex-specific metabolic outcomes elicited by perinatal TF exposure. It is possible that the placenta expresses drug transporters in a sex-specific manner and lead to differential fetal TF exposure. One initial experiment could test for differences in fetal TF and TF metabolite levels between male and female fetuses with mass spectrometry. Second, sex-differences in GR-mediated placental response to an acute TF exposure could be assessed. To test this, whole placental tissue could be analyzed for placenta-specific GR-mediated endpoints such as phosphorylated GR, 11β-HSD2 expression, and apoptosis markers by western blot or histology following an oral gavage of TF dissolved in corn oil. Placental sex would be determined by genotyping the associated fetus for SRY. Sex-specific differences in any of the observed GR-mediated endpoints would warrant

further studies to characterize other potential placental responses that could alter fetal development, such as changes in placental vasculature, expression of nutrient transporters, and synthesis of hormones and growth factors. An assessment of these endpoints could also be carried out during gestation after the same dietary exposure paradigm. Alternatively, a validated *ex vivo* placental perfusion system [369] could be employed to directly expose the placenta to TF and test for differences in gene expression and TF-metabolism. Overall, sex-specific placental responses could be interrogated to assess whether the placenta is responsible for mediating differential metabolic outcomes in developmentally exposed offspring.

Section 4.3.2: Uncovering Potential Crosstalk Between GCs and Sex Steroid Hormones During Liver Development

Definitive evidence shows that GCs crosstalk with androgens and estrogens in several tissues by numerous mechanisms, yet the extent and role of GR crosstalk with AR and ER during fetal development remains largely unknown. Further, whether disrupting GR signaling can lead to sex-specific differences by disrupting crosstalk with AR and ER has not been assessed. Comparing differences in global gene expression and hormone receptor chromatin binding in rodent fetal hepatocytes co-treated *ex vivo* with DEX and DHT, as well as DEX and E2, would answer these questions. Specifically, fetal hepatocytes isolated at the same gestational day from the same sex would first be treated with either DEX, DHT, or E2 to obtain a transcriptional and genome binding signature for activated GR, AR, and ER during a specific developmental stage. Steroid receptor DNA-binding depends on chromatin accessibility of the respective hormone response elements [170-172], which is markedly different depending on developmental stage. Thus, testing primary fetal hepatocytes would capture the chromatin landscape necessary to answer whether these

nuclear receptors can influence each other's gene-regulatory activity specifically during development. ChIP-Seq and RNA-Seq data from each treatment could be compared to assess differences and overlap in gene expression and DNA-binding patterns. After this initial assessment, fetal hepatocytes from the same gestational day and sex would be co-treated with DEX and DHT, as well as DEX and E2 to test how co-treatments affect gene expression and DNA-binding in comparison to the single treatments. As with similar experiments performed with adult cell lines, these experiments would provide information about new DNA-binding and transcriptional outcomes not observed during single hormone receptor activation, which has never been assessed during fetal development. Further, knowing which genes are differentially regulated during co-exposures would provide insight into which sex-specific metabolic outcomes observed in published GC overexposure studies may be attributed to such differential expression.

Considerable differences in outcomes measured during the co-treatment experiments would strongly suggest that disrupting GR signaling in times where fetal T or E2 levels differ between male and female fetuses could result in sex-specific programming differences. To directly test this hypothesis, similar co-treatment experiments could be performed with doses designed to reflect sex-differences in T during fetal development between male and female fetuses, while keeping the DEX dose constant. Alternatively, a similar experiment with E2 instead of T could be performed given that T could be aromatized to E2 in the fetal liver as it is done in other tissues. If significant differences in global gene expression are observed between the DEX/female T concentration, and DEX/male T concertation co-treatment experiments, then it is possible that GC crosstalk during times where sex hormone levels are different between male and female fetuses could lead to sex differences in programming. Importantly, all prenatal DEX studies that have shown sex differences in metabolic outcomes have administered DEX during the last week of

gestation, which is when female and male rats have differences in circulating T [209]. Moreover, a longitudinal assessment at different points during fetal development would determine how steroid hormone crosstalk dynamics change, and importantly, identify critical windows of susceptibility to disruption. Overall, the outlined experiments would directly test whether GR disruption during developmental windows where circulating fetal sex hormone differ in male and female fetuses could result in sex-specific differences in developmental programming. Future studies could test whether similar results are obtained with weaker GR agonists, such as TF or other GR-modulating EDCs.

Section 4.4: Addressing Exposure Disparities Through Community-Based Collaborations

The gradual and cumbersome nature of the EPA's chemical regulatory process warrants additional means to end the higher exposures to EDCs amongst disproportionately exposed communities. This point is underlined by the shortcomings of the Toxic Substances Control Act (TSCA), which guides the EPA's toxic chemical regulatory practices. The TSCA places the "burden of proof" on researchers rather than chemical manufacturers, resulting in an unsustainable system that requires the investment of millions of dollars, decades of research, and an unknown amount of affected lives to prove that a suspected chemical is hazardous. The EPA must also weigh the economic benefits of a potentially hazardous chemical in their regulatory decision-making, which can prevent adequate regulation of lucrative chemicals that vulnerable populations are unequally exposed to. Thus, proactive place-based measures in addition to research-guided legislation are needed to address EDC exposures in disproportionately exposed communities.

Equitable academic collaborations with non-profit and autonomous environmental organizations can optimize efforts to locally address environmental injustices by combining

resources that are specific to each of these entities. For decades, environmental non-profit groups along with autonomous environmental groups have pushed for preventive health by realizing changes that result in tangible reductions in pollution exposures. These groups create change by engaging the youth, adults, and elders of affected neighborhoods in campaigns that combine community organizing, media communications, legal and electoral strategies, and research. Some organizations realizing environmental justice in their communities include Communities for a Better Environment (CBE) and East Yard Communities for a Better Environment (EYCEJ) in California, and Little Village Environmental Justice Organization (LVEJO) in Chicago, IL. CBE has prevented the siting and expansion of various polluting sources, has won several settlements against polluters and influenced the reduction of their emissions, and continues to support the resilience of communities of color by tackling issues dealing with food justice, transportation, and green zoning. Some of LVEJO's distinguished accomplishments is the closure of two of major coal plants sited in a predominantly Latinx neighborhood, expanding a bus route in an area deprived of public transportation, and the conversion of a superfund site into their neighborhood's a largest park. Environmental community groups can serve as central entities that can guide initiatives with external collaborators given the strong community ties and understanding of the neighborhood dynamics that environmental community groups belong to.

Academic collaborations through Community Based Participatory Research (CBPR) can comprehensively identify unequal exposure sources and help implement successful interventions that will reduce these exposures. CBPR is a model based on the sharing of power in all aspects of the research process in academic–community partnerships and benefits communities through interventions or policy change [370]. A successful CBPR collaboration is exemplified by the California Household Exposure study, which investigated socioeconomic influences behind the

exposure of EDCs linked to breast cancer. This collaboration unraveled the disproportionate exposure to oil combustion air pollution inside the homes of low-income people of color in Richmond, CA, it elucidated of some of the highest levels of PBDEs ever recorded in human samples, and discovered that indoor products significantly contribute to indoor air levels of EDCs [121, 371, 372]. These breakthroughs influenced litigation against Chevron refinery and the prevention of its proposed expansion, and also influenced PBDE regulation in CA [372, 373]. Similar academic-community partnerships can help identify sources of other EDCs that vulnerable communities are disproportionately exposed to. Just previous studies have identified increased packaged and processed food consumption as drivers of BPA and phthalate exposure amongst people with lower income [374-376], community-wide studies could assess how commercial product consumption patterns can lead to unequal exposures to other EDCs. These types of projects can lead to interventions aimed to build local consumer consciousness about EDC exposures and increase the demand for cleaner and healthier alternatives. Consumer pressure can then influence local businesses to adopt healthier products in their communities, and thus bypass the bureaucratic process of banning potentially harmful chemicals. For example, the Campaign for Safe Cosmetics has increased awareness about EDC additives in cosmetic products, and may be responsible for the voluntarily removal of several phthalates from cosmetic products in the recent years. Accordingly, exposure levels of these EDCs have dropped in the recent years [96]. Similar initiatives focused in communities of color may result in lower exposure levels of EDCs found in consumer products. Increased funding for academic-community initiatives can be a priority for funding agencies that aim to address socioeconomic disparities in health. Despite lack of funding, environmental non-profits creating change within their communities, thus, increasing funding to these entities will only result in greater tangible health and environmental outcomes.

Section 4.5: Conclusion

In conclusion, disruption of GR signaling during fetal development misprograms metabolism in the offspring in a sex-specific manner as evidenced by different methods of gestational GC overexposure. Potential reasons explaining sex-specific outcomes following *in utero* GC overexposures include hormone crosstalk between GR sex hormone receptors during development, as well as differential placental responses. Developmental exposure to the GR-modulating EDC TF leads to various metabolic derangements in the offspring in a sex-specific manner, some of which parallel outcomes observed in other *in utero* GC overexposure models. These results warrant further efforts to assess how exposure to other GR-modulating EDCs of known public health relevance are influencing long-term metabolic disease risk in the offspring.

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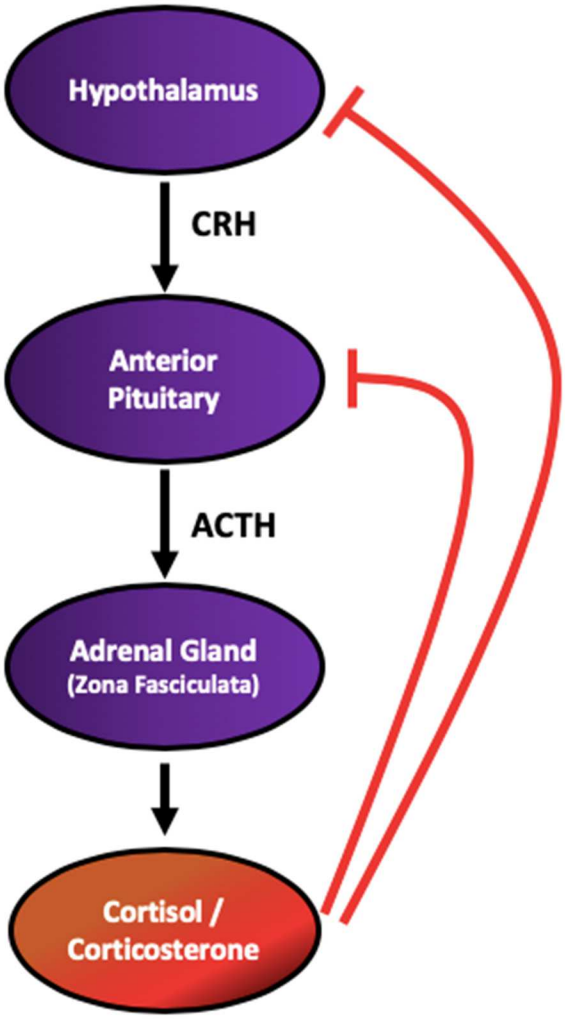
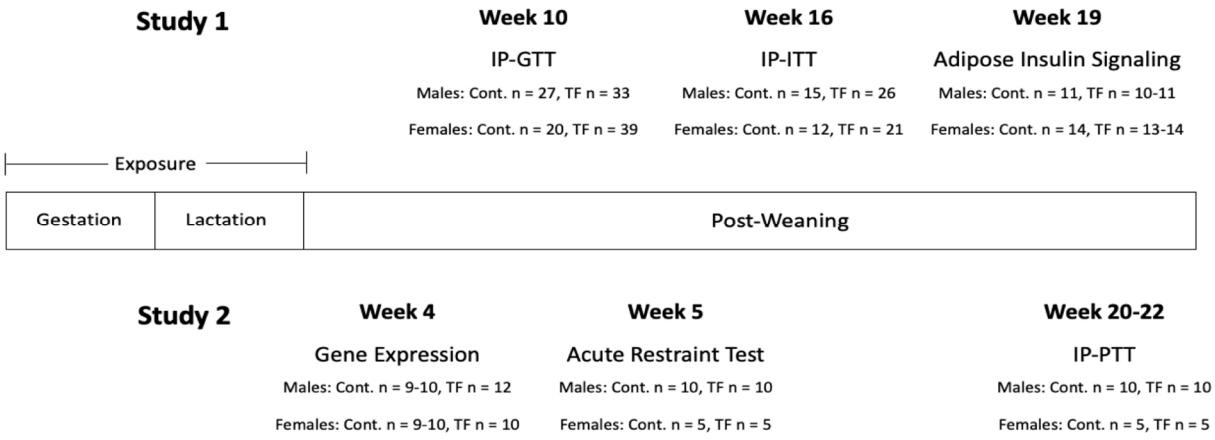
Appendix A: Figures

