Impact of Perinatal Bisphenol A and High Fat Diets on Non-Alcoholic Fatty Liver Disease

by

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DEDICATION

This PhD research allowed me to combine two of my passions:

environmental preservation and maternal & child health.

Building on the lessons I have learned during graduate school,

my career ambition is to extend the practical applications of environmental health,

working to create a world where health is the default option,

even for the world's most vulnerable and underserved populations.

I dedicate this thesis to my family,

who taught me to treasure the tranquil beauty of nature

and to listen to others with an open heart.

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LIST OF ABBREVIATIONS

10M	10 Months (mouse offspring are 10 months old)
a/a	Agouti mouse, wild type genotype
$A^{vy/a}$	Agouti mouse, viable yellow genotype
BMI	Body mass index
BPA	Bisphenol A
CDC	Centers for Disease Control and Prevention
CBPA	Control diet + 50μg BPA / kg diet
Con	Mouse Control diet group
Cys	Cysteine
CysS	Cystine
DBP	Diastolic blood pressure
DOHaD	Developmental origins of health and disease
EDC	Endocrine disrupting chemical
Eh	Redox potential
EIA	Enzyme Immunoassay
ELEMENT	Early Life Exposures in Mexico to ENvironmental Toxicants
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
FFQ	Food Frequency Questionnaire
GSH	Glutathione
GSSG	Glutathione disulfide
HCC	Hepatocellular carcinoma
HDL-C	High-density lipoprotein – cholesterol
HFD	High fat diet
HPLC	High performance liquid chromatography
IAP	Intracisternal A Particle, a retrotransposon in the mouse genome
ID-LC-MS/MS	Isotope dilution-liquid chromatography-tandem mass spectrometry
INSP	Instituto Nacional de Salud Publica
IR	Insulin Resistance
LMM	Linear mixed models
MDS	Mediterranean Diet Score
MBPA	Mediterranean diet + 50μg BPA / kg diet
Med	Mouse Mediterranean high fat diet group
MetSyn	Metabolic Syndrome
MUFA	Monounsaturated fatty acid
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NHANES	National Health and Nutrition Examination Survey
NIEHS	National Institute of Environmental Health Sciences

NIH	National Institutes of Health
PND10	Postnatal day 10 (mouse offspring are 10 days old)
PND21	Postnatal day 21 (mouse offspring are 21 days old)
PUFA	Polyunsaturated fatty acid
RIA	Radio Immunoassay
SBP	Systolic blood pressure
SFA	Saturated fatty acid
S-glut	S-glutathionylation
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
WBPA	Western diet + 50μg BPA / kg diet
West	Mouse Western high fat diet group

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is now the leading cause of chronic liver disease among youth in the United States. This recent rise of NAFLD may be partially due to perinatal programming, where *in utero* exposures alter the lifelong health trajectory of offspring. Maternal pregnancy diet and endocrine disrupting chemical exposure have been identified as drivers of perinatal programming. However, the potential for maternal diet to modify the impact of perinatal chemical exposure is not well understood. This dissertation examined whether perinatal exposure to two common environmental toxicants, bisphenol A (BPA) and high fat diets (HFDs), would affect NAFLD incidence in offspring. A longitudinal mouse exposure study and a human birth cohort were used to investigate this hypothesis and to evaluate the translation of findings across species.

Oral exposure to one of six diets: Control, Western HFD, Mediterranean HFD or each diet with 50μg BPA/kg added, occurred pre-gestation through lactation. All mice were weaned onto the Control diet, thus isolating exposure to the perinatal period. Offspring NAFLD was assessed via hepatic steatosis and hepatic oxidative response at postnatal day 10 (PND10) and 10-months. Hepatic triglyceride (TG) levels were altered by perinatal HFD in dams, but in offspring perinatal exposures affected metabolic outcomes not hepatic TGs. Hepatic histology from 10-month offspring highly correlated with hepatic TG levels, validating the TG findings. Hepatic 8-isoprostane (8-iso) levels differed by perinatal exposure in PND10 and 10-month offspring, but alterations were age and sex-specific. Perinatal HFD and BPA minimally impacted

offspring redox parameters (*Eh*GSH, *Eh*Cys, S-glut), suggestive of greater homeostatic control of these parameters compared to lipid oxidation. Dam metabolic phenotype significantly altered offspring hepatic steatosis and oxidative response, even when perinatal HFD and BPA did not, emphasizing the critical role of the maternal environment on offspring health.

The impact of maternal BPA exposure and gestational Mediterranean diet adherence (MDS) on the metabolic health of peripubertal youth was examined in a well-established human birth cohort. Youth metabolic and oxidative health was assessed via metabolic risk score (MRS) and serum 8-iso. Maternal pregnancy average and Trimester 2 BPA were associated with a suggestive decrease in youth MRS driven by boys, but a suggestive increase in 8-iso levels driven by girls. Maternal MDS did not affect youth MRS, but altered youth serum 8-iso in opposite directions based on sex. Additional youth characteristics (peripubertal BPA, MDS, vigorous activity, and pubertal status) contributed to predictive models of MRS and 8-iso, underscoring the impact healthy lifestyle behaviors may have, potentially even modifying perinatal programming.

The unexpected lack of protection exerted by the Mediterranean diet in both mouse and human studies, suggests the beneficial effect observed in adults may not apply to perinatal exposure. Greater impact of HFDs in mice but BPA in humans highlights the need to carefully scrutinize findings before translating across species. Despite this difference, sex-specific effects occurred in both species, emphasizing the importance of investigating perinatal programming in all offspring. This research suggests that perinatal BPA and HFD exposure may be insufficient to induce perinatal programming of NAFLD. The significant impact of dam metabolic phenotype in mice and peripubertal behaviors in humans on metabolic and oxidative outcomes suggest NAFLD risk can be altered and potentially prevented at multiple life stages.

CHAPTER 1

Introduction

Rationale & Significance

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease among children and adolescents in the U.S. [1–3]. NAFLD prevalence has increased with the concomitant rise in worldwide obesity and insulin resistance (IR); it is now regarded to be the hepatic precursor of metabolic syndrome [4]. Rising rates of childhood obesity are concerning due to the 2-6 fold increased risk of obese children becoming obese adults [5,6]. An estimated 9% of U.S. children have NAFLD, but prevalence rises to 38% among obese children [7–9]. NAFLD describes a continuum of liver diseases, beginning with simple hepatic steatosis and steatohepatitis, advancing to fibrosis, cirrhosis and in some cases hepatocellular carcinoma [10–12]. Of concern, hepatocellular carcinoma incidence has increased, now occurring in the absence of cirrhosis in up to 50% of cases [13,14].

Simple steatosis and steatohepatitis are reversible with intensive lifestyle change, similar to current recommendations for pre-diabetes and early type 2 diabetes (T2DM): regular exercise and a healthy diet [15,16]. NAFLD prevalence is higher among: males than females, individuals of Mexican-American descent compared to those of non-Hispanic African and Caucasian descent, and individuals with concomitant metabolic disease (e.g. obesity, dyslipidemia, T2DM

and IR in the absence of overt T2DM) [17]. Improved understanding of contributors to NAFLD could help identify early biomarkers of disease. Early detection, while hepatic damage is still reversible, could reduce morbidity and improve quality of life for the 75-100 million individuals estimated to have NAFLD in the U.S. [18].

NAFLD: Pathogenesis, Diagnosis, and Treatment

Pathogenesis of NAFLD

The liver plays a central role in energy homeostasis and nutrient metabolism. These functions are so energy-intensive, the liver accounts for almost 25% of the body's total metabolic rate under basal conditions [19]. In healthy, fasted individuals, the liver relies on β-oxidation of free fatty acids (FFAs) as it's primary fuel source, while releasing glucose into circulation to supply metabolic fuel to peripheral, glucose-dependent tissues [19]. In healthy livers, uptake of circulating non-esterified fatty acids is mediated by fatty acid transport proteins (FATPs) and CD36; in the hepatocyte cytosol, the fatty acids are converted to acyl-CoAs, facilitated by fatty acylCoA synthetases (ACSs). Acyl-CoAs are also formed via *de novo* lipogenesis (DNL), the conversion of glucose to palmitic acid, catalyzed by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). ACS activates palmitic acid to palmitoyl-CoA. Acyl-CoAs are progressively esterified to a glycerol backbone resulting in triglyceride (TG) synthesis. These TGs can be packaged with apolipoproteinB 100 (apoB 100) to form very low density lipoproteins (VLDLs), which are secreted into circulation thus transporting TGs to peripheral tissues [20].

NAFLD begins as simple steatosis, lipid accumulation in hepatocytes. This hepatic lipid accumulation can result from increased FFA uptake from peripheral tissue and a higher rate of

DNL, both resulting in increased hepatic triglyceride levels. Elevated rates of β-oxidation and higher VLDL production are not capable of compensating for the increased TG synthesis, which results in hepatic TG accumulation [20–23]. Hepatic alterations associated with NAFLD, such as increased FFAs, ceramide, JNK1, SOCS, TNFα, and NF-κB levels, interfere with intracellular insulin signaling cascades [24–26]. In healthy individuals, insulin binding to the insulin receptor triggers an intracellular phosphorylation cascade (e.g. PI3K, AKT/PKB). Insulin signaling cascades induce GLUT4 translocation to the cellular membrane of skeletal muscle, facilitating glucose uptake, they also increase lipogenic gene expression and decrease gluconeogenic gene expression. In adipose tissue, insulin has a potent inhibitory affect on lipolysis, but this inhibitory effect is suppressed in NAFLD patients, resulting in greater efflux of FFA out of adipose tissue [27]. Thus, NAFLD and IR appear to be mutually exacerbating conditions.

Although NAFLD is known to run in families, it appears that heritability accounts for only 0.26-0.27 variance in disease prevalence in people of African, Caucasian, and Hispanic descent [28]. The gene locus, *PNPLA3,* is associated with a 3.24 increased in odds of NAFLD; single nucleotide polymorphisms (SNPs) vary by racial-ethnic background and likely impacts the difference in prevalence rates [29,30]. More than 10 SNPs have now been associated with increased incidence of NAFLD, but combined they only account for ~5% variance [29,31]. This leaves a large potential role for environmental influences, gene x environment interactions and epigenetic programming in NAFLD.

Animal models have been used to investigate the molecular, tissue-specific changes that occur during NAFLD development and progression. Although rodents develop hepatic steatosis, the full range of human NAFLD phenotypes are not replicated in murine models [32,33], which has complicated the translation of findings from animal models to relevant clinical outcomes.

Many genetically modified mouse models mimic the histologic appearance of steatosis; for example, *ob/ob* and *db/db* mice exhibit steatosis resulting from obesity [32,34], increased levels of long-chain fatty acids and TGs occur in *CD36*$^{-/-}$ mice leading to steatosis and unchecked hepatic gluconeogenesis [35], additional mouse knock outs promote steatosis by promoting hepatic *de novo* lipogenesis. HFD-feeding of adult C57BL6/J mice induces hepatic steatosis, insulin resistance, and induction of multiple lipogenic transcription factors: *SREBP-1c*, *PPAR*γ, and *LXR*α [33,36].

Diagnosis of NAFLD

Clinical diagnosis of NAFLD is often challenging, since many patients are asymptomatic or have generic symptoms, like fatigue or mild abdominal discomfort in the upper right quadrant [18,37–40]. NAFLD is often discovered during clinical treatment for other metabolic diseases, such as diabetes mellitus, hyperlipidemia, hypertension or as an incidental finding during abdominal imaging for an unrelated condition [18,38–40]. Liver biopsy is the current, gold standard diagnostic measure of NAFLD [41]. Less invasive methods of diagnosis like radiologic procedures (e.g. ultrasound, CT, MRI) and common serum metabolite measures (e.g. ALT, AST, ALP, ferritin) have been investigated. Unfortunately, current serum metabolite panels are only predictive of NAFLD in 50% of patients [41–46], so are unable to predict early steatosis better than the odds of chance alone.