Figure 2.1: Overview of the Hypothalamic-Pituitary-Adrenal (HPA) Axis.



Glucocorticoids (GCs) are synthesized under control of the Hypothalamic-Pituitary-Adrenal (HPA) axis. In response to HPA activation, the paraventricular nucleus of the hypothalamus releases Corticotropin-Releasing Hormone (CRH), which signals corticotropic cells in the anterior pituitary to release Adrenocorticotropic hormone (ACTH). ACTH promotes the synthesis of GCs in the zone fasciculata of the adrenal cortex, namely cortisol in humans and corticosterone in rodents, which negatively feedback on the HPA axis.

Figure 3.1: Perinatal TF study timeline.



Eight-week old C57BL/6J mice were mated and fed a standard chow diet or an identical diet supplemented with TF at 100 g/kg diet throughout gestation and lactation. Offspring were weaned at 3 weeks and pair housed by sex. In Study 1, offspring were subjected to IP-GTT at week 10, IP-ITT at week 16, and were sacrificed at 19 weeks. Fat from a subset of these mice was assayed *ex vivo* for adipose insulin sensitivity. In Study 2, hepatic gene expression of overnight-fasted mice was assessed at week 4, and HPA axis reactivity was assessed at week 5. Littermates were subjected to an IP-PTT at week 20-22.

Figure 3.2: Litter and growth outcomes of perinatal TF exposure in female and male offspring.

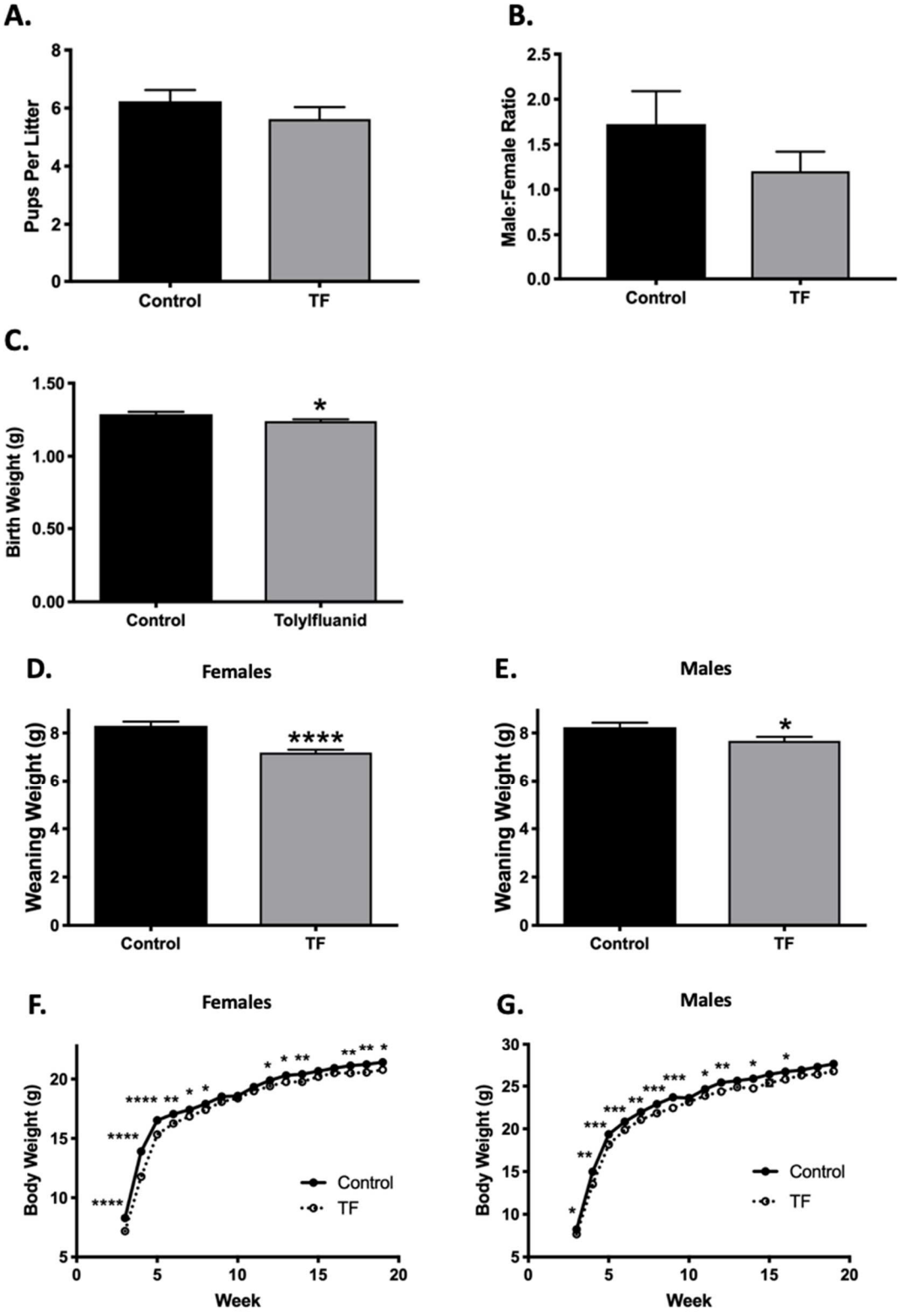
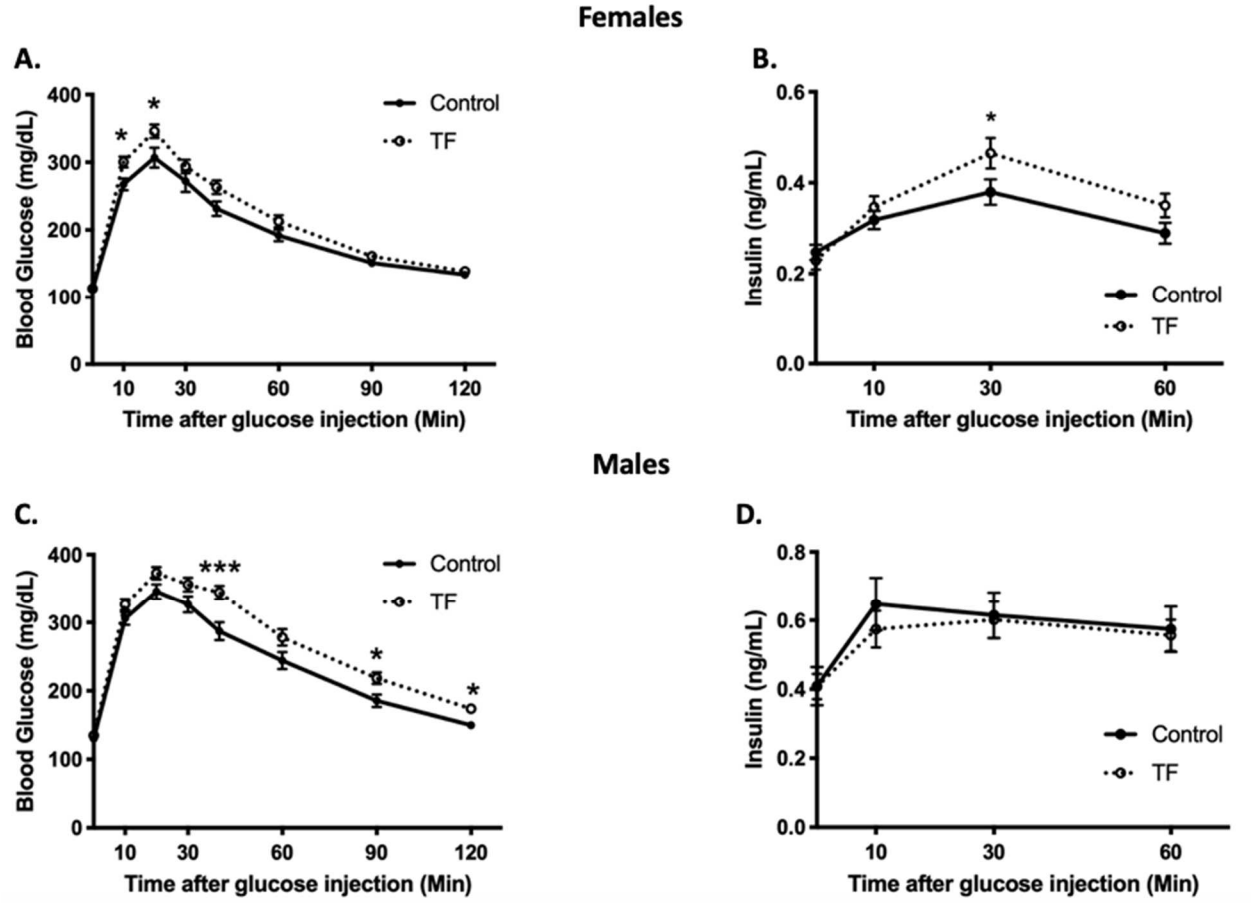


Figure 3.2, continued

Pregnant C57BL/6J mice were provided a standard chow with or without TF added at the time of manufacturing at a concentration of 100 mg/kg throughout pregnancy and lactation. Litter size (**Panel A**) and offspring sex-ratio (**Panel B**) was assessed at weaning), Control n=17 litters, TF n=24 litters. Body weight was measured at birth (**Panel C**), Control n=30 pups, TF n=25 pups. Offspring were weaned at 3 weeks and weighed until they reached 19 weeks of age. Weaning weight for females (**Panel D**) and males (**Panel E**). Weekly body weight for females (**Panel F**) and males (**Panel G**). Control n=25, TF n=53 for female offspring weaning and weekly body weights. Control n=36, TF n=43 for male offspring weaning and weekly body weights. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

Figure 3.3: Perinatal TF exposure impairs glucose clearance during acute glucose challenge.



An IP-GTT was performed in the offspring at week 10 by IP injection of dextrose (2 g/kg). Serial blood glucose measurements were taken for 120 minutes in females (**Panel A**) and males (**Panel C**). Control n=20, TF n=39 for female offspring. Control n=27, TF n=33 for male offspring. Plasma insulin during IP-GTT was assessed at baseline and 10, 30, and 60 minutes after dextrose injection in females (**Panel B**) and males (**Panel D**). Control n=11, TF n=20 for female offspring; control n=20, TF n=20 for male offspring. GTT, glucose tolerance test; IP, intraperitoneal; *p<0.05; ***p<0.001.

Figure 3.4: Perinatal TF exposure results in sex-specific differences in insulin sensitivity.

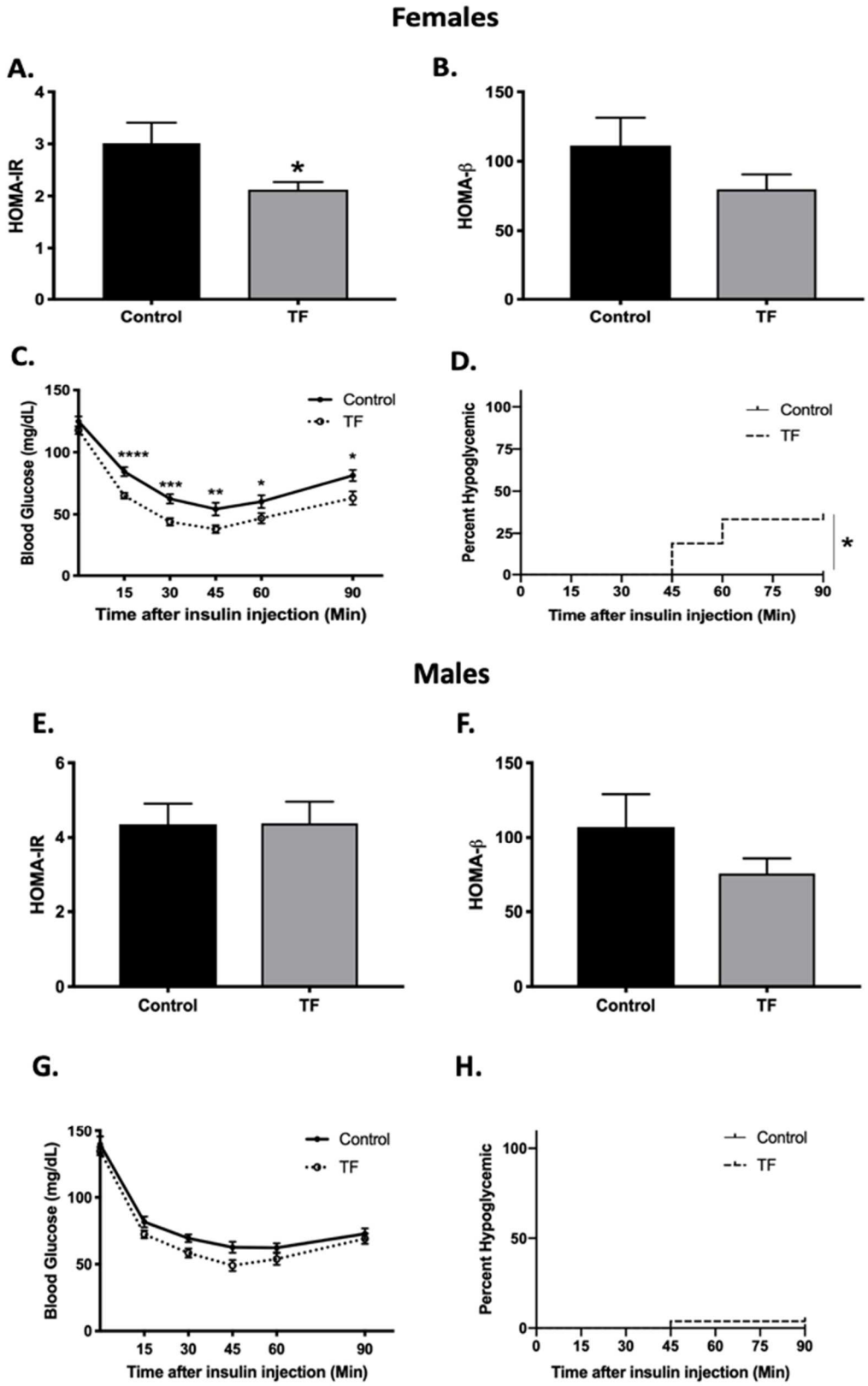
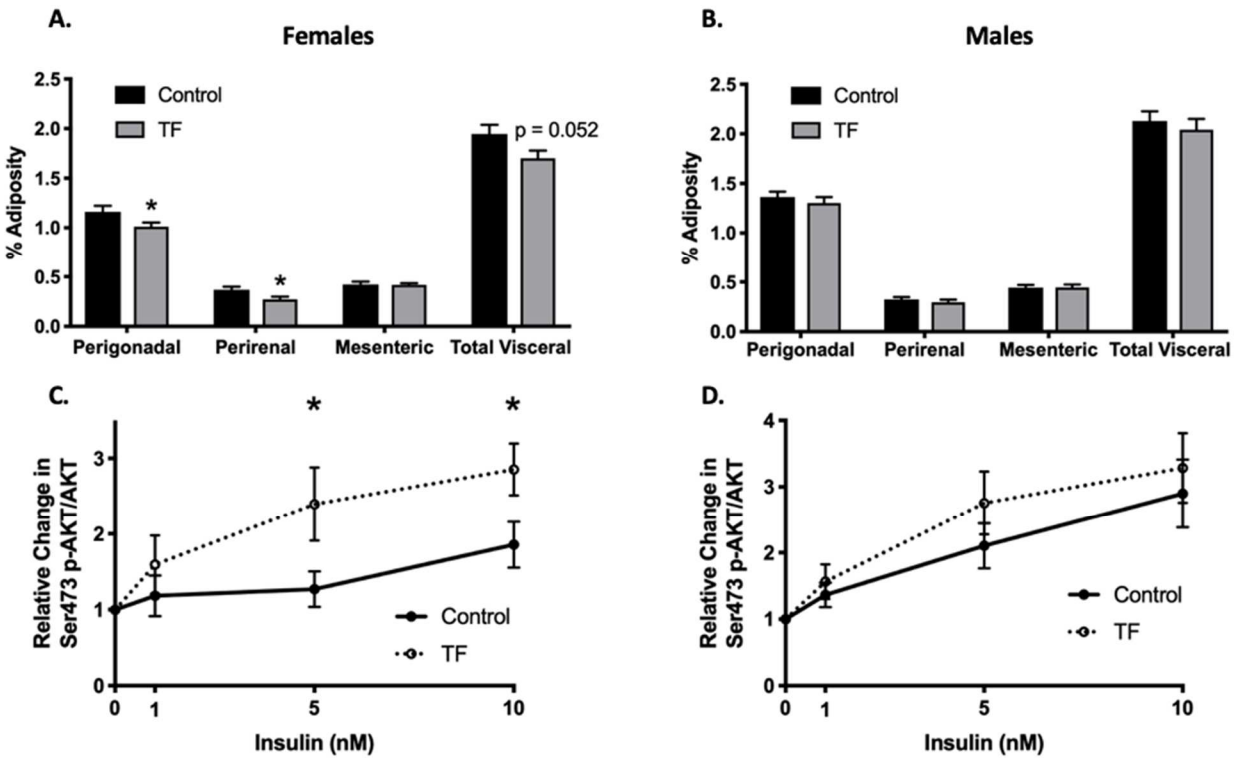


Figure 3.4, continued

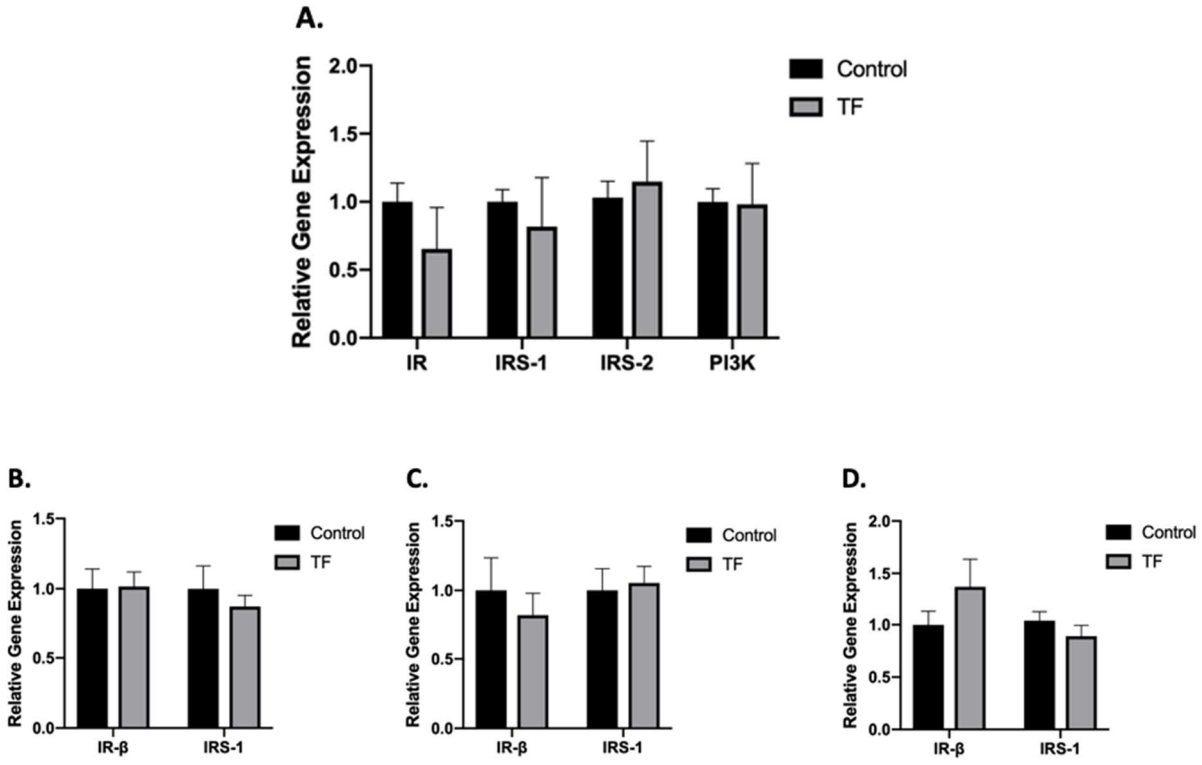
HOMA-IR (**Panel A**) and HOMA-β (**Panel B**) were calculated for female offspring using fasting blood glucose and plasma insulin levels at week 10 after a 6-hour fast, (Control n=21, TF n=31). An IP-ITT was performed at week 16 by IP injection of insulin (0.4 U/kg), and serial blood glucose measured for 90 minutes in female offspring (Control n=12; TF n=21) (**Panel C**). HOMA-IR (**Panel E**) and HOMA-β (**Panel F**) were calculated for male offspring using fasting blood glucose and plasma insulin levels at week 10 after a 6-hour fast (Control n=28; TF n=19). An IP-ITT was performed at week 16 of exposure by IP injection of insulin (0.5 U/kg for males), and serial blood glucose was measured for 90 minutes for male offspring (Control n=15; TF n=26) (**Panel G**). Survival curves for females (**Panel D**) and males (**Panel H**) show the percentage of mice that experienced severe hypoglycemia (<20 mg/dL) during IP-ITT at week 16. ITT, insulin tolerance test. *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001.