Following a clinical diagnosis of NAFLD in human patients, staging of the disease is performed based on three main criteria: (1) the amount of hepatocyte ballooning, a measure of lipid accumulation per cell, (2) the level of hepatic inflammation, and (3) the extent of fibrosis that has occurred in the liver [41]. Simple steatosis, the early stage of NAFLD, is characterized

by macrovesicular triglyceride accumulation localized to the perivenular region in more than 5% of hepatocytes [47–51]. Progression of NAFLD from simple steatosis to steatohepatitis (NASH) occurs in about 1/3 of cases, and is characterized by scattered intralobular and mild portal inflammation, hepatocellular ballooning, increased steatosis in up to 66% of hepatocytes, and mitochondrial abnormalities. As NASH progresses, steatosis becomes panacinar, portal and intralobular inflammation increase, cellular disarray and early fibrosis become apparent [47,51–53]. NASH can lead to hepatic cirrhosis and hepatocellular carcinoma (HCC). Over a 10 year period, hepatic cirrhosis develops in 5-20% of NASH cases [51]. At these more advanced stages of NAFLD, steatosis and inflammatory markers often become less apparent [54–56].

Interestingly, pediatric NAFLD can have a different histological presentation than the disease in adults. Although simple steatosis presents similarly in people of all ages, pediatric NASH occurs in two distinct patterns. Type I NASH mimics adult histopathology with increasing steatosis, hepatocellular ballooning, and perisinusoidal fibrosis, without portal involvement. In contrast, Type 2 NASH presents as steatosis accompanied by portal inflammation and fibrosis in the absence of hepatocellular ballooning and perisinusoidal fibrosis. Type 2 NASH is unique among children, with one study of pediatric NAFLD, reporting 16% of cases as simple steatosis, 17% as Type 1 NASH, but 51% as Type 2 NASH [57]. Compared to Type 1 NASH, cases of Type 2 NASH were more common among children who were younger, had more severe obesity, were male, or were of Asian, Hispanic, or Native American descent [51].

Treatment of NAFLD

Pharmaceutical efforts to create a therapy for NAFLD, including pioglitazone, pentoxifylline, and vitamin E, have been less than 50% effective [31,37]. A confluence of multiple lipogenic, inflammatory, and fibrogenic pathways in the development and progression of NAFLD may explain the difficulty in diagnosis and treatment of NAFLD [58]. While efforts to find a pharmaceutical therapy for NAFLD continues, lifestyle intervention studies have reported some success. A systematic review of exercise intervention studies (8-48 week long) in NAFLD patients reported reduction of intrahepatic triglyceride levels in exercising participants independent of weight loss. The effect on fat mobilization was 30.2% in exercise interventions, but increased to 49.8% in participants assigned to diet and exercise interventions, suggesting an additional benefit from the combined intervention [59].

Prevalence of NAFLD

Many chronic diseases, including NAFLD, may result from fetal epigenetic reprogramming [60–62]. The Developmental Origins of Health and Disease (DOHaD) paradigm states that the fetal environment impacts gene regulation via epigenetic mechanisms, to optimally prepare the fetus for life in the environment into which it will be born [63,64]. Despite evidence that even healthy pregnancies produce an oxidizing state [65], early life programming as a driver of oxidative stress (OS) response and later chronic disease development has not been studied. One murine study found maternal malnutrition during pregnancy resulted in increased lipid oxidation in prostate tissue of young adult male offspring [14], but few studies have examined the impact on offspring hepatic function or the potential for prenatal exposures to reprogram redox-regulated IR gene expression. In utero diet and chemical exposures have been implicated as modifiers of fetal epigenetic programming [66–68]. Exposure to endocrine disrupting

chemicals (EDCs) is especially concerning since reprogramming can impact enzyme expression levels, hormone regulation, and homeostatic processes across the lifespan.

Perinatal Programming of NAFLD

Metabolic diseases, including NAFLD, can be developmentally programmed. Maternal pre-gestational obesity is associated with increased hepatic lipid storage in infants [69,70]. Elevated placental transfer of fatty acids and glucose, early in pregnancy before subcutaneous adipose tissue develops to store excess nutrients, has been proposed as a potential rationale for the intrahepatic storage of excess lipids in infants of obese mothers [71]. In non-human primates perinatally exposed to a HFD, third trimester fetuses have 3-fold greater hepatic TGs and elevated levels of nuclear (8-OH—dG) and cytosolic (4-hydroxy-2-nonenal, HNE) oxidative stress, without increased body adiposity [72]. Fetal hepatic steatosis not only poses a health threat to young offspring, it also increases NAFLD risk into adulthood, with reports of worsened NAFLD phenotypes in adult mice [73,74]. Some researchers have proposed that the alterations in fetal liver function may be central to metabolic programming [74–76]

Originally proposed in the mid-1990s, the Barker Hypothesis posits that offspring health can be altered by the *in utero* environment [67,77,78]. A mismatch between the fetal and postnatal environments increases the risk of chronic disease later in life; this paradigm is now known as prenatal programming. Although Barker's original hypothesis focused on the effect of maternal diet during pregnancy on offspring's cardiovascular health, the hypothesis has been expanded to include exposure to chemicals [79]. As the field of Environmental Health Sciences incorporated the concept of prenatal programming into its list of potential exposure-disease

relationships, the Barker Hypothesis became more widely known as the Developmental Origins of Health and Disease (DOHaD) hypothesis [80].

Epigenetics, heritable alterations in gene regulation without changing the genetic code, has been proposed as the underlying mechanism, by which these altered phenotypes may occur on a molecular basis. Epigenetics includes multiple types of gene regulatory mechanisms, including DNA methylation, histone modification, and non-coding RNA [81,82], all of which can be affected by the intrauterine environment [83]. DNA methylation is the most thoroughly studied mechanisms and refers to the bonding of a methyl group to a cytosine followed by a guanine (CpG) residue in the DNA code [82,84]. Methylation of CpG sites is generally associated with decreased gene expression; thus, DNA methylation is a method of regulating gene transcription via repression of RNA transcripts [84].

This epigenetic regulation of gene expression is especially sensitive to external exposures (e.g. diet, chemicals, stress) during periods of reprogramming. There are two waves of extensive epigenetic reprogramming that occur during early fetal development. Shortly after fertilization the first wave of global demethylation begins with active demethylation of the paternal genome begins, followed by the slower, passive demethylation of the maternal genome [63,85]. Repetitive elements and some specific gene loci do not completely demethylate during this first wave [86]; imprinted genes retain their parent-of-origin methylation marks, while transposons only partially demethylate on both parental genomes [63]. The second wave of epigenetic reprogramming only occurs in the primordial germ cells of the fetus, so will impact the next generation, if the fetus has offspring of its own. This reprogramming occurs while the primordial germ cells migrate to the genital ridge, occurring between gestational days 11.5-12.5 in mice. All parental, imprinted marks are erased; de novo methylation is established according to fetal sex

[87,88]. In mice, male de novo methylation occurs between gestational days 16.0-18.5. In females, germ cell development is halted following meiosis I; re-methylation occurs after birth in the mature oocyte [89]. Tissue-specific DNA methylation, which drives the specialized structure and function of different tissues, occurs later in development, during organogenesis [85]. These tissue-specific marks are relatively stable, however, fluctuation of DNA methylation in somatic tissues happens during sensitive periods of rapid growth and development, including pregnancy and puberty [63].

Recently, a few new concepts have been posited to advance the understanding of epigenetics as a mechanism underlying environmental exposures and subsequent health effects. One such paradigm is the 'Seed and Soil Model' where 'soil' represents the potential epigenetic modifications in a genomic region, that will impact gene expression via alteration of basal gene expression or modification of the magnitude of response of the gene to an acute stimulus. The 'seed' signifies a cellular signal, which could be homeostatic, toxicant-, pathogen-, pathology-induced [90]. This paradigm emphasizes the importance of considering the cumulative effects of epigenetic changes at a particular genic locus to elucidate the impact of environmental exposures on exposure-triggered disease states. For instance, environmental exposures could lead to changes in basal gene expression via epigenetic reprogramming of the gene's response to a normal, homeostatic stimulus. Or environmental exposures could reprogram a genic locus' magnitude of response to a toxicant or other acute stimulus. In this paradigm, a receptive epigenetic soil will produce a more robust response following stimulus activation, due to the cumulative effect of epigenetic modifications favoring gene expression; non-receptive soil will produce a more moderate response [91].

A second, recently proposed conceptual framework suggests that non-genomic signaling, acting as 'readers, writers, and erasers' of epigenomic signals can alter developmental reprogramming. These processes are controlled by proteins: histone methyltransferase (HMT, 'writers'), histone demethylases (HDM, 'erasers'), and effector proteins ('readers'). Intracellular phosphorylation cascades can regulate several HMTs; post-translational phosphorylation of HDMs alters their function, often triggering their dissociation from chromatin. Finally, phosphorylation of effector proteins alters their intracellular localization. This non-genomic regulation of enzymes that control epigenetic modifications is posited as a mechanism by which endocrine disrupting chemicals may alter epigenetic marks [92]. Non-genomic activity of the estrogen receptor, for instance, includes rapid kinase activation, triggering intracellular phosphorylation cascades [93]. These signals begin by ER activation on the extracellular membrane, thus triggering signaling pathways that are independent of ER localization to the nucleus. This type of non-genomic ER signaling has a demonstrated role in hormone-mediated gene transcription [29], and has been linked to alterations in health of the reproductive tract and mammary gland development.

Bisphenol A Impacts Perinatal Programming

Over 70,000 new synthetic chemicals have been introduced to the market since 1970, less than 10% of which have undergone rigorous, unbiased analysis of their potential impacts on human health [94]. Considered a high production volume chemical by the U.S. EPA, produced in excess of 1 million pounds per year in the U.S., bisphenol A (BPA) [95] is now classified as an endocrine disrupting chemical (EDCs) [68,96–98]. Exposure to EDCs, exogenous chemicals that alter normal hormonal function, is especially concerning due to the potential for developmental

endocrine dysregulation with metabolic repercussions later in life. More than 95% of U.S. adults and children have detectable levels of BPA in their urine [99], suggesting exposure is widespread and likely chronic in the general population.

BPA has been associated with increased risk of obesity and associated metabolic diseases in both animal and human studies. In adults, urinary BPA levels are associated with increased 1.5 odds of obesity, 1.28 odds of abdominal obesity, and 1.37 odds of IR [100]. Among pre-menopausal women, urinary BPA levels were linked to increased fat mass and serum leptin levels [101]. In NHANES analyses from 2003-2006, adults with higher urinary BPA were more likely to be obese and have more abdominal adiposity compared to those with the lowest quartile of BPA levels [102]. Independent of risk factors, such as age, gender, race-ethnicity, BMI, diabetes, and smoking status, urinary BPA was also positively associated with hypertension [103]. The same trend was observed in U.S. children, with odds of obesity 2.5 times higher amongst youth in the top quartile of urinary BPA level compared to the lowest quartile [104]. Higher quartiles of urinary BPA in 6-18 year old children were associated with 1.64-2.01 greater odds of obesity, and 1.37-1.55 greater odds of increased waist circumference to height ratio, without impacting circulating glucose, insulin, or cholesterol levels [105]. These studies were all conducted with cross-sectional data, so causality cannot be determined; it is possible that individuals with greater adiposity are exposed to higher BPA levels.

Prenatal BPA exposure in human birth cohorts has been associated with decreased BMI early in life. In 2-5 year old children, maternal urinary BPA from pregnancy was associated with a non-significant lower BMI, especially in girls, but urinary BPA at 2 years was linked to a more rapid increase in BMI from 2-5 years [106]. Another cohort confirmed these findings, with prenatal urinary BPA from mothers was inversely associated with BMI in girls at 9 years;

urinary BPA in 9 year olds was associated with waist circumference, overall fat mass, and overweight-obesity status in youth of both sexes [107]. Trimester 2 maternal urinary BPA has also been linked to a 7.9 mmHg increase in diastolic blood pressure (DBP) in children at 4 years [108]. Sex-specific impacts of perinatal BPA exposure have been observed in murine studies also. Adult male mice perinatally exposed to human-relevant doses of BPA resulted in dose dependent increases in body and liver weights; however, in adult female mice BPA exposure was associated with decreased body and liver weights, serum leptin and lipids [109]. Male mice prenatally exposed to BPA from gestational days 9-16 had increased IR, reduced glucose tolerance, and altered calcium signaling in the pancreatic islets of Langerhans at 6 months [110]. In 30-day old male offspring, pancreatic β-cell mass and proliferation were greater and apoptosis lower if perinatally exposed to BPA; these pancreatic alterations were accompanied by increased circulating insulin, c-peptide, and leptin levels in these fasted, PND30 offspring. By PND120, pancreatic β-cell mass was decreased in BPA-exposed male offspring [111], suggestive of pancreatic burnout early in life, which may explain diabesogenic outcomes observed following prenatal BPA exposure [112].