Figure 3.5: Perinatal TF exposure results in sex-specific differences in adiposity and adipose insulin sensitivity.



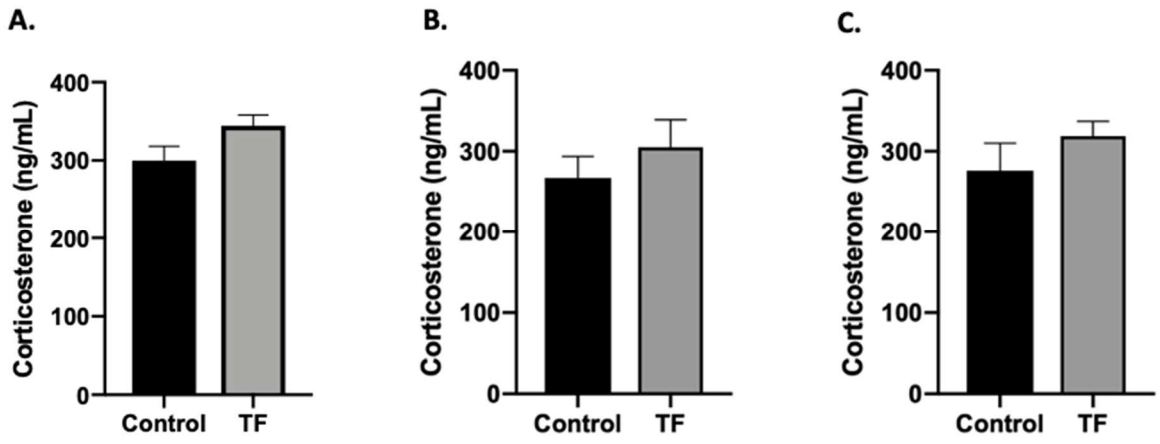
Visceral adipose depots were collected and weighed at sacrifice week 19. Perigonadal (PGF), perirenal, and mesenteric fat depot weight was normalized to total body weight. Total visceral adiposity was calculated by adding all of the individual depots and normalizing to body weight for female offspring (**Panel A**), Control n=25, TF n=50; and male offspring (**Panel C**), Control n=35, TF n=31**.** A subset of the PGF tissue was stimulated with different insulin concentrations for 10 minutes, and insulin sensitivity was assessed as the ratio of the band sizes of phosphorylated-to-total Akt at the S473 site for female offspring (**Panel B**) Control n=14, TF n=14 (except for 10 nM, n=13); and male offspring (**Panel D**), Control n=11, TF n=11 (except for nM 10, n=10). *p<0.05.

Figure 3.6: Insulin signaling mediator gene expression is not altered in TF exposed female offspring.



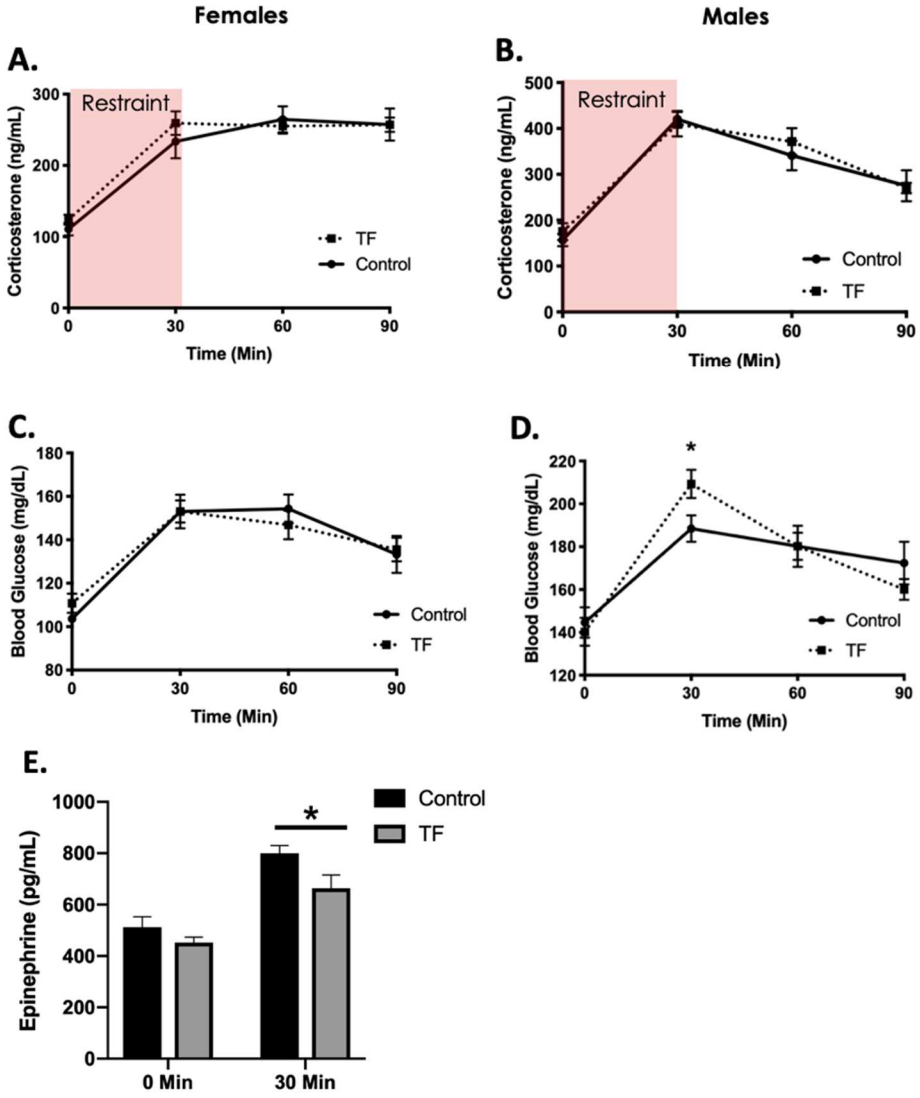
Perigonadal adipose tissue was collected at week 19 from female offspring after a 3-hour fast and gene expression for insulin signaling mediators upstream of Akt was assessed with RT-qPCR (**Panel A**) control n=8-13, TF n=8-14. Perigonadal adipose tissue (**Panel B**), skeletal muscle (**Panel C**), and liver (**Panel D**) was collected from female offspring at week 4 after an overnight fast and gene expression for the insulin receptor and IRS-1 was assessed with RT-qPCR control n=10, TF n=10. IR, insulin receptor; IRS, insulin receptor substrate; PI3K, Phosphatidylinositol 3 phosphate kinase.

Figure 3.7: Perinatal TF exposure does not alter fasting circulating corticosterone in offspring.



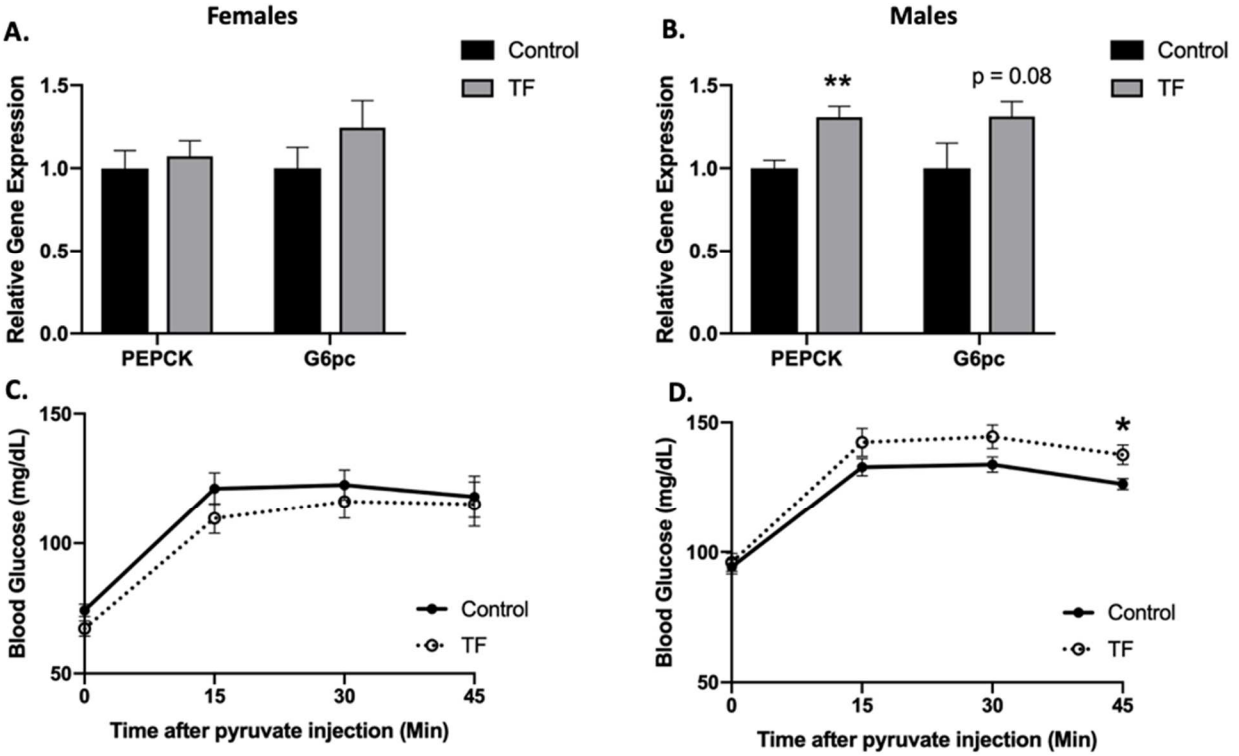
Offspring were fasted for 16 hours (5:00pm – 9:00am) at week 4 and sacrificed by cardiac puncture. Serum corticosterone was measured in total offspring (**Panel A**) Control n=18, TF n=20; female offspring (**Panel B**) Control n=9, TF n=11; and male offspring (**Panel C**) Control n=9, TF n=9.

Figure 3.8: Perinatal TF exposure does not increase HPA reactivity, but increases blood glucose levels in male offspring only.



Offspring HPA axis reactivity was assessed at week 5 by acute restraint test. Circulating corticosterone was measured in females (**Panel A**), Control n=5, TF n=5; and male offspring (**Panel B**), Control n=10, TF n=10**.** Blood glucose levels were measured in females (**Panel C**), Control n=5, TF n=5; and male offspring (**Panel D**), Control n=10, TF n=10**.** Circulating epinephrine was measured in males only (**Panel E**), Control n=10, TF n=10**.** *p<0.05.

Figure 3.9. Perinatal TF exposure results in sex-specific differences in gluconeogenic capacity.



Hepatic gene expression was measured with qPCR at week 4 after an overnight fast (16 hour) for females (**Panel A**) (n=9-10, TF n=10) and males (**Panel B**) (control n=9-10, TF n=12) per group. An IP-PTT was performed at week 20-22 after an overnight fast by IP injection of sodium pyruvate (1 g/kg) and serial blood glucose measured for 75 minutes in female offspring (**Panel C**) (n=5 per group) and male offspring (**Panel D**) (n=10 per group). PTT, pyruvate tolerance test; PEPCK, phosphoenolpyruvate carboxykinase; G6pc, glucose-6-phosphatase; *p<0.05; **p<0.01.

Appendix B: Tables

Table 1.1: Prospective studies documenting associations between EDC exposures and diabetes risk.

Polychlorinated Biphenyls (PCBs)			
Ref.	**Population**	**Results**	**Effect Estimate with Confidence Interval**
[45]	1384 subjects without diabetes in the Michigan PBB Cohort followed for 25 years	Women with the highest (vs. lowest) PCB serum levels had increased incidence of diabetes.	Incidence Density Ratio (IDR): 2.33 [1.25-4.34]
[46]	378 subjects and 370 matched references from the Yucheng Poisoning in Taiwan in the 1970s	Increased risk of incident diabetes in women who consumed rice bran oil laced with PCBs. Women with chloracne, a severe skin manifestation of dioxin-like PCB exposure, had an increased risk of diabetes.	Odds Ratio (OR) for PCBs: 2.1 [1.1-4.5]; OR for chloracne: 5.5 [2.1-13.4]
[51]	471 Great Lakes sport fish consumers without diabetes followed from 1994-5 to 2005	Highest tertile of PCB levels were not associated with incident diabetes.	Incident Rate Ratio (IRR) for diabetes: 1.8; [0.6-5.0] for total PCBs; IRR: 1.3 [0.5–3.0] for PCB 118
[47]	Two case-control studies of women without diabetes from the Nurses' Health Study (NHS), and a meta-analysis of pooled data with six additional prospective studies	After pooling of data, total PCBs were associated with incident diabetes.	Pooled OR for diabetes: 1.70 [1.28-2.27]
[50]	Case-control study of women aged 50-59 years in Southern Sweden	PCB 153 not associated with T2D in women in the highest quartile of exposure examined more than 6 years after study entry.	OR for T2D: 1.6 [0.61-4.0]
[49]	90 cases and controls in a nested case-control study followed for approximately 18 years	Highly chlorinated PCBs showed nonlinear associations with incident diabetes risk.	OR of 5.3 for second sextile vs. lowest sextile of sum of 16 persistent organic pollutants (POPs), including 12 PCBs

Table 1.1 continued

[48]	725 participants from the Prospective Investigation of the Vasculature of Uppsala Seniors (PIVUS) study	Increased OR for T2D across quintiles of a summary measure of PCBs vs. the lowest quintile.	OR: 4.5 [0.9-23.5], 5.1 [1.0-26.0], 8.8 [1.8-42.7], and 7.5 [1.4-38.8]; P_{trend} <0.01
[53]	Meta-analysis of cross-sectional and prospective studies published before March 8, 2014 examining links between various EDCs and diabetes risk	Increased risk of diabetes in the highest exposure to lowest exposure group for serum concentrations of total PCBs.	Relative Risk (RR) for diabetes: 2.39 [1.86-3.08]
Chemical Constituents of Air Pollution			
Ref.	**Population**	**Results**	**Effect Estimate with Confidence Interval**
[64]	65 adults with metabolic syndrome and insulin resistance from the Air-Pollution and Cardiometabolic Diseases (AIRCMD) - China Study	$PM_{2.5}$ exposure during the 4th and 5th day prior to metabolic assessment.associated with worsening insulin resistance.	0.18 (0.02-0.34) and 0.22 (0.08–0.36) HOMA-IR unit increase per standard deviation (SD) increase of $PM_{2.5}$
[66]	3607 individuals from the Heinz Nixdorf Recall Study in Germany followed for an average of 5.1 years	PM exposure associated with incident diabetes.	RRs for PM_{10}: 1.20 [1.00-1.31]; RR for $PM_{2.5}$: 1.11 (0.99-1.23); RR for individuals living closer than 100 m to a busy road relative to those >200 m away: RR: 1.37 (1.04-1.81)
[377]	29,549 women from the Canadian National Breast Screening Study	Increased prevalence of diabetes with increasing $PM_{2.5}$ exposure	Prevalence rate ratio (PR) of diabetes: 1.28 [1.16-1.41] per 10 $\mu g/m^3$ increase in $PM_{2.5}$

Table 1.1 continued

[69]	5839 subjects in the Multi-Ethnic Study of Atherosclerosis (MESA) Cohort	No air pollution measures were signifiantly associated with *incident* DM over 9 years of follow-up; however, *prevalent* diabetes OR per IQR increase showed an association	$PM_{2.5}$ 1.09 [1.00-1.17]; nitrogen oxides 1.18 [1.01-1.38].
[71]	669,046 participants from the American Cancer Society Cancer Prevention Study II	Deaths linked to diabetes (on death certificates) were associated with increased $PM_{2.5}$.	Hazard Ratio (HR): 1.13 [1.02–1.26] per 10 $\mu g/m^3$ increment of $PM_{2.5}$
[72]	2.1 milion adults from the 1991 Canadian Census Mortality Follow-Up Study	A 10 μ/m^3 increase in $PM_{2.5}$ was associated with an increase in diabetes-related mortality.	HR: 1.49 [1.37-1.62]
[65]	Fasting blood from 397 10-year old children in 2 prospective German birth cohort studies	Insulin resistance (IR) increased for every 2 SD increase in ambient NO_2, PM_{10}, and for every 500 m to nearest major road.	17.0% [5.0-30.3] for NO_2; 18.7% [2.9-36.9] for PM_{10}; and 7.2% [0.8-14.0] for proximity to nearest road
[67]	3992 black women living in Los Angeles followed for 10 years	Increase in nitrogen oxides (NOx) were associated with diabetes.	IRR: 1.63 [0.78-3.44] for each 10 $\mu g/m^3$ increase in $PM_{2.5}$; 1.25 [1.07-1.46] for the IQR of NOx (12.4 ppb).
[70]	43,003 participants in the Black Women's Health Study (BWHS) followed from 1995 to 2011	After adjustment for age, metropolitan area, education, vigorous exercise, BMI, smoking, and diet, NO_2 was not associated with diabetes.	HR per IQR of NO_2: 0.96 [0.88-1.06] for a land use regression model for participants living in 56 metropolitan areas; HR: 0.94 [0.80-1.10] using a dispersion model for participants living in 27 cities.

Table 1.1 continued

[68]	1775 non-diabetic women aged 54-55 followed for 16 years in West Germany	HR for diabetes was increased per IQR of traffic-related PM or NO_2 exposure.	HR for PM and NO_2: 1.15 [1.04–1.27]; HR for NO_2: 1.42 [1.16–1.73] using land-use regression.
[73]	22 people with T2D living in North Carolina	Increased ambient $PM_{2.5}$ prior to clinical assessment was associated with impairment in endothilial function, such as decreased flow-mediated dilitation (FMD) and small-artery elasticity index (SAEI).	FMD: –17.3 [–34.6 to 0.0] during the first 24 hours of clinical assessment; SAEI –17.0 [–27.5 to –6.4] and –15.1 [–29.3 to –0.9] 1-3 days prior to clinical assessment
[74]	9202 patients hospitalized with ischemic stroke	Among patients with diabetes, $PM_{2.5}$ was associated with an increased risk of ischemic stroke.	11% [1-22%] increased risk per 10 $\mu g/m^3$ increase in $PM_{2.5}$
[63]	25 healthy adults from rural Michigan brought to an urban location for 5 consecutive days	$PM_{2.5}$ associated with increased insulin resistance.	0.7 [0.1 to 1.3] increase in HOMA-IR per 10 $\mu g/m^3$ increase in $PM_{2.5}$.
Bisphenol A (BPA)			
Ref.	**Population**	**Results**	**Effect Estimate with Confidence Interval**
[88]	971 incident T2D case-control pairs from the NHS II	Extremes of BPA quartiles associated with increased incident T2D after adjusting for BMI.	OR: 2.08 [1.17-3.69].
[89]	2209 non-diabetic middle-aged and elderly subjects followed for 4 years	Individuals with genetic susceptibility to diabetes with high BPA levels showed a greater increase in fasting plasma glucose compared with individuals with low BPA.	Increase in fasting plasma glucose: 0.85 ± 0.07 vs. 0.59 ± 0.04 mmol/L.

Table 1.1 continued

[90]	121 patients with T2D followed for 6 years	Individuals with diabetes and high BPA had increased risk of developing chronic kidney disease vs. subjects with low BPA.	OR: 6.65 [1.47-30.04].
[53]	Meta-analysis of cross-sectional and prospective studies published before March 8, 2014 examining links between various EDCs and diabetes risk	Higher urinary BPA concentrations.were positively associated with diabetes risk.	RR: 1.45 [1.13-1.87].
Phthalates			
Ref.	**Population**	**Results**	**Effect Estimate with Confidence Interval**
[88]	971 incident T2D case-control pairs from the Nurses' Health Study II	Incident T2D in NHSII associated with summed metabolites of butyl phthalates and diethylhexyl phthalate (DEHP).	OR for metabolites of butyl phthalate: 3.16 [1.68-5.95]; OR for DEHP: 1.91 [1.04-3.49].
[103]	250 children of women enrolled in Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) cohort	*In utero* monoethyl phthalate (MEP) associated with lower insulin secretion among pubertal boys. Peripubertal DEHP associated with increased insulin resistance and higher insulin secretion.	17% [-29 to -3.3%] lower insulin secretion among pubertal boys exposed to MEP *in utero*. 20% [2.5-41] higher insulin secretion for peripubertal DEHP exposure.
[53]	Meta-analysis of cross-sectional and prospective studies published before March 8, 2014 examining links between various EDCs and diabetes risk	Group with highest urinary concentrations of phthalates had increased risk for diabetes.	RR: 1.48 [0.98-2.25].