BPA exposure has been shown to alter epigenetic marks, suggesting that developmental epigenetic programming may underlie the increased metabolic risks observed in perinatally exposed offspring. In viable yellow agouti (A^{vy}) mice, hypomethylation was observed at the A^{vy} locus the [113], and later global hypomethylation was reported in PND22 offspring, perinatally exposed to 50ng, 50μg, or 50mg BPA/kg diet [114]. Female 10-month offspring from this same perinatal mouse exposure study, had altered DNA methylation at *Jak-2, Rxr, Rfxap,* and *Tmem238*; methylation at all of these loci mediated the connection between perinatal BPA and metabolic outcomes, including body weight, body fat phenotype, and energy expenditure

[115]. Investigation of differential DNA methylation following perinatal BPA reported hypomethylation in neuronal pathways associated with metabolic function and energy regulation [116], further supporting the potential mechanistic role of epigenetic modification in offspring altered metabolic function. Genome-wide analyses have also reported altered DNA methylation by perinatal BPA in both mice and humans [117,118]. BPA exposure has been linked to epigenetic modifications in other mouse and human cohorts [119–124], suggesting widespread physiologic impacts may result from epigenetic alterations during development.

High Fat Diets and Perinatal Programming

Increasing evidence shows that maternal nutrition impacts the intrauterine metabolic environment and successive fetal epigenetic programming [66,125–127]. For example, studies in animal models demonstrate that maternal diet modulation, including caloric [128,129] and protein restriction [130,131], over-nutrition [132,133], and micronutrient supplementation [134], alters DNA methylation at candidate gene loci and globally at cytosine-guanine (CpG) dinucleotides and repetitive elements.

Maternal high fat diet (HFD), in murine and non-human primate models, is associated with offspring adult obesity due to hyperphagia and a preference for high fat, high sugar foods [133,135]. Further work in rodents found that offspring prenatally exposed to HFD had increased hepatic steatosis in adulthood [136]. Thus, intrauterine exposure to a maternal HFD is known to increase risk of metabolic disease and NAFLD development in offspring. It then follows that maternal diet could modify the metabolic health effects of perinatal EDC exposures (like BPA). Interest in this synergy led to the nutrient-toxicant study design of this thesis research. All HFDs used in murine models examining diet-induced-obesity, NAFLD, and other metabolic outcomes

mimic a human Western diet, with a fat content of 45-65% total calories, composed largely of saturated fatty acids (SFA).

Epidemiologic studies suggest that Western and Mediterranean HFDs are both associated with altered risk of NAFLD. A prospective study of adolescents found a Western diet to be associated with NAFLD [137]. Conversely, consumption of a Mediterranean diet among adults with biopsy-confirmed NAFLD reduced hepatic steatosis and improved insulin sensitivity even without weight loss [138]. These findings suggest that composition of the HFD may be a critical component of disease risk. This was the basis for my hypothesis that Western HFD would exacerbate hepatic steatosis and lipid peroxidation resulting from perinatal BPA exposure, while Mediterranean HFD would have a mitigating effect.

Study Overview

Research Objectives

The early stage of NAFLD, 'simple steatosis', is characterized by hepatic lipid accumulation and increased hepatocellular oxidation [58]. Thus, this dissertation was designed to examine the ***hypothesis that perinatal BPA exposure, modified by maternal diet, would increase hepatic lipid accumulation and hepatic oxidative response in offspring, thereby raising lifetime risk of NAFLD*** (Figure 1.1)**.** Further, it was hypothesized that maternal consumption of a Western-style HFD concurrent with the perinatal BPA exposure would increase offspring NAFLD incidence, via either greater magnitude of response or earlier onset of morbidity. On the other hand, maternal Mediterranean HFD intake during perinatal BPA exposure was hypothesized to protect offspring against hepatic lipid accumulation and oxidative responses (Figure 1.2).

These hypotheses were examined in both a perinatal mouse exposure study and an established human birth cohort study, providing an opportunity to compare results across species and to determine if mouse study findings would translate to a human population. This dissertation aimed to examine (1) whether perinatal BPA and HFD exposures would increase hepatic lipid accumulation in mouse offspring, (2) if the exposures would alter hepatic oxidative response in these mouse offspring, and (3) whether maternal BPA levels and Mediterranean diet adherence during pregnancy would impact metabolic health and serum lipid oxidation in peripubertal human youths (Figure 1.3).

Mouse Experimental Design

The perinatal mouse exposure study was conducted using a well-established viable yellow agouti (A^{vy}) mouse colony, in which 250 generations of sibling mating, with forced heterozygosity for the A^{vy} allele, have produced a genetically invariant background. At 8-10 weeks of age, wild-type (*a/a*), virgin dams were randomized to one of six experimental diets: Control, Control + 50 μg BPA/kg diet, Western HFD, Western + 50 μg BPA/kg diet, Mediterranean HFD, or Mediterranean + 50 μg BPA/kg diet. BPA, supplied by the National Toxicology Program (NTP, Durham, NC), was mixed into sucrose, creating a 0.1% BPA/sucrose mixture, which was mailed to Harlan Teklad for incorporation into the pellets of the three BPA-containing experimental diets. After two weeks on their respective diets, dams (n=122) were mate-paired with virgin A^{vy}/a colony males (average: 7.5 weeks old). Dams remained on their assigned diets through pregnancy and lactation.

On postnatal day 21 (PND21), all offspring were weaned onto the Control diet, which they remained on for the duration of the study. In each litter, one male and one female *a/a*

offspring was randomly selected for longitudinal follow-up to 10 months. All remaining *a/a* offspring were sacrificed at postnatal day 10 (PND10). Outcome measurements, hepatic lipid accumulation and oxidative response, were assessed in offspring at both PND10 and 10-months (Figure 1.4).

Mouse Design Decisions

The selection of a representative model species for physiologic research is imperative, to facilitate data interpretation and translation to human health outcomes. Agouti mice are 93% homologous with C57BL/6J and 7% homologous with C3H/HeJ mouse strains [139,140]. C57BL/6J are prone to aging associated obesity, glucose intolerance, insulin resistance, and readily develop diet-induced hepatic steatosis [32,141,142]. Additionally, reactive oxygen species (ROS) and gene expression of oxidative stress loci in liver and retroperitoneal adipose tissues have been observed to increase in HFD-fed male C57BL/6J mice prior to the development of insulin resistance or obesity [143]. Thus, it is reasonable to expect that diet-induced hepatic steatosis and an early hepatic oxidative response could occur in the *a/a* offspring followed in this study. This viable yellow agouti mouse model has been extensively used to examine the epigenetic and phenotypic effects of perinatal BPA exposure [113,114,117,140,144–146], including a BPA dose-range finding study to determine which mouse perinatal exposure level would result in human-relevant levels in mouse offspring liver samples [114]. Further, concurrent nutrient exposure has been shown to modify the impact of perinatal BPA exposure in these mice [113]. So agouti mice are not only expected to develop the physiologic outcomes of interest, but responsiveness to perinatal BPA and dietary exposures have previously been

demonstrated in this strain. This suggests the viable yellow agouti mouse model is a good choice for examining the potential for perinatal programming of NAFLD.

Toxicology studies often examine chemicals at doses levels of magnitude above than common human exposure dose. While this is helpful for determining overt toxicity, studies have suggested that endocrine disrupting chemicals, like BPA, often follow non-monotonic dose response curves; low dose exposures can exert hormone-like effects, triggering physiological cascades of response [147–149]. The BPA dose in this study was chosen based on offspring hepatic BPA levels in a previous perinatal exposure study in this agouti mouse colony [114]. This previous study used the same experimental design, with oral BPA introduced pre-gestation through lactation via the maternal diet. In 10-month mouse offspring, hepatic BPA levels averaged 2.0 ng/g, which is within the range of human fetal liver tissue (mean: 10.8 ng/g) and human maternal serum at delivery (mean: 5.9 ng/mL) (Table 1.1). Ingestion is a major route of BPA exposure in humans [147,150,151], and since this study examined potential modification of BPA-related health effects by diet, BPA was incorporated into the mouse food pellets. Many murine models of BPA exposure introduce the chemical via intraperitoneal (IP) injection or gavage a bolus dose directly into the stomach; these methods ensure exposure dose is identical across animals and consistent daily in each individual animal. Unlike carefully quantified injected doses, ingestion inherently introduces variation into animal dosing due to inter-individual differences in intestinal absorption, uptake, and hepatic processing, but this more realistically mimics human BPA exposure. This study was designed to ensure BPA introduction in the mouse study was relevant to human exposure in route as well as dose.

When animal models are used to examine the impact of diet on a particular health outcome, traditionally one aspect of the diet is altered at a time. For instance, if comparing the

contribution of various lipid species in a diet, two diets composed of 45% calories from fat would be designed with one diet composed entirely of saturated fatty acids (SFA) and the second composed of monounsaturated fatty acids (MUFA); this allows direct comparison of the health effects of SFA vs. MUFA. This type of dietary comparison, altering only lipid composition, was considered for this dissertation research. However, since the perinatal mouse exposure study was designed to mimic human exposure (already discussed regarding BPA), it was determined that designing diets representative of human consumption patterns would provide more translatable results. Protein source and relative amount within the diet (20g/100g food) were held constant between the Control, Western HFD, and Mediterranean HFD. Lipid composition, carbohydrate content, vitamin and mineral levels were altered to reflect the differences observed in traditional Western and Mediterranean-style diets among free-living human populations. These diets are described in depth in Chapter 2. A challenge and common criticism of using human-relevant, experimental diets like this is that if differences are observed between dietary groups, it will be difficult to determine which aspect of the diet is driving the effect. However, the aim of this study was to examine if a common dietary pattern as a whole affected metabolic health outcomes in offspring, which component of the diet drove the effect was not part of the question. Humans eat complex diets with nutrients coming from many distinct food sources, so studies that use high levels of supplementation of a specific dietary nutrient are not able to determine if a complete human diet would result in a similar health effect. For example, a mouse consuming standard chow (e.g. AIN93-G) with omega-3 fatty acids added is not equivalent to a mouse consuming a complete Mediterranean-style diet.

Human Birth Cohort Study

This dissertation was built upon an existing, well-established human birth cohort study, which initially recruited mothers in 1997-2005 at hospitals serving low-to-moderate income areas. The Early Life Exposures in Mexico to ENvironmental Toxicants (ELEMENT), a more than 20 year collaboration with the Instituto Nacional de Salud Publica (INSP) in Mexico, recruited 236 mothers during a Trimester 1 (T1) clinic visit and an additional 14 mothers at delivery. Trimester-specific maternal BMI, urine samples, and dietary intake data were collected for the 236 mothers. Urinary BPA levels were quantified in spot urine samples from all three trimesters. Mediterranean diet scores (MDS) were computed from self-reported food frequency questionnaire data also collected in all three trimesters. A pregnancy average value was calculated for each mother's urinary BPA and MDS in order to make data comparisons between this human birth cohort and the mouse exposure study, which provided experimentally constant exposures across pregnancy.