Table 1.1 continued

Organochlorine (OC) Pesticides			
Ref.	**Population**	**Results**	**Effect Estimate with Confidence Interval**
[47]	Two case-control studies of women without diabetes from the Nurses' Health Study (NHS), and a meta-analysis of pooled data with six additional prospective studies	Highest tertile of plasma hexachlorobenzene (HCB) was positively associated with incident T2D. After pooling results from 6 published prospective studies, HCB was associated with incident diabetes.	OR: 3.59 [1.49-8.64]; Pooled OR: 2.00 [1.13-3.53].
[51]	471 Great Lakes sport fish consumers without diabetes followed from 1994-5 to 2005	Higher levels of dichlorodiphenyldichloroethylene (DDE), a metabolite of dichlorodiphenyltrichloroethane (DDT), were associated with incident diabetes.	IRR: 7.1 [1.6–31.9].
[50]	Case-control study of women aged 50-59 years in Southern Sweden	DDE was associated with T2D in women in the highest quartile of exposure followed for more than 6 years.	OR: 5.5 [1.2-25].
[49]	90 cases and controls in a nested case-control study followed for approximately 18 years	Sum of 16 POPs (including 3 OC pesticides) were associated with T2D.	OR (second sextile vs. lowest sextile): 5.4 [1.6-18.4].
[48]	725 participants from the Prospective Investigation of the Vasculature of Uppsala Seniors (PIVUS) study	*Trans*-nonachlor was non-linearly associated with incident diabetes risk (P_{trend}=0.03). Summary measure of 3 OC pesticides non-linearly associated with diabetes risk (P_{trend}=0.03).	OR (fourth quintile of *Trans*-nonachlor vs. first): 4.2 [1.3-13.3]. OR (fifth quintile of OC exposure vs. first): 3.4 [1.0-11.7].
[52]	973 participants of the Flemish Environment and Health Survey	Doubling serum HCB was positively associated with diabetes risk. Doubling DDE was positively associated with diabetes in men but not women.	OR for HCB: 1.61 [1.07-2.42]; OR for DDE: 1.67 [1.25-2.24].
[118]	13,637 women from the Agricultural Health Study	Incident diabetes was positively associated with dieldrin.	HR: 1.99 [1.12-3.54].

Table 1.1 continued

[53]	Meta-analysis of cross-sectional and prospective studies published before March 8, 2014 examining links between various EDCs and diabetes risk	Comparing the highest exposure group to the lowest exposure group, serum concentrations of chlorinated pesticides were positively associated with diabetes.	RR: 2.30 [1.81-2.93].

Table 1.2: Studies documenting racial, ethnic, and/or socioeconomic disparities in exposures to EDCs associated with metabolic disease.

Polychlorinated Biphenyls (PCBs)					
Ref.	**Population**	**Assessment**	**Comparisons**	**Pollutants**	**Differences**
[23]	Pregnant Women from the Child Health and Development study cohort 1963-1967	Percent difference of serum PCBs between [95% CI]	non-white vs. white	PCB 105	6.57 [-7.32-22.1]†
				PCB 110	-1.84 [-20.5-21.2]†
				PCB 118	-1.37 [-11.8-10.2]†
				PCB 137	-15.1 [-30.9-3.12]†
				PCB 138	9.5 [-1.69-22.2]†
				PCB 153	5.35 [-4.3-15.9]†
				PCB 170	6.68 [-3.19-18.5]†
				PCB 180	12.8 [2.52-24.2]†*
				PCB 187	17.9 [5.83-31.9]†*
				Sum PCBs	6.28 [-2.05-15.9]
[378]	Women from the Northern California Region Kaiser Permanente Medical Care Program, 1964-1971	Adjusted mean difference of serum PCBs (ppb) [95% CI]	AA vs. NHW	PCBs (not specified)	1.4 [0.7, 2.1]*
			AA vs. NHW (Breast Cancer patients)		1.7 [0.8, 2.5]*
			AA vs. NHW (Control patients)		1.1 [0.0, 2.2]
[28]	National Adipose Tissue Survey 1972-1979	Population percentage with >3 ppm PCB in adipose tissue; no statistical comparisons are reported	Non-white vs. white	Total PCBs	5.05 vs. 4.52 (1972)†
					11.0 vs. 4.68 (1973)†
					5.58 vs. 4.89 (1974)†

Table 1.2 continued

					12.6 vs. 7.00 (1975)†
					12.6 vs. 6.03 (1976)†
					14.6 vs. 8.96 (1977)†
					10.1 vs. 8.02 (1978)†
					6.11 vs. 4.68 (1979)†
					9.71 vs. 6.10 (Average, ’72-’79)*
[379]	National Human Adipose Tissue Survey 1986	Average adipose concentrations (ng/g) [RSE]	Non-white vs. white	Tetrachloro biphenyl	73.0 [22] vs. 53.0 [11]
				Pentachlor obiphenyl	141 [30] vs. 133 [14]
				Hexachloro biphenyl	435 [15] vs. 289 [8]
				Heptachlor obiphenyl	195 [31] vs. 111 [24]
[105]	Pregnant women from NHANES 1999-2002	GM for serum lipid adjusted PCBs [95% CI]	AA vs NHW	PCB-126 (pg/g)	20.3 [16.9–24.5] vs. 13.9 [12.4–15.6]*
				PCB-138/158 (ng/g)	21.7 [19.4–24.2] vs. 16.2 [15.1–17.3]*
				PCB-153 (ng/g)	30.5 [28–33.2] vs. 22.8 [21.5–24.2]*
				PCB-169 (pg/g)	13.4 [12.1–14.9] vs. 10.9 [9.9–12]*

Table 1.2 continued

				PCB-180 (ng/g)	17.2 [16.1–18.4] vs. 14.1 [13.2–15]*
			MA vs. NHW	PCB-126 (pg/g)	15.9 [14.2–17.7] vs. 13.9 [12.4–15.6]
				PCB-138/158 (ng/g)	13.9 [12.6–15.4] vs. 16.2 [15.1–17.3]*
				PCB-153 (ng/g)	18.2 [16.5–20] vs. 22.8 [21.5–24.2]*
				PCB-169 (pg/g)	9.4 [8.7–10.2] vs. 10.9 [9.9–12]*
				PCB-180 (ng/g)	12.3 [11.5–13.2] vs. 14.1 [13.2–15]*
[380]	NHANES 1999-2002 Elders 60-84 years old	GM [GSD] of serum PCBs (ng/g lipid)	AA vs. NHW	Sum of 12 non–dioxin and dioxin-like PCBs	410 [1.74] vs. 283 [1.67]*
			MA vs. NHW		206 [1.76] vs. 283 [1.67]*
			First PIR quartile vs. fourth quartile		244 [1.80] vs. 294 [1.72]
[381]	NHANES 2001-2004	Total blood concentration of 30 PCB congeners (ng/g lipid)	AA vs. NHW (> 30 years old)	30 PCB congeners	1.97 vs. 1.54†
			AA vs. NHW (+50 years old)		3.08 vs. 2.02
			MA vs. NHW (> 30 years old)		1.50 vs. 1.54†
			MA vs. NHW (+50 years old)		1.57 vs. 2.02

Table 1.2 continued

			AA vs. NHW (+50 years old, female, 95th percentile)		7.68 vs. 4.72
			AA vs. NHW (+50 years old, male, 95th percentile)		7.70 vs. 4.21
[22]	NHANES 2003-2008 Women and men > 60 years of age	Serum PCB 153 (ng/g lipid) ±95%CI	AA vs. NHW Females (‘03-‘04)	PCB 153 in people ≥ 60 years old	146.5 ± 27.7 vs. 62.1 ± 7.8*
			AA vs. NHW Females (‘05-‘06)		129.5 ± 74.8 vs. 58.0 ± 10.8
			AA vs. NHW Females (‘07-‘08)		102.4 ± 15.6 vs. 56.4 ± 7.8*
			AA vs. NHW Males (‘03-‘04)		153 ± 53.6 vs. 65.0 ± 11.0*
			AA vs. NHW Males (‘05-‘06)		103.5 ± 40.9 vs. 60.4 ± 6.2
			AA vs. NHW Males (‘07-‘08)		94.5 ± 29.3 vs. 63.9 ± 10.6
			MA vs. NHW Females (‘03-‘04)		39.5 ± 11.4 vs. 62.1 ± 7.8*
			MA vs. NHW Females (‘05-‘06)		36.2 ± 9.3 vs. 58.0 ± 10.8*
			MA vs. NHW Females (‘07-‘08)		40.9 ± 32.1 vs. 56.4 ± 7.8
			MA vs. NHW Males (‘03-‘04)		36.7 ± 5.5 vs. 65.0 ± 11.0*
			MA vs. NHW Males (‘05-‘06)		37.3 ± 16.8 vs. 60.4 ± 6.2

Table 1.2 continued

			MA vs. NHW Males (‘07-‘08)		39.5 ± 8.9 vs. 63.9 ± 10.6*
			AA vs. NHW Females (‘03-‘04)	PCB 153 in people 40-59 years old	53.2 ± 11.9 vs. 34.2 ± 3.5*
			AA vs. NHW Females (‘05-‘06)		41.2 ± 10.1 vs. 27.8 ± 2.5*
			AA vs. NHW Females (‘07-‘08)		35.7 ± 8.0 vs. 27.7 ± 2.3
			AA vs. NHW Males (‘03-‘04)		59.9 ± 27.2 vs. 38.2 ± 9.4
			AA vs. NHW Males (‘05-‘06)		38.8 ± 15.3 vs. 36.4 ± 16.4
			AA vs. NHW Males (‘07-‘08)		41.0 ± 18.6 vs. 28.2 ± 4.8
			MA vs. NHW Females (‘03-‘04)		23.7 ± 10.5 vs. 34.2 ± 3.5
			MA vs. NHW Females (‘05-‘06)		19.1 ± 3.3 vs. 27.8 ± 2.5*
			MA vs. NHW Females (‘07-‘08)		20.9 ± 6.0 vs. 27.7 ± 2.3
			MA vs. NHW Males (‘03-‘04)		26.5 ± 7.3 vs. 38.2 ± 9.4

Table 1.2 continued

			MA vs. NHW Males (‘05-‘06)		16.5 ± 2.6 vs. 36.4 ± 16.4*
			MA vs. NHW Males (‘07-‘08)		22.4 ± 6.1 vs. 28.2 ± 4.8
[382]	NHANES 2003-2004	Serum PCBs (ng/g lipid) [95% CI]	AA vs. NHW (GM)	Sum of 35 PCBs	148.3 [129.0-170.5] vs. 142.7 [134.2-151.9]
			MA vs. NHW (GM)		71.2 [61.0-83.1] vs. 142.7 [134.2-151.9]*
			AA vs. NHW (90^{th} percentile)		604.6 [454.4-830.6] vs. 406.0 [363.9-433.8]*
			MA vs. NHW (90^{th} percentile)		188.2 [155.8-220.3] vs. 406.0 [363.9-433.8]*
			AA vs. NHW (95^{th} percentile)		984.3 [631.1-1426.9] vs. 508.8 [461.8-539.2]*

Table 1.2 continued

			MA vs. NHW (95th percentile)		245.1 [192.7-323.9] vs. 508.8 [461.8-539.2]*
[383]	6-8 year old girls from California and Ohio 2005-2007	Serum PCB GM (ng/g lipid)	AA vs. NHW	PCB 118	2.4 vs. 3.0*
				PCB 138/158	3.6 vs. 4.5*
				PCB 153	4.2 vs. 6.0*
				PCB 170	1.0 vs. 1.4*
				PCB 180	2.2 vs. 3.2*
			Latinas vs. NHW	PCB 118	2.4 vs. 3.0*
				PCB 138/158	3.6 vs. 4.5*
				PCB 153	4.4 vs. 6.0*
				PCB 170	0.9 vs. 1.4*
				PCB 180	2.1 vs. 3.2*
Chemical Constituents of Air Pollution					
Ref.	**Population**	**Assessment**	**Comparisons**	**Pollutants**	**Differences**
[19]	215 U.S. Census tracts from 2000-2006	Percent increase in long-term average exposure per an additional 10% increase in demographic	AA	$PM_{2.5}$	1.88*
			Latino		0.13
			NHW		-1.37*
[384]	5921 participants from the Multi-Ethnic Study of Atherosclerosis, 2000-2002	Ambient GM for $PM_{2.5}$ ($\mu g/m^3$), and NOx (ppb)	AA vs. NHW	$PM_{2.5}$	16.5 [16.4, 16.6] vs. 15.7 [15.6, 15.8]*
			Latinos vs. NHW		16.9 [16.8, 17.1] vs. 15.7 [15.6, 15.8]*
			AA vs. NHW	NOx	43.3 [42.2, 44.4] vs. 33.6 [33.0, 34.4]*

Table 1.2 continued

			Latinos vs. NHW		58.7 [57.1, 60.4] vs. 33.6 [33.0, 34.4]*
[54]	80 metropolitan areas in the U.S.	Coefficient of total exposure: Log (μ x p x e) +1); μ = concentration at pollution monitor, p = population living within ½ mile of monitor, e = total number of days monitor reported levels higher than federal standards from 2001-2003	% AA	$PM_{2.5}$	3.82*
			% Latino		0.23
			%Poverty		8.85*
			% AA	Ozone	2.37*
			% Latino		0.02
			% Poverty		1.77*
[385]	U.S. Census demographics from 2000, air quality data from 587 U.S. counties from 2005-2007	Odds Ratio for a county being in the worst 20% vs. best 20% of counties for each pollution metric per increase in IQR for each demographic across all U.S. counties	% AA	Annual $PM_{2.5}$	2.73*
			% Latino		0.83
			% living in poverty		3.95*
			% AA	Daily $PM_{2.5}$	1.58*
			% Latino		1.13
			% living in poverty		1.92*
[55]	U.S. population demographics from 2000, air pollution data from 2006	Population-weighted mean (ppb)	Non-white vs. white	NO_2	14.5 vs. 9.9, 38% Relative Difference*
			AA vs. NHW		13.3 vs. 9.9
			Latinos vs. NHW		15.6 vs. 9.9
			Non-white vs. white (Children below the poverty level)		14.3 vs. 9.1

Table 1.2 continued

			Non-white vs. white (Elderly below the poverty level)		14.5 vs. 9.9
Bisphenol A (BPA)					
Ref.	**Population**	**Assessment**	**Comparisons**	**Pollutants**	**Differences**
[386]	NHANES 2003-2004	Adjusted LSGM [95% CI] of Total urinary BPA (µg/L)	Income <$20,000 vs. >$45,000	BPA	3.1 [2.7–3.5] vs. 2.5 [2.3–2.7]*
[387]	NHANES 2005-2006	Total urinary BPA (ng/mL)	AA vs. NHW	BPA	Higher urinary BPA levels in AA than NHW, (Wilcoxon test, P< 0.00001); Note: Original article does not provide urinary concentrations
[79]	NHANES 2003-2006	Total urinary median BPA (µg/g creatinine)	Emergency food assistance vs. no food assistance (Children, 6-11 years olds)	BPA	Percent change 54 [13 to 112]*
			Lowest family Income vs. highest family income		2.5 vs. 1.8 µg/g; Percent Change: 22.8 [10.6, 36.4]*

Table 1.2 continued

			Very low food security vs. full food security		2.6 vs. 2.0 µg/g; Percent change: 19.6 [5.6, 35.5]*
			AA vs NHW		2.2 vs. 2.2 µg/g
			MA vs NHW		1.9 vs. 2.2 µg/g
[388]	South Carolina Pilot Study of 27 pregnant women	Total serum median [range] BPA (ng/mL)	AA vs. NHW	BPA	30.13 [0–134.8] vs. 3.14 [0–37.1]*
			Latinas vs. NHW		24.46 [0.2–153.5] vs. 3.14 [0–37.1]
			Unemployed vs. employed		41 [8.55–153] vs. 7.45 [0–43.7]†*
Phthalates					
Ref.	**Population**	**Assessment**	**Comparisons**	**Pollutants**	**Differences**
[389]	NHANES 1988-1994	Relation between the log of exposure estimates for phthalates and demographic factors	Monthly family income <$1,500 vs. ≥$1,500	BBP	0.23*
			Monthly family income <$1,500 vs. ≥$1,500	DEHP	0.68*
[390]	NHANES 1999-2000	LSGM of urinary phthalates (µg/L)	AA vs. NHW	MEP	237.8 vs. 162.1*
			MA vs. NHW		191.9 vs. 162.1
			AA vs. NHW	MBzP	14.7 vs. 15.5

Table 1.2 continued

			MA vs. NHW		13.1 vs. 15.5*
[97]	2001-2004 NHANES, (20-49 year old women)	GM [GSE] of urinary phthalates (ng/mL); percent change [95% CI]	AA vs. NHW	MEP	268 [26.5] vs. 127 [10.7]; Percent change: 48.4 [16.8-88.6]*
			MA vs. NHW		247 [26.1] vs. 127 [10.7]; Percent change: 58 [24.7-100.8]*
			AA vs. NHW	MnBP	32.3 [2.0] vs. 18.2 [1.0] §
			MA vs. NHW		23.7 [2.3] vs. 18.2 [1.0] §
[150]	2003-2008 NHANES, (12-19 years old)	Mean urinary phthalates (μM)	AA vs. NHW	Low molecular weight phthalates	1.010 vs. 0.662*
			MA vs. NHW		0.891 vs. 0.662*
			First PIR quartile (poor) vs. fourth PIR quartile		0.982 vs. 0.727*
[391]	NHANES 2001-2008, (20-39 year old women)	Multiplicative differences in urinary phthalate concentrations [95% CI]	Non-white vs. white	DBP molar sum	1.26 [1.12-1.40]*
			Income-to-poverty ratio 0-1 (most poor) vs. 4-5		1.16 [1.03-1.32]*
			Non-white vs. white	MEP	1.44 [1.24-1.68]*
			Income-to-poverty ratio 0-1 (most poor) vs. 4-5	MBzP	1.62 [1.37-1.91]*

Table 1.2 continued

			Food Security (Full, Marginal, Low, Very Low)	DBP molar sum	1 (ref), 1.07 [0.87, 1.31], 1.19 [0.97, 1.46], 1.30 [0.98, 1.73]; Trend*
				MBzP	1 (ref), 1.14 [0.97, 1.35], 1.17 [0.95, 1.45] 1.24 [0.98, 1.56]; Trend*
Organochlorine (OC) Pesticides					
Ref.	**Population**	**Assessment**	**Comparisons**	**Pollutants**	**Differences**
[378]	Women from the Northern California Region Kaiser Permanente Medical Care Program, 1964-1971	Adjusted mean difference (ppb) [95% CI]	AA vs. NHW	DDE	13.2 [5.6, 20.9]*
			AA vs. NHW (Breast Cancer patients)		15.5 [4.0, 26.9]*
			AA vs. NHW (Control patients)		11.6 [1.4, 21.8]**
[392]	Dade County, FL, study population, 1965-1967	Mean adipose (ppm) and whole blood (ppb) DDE	DDE	AA vs. NHW (adipose)	10.8 vs. 5.5*
				AA vs. NHW (serum)	16 vs. 8*
[393]	Dade County, FL, residents, 1970-1971	Mean [SD] serum DDT and DDE (ppb)	Lowest social classes vs. highest social classes (AA)	DDT	10.4 vs. 8.0*
			Lowest social classes vs. highest social classes (NHW)		7.4 vs. 5.1*
			AA vs. NHW (highest social class)		7.7 [2.6] vs. 5 [2.7] ‡
			AA vs. NHW (Lowest social class)		11.4 [7.0] vs. 7.9 [6.0] ‡

Table 1.2 continued

			Lowest social classes vs. highest social classes (AA)	DDE	46.8 vs. 35.3*
			Lowest social classes vs. highest social classes (NHW)		31.2 vs. 24.3*
			AA vs. NHW (highest social class)		33.1 [11.3] vs. 22.3 [10.4]
			AA vs. NHW (Lowest social class)		50.5 [30.1] vs. 33.9 [25.2]
[23]	Pregnant Women from the Child Health and Development Study Cohort 1963-1967	Percent difference of serum [95% CI]	Non-white vs. white	pp'-DDE	53.4 [38.3-70.8]†*
				op'-DDT	24.5 [6.53-44.2]†*
				pp'-DDT	48.0 [32.9-64.2]†*
				sum DDTs	53.5 [38.6-69.9]†*
[379]	National Human Adipose Tissue Survey 1986	Average adipose concentrations (ng/g) [RSE]	Non-white vs. white	pp'-DDT	301 [25] vs. 152 [15]
				pp'-DDE	2780 [25] vs. 2250 [13]
				β-HCB	212 [32] vs. 146 [21]
				Heptachlor epoxide	51.6 [19] vs. 58.8 [8]
				Oxychlordane	103 [22] vs. 116 [8]
				Trans-nonachlor	131 [32] vs. 130 [14]
				Dieldrin	54.1 [41] vs. 45.6 [21]

Table 1.2 continued

[105]	Pregnant women in NHANES 1999-2002	GM for serum lipid adjusted pesticides [95% CI]	AA vs. NHW	β-HCH (ng/g)	7.3 [6.5–8.3] vs. 6.7 [6.2–7.2]
				p,p´-DDE (ng/g)	311.6 [253.2–383.4] vs. 177.2 [156.7–200.3]*
				trans-Nonachlor (ng/g)	18.2 [16–20.8] vs. 13.9 [12.7–15.2]*
			MA vs. NHW	β-HCH (ng/g)	19 [16–22.5] vs. 6.7 [6.2–7.2]*
				p,p´-DDE (ng/g)	806.8 [674.6–964.8] vs. 177.2 [156.7–200.3]*
				trans-Nonachlor (ng/g)	14.8 [13.2–16.7] vs. 13.9 [12.7–15.2]
[394]	Pregnant women in in the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) cohort, 1999-2000	GM (ng/g lipid) [range] for CHAMACOS cohort, Median (ng/g lipid) [range] for NHANES	CHAMACOS vs. NHANES	*p,p′*-DDE	1,500 [49 - 159,303] vs. 210.5 [5.4 - 17,900] ‡
			CHAMACOS vs. NHANES	*p,p′*-DDT	24 [2 - 33,174] vs. 6.8 [3.3 - 1,070] ‡
			CHAMACOS vs. NHANES	*o,p′*-DDT	2 [0.1 - 1,878] vs. <LOD‡
[104]	Pregnant women in the CHAMACOS cohort, 1999-2000	Median serum pesticides (ng/g lipid)	CHAMACOS vs. NHANES	HCB	64.9 vs. <LOD‡