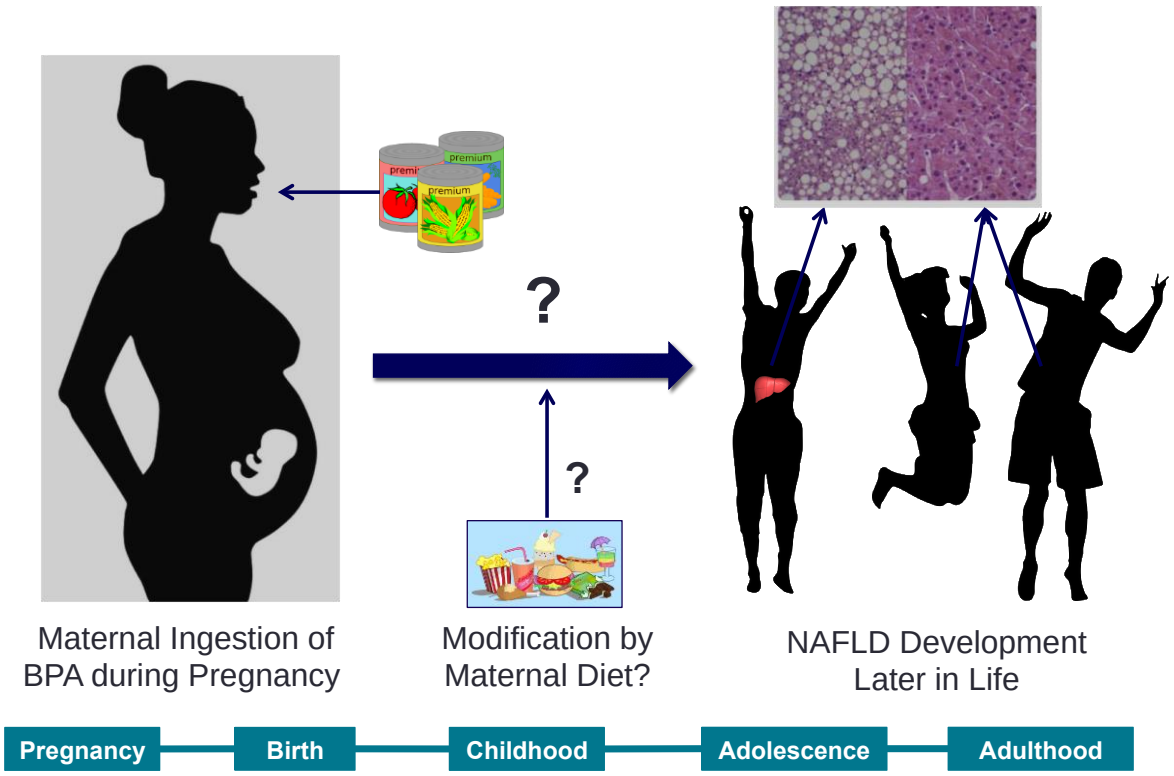
The 250 children followed from birth, in this study, were re-recruited for peripubertal clinic visits in 2010-2012, when they were 8-14 years old. Although research data was collected on these children at multiple clinic visits from birth through early childhood, this study only focused on the impact of prenatal exposures on peripubertal outcomes. Metabolic health in peripubertal youth was assessed via Metabolic Risk Score (MRS) and serum 8-isoprostane. The use of 8-isoprostane as a measure of lipid oxidation in this human cohort was a key determinant in the decision to include an 8-isoprostane outcome measure in the perinatal mouse exposure study, to improve data interpretation and translation from the mouse to human study in this dissertation (Figure 1.4).

Species Specificity & Translation

Mouse studies can provide insight into mechanisms and pathways that are not easily accessible via bioavailable and ethical testing in humans. However, consideration of the translatability of animal results to human studies is critical for improvements in clinical and public health measures. Understanding human health is the main goal of biomedical research. Animal models are used to access tissues, time points, and to undergo exposures that would be unethical to conduct in people. This thesis aimed to make experimental study design between the longitudinal mouse exposure study and human birth cohort as comparable as possible, in order to provide clinically relevant, translatable results. This dissertation included multiple efforts to enhance data translation between the mouse and human findings. Design decisions in the perinatal mouse exposure study that were planned to mimic the ELEMENT birth cohort, include: (1) using an oral route of BPA exposure, (2) creating humanized HFDs for the mice, (3) using a Mediterranean diet score (MDS) to quantify human diet adherence, and (4) measuring the same marker of lipid peroxidation, 8-isoprostane, in both species.

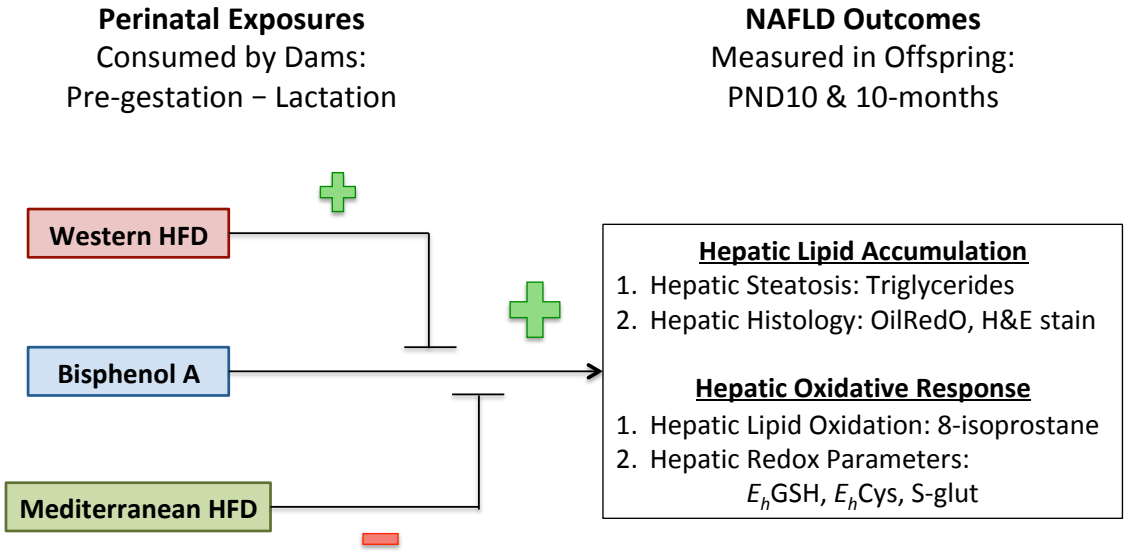
Another benefit of simultaneously investigating a scientific question across multiple species is the ability to compare the effect genetics may play on the exposure-health outcome relationship. The mice used for this study are genetically identical [153], so the genetic variability observed in humans is automatically controlled for in the mouse study. On the other hand, translating the mouse findings to an actual human birth cohort is instructive, because it allows findings to be investigated within the inherent genetic heterogeneity of human populations. The insights gained from examining mice and humans in a similar experimental design can provide a more complete picture of the exposure-health outcome relationship from the molecular level of physiologic mechanisms in the mice to biomarker trends that can be applied in human epidemiologic studies.

Figure 1.1 Conceptual Framework of this Dissertation



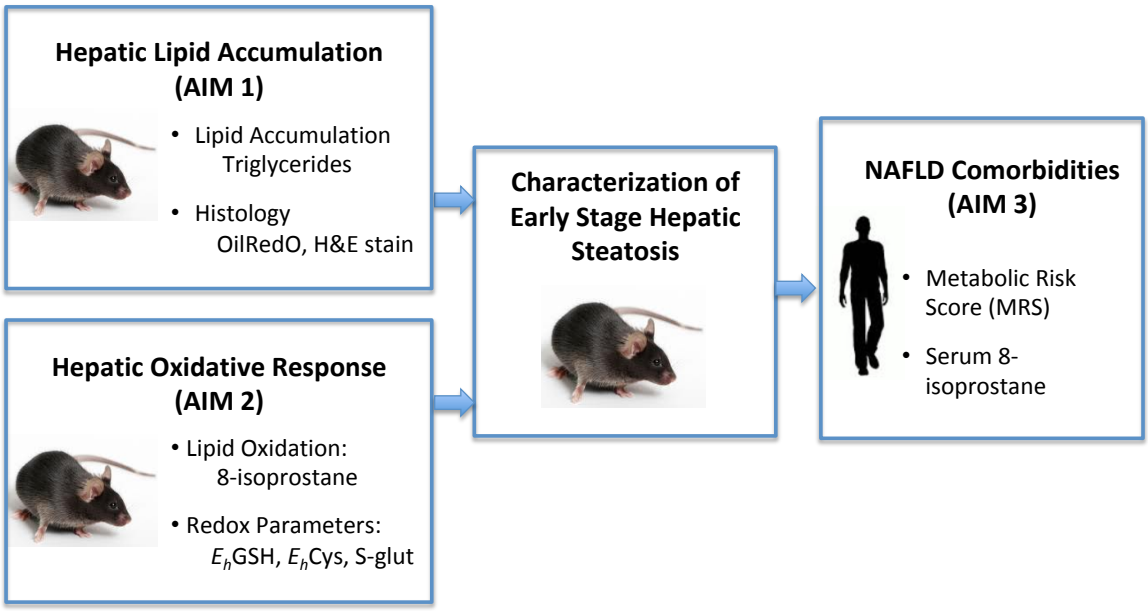
This dissertation aimed to investigate if perinatal BPA exposure would be associated with increased incidence of NAFLD among offspring later in life, and whether this association could be modified by maternal diet during pregnancy.

Figure 1.2 Original Dissertation Hypothesis



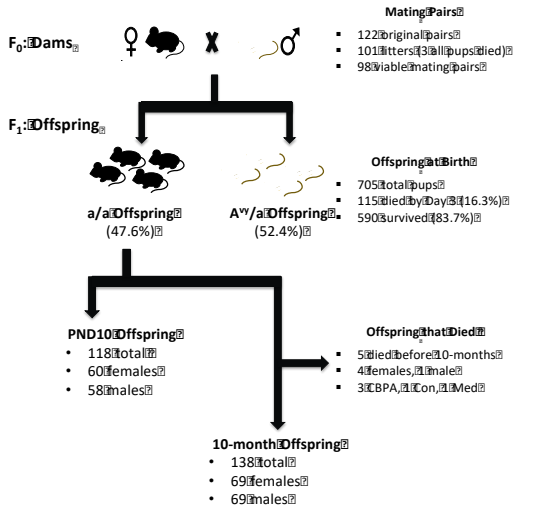
The original hypothesis of this dissertation research was that perinatal BPA exposure, modified by maternal diet, would increase hepatic lipid accumulation and hepatic oxidative response in offspring, thereby raising lifetime risk of NAFLD**.** Perinatal Western HFD exposure was expected to increase the steatotic and oxidative effects of BPA, while Mediterranean HFD exposure was predicted to prevent the NAFLD-inducing impact of perinatal BPA.

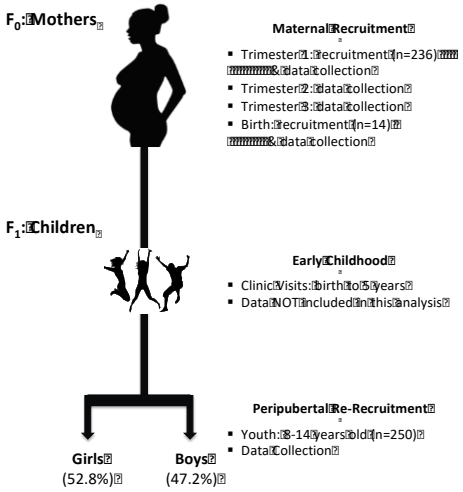
Figure 1.3 Conceptual Diagram of Dissertation Aims



Parallel investigation of a mouse model and human birth cohort study provides the opportunity to examine perinatal programming of NAFLD across species. Invasive, tissue-specific measurements were possible in the mouse offspring, while only minimally invasive techniques were used in the human study. However, examining the same type and timing of exposures between species allowed a comparison of findings, to determine if mouse study results would translate to a human population.

Figure 1.4 Comparison of Mouse and Human Experimental Designs and Study Measurement





Measure Category	Mice by Age		
	Dams	PND10	10-month
Reproductive Outcomes (Chapter 2)	• Time to Delivery • Litter Size • % Live Pups • % Female Pups • % $A^{vy/a}$ Pups	Same as Dams	Same as Dams
Metabolic Phenotype (Chapter 2 & 3)	• Exposure Weight • Mate-Pair Weight • Exposure Weight Change • Gestational Weight Gain • Sacrifice Body Weight • Relative Liver Weight • Relative InWAT Weight • Serum Leptin • Serum Insulin	• Body Weight • Relative Liver Weight • Serum Leptin	• Body Weight • Relative Liver Weight • Relative InWAT Weight • Serum Leptin • Serum Insulin • Serum Resistin • Serum PAI-1
Hepatic Lipid Outcomes (Chapter 2)	• Liver Triglycerides	• Liver Triglycerides	• Liver Triglycerides • Histologic Analysis
Hepatic Oxidative Outcomes (Chapter 3)	• Liver 8-isoprostane • E_hGSH (GSH & GSSG) • E_hCys (Cys & CysS) • S-glutathionylation	• Liver 8-isoprostane • E_hGSH (GSH & GSSG) • E_hCys (Cys & CysS) • S-glutathionylation	• Liver 8-isoprostane • E_hGSH (GSH & GSSG) • E_hCys (Cys & CysS) • S-glutathionylation

Measure Category	Maternal-Child Dyads	
	Mothers	Peripubertal Youth
Environmental Exposures (Chapter 4)	• Urinary BPA (specific-gravity adjusted) [illegible] 1, T2, T3, pregnancy average • Mediterranean Diet Score [illegible] 1, T2, T3, pregnancy average	• Urinary BPA (specific-gravity adjusted) [illegible] peripubertal clinic visit • Mediterranean Diet Score [illegible] peripubertal clinic visit
Demographic & Metabolic Characteristics (Chapter 4)	• Maternal Age • Educational Attainment • Trimester 1 BMI • Gestational Weight Gain	• Youth Age • Pubertal status • BMI • Skinfold thicknesses • Physical Activity: Vigorous & Non
Metabolic Outcome (Chapter 4)	Not Applicable	• Metabolic Risk Score
Oxidative Outcome (Chapter 4)	Not Applicable	• Serum 8-isoprostane

Table 1.1 Comparison of Mouse Hepatic BPA Levels with Human BPA Exposure Levels

Subjects	Tissue	Age	Range Total BPA	Conjugated BPA Mean (SD)	Total BPA Mean (SD)	Reference
Mice (50µg BPA)	Liver	10 mos.	<LOQ – 11.3 ng/g	0.3 (0.3) ng/g	2.0 (3.5) ng/g	Anderson et al, 2012
Human: M&F	Liver	Fetal	<LOQ – 96.8 ng/g	3.2 (8.0) ng/g	10.8 (18.5) ng/g	Nahar et al, 2013
Human: maternal	Serum	Delivery	0.5 – 22.3 ng/mL	n/a	5.9 (0.94) ng/mL	Padmanabhan et al, 2008
Human: M&F	Urine	40-59 yr	<LOQ – 28.0 ng/mL	n/a	3.5 (1.3) ng/mL	LaKind & Naiman, 2015

REFERENCES

1. Loomba R, Sirlin CB, Schwimmer JB, Lavine JE. Advances in Pediatric Nonalcoholic Fatty Liver Disease. Hepatology. 2009;50: 1282–93.

2. Lindbäck SM, Gabbert C, Johnson BL, Smorodinsky E, Sirlin CB, Garcia N, et al. Pediatric Nonalcoholic Fatty Liver Disease: A Comprehensive Review. Adv Pediatr. 2010;57: 85–140.

3. Giorgio V, Prono F, Graziano F, Nobili V. Pediatric Non Alcoholic Fatty Liver Disease: Old and New Concepts on Development, Progression, Metabolic Insight and Potential Treatment Targets. BMC Pediatr. BMC Pediatrics; 2013;13: 40.

4. Lonardo A, Ballestri S, Marchesini G, Angulo P, Loria P. Nonalcoholic Fatty Liver Disease: A Precursor of the Metabolic Syndrome. Dig Liver Dis. Editrice Gastroenterologica Italiana; 2015;47: 181–190.

5. Guo SS, Huang C, Maynard LM, Demerath E, Towne B, Chumlea WC, et al. Body Mass Index during Childhood, Adolescence and Young Ddulthood in Relation to Adult Overweight and Adiposity: The Fels Longitudinal Study. Int J Obes Relat Metab Disord. 2000;24: 1628–35.

6. Guo SS, Wu W, Chumlea WC, Roche AF. Predicting Overweight and Obesity in Adulthood from Body Mass Index Values in Childhood and Adolescence 1 – 3. Am J Clin Nutr. 2002;76: 653–658.

7. Schwimmer JB, Deutsch R, Kahen T, Lavine JE, Stanley C, Behling C. Prevalence of Fatty Liver in Children and Adolescents. Pediatrics. 2006;118: 1388–93.

8. Patton HM, Sirlin C, Behling C, Middleton M, Schwimmer JB, Lavine JE, et al. Pediatric Nonalcoholic Fatty Liver Disease: A Critical Appraisal of Current Data and Implications for Future Research. J Pediatr Gastro Nutr. 2006;43: 413–427.

9. Anderson EL, Howe LD, Jones HE, Higgins JPT, Lawlor A, Fraser A. The Prevalence of Non-Alcoholic Fatty Liver Disease in Children and Adolescents: A Systematic Review and Meta-Analysis. PLoS One. 2015;10: e0140908.

10. Angulo P. Nonalcoholic Fatty Liver Disease and Liver Transplantation. Liver Transplant. 2006;12: 523–534.

11. Angulo P. Obesity and Nonalcoholic Fatty Liver Disease. Nutr Rev. 2007;65: 57–63.

12. Freedman DS, Khan LK, Serdula MK, Dietz WH, Srinivasan SR, Berenson GS. The Relation of Childhood BMI to Adult Adiposity: The Bogalusa Heart Study. Pediatrics. 2005;115: 22–7.

13. Mittal S, El-serag HB. Epidemiology of Hepatocellular Carcinoma Consider the

Population. J Clin Gastroenterol. 2013;47: S2–S6.

14. Yasui K, Hashimoto E, Komorizono Y, Koike K, Arii S, Imai Y, et al. Characteristics of Patients With Nonalcoholic Steatohepatitis Who Develop Hepatocellular Carcinoma. Clin Gastro Hepatol. 2011;9: 428–433.

15. Wong V, Chitturi S, Wong G, Yu J, Chan H, Farrell G. Pathogenesis and Novel Treatment Options for Non-Alcoholic Steatohepatitis. Lancet Gastroenterol Hepatol. 2016;1: 56–67.

16. Sevastianos V a, Hadziyannis SJ. Nonalcoholic Fatty Liver Disease: From Clinical Recognition to Treatment. Expert Rev Gastroenterol Hepatol. 2008;2: 59–79.

17. Lazo M, Hernaez R, Eberhardt MS, Bonekamp S, Kamel I, Guallar E, et al. Prevalence of Nonalcoholic Fatty Liver Disease in the United States: The Third National Health and Nutrition Examination Survey, 1988-1994. Am J Epidemiol. 2013;

18. Rinella ME. Nonalcoholic Fatty Liver Disease: A Systematic Review. J Am Med Assoc. 2015;313: 2263–2273.

19. Corless JK, Middleton III HM. Normal Liver Function: A Basis for Understanding Hepatic Disease. Arch Intern Med. 1983;143: 2291–2294.

20. Kawano Y, Cohen DE. Mechanisms of Hepatic Triglyceride Accumulation in Non-Alcoholic Fatty Liver Disease. J Gastroenterol. 2013;48: 434–41.

21. Dowman JK, Tomlinson JW, Newsome PN. Pathogenesis of Non-Alcoholic Fatty Liver Disease. QJ Med. 2010;103: 71–83.

22. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of Fatty Acids Stored in Liver and Secreted via Lipoproteins in Patients with Nonalcoholic Fatty Liver Disease. J Clin Invest. 2005;115: 1343–1351.

23. Tilg H, Moschen AR. Evolution of Inflammation in Nonalcoholic Fatty Liver Disease: The Multiple Parallel Hits Hypothesis. Hepatology. 2010;52: 1836–1846.

24. McCullough AJ. The Clinical Features, Diagnosis and Natural History of Nonalcoholic Fatty Liver Disease. Clin Liver Dis. 2004;8: 521–33, viii.

25. McCullough A. Pathophysiology of nonalcoholic steatohepatitis. J Clin Gastroenterol. 2006;40: S17–S29.

26. Taniguchi C, Emanuelli B, Kahn C. Critical Nodes in Signalling Pathways: Insights into Insulin Action. Nat Rev Mol Cell Biol. 2006;7: 85–96.

27. Lewis G, Carpentier A, Adeli K, Giacca A. Disordered Fat Storage and Mobilization in the Pathogenesis of Insulin Resistance and Type 2 Diabetes. Endocr Rev. 2002;23: 201.

28. Palmer ND, Musani SK, Yerges-armstrong LM, Feitosa MF, Bielak LF, Hernaez R, et al. Characterization of European Ancestry Nonalcoholic Fatty Liver Disease-Associated Variants in Individuals of African and Hispanic Descent. Hepatology. 2013;58: 966–975.

29. Kahali B, Halligan B, Speliotes EK. Insights from Genome-Wide Association Analyses of Nonalcoholic Fatty Liver Disease. Semin Liver Dis. 2015;35: 375–391.

30. Speliotes EK, Yerges-Armstrong LM, Wu J, Hernaez R, Kim LJ, Palmer CD, et al. Genome-Wide Association Analysis Identifies Variants Associated with Nonalcoholic Fatty Liver Disease that have Distinct Effects on Metabolic Traits. PLoS Genet. 2011;7: e1001324.

31. Rinella ME. Nonalcoholic Fatty Liver Disease A Systematic Review. JAMA. 2015;313: 2263–2273.

32. Anstee QM, Goldin RD. Mouse Models in Non-Alcoholic Fatty Liver Disease and Steatohepatitis Research. Int J Exp Pathol. 2006;87: 1–16.

33. Nakamura A, Terauchi Y. Lessons from Mouse Models of High-Fat Diet-Induced NAFLD. Int J Mol Sci. 2013;14: 21240–21257.

34. Chalasani N, Crabb DW, Cummings O, et al. Does Leptin Play a Role in the Pathogenesis of Human Nonalcoholic Steatosis? Am J Gastroenterol. 2003;98: 2771–2776.

35. Gourdriaan J, Dahlmans V, Teusink B, Al E. CD36 Deficiency Increases Insulin Sensitivity in Muscle, but Induces Insulin Resistance in the Liver in Mice. J Lipid Res. 2003;44: 2270–2277.

36. Deng Q, She H, Cheng J, Al E. Steatohepatitis Induced by Intragastric Overfeeding in Mice. Hepatology. 2005;42: 905–914.

37. Torres DM, Williams CD, Harrison SA. Features, Diagnosis, and Treatment of Nonalcoholic Fatty Liver Disease. Clin Gastroenterol Hepatol. Elsevier Inc.; 2012;10: 837–858.

38. Paredes AH, Torres DM, Harrison SA. Nonalcoholic Fatty Liver Disease. Clin Liver Dis. Elsevier Inc; 2012;16: 397–419.

39. Karlas T, Wiegand J, Berg T. Gastrointestinal Complications of Obesity: Non-Alcoholic Fatty Liver Disease (NAFLD) and its Sequelae. Best Pract Res Clin Endocrinol Metab. 2013;27: 195–208.

40. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The Diagnosis and Management of Non-Alcoholic Fatty Liver Disease: Practice Guideline by the American Association for the Study of Liver Diseases, American College of

Gastroenterology, and the American Gastroenterological Association. Hepatology. 2012;55: 2005–2023.

41. Dowman JK, Tomlinson JW, Newsome PN. Systematic Review: The Diagnosis and Staging of Non-Alcoholic Fatty Liver Disease and Non-Alcoholic Steatohepatitis. Aliment Pharmacol Ther. 2011;33: 525–540.

42. Mofrad P, Contos MJ, Haque M, Sargeant C, Fisher RA, Luketic VA, et al. Clinical and Histologic Spectrum of Nonalcoholic Fatty Liver Disease Associated with Normal ALT values. Hepatology. 2003;37: 1286–1292.

43. Amarapurkar D, Patel N. Clinical Spectrum and Natural History of Non-Alcoholic Steatohepatitis with Normal Alanine Aminotransferase Values. Trop Gastroenterol. 2004;25: 130–134.

44. Portillo-Sanchez P, Bril F, Maximos M, Lomonaco R, Biernacki D, Orsak B, et al. High Prevalence of Nonalcoholic Fatty Liver Disease in Patients With Type 2 Diabetes Mellitus and Normal Plasma Aminotransferase Levels. J Clin Endocrinol Metab. 2015;100: 2231–2238.

45. Maximos M, Bril F, Portillo Sanchez P, Lomonaco R, Orsak B, Biernacki D, et al. The Role of Liver Fat and Insulin Resistance as Determinants of Plasma Aminotransferase Elevation in Nonalcoholic Fatty Liver Disease. Hepatology. 2015;61: 153–160.

46. Molleston JP, Schwimmer JB, Yates KP, Murray KF, Cummings OW, Lavine JE, et al. Histological Abnormalities in Children with Nonalcoholic Fatty Liver Disease and Normal or Mildly Elevated Alanine Aminotransferase Levels. J Pediatr. Elsevier Ltd; 2014;164: 707–713.e3.

47. Hubscher S. Histological Assessment of Non-Alcoholic Fatty Liver Disease. Histopathology. 2006;49: 450–465.

48. Oh M, Winn J, Poordad F. Review: Diagnosis and Treatment of Non-Alcoholic Fatty Liver Disease. Aliment Pharmacol Ther. 2008;28: 503–522.

49. Larter CZ, Yeh MM, Cheng J, Williams J, Brown S, Dela Pena A, et al. Activation of Peroxisome Proliferator-Activated Receptor α by Dietary Fish Oil Attenuates Steatosis, but Does not Prevent Experimental Steatohepatitis because of Hepatic Lipoperoxide Accumulation. J Gastroenterol Hepatol. 2008;23: 267–275.

50. Yeh MM, Brunt EM. Pathological Features of Fatty Liver Disease. Gastroenterology. Elsevier, Inc; 2014;147: 754–764.

51. Takahashi Y, Fukusato T. Histopathology of Nonalcoholic Fatty Liver Disease/Nonalcoholic Steatohepatitis. World J Gastroenterol. 2014;20: 15539–15548.

52. Pessayre D, Fromentry B. NASH: A Mitochondrial Disease. J Hepatol. 2005;42: 928–940.

53. Sanyal A, Campbell-Sargent C, Mirshahi F, et al. Nonalcoholic Steatohepatitis: Association of Insulin Resistance and Mitochondrial Abnormalities. Gastroenterology. 2001;120: 1183–1192.

54. Powell E, Cooksley W, Hanson R, Searle J, Halliday J, Powell L. The Natural History of Nonalcoholic Steatohepatitis: A Follow-Up Study of Forty Two Patients for Up to 21 Years. Hepatology. 1990;11: 74–80.

55. Marrero J, Fontana R, Su G, Conjeevaram HS, Emick D, Lok A. NAFLD May be a Common Underlying Liver Disease in Patients with Hepatocellular Carcinoma in the United States. Hepatology. 2002;36: 1349–1354.

56. Bugianesi E. Review: Steatosis, the Metabolic Syndrome and Cancer. Aliment Pharmacol Ther. 2005;22: 40–43.

57. Schwimmer JB, Behling C, Newbury R, Deutsch R, Nievergelt C, Schork NJ, et al. Histopathology of Pediatric Nonalcoholic Fatty Liver Disease. Hepatology. 2005;42: 641–649.

58. Berlanga A, Guiu-jurado E, Porras JA, Auguet T. Molecular Pathways in Non-Alcoholic Fatty Liver Disease. Clin Exp Gastroenterol. 2014;7: 221–239.

59. Golabi P, Locklear CT, Austin P, Afdhal S, Byrns M, Gerber L, et al. Effectiveness of Exercise in Hepatic Fat Mobilization in Nonalcoholic Fatty Liver Disease: Systematic Review. World J Gastroenterol. 2016;22: 6318–6327.

60. Eberle C, Ament C. Diabetic and Metabolic Programming: Mechanisms Altering the Intrauterine Milieu. ISRN Pediatr. 2012;2012: 975685.

61. Ornoy A. Prenatal Origin of Obesity and their Complications: Gestational Diabetes, Maternal Overweight and the Paradoxical Effects of Fetal Growth Restriction and Macrosomia. Reprod Toxicol. 2011;32: 205–12.

62. Tamashiro KLK, Moran TH. Perinatal Environment and its Influences on Metabolic Programming of Offspring. Physiol Behav. Elsevier Inc.; 2010;100: 560–6.

63. Faulk C, Dolinoy DC. Timing is Everything: The When and How of Environmentally Induced Changes in the Epigenome of Animals. Epigenetics. 2011;6: 791–797.

64. Bernal AJ, Jirtle RL. Epigenomic Disruption: The Effects of Early Developmental Exposures. Birth Defects Res A Clin Mol Teratol. 2010;88: 938–44.

65. Agarwal A, Gupta S, Sharma RK. Role of Oxidative Stress in Female Reproduction. Reprod Biol Endocrinol. 2005;21: 1–21.

66. Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley R a, et al. Developmental Plasticity and Human Health. Nature. 2004;430: 419–21.

67. Barker DJ. The fetal and infant origins of disease. Eur J Clin Invest. 1995;25: 457–63.

68. Schug TT, Janesick A, Blumberg B, Heindel JJ. Endocrine Disrupting Chemicals and Disease Susceptibility. J Steroid Biochem Mol Biol. Elsevier Ltd; 2011;127: 204–15.

69. Brumbaugh DE, Tearse P, Cree-green M, Fenton LZ, Brown M, Scherzinger A, et al. Intrahepatic Fat is Increased in Neonatal Offspring of Obese Women with Gestational Diabetes. J Pediatr. 2013;162: 930–936.

70. Modi N, Murgasova D, Ruager-Martin R, Thomas EL, Hyde MJ, Gale C, et al. The Influence of Maternal Body Mass Index on Infant Adiposity and Hepatic Lipid Content. Pediatr Res. 2011;70: 287–291.

71. Brumbaugh DE, Friedman JE. Developmental Origins of Nonalcoholic Fatty Liver Disease. Pediatr Res. 2014;75: 140–7.

72. Mccurdy CE, Bishop JM, Williams SM, Grayson BE, Smith MS, Friedman JE, et al. Maternal High-Fat Diet Triggers Lipotoxicity in the Fetal Livers of Nonhuman Primates. J Clin Invest. 2009;119: 323–335.

73. Bayol S a, Simbi BH, Fowkes RC, Stickland NC. A Maternal "junk food" Diet in Pregnancy and Lactation Promotes Nonalcoholic Fatty Liver Disease in Rat Offspring. Endocrinology. 2010;151: 1451–61.

74. Bruce KD, Cagampang FR, Argenton M, Zhang J, Ethirajan PL, Burdge GC, et al. Maternal High-Fat Feeding Primes Steatohepatitis in Adult Mice Offspring, Involving Mitochondrial Dysfunction and Altered Lipogenesis Gene Expression. Hepatology. 2009;50: 1796–1808.

75. Benatti RO, Melo AM, Borges FO, Ignacio-Souza LM, Simino LAP, Milanski M, et al. Maternal High-Fat Diet Consumption Modulates Hepatic Lipid Metabolism and microRNA-122 (miR-122) and microRNA-370 (miR-370) Expression in Offspring. Br J Nutr. 2014;111: 2112–2122.

76. Gregorio BM, Souza-Mello V, Carvalho JJ, Mandarim-De-Lacerda CA, Aguila MB. Maternal High-Fat Intake Predisposes Nonalcoholic Fatty Liver Disease in C57BL/6 Offspring. Am J Obstet Gynecol. Elsevier Inc.; 2010;203: 495.e1-8.

77. Phillips DI, Barker DJ. Association Between Low Birthweight and High Resting Pulse in Adult Life: Is the Sympathetic Nervous System Involved in Programming the Insulin Resistance Syndrome? Diabetes Med. 1997;14: 673–677.

78. Barker DJ. Fetal Origins of Cardiovascular Disease. Ann Med. 1999;31: 3–6.

79. Haugen AC, Schug TT, Collman G, Heindel JJ. Evolution of DOHaD: The Impact of Environmental Health Sciences. J Dev Orig Health Dis. 2015;6: 55–64.

80. Barker DJ. The Origins of the Developmental Origins Theory. J Intern Med. 2007;261: 412–417.

81. Holliday R. Epigenetics: An Overview. Dev Genet. 1994;15: 453–457.

82. Holliday R. Epigenetics: A Historical Overview. Epigenetics. 2006;1: 76–80.

83. Gicquel C, El-Osta A, Bouc Y Le. Epigenetic Regulation and Fetal Programming. Best Pract Res Clin Endocrinol Metab. 2008;22: 1–16.

84. Bird A. DNA Methylation Patterns and Epigenetic Memory. Genes Dev. 2002;16: 6–21.

85. Jirtle RL, Skinner MK. Environmental Epigenomics and Disease Susceptibility. Nat Rev Genet. 2007;8: 253–62.

86. Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, Li E, et al. Essential Role for de novo DNA Methyltransferase Dnmt3a in Paternal and Maternal Imprinting. Nature. 2004;429: 900–903.

87. Lees-Murdock D, Walsh C. DNA Methylation Reprogramming in the Germ Line. Epigenetics. 2008;3: 5–13.

88. Sasaki H, Matsui Y. Epigenetic Events in Mammalian Germ-Cell Development: Reprogramming and Beyond. Nat Rev Genet. 2008;9: 129–140.

89. Kota S, Feil R. Epigenetic Transitions in Germ Cell Development and Meiosis. Dev Cell. 2010;19: 675–686.

90. Bowers EC, McCullough SD. Linking the Epigenome with Exposure Effects and Susceptibility: The Epigenetic Seed and Soil Model. Toxicol Sci. 2017;155: 302–314.

91. McCullough SD, Bowers EC, On DM, Morgan DS, Dailey LA, Hines RN, et al. Baseline Chromatin Modification Levels May Predict Interindividual Variability in Ozone-Induced Gene Expression. Toxicol Sci. 2016;150: 216–224.

92. Wong RLY, Walker CL. Molecular pathways: Environmental Estrogens Activate Nongenomic Signaling to Developmentally Reprogram the Epigenome. Clin Cancer Res. 2013;19: 3732–3737.

93. Treviño LS, Wang Q, Walker CL. Hypothesis: Activation of Rapid Signaling by Environmental Estrogens and Epigenetic Reprogramming in Breast Cancer. Reprod Toxicol. 2015;54: 136–140.

94. Colborn T. Neurodevelopment and Endocrine Disruption. Environ Health Perspect.

2003;112: 944–949.

95. EPA. Integrated risk information system (IRIS): Bisphenol A [Internet]. 2012. Available: http://www.epa.gov/iris/subst/0356.htm

96. De Coster S, van Larebeke N. Endocrine-Disrupting Chemicals: Associated Disorders and Mechanisms of Action. J Environ Public Health. 2012;2012: 713696.

97. Halden RU. Plastics and Health Risks. Annu Rev Public Health. 2010;31: 179–94.

98. Waring RH, Ayers S, Gescher a J, Glatt H-R, Meinl W, Jarratt P, et al. Phytoestrogens and Xenoestrogens: The Contribution of Diet and Environment to Endocrine Disruption. J Steroid Biochem Mol Biol. 2008;108: 213–20.

99. Calafat A, Ye X, Wong L, Reidy J, Needham L. Exposure of the U.S. Population to Bisphenol A and 4-tertiary-octylphenol: 2003-2004. Environ Health Perspect. 2008;116: 39–44.

100. Wang T, Li M, Chen B, Xu M, Xu Y, Huang Y, et al. Urinary Bisphenol A (BPA) Concentration Associates with Obesity and Insulin Resistance. J Clin Endocrinol Metab. 2012;97: E223–E227.

101. Zhao H-Y, Bi Y-F, Ma L-Y, Zhao L, Wang T-G, Zhang L-Z, et al. The Effects of Bisphenol A (BPA) Exposure on Fat Mass and Serum Leptin Concentrations have no Impact on Bone Mineral Densities in Non-Obese Premenopausal Women. Clin Biochem. Elsevier B.V.; 2012.

102. Carwile JL, Michels KB. Urinary Bisphenol A and Obesity: NHANES 2003-2006. Environ Res. Elsevier; 2011;111: 825–30.

103. Shankar A, Teppala S. Urinary Bisphenol A and Hypertension in a Multiethnic Sample of US Adults. J Environ Public Health. 2012; 1–5.

104. Bhandari R, Xiao J, Shankar A. Urinary Bisphenol A and Obesity in US Children. Am J Epidemiol. 2013;18: 1–8.

105. Eng DS, Lee JM, Gebremariam A, Meeker JD, Peterson K, Padmanabhan V. Bisphenol A and Chronic Disease Risk Factors in US Children. Pediatrics. 2013;132: e637–e645.

106. Braun JM, Lanphear BP, Calafat AM, Deria S, Khoury J, Howe CJ, et al. Early-Life Bisphenol A Exposure and Child Body Mass Index: A Prospective Cohort Study. Env Heal Perpsect. 2014;122: 1239–1245.

107. Harley KG, Schall RA, Chevrier J, Tyler K, Aguirre H, Bradman A, et al. Prenatal and Postnatal Bisphenol A Exposure and Body Mass Index in Childhood in the CHAMACOS Cohort. Env Heal Perpsect. 2013;121: 514–520.

108. Bae S, Lim YH, Lee YA, Shin CH, Oh SY, Hong YC. Maternal Urinary Bisphenol A Concentration during Midterm Pregnancy and Children's Blood Pressure at Age 4. Hypertension. 2017;69: 367–374.

109. van Esterik J, Dollé M, Lamoree M, van Leeuwen S, Hamers T, Legler J, et al. Programming of Metabolic Effects in C57BL/6JxFVB Mice by Exposure to Bisphenol A during Gestation and Lactation. Toxicology. Elsevier Ireland Ltd; 2014;321: 40–52.

110. Alonso-Magdalena P, Vieira E, Soriano S, Menes L, Burks D, Quesada I, et al. Bisphenol A Exposure during Pregnancy Disrupts Glucose Homeostasis in Mothers and Adult Male Offspring. Environ Health Perspect. 2010;118: 1243–50.

111. García-Arévalo M, Alonso-Magdalena P, Servitja JM, Boronat-Belda T, Merino B, Villar-Pazos S, et al. Maternal Exposure to Bisphenol-A during Pregnancy Increases Pancreatic β-cell Growth during Early Life in Male Mice Offspring. Endocrinology. 2016;157: 4158–4171.

112. Alonso-Magdalena P, Quesada I, Nadal A. Prenatal Exposure to BPA and Offspring Outcomes: The Diabesogenic Behavior of BPA. Dose Response. 2015; 1–8.

113. Dolinoy DC, Huang D, Jirtle RL. Maternal Nutrient Supplementation Counteracts Bisphenol A-Induced DNA Hypomethylation in Early Development. Proc Natl Acad Sci. 2007;104: 13056–13061.

114. Anderson OS, Nahar MS, Faulk C, Jones TR, Liao C, Kannan K, et al. Epigenetic Responses Following Maternal Dietary Exposure to Physiologically Relevant Levels of Bisphenol A. Environ Mol Mutagen. 2012;53: 334–342.

115. Anderson OS, Kim JH, Peterson KE, Sanchez BN, Sant KE, Sartor MA, et al. Novel Epigenetic Biomarkers Mediating Bisphenol A Exposure and Metabolic Phenotypes in Female Mice. Endocrinology. 2017;158: 31–40.

116. Weinhouse C, Sartor MA, Faulk C, Anderson OS, Sant KE, Harris C, et al. Epigenome-Wide DNA Methylation Analysis Implicates Neuronal and Inflammatory Signaling Pathways in AdultMurine Hepatic Tumorigenesis Following Perinatal Exposure to Bisphenol A. Environ Mol Mutagen. 2016;57: 435–446.

117. Kim JH, Sartor M a, Rozek LS, Faulk C, Anderson OS, Jones TR, et al. Perinatal Bisphenol A Exposure Promotes Dose-Dependent Alterations of the Mouse Methylome. BMC Genomics. 2014;15: 1–15.

118. Faulk C, Kim JH, Jones TR, McEachin RC, Nahar MS, Dolinoy DC, et al. Bisphenol A-Associated Alterations in Genome-Wide DNA Methylation and Gene Expression Patterns Reveal Sequence-Dependent and Non-Monotonic Effects in Human Fetal Liver. Environ Epigenetics. 2015;1: 87–92.

119. Kundakovic M, Champagne FA. Epigenetic Perspective on the Developmental Effects

of Bisphenol A. Brain Behav Immun. 2011;25: 1084–93.

120. Kundakovic M, Gudsnuk K, Franks B, Madrid J, Miller RL, Perera FP, et al. Sex-Specific Epigenetic Disruption and Behavioral Changes Following Low-Dose *In Utero* Bisphenol A Exposure. Proc Natl Acad Sci. 2013;110: 9956–9961.

121. Singh S, Li SS-L. Epigenetic effects of environmental chemicals bisphenol a and phthalates. Int J Mol Sci. 2012;13: 10143–53. doi:10.3390/ijms130810143

122. Wolstenholme JT, Rissman EF, Connelly JJ. The Role of Bisphenol A in Shaping the Brain, Epigenome and Behavior. Horm Behav. Elsevier Inc.; 2011;59: 296–305.

123. Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK. Plastics Derived Endocrine Disruptors (BPA, DEHP and DBP) Induce Epigenetic Transgenerational Inheritance of Obesity, Reproductive Disease and Sperm Epimutations. PLoS One. 2013;8: e55387.

124. Ito Y, Nakamura T, Yanagiba Y, Ramdhan DH, Yamagishi N, Naito H, et al. Plasticizers May Activate Human Hepatic Peroxisome Proliferator-Activated Receptor α Less Than That of a Mouse but May Activate Constitutive Androstane Receptor in Liver. PPAR Res. 2012;2012: 201284.

125. Lillycrop K a, Burdge GC. The Effect of Nutrition during Early Life on the Epigenetic Regulation of Transcription and Implications for Human Diseases. J Nutrigenet Nutrigenomics. 2011;4: 248–60.

126. Canani RB, Costanzo M Di, Leone L, Bedogni G, Brambilla P, Cianfarani S, et al. Epigenetic Mechanisms Elicited by Nutrition in Early Life. Nutr Res Rev. 2011;24: 198–205.

127. El Hajj N, Schneider E, Lehnen H, Haaf T. Epigenetics and Life-Long Consequences of an Adverse Nutritional and Diabetic Intrauterine Environment. Reproduction. 2014;148: R111–R120.

128. Gluckman PD, Lillycrop K a, Vickers MH, Pleasants AB, Phillips ES, Beedle AS, et al. Metabolic Plasticity during Mammalian Development is Directionally Dependent on Early Nutritional Status. Proc Natl Acad Sci U S A. 2007;104: 12796–800.

129. Palou M, Priego T, Sánchez J, Palou A, Picó C. Sexual Dimorphism in the Lasting Effects of Moderate Caloric Restriction during Gestation on Energy Homeostasis in Rats is Related with Fetal Programming of Insulin and Leptin Resistance. Nutr Metab (Lond). 2010;7: 69–79.

130. Altobelli G, Bogdarina IG, Stupka E, Clark AJL, Langley-Evans S. Genome-Wide Methylation and Gene Expression Changes in Newborn Rats Following Maternal Protein Restriction and Reversal by Folic Acid. PLoS One. 2013;8: e82989.

131. Oster M, Murani E, Metges CC, Ponsuksili S, Wimmers K. A Low Protein Diet during Pregnancy Provokes a Lasting Shift of Hepatic Expression of Genes Related to Cell Cycle Throughout Ontogenesis in a Porcine Model. BMC Genomics. BioMed Central Ltd; 2012;13: 93.

132. Burgueño AL, Cabrerizo R, Gonzales Mansilla N, Sookoian S, Pirola CJ. Maternal High-Fat Intake during Pregnancy Programs Metabolic-Syndrome-Related Phenotypes through Liver Mitochondrial DNA Copy Number and Transcriptional Activity of Liver PPARGC1A. J Nutr Biochem. 2013;24: 6–13.

133. Samuelsson A-M, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EHJM, et al. Diet-Induced Obesity in Female Mice Leads to Offspring Hyperphagia, Adiposity, Hypertension, and Insulin Resistance: A Novel Murine Model of Developmental Programming. Hypertension. 2008;51: 383–392.

134. Gao R, Ding Y, Liu X, Chen X, Wang Y, Long C, et al. Effect of Folate Deficiency on Promoter Methylation and Gene Expression of Esr1, Cdh1 and Pgr, and its Influence on Endometrial Receptivity and Embryo Implantation. Hum Reprod. 2012;27: 2756–65.

135. Sullivan EL, Smith MS, Grove KL. Perinatal Exposure to High-Fat Diet Programs Energy Balance, Metabolism and Behavior in Adulthood. Neuroendocrinology. 2011;93: 1–8.

136. Oben JA, Mouralidarane A, Samuelsson A, Matthews PJ, Morgan ML, Mckee C, et al. Maternal Obesity during Pregnancy and Lactation Programs the Development of Offspring Non-Alcoholic Fatty Liver Disease in Mice. J Hepatol. European Association for the Study of the Liver; 2010;52: 913–920.

137. Oddy WH, Herbison CE, Jacoby P, Ambrosini GL, O'Sullivan T a, Ayonrinde OT, et al. The Western Dietary Pattern Is Prospectively Associated With Nonalcoholic Fatty Liver Disease in Adolescence. Am J Gastroenterol. 2013; 1–8.

138. Ryan MC, Itsiopoulos C, Thodis T, Ward G, Trost N, Hofferberth S, et al. The Mediterranean Diet Improves Hepatic Steatosis and Insulin Sensitivity in Individuals with Non-Alcoholic Fatty Liver Disease. J Hepatol. 2013;59: 138–143.

139. Waterland RA, Jirtle RL. Transposable Elements: Targets for Early Nutritional Effects on Epigenetic Gene Regulation. Mol Cell Biol. 2003;23: 5293–5300.

140. Weinhouse C, Anderson O, Bergin I, Vandenbergh D, Gyekis J, Dingman M, et al. Dose-Dependent Incidence of Hepatic Tumors in Adult Mice following Perinatal Exposure to Bisphenol A. Env Heal Perpsect. 2014;122: 485–491.

141. Fengler VHI, Macheiner T, Kessler SM, Czepukojc B, Gemperlein K, Muller R, et al. Susceptibility of Different Mouse Wild Type Strains to Develop Diet-Induced NAFLD / AFLD-Associated Liver Disease. PLoS One. 2016;11: 1–21.

142. Hansen HH, Feigh M, Veidal SS, Rigbolt KT, Vrang N, Fosgerau K. Mouse Models of Nonalcoholic Steatohepatitis in Preclinical Drug Development. Drug Discov Today. Elsevier Ltd; 2017;22: 1707–1718.

143. Matsuzawa-Nagata N, Takamura T, Ando H, Nakamura S, Kurita S, Misu H, et al. Increased Oxidative Stress Precedes the Onset of High-Fat Diet-Induced Insulin Resistance and Obesity. Metab Clin Exp. 2008;57: 1071–1077.

144. Anderson OS, Peterson KE, Sanchez BN, Zhang Z, Mancuso P, Dolinoy DC. Perinatal Bisphenol A Exposure Promotes Hyperactivity, Lean Body Composition, and Hormonal Responses across the Murine Life Course. FASEB J. 2013;27: 1784–1792.

145. Faulk C, Kim JH, Anderson OS, Nahar MS, Jones TR, Sartor MA, et al. Detection of Differential DNA Methylation in Repetitive DNA of Mice and Humans Perinatally Exposed to Bisphenol A. Epigenetics. 2016;11: 489–500.

146. Kochmanski J, Marchlewicz EH, Savidge M, Montrose L, Faulk C, Dolinoy DC. Longitudinal Effects of Developmental Bisphenol A and Variable Diet Exposures on Epigenetic Drift in Mice. Reprod Toxicol. 2017;68: 154–163.

147. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons W V. Human Exposure to Bisphenol A (BPA). Reprod Toxicol. 2007;24: 139–177.

148. Vandenberg LN. Non-Monotonic Dose Responses in Studies of Endocrine Disrupting Chemicals: Bisphenol A as a Case Study. Dose-Response. 2014;12: 259–276.

149. Vandenberg LN, Gerona RR, Kannan K, Taylor JA, van Breemen RB, Dickenson CA, et al. A Round Robin Approach to the Analysis of Bisphenol A (BPA) in Human Blood Samples. Environ Heal. 2014;13: 25–45.

150. Schecter A, Malik N, Haffner D, Smith S, Harris TR, Paepke O, et al. Bisphenol A (BPA) in U.S. Food. Env Sci Technol. 2010;44: 9425–9430.

151. Rudel RA, Gray JM, Engel CL, Rawsthorne TW, Dodson RE, Ackerman JM, et al. Food Packaging and Bisphenol A and Bis(2-Ethyhexyl) Phthalate Exposure: Findings from a Dietary Intervention. Env Heal Perpsect. 2011;119: 914–920.

152. Trichopoulou A, Katsouyanni K, Gnardellis C. The Traditional Greek Diet. Eur J Clin Nutr. 1993;47: S76–S81.

153. Dolinoy DC. The Agouti Mouse Model: An Epigenetic Biosensor for Nutritional and Environmental Alterations on the Fetal epigenome. Nutr Rev. 2008;66: S7–S11.

CHAPTER 2

Perinatal Exposure to Experimental Diets and Bisphenol A Alters Metabolic Outcomes in Dams and Offspring, but Increases Hepatic Steatosis Only in Dams

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) prevalence is increasing worldwide. Perinatal development has been identified as a critical window for altered lifelong health trajectory, and evidence supports a role for perinatal programming in chronic metabolic diseases, including obesity, type 2 diabetes, and metabolic syndrome. To examine the role two common exposures, diet and bisphenol A (BPA), play on perinatal programming of NAFLD across the life course, we developed a longitudinal mouse model that uses a human-relevant dose of oral BPA coupled with intake of Western or Mediterranean style diets in dams, from pre-gestation through lactation. We assessed hepatic steatosis via quantification of hepatic triglycerides (TGs) and metabolic health via body weight, relative organ weights, and serum hormone levels in dams and offspring, at postnatal day 10 (PND10) and 10-months. In dams, consumption of the Western or Mediterranean diets increased hepatic TGs 1.7-2.4 fold ($p < 0.005$), independent of BPA intake. However, among offspring, perinatal exposures had a greater impact on metabolic outcomes than on hepatic steatosis. At PND10, serum leptin levels were elevated (2.6-4.8 fold, $p < 0.03$) in pups

exposed to the Mediterranean diet, with sex-specific effects on body and organ weights ($p < 0.10$). Similarly, at 10-months sex-specific increases in body ($p < 0.10$), organ weight ($p < 0.02$), and hormone levels ($p < 0.005$) were observed; however, they occurred mainly in mice perinatally exposed to the combination diets, Western+BPA or Mediterranean+BPA. These findings suggest that perinatal exposure to experimental diets and low-dose BPA may not be sufficient to program NAFLD later in life. Further, the impact of perinatal exposures on offspring metabolic health may differ by offspring life stage. Finally, alterations in dam phenotype by exposure appear to impact offspring health trajectory, suggestive of a need to better characterize mothers in future perinatal exposure studies.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is now the leading cause of liver disease among U.S. children and adults [1–3]. NAFLD prevalence has increased coincident with the rise in global obesity and insulin resistance (IR) and is likely a metabolic precursor to type 2 diabetes (T2DM) and metabolic syndrome (MetSyn) [4]. An estimated 9% of U.S. children have NALFD, but prevalence estimates rise to 38% among obese children [5], who will suffer a prolonged morbidity from these early-life alterations in hepatic function. NAFLD describes a range of pathologic states, beginning with simple hepatic steatosis and steatohepatitis, advancing to fibrosis, cirrhosis and in some cases hepatocellular carcinoma. If detected early at the simple steatosis stage, the condition is reversible [6], making steatosis a prime target for public health intervention. Thus, improved understanding of early life factors that contribute to hepatic steatosis is critical for slowing the incidence of youth NAFLD.

Many perinatal conditions alter fetal developmental trajectory and culminate in adult metabolic pathologies. Maternal disease states, nutritional deficit or excess, lifestyle choices, substance abuse, and environmental exposure to endocrine disrupting chemicals (EDCs) have all been associated with increased risk of offspring metabolic disease [7]. For instance, maternal pre-gestational weight/obesity and gestational weight gain are associated with increased risk of metabolic syndrome, insulin resistance, and obesity in offspring, in animal models and epidemiologic studies [8–11]. Perinatal diet, a contributing factor in maternal obesity, plays a major role in organizing the fetal developmental trajectory and offspring metabolism and health later in life [12,13]. In particular, *in utero* overnutrition has been associated with increased hepatic steatosis in offspring, suggestive of a prenatal programming component to NAFLD [14–16]. However, based on these studies, it is unclear if all hypercaloric diets would have the same steatotic effect.

Perinatal exposure to a Western-style high fat diet (HFD) is associated with a well-documented increase in offspring hepatic steatosis and metabolic alterations, in animal models [17–21]. Similarly, in human adolescents consumption of a Western HFD is associated with increased risk of steatosis [22,23]. On the other hand, adherence to a Mediterranean HFD in human adults is associated with reversal of biopsy-confirmed NAFLD, significantly reducing hepatic steatosis and improving insulin sensitivity, even without weight loss [24–26]. A recent article even proposed the Mediterranean diet as the "diet of choice" for NAFLD patients [27]. However, perinatal exposure to a Mediterranean-style HFD has never been conducted in a murine model. The potential ability of a maternal Mediterranean HFD to protect against hepatic steatosis in offspring is appealing as a low-cost, high-gain public health intervention.

In addition to diet, perinatal exposure to the EDC bisphenol A (BPA) has been associated with fetal programming of metabolic diseases [28], including NAFLD [29–31]. BPA, a high production volume synthetic chemical found predominantly in polycarbonate plastics and epoxy resins [32], has been shown to cross the placenta in both humans and murine models [33–35]. We previously reported reduced expression of BPA-specific biotransformation enzymes (e.g. *UGT2B15, SULT1A1,* and *STS*) in human fetal tissue [35], suggestive of impaired fetal ability to metabolize BPA, making even low-dose exposures concerning. Perinatal exposure to low-dose BPA via either dam drinking water or subcutaneous pump is associated with greater body weight and hepatic lipid accumulation in offspring [36,37]. In both of these studies, dam BPA exposure began after conception, but a wave of fetal reprogramming occurs in the first few hours to days post-fertilization [38,39]; the later BPA exposures miss this critical window.

Recognizing that human BPA exposure does not occur in a sterile environment, recent studies have begun to investigate the potential interaction between BPA and diet. Two murine models of perinatal oral BPA exposure with a post-weaning HFD challenge have reported greater hepatic damage in offspring exposed to both [40,41]. We built on these experiments by providing simultaneous perinatal exposure to diet and BPA, thus mimicking the concurrent exposure human mothers experience. Furthermore, to assess the potential impact of a diet-BPA interaction when background diet varied, we added low-dose BPA to a control diet and to Western and Mediterranean experimental diets.

We examined the impact of perinatal experimental diets, with and without BPA, on hepatic steatosis and metabolic outcomes in dams and in offspring across the life-course (PND10 and 10-months). Hepatic steatosis was assessed via hepatic triglyceride (TG) quantification in dams, PND10 and 10-month offspring; liver histology (OilRedO and H&E vacuolation) was

used to validate hepatic TG levels in 10-month offspring. Metabolic outcomes included body weight, relative organ weights, and serum hormone levels, for all mice. We hypothesized that perinatal Western diet would increase hepatic TGs and trigger metabolic alterations, while Mediterranean diet would minimize these changes. We also evaluated whether adding BPA to experimental diets would exacerbate hepatic steatosis. This study advances our understanding of factors that contribute to developmental programming of hepatic steatosis and related metabolic alterations in five ways, investigating: timing of exposure, comparison of two different experimental diets, effects of concurrent diet and BPA intake, impact of these exposures and interactions at two offspring life stages, and importance of maternal phenotype in offspring health trajectory.

METHODS

Experimental Design

Mice used for this study originated in the Dolinoy Lab viable yellow agouti (A^{vy}) mouse colony. The colony has been maintained by sibling mating for over 250 generations with forced heterozygosity for the A^{vy} allele, producing a genetically invariant background with 93% homology to C57BL/6J and 7% homology to C3H/HeJ [42,43]. Mice were housed in polycarbonate-free cages with enrichment, in a climate-controlled room with a 12hr light-dark cycle, in accordance with the Institute for Laboratory Animal Research (ILAR) guidelines [44]. Mice were treated humanely and provided *ad libitum* access to food and water 24-hours a day, in accordance with the University of Michigan's University Committee on Use and Care of Animals (UCUCA) policies on cage enrichment, cleaning, maintenance, and daily mouse health checks [45]. This study protocol (PRO00004797) was approved by UCUCA.

To reduce the effect of parity and age, virgin, 8-10 week old, wild-type (*a/a*) dams were randomly assigned to one of six exposure groups: Control, Control + 50 μg BPA/kg diet, Western, or Western + 50 μg BPA/kg diet, Mediterranean, or Mediterranean + 50 μg BPA/kg diet. After two weeks on their respective diets, virgin *a/a* dams (10-12 weeks of age) were mated with young, virile A^{vy}/a males (7.5 weeks old on average). Dams remained on their assigned diets from pre-gestation through lactation (Figure 2.1), such that dams were directly exposed to their assigned diet for an average of 8-9 weeks. All offspring were weaned onto the Control diet at postnatal day 21 (PND21).

Mating *a/a* females with A^{vy}/a males generates litters of approximately 50% *a/a* and 50% A^{vy}/a pups. Mice with the A^{vy} allele have a range of coat colors from yellow to pseudoagouti (brown), that phenotypically display differences in epigenetic marks in the Agouti gene promoter [46–48]; wild type *a/a* mice have black coats and are thus easily distinguishable by eight days of age. To avoid confounding from the obesity and metabolic abnormalities observed in the A^{vy}/a mice [49–51], only *a/a* pups were followed in this study. Offspring coat color was recorded at postnatal day 8 to determine which pups would be followed longitudinally. One male and one female *a/a* pup per litter were maintained for longitudinal testing to 10 months of age (10-month). All other *a/a* pups were sacrificed at postnatal day 10 (PND10). Offspring sacrifices were conducted in the afternoon (2-5pm) to normalize diurnal hormone fluctuations. To further standardize measurements, 10-month females were only sacrificed when in estrus, confirmed by vaginal cytology [52]. Sacrificing offspring at 10 months approximates human middle age, prior to aging-related health decline [53].

Composition of Experimental Diets

Experimental diets were modifications of the standard AIN93G mouse diet [54,55]. Like AIN93G, the Control diet contains casein as the sole protein source, but the soybean oil was replaced with corn oil to remove the potential epigenetic programming effect of phytoestrogens in the soybean oil [56,57]. The Mediterranean and Western diets differed from the Control diet in their lipid ratio, carbohydrate profile, vitamin and mineral content; protein content was kept constant between all three diets.

Mouse diets were formulated on a per weight basis at the University of Michigan and manufactured by Harlan Teklad (Madison, WI). The Mediterranean diet was based on a traditional Cretan diet which included high nut, fruit and vegetable content [58–60]. The Western diet was based on U.S. dietary intake as recorded in NHANES II [61,62]. This resulted in a Western diet composed of more saturated fats (butter and palm oil), lower fiber / higher sugar carbohydrates, higher salt, and lower antioxidant content [61,62] (Table S2.1, Figure 2.2). Both experimental diets were formulated by nutrient content (grams) per kilogram diet.

Bisphenol A (BPA) Exposure

The choice of 50 ug BPA/kg dose was based on a previous BPA dose range finding study [63], which found an intake of 50 μg BPA/kg diet produced on average 2.02 ng BPA/g liver. This is within the human exposure levels measured in in human fetal liver samples (range: below limit of quantification to 96.8 ng BPA/g liver) [63]. BPA was supplied by the National Toxicology Program (NTP, Durham, NC). To create a 0.1% BPA/sucrose mixture, BPA (0.01 g) was mixed into sucrose (9.99 g) in glass containers. Harlan Teklad incorporated this mixture at 0.05 g/kg into three of the six experimental diets: Control+BPA, Western+BPA, and Mediterranean+BPA.

Body and Tissue Weights

To decrease variation and reduce human error, a strict protocol was followed for all sacrifices, necropsies, animal and tissue weighing [45]. The liver was dissected and separated by lobe; all analyses were run on the left lobe. Dam body weight was recorded three times throughout the study: (1) pre-gestation, at initial exposure to the study diets, (2) at mate-pairing, two weeks later, and (3) at sacrifice, four days post-weaning. Two weight change periods were calculated: dam pre-pregnancy weight change (pre-gestation to mate-pairing), and gestational weight gain (mate-pairing to sacrifice). Organ weights were measured for dam liver and mesenteric white adipose tissue (mWAT). mWAT, a component of visceral WAT that does not include gonadal fat, was separated from abdominal organs (stomach, pancreas, spleen, intestines) prior to weighing.

Total body and liver weights were also recorded for PND10 offspring. Mice at PND10 have negligible mWAT, so no adipose weights were recorded for the pups. 10-month offspring body, liver, and mWAT weights were measured during necropsy. Relative liver and mWAT weights were calculated as a ratio of: absolute organ weight / total body weight. All weights were measured on a SLF103 balance (Fisher Scientific) to the hundredths digit.

Hepatic Triglycerides (TG)

TG levels were quantified via the previously published TG extraction protocol [64], using the Sigma Triglyceride Determination Kit (TRO100). Samples were analyzed in triplicate (sensitivity 0.0625 mg/mL, 10.5% CV).

Liver Histology

Two histologic staining methods were used to validate hepatic TG quantification and to independently analyze hepatic lipid accumulation and lesions in 10-month offspring liver tissue: (1) OilRedO, and (2) hematoxylin and eosin (H&E). Tissue samples were frozen or fixed (respectively) during necropsy, then transferred to the University of Michigan In-Vivo Animal Core (IVAC) for histologic staining and analysis. Slides were read by a board-certified veterinary pathologist.

Tissue reserved for OilRedO staining was embedded in optimal cutting temperature (OCT) compound, flash frozen in liquid nitrogen, and stored at -80^{o}C. Frozen, OCT-embedded tissue was thinly sliced by microtome. The standard AbCam OilRedO kit (ab150678) protocol was followed to stain lipids on each slide. OilRedO staining was scored from 1-4 based on the amount of micro- and/or macro-vesicular vacuolation, multifocal or diffuse: 1 = minimal, 2 = mild, 3 = moderate, 4 = severe.

To prepare liver samples for H&E staining