Table 1.2 continued

			CHAMACOS vs. NHANES	β-HCH	36.9 vs. 5‡
[383]	6-8 year old girls from California and Ohio 2005-2007	GM (ng/g lipid)	AA vs. NHW	HCB	6.6 vs. 7.8*
			Latinas vs. NHW		7.8 vs. 7.8
			AA vs. NHW	*trans*-nonachlor	3.4 vs. 4.7*
			Latinas vs. NHW		4.3 vs. 4.7
			AA vs. NHW	p,p-DDE	69.1 vs. 72.1
			Latinas vs. NHW		110.7 vs. 72.1*
[382]	NHANES 2003-2004	LSGM of serum pesticides (ng/g lipid) [95% CI]	AA vs. NHW	HCB	14.8 [14.3-15.3] vs. 15.0 [14.2-15.8]
			MA vs. NHW		17.2 [15.9-18.6] vs. 15.0 [14.2-15.8]*
			AA vs. NHW	GM of pp'-DDE	262.4 [233.38-294.98] vs. 208.2 [165.00-262.54]
			MA vs. NHW		444.2 [361.72-545.43] vs. 208.2 [165.00-262.54]*
			AA vs. NHW	β-HCH at the 75th percentile	9.60 [8.30-11.90] vs. 12.80 [10.90-14.70]

Table 1.2 continued

			MA vs. NHW		23.50 [17.50-29.90] vs. 12.80 [10.90-14.70]*
			AA vs. NHW	pp'-DDT at the 90th percentile	17.50 [14.80-25.40] vs. 9.70[8.50-11.20]*
			MA vs. NHW		24.00 [18.50-33.30] vs. 9.70[8.50-11.20]*
			AA vs. NHW	pp'-DDT at the 95th percentile	30.70 [19.00-53.40] vs. 12.90 [10.70-16.60]
			MA vs. NHW		48.60 [31.00-71.10] vs. 12.90 [10.70-16.60]*
			AA vs. NHW	GM of *trans*-nonachlor	14.4 [12.24-16.98] vs. 15.8 [13.72-18.21]
			MA vs. NHW		10.2 [7.68 - 13.24] vs. 15.8 [13.72-18.21]*

Table 1.2 continued

‡: Statistical comparisons between groups not reported, or not possible due to varying detection limits and high non-detect frequency. §: group differences are significant, but single comparisons between groups were not reported. †: Values were estimated from graphs using DigitizeIt software (http://www.digitizeit.de). * Denotes significantly different comparisons where $p < 0.05$ or lower.
Abbreviations: AA: African-American. MA: Mexican-American. NHW: Non-Hispanic White. GM: Geometric Mean. LSGM: Least Square Geometric Mean. GSD: Geometric Standard Deviation. GSE: Geometric Standard Error. RSE: Relative Standard Error. CI: Confidence Interval. NHANES: National Health and Nutrition Examination Survey. $PM_{2.5}$: Particulate Matter <2.5 µm. NOx: Nitrogen oxides. ppm: parts per million. ppb: parts per billion. MnB: Mono-n-butyl phthalate. MEP: monoethyl phthalate, MBzP: Mono-benzyl phthalate. DEHP: Bis(2-ethylhexyl) phthalate. BBP: Benzyl butyl phthalate. DBP: Dibutyl phthalate. DDT: Dichlorodiphenyltrichloroethane. DDE: dichlorodiphenyldichloroethylene. β-HCH: beta-hexachlorocyclohexane. HCB: Hexachlorobenzene.

Table 1.3: Representative animal and cellular studies linking EDCs with metabolic dysfunction.

Metabolic Alterations	PCBs	Chemicals Constituents of Air Pollution	BPA	Phthalates	Organochlorine (OC) Pesticides
Weight Gain and/or Increased Adiposity	**[32]**	**[395, 396]**	**[397]**	**[398]**	**[113]**
Glucose Intolerance	**[34]**		**[399]**	**[100]**	**[113]**
Systemic and/or Cellular Insulin Resistance or Hyperinsulinemia	**[34]**	**[400]**	**[399, 401, 402]**	**[403, 404]**	**[113]**
Altered β-cell Function, Reduced β-cell Mass, or Increased Insulitis	**[405]**		**[402, 406, 407]**	**[100]**	**[408]**
Altered Hepatic Gene Expression, Lipid Handling, and Steatosis	**[409, 410]**	**[396]**	**[411]**	**[412]**	**[410]**
Altered Adipocyte Differentiation and Adipose Gene Expression, including Inflammatory Mediators	**[32]**	**[400]**	**[401, 413, 414]**	**[398]**	**[415]**
Alterations α-cell Signaling			**[416]**		

Table 2.1: Summary of prenatal dexamethasone exposure studies that reported metabolic outcomes in rat offspring.

Ref.	Animal	DEX Treatment	Offspring Sex	Metabolic Outcomes
[251]	Wistar Rat	100 µg/kg/day during third trimester	Male	Decreased glucose tolerance and increased insulin levels during OGTT, increased glucose levels after corticosterone implant, increased hepatic PEPCK and GR expression at 5 months
[242]	Wistar Rat	100 µg/kg/day from GD15-21	Male	Decreased epididymal adiposity, higher 2-deoxyglucose uptake in EDL muscle (at 7-8 months old), increased GR and less lipoprotein lipase in retroperitoneal fat
[238]	Sprgue-Dawley Rat	100 µg/kg/day on last week of gestation	Male	Decreased glucose tolerance and insulin sensitivity at weaning. Increased PEPCK and G6pc gene expression and lower number of islets on PD7 Increased adipose leptin and CEBPα expression, but no differences in adiposity at weaning.
[245]	Wistar Rat	100 µg/kg/day from GD15-21	Male	No differences in glucose tolerance during O-GTT, no differences in retroperitoneal fat pad weight, upregulation of FAS, HSL, and SREBP1c in adipose at 6 months. Increased liver triglyceride content in DEX offspring fed a high fat diet
[249]	Wistar Rat	100 µg/kg/day from GD 14-21	Male and Female	Increased PEPCK activity at weaning in males, evidence of insulin resistance during O-GTT only in males at 6 months. Increased morning ACTH and cort. in DEX-treated males. DEX-exposed female offspring were hypertensive, but showed no metabolic differences.
[237]	Sprague-Dawley Rat	100 mg/kg/day IP from GD 14-20	Male	No differences in blood glucose during GTT, decreased insulin secretion during GTT, trending lower blood glucose levels during IP-ITT at PD120, fewer beta cell pancreatic fractions

Table 2.1 continued

[243]	Wistar Rat	200 µg/kg/d SC on GD 15-19	Male and Female	Decreased glucose tolerance in females during IP-GTT at week 12-13, less visceral adiposity by weight in males, lower fasting insulin in males, increased hepatic PEPCK activity in total offspring at week 14-15
[250]	Sprague-dawley Rat	150 µg/kg/d GD 14-21 DEX	Sex not specified	Lower systemic insulin sensitivity during clamp, increased hepatic PEPCK expression and lower insulin supression of hepatic glucose output, increased circulating corticosterone
[366]	Wistar Rat	200 mg/kg/d from GD 14-19	Sex not specified	Decreased glucose tolerance during IP-GTT at 12 weeks, no difference in insulin tolerance during IP-ITT, increased PEPCK expression after 12-hour fast. Increased hepatic lipid accumulation after 60-hour fast.
[244]	Wistar Rat	100 µg/kg/d DEX on last week of gestation	Male and Female	Lower placental leptin expression. At 1 year, plasma leptin was higher for exposed offspring. Exposed male offspring had lower epididymal adiposity and higher plasma insulin at 1 year.
[417]	Sprague-dawley Rat	100 mg/kg/day IP during GD14-20	Male	Increased hepatic hexokinase 2 and reduced skeletal muscle insulin receptor expression. High fat diet plus prenatal DEX exposure resulted in insulin resistance and glucose intolerance compared to high fat control group
[239]	ICR Mice	600 µg/kg/day BETA SQ for 4 days, GD 8.5, GD 9.5, GD 10.5 and GD 14.5	Sex not specified	Decreased glucose intolerance, and lower insulin secretory capacity during GTT. Higher circulating triglycerides.
[246]	Sprague-dawley Rat	400 µg/kg/d DEX from GD18-23	Male and Female	More severe liver steatosis in DEX-exposed female offspring fed a high-fat diet possibly mediated by suppressed GH axis.

Table 2.1 continued

[247]	Sprague-dawley Rat	100 μg/kg/d DEX from GD 14-21	Male	Increased hepatic lipid accumulation in DEX vs control group. Worse hepatic steatosis and apoptosis in DEX-exposed offspring fed a high-fat diet compared to high fat control group.
[248]	Sprague-dawley Rat	100 μg/kg/d DEX from GD 14-21	Male	Increased hepatic lipid accumulation, apoptosis, and TNF-α expression in DEX vs control group. Worse hepatic steatosis and apoptosis in DEX-exposed offspring fed a high-fat diet compared to high fat control group.

Abbreviations: BETA: betamethasone. Cort: corticosterone. CEBP: CCAAT/enhancer-binding protein. EDL: extensor digitorum longus. FAS: fatty acid synthase. GD: gestational day. HSL: hormone sensitive lipase. OGTT: oral glucose tolerance test. SQ: subcutaneous. SREBP: sterol regulatory element-binding protein 1. TNF: tumor necrosis factor.

Table 3.1: Primer sequences used for qPCR.

Gene	Direction	Primer sequence; 5' → 3'	Amplicon size, bp
G6pc	Forward	TGCAAGGGAGAACTCAGCAA	145
	Reverse	TTGCGCTCTTGCAGAAAGAC	
PEPCK	Forward	TGGTGGGAACTCACTACTCGG	105
	Reverse	ATGCCCAGGATCAGCATATGC	
IR	Forward	TTCAGGAAGACCTTCGAGGATTACCTGCAC	218
	Reverse	AGGCCAGAGATGACAAGTGACTCCTTGTT	
IRS-1	Forward	GCCAGAGGATCGTCAATAGC	140
	Reverse	GAGGAAGACGTGAGGTCCTG	
IRS-2	Forward	AACCTGAAACCTAAGGGACTGG	140
	Reverse	CGGCGAATGTTCATAAGCTGC	
PI3K-p85β	Forward	GGACAGTGAATGCTACAGTAAGC	189
	Reverse	CCTGCAACCTCTCGAAGTG	

Table 3.2: Perinatal TF exposure does not alter pancreatic endocrine cell area.

	Females			Males		
	Control	**TF**	**p-value**	**Control**	**TF**	**p-value**
β-cell area (%)	0.39 ± 0.07	0.28 ± 0.05	0.23	0.22 ± 0.05	0.33 ± 0.04	0.11
α-cell area (%)	0.07 ± 0.02	0.08 ± 0.02	0.67	0.05± 0.02	0.03 ± 0.01	0.35
δ-cell area (%)	0.15 ± 0.07	0.29 ± 0.22	0.57	0.09 ± 0.04	0.04 ± 0.02	0.34
Islet area (%)	0.61 ± 0.12	0.65 ± 0.21	0.87	0.36 ± 0.09	0.41 ± 0.07	0.64

Pancreas was collected at sacrifice (week 19). Histological slides were immunostained for insulin, glucagon, and somatostatin. β-cell, α-cell, and δ-cell areas were calculated as the percent cell area relative to the pancreatic area analyzed, and islet area was calculated as the sum of three endocrine cell types relative to analyzed area. For females, control n = 9, TF n= 8. For males n = 9 per group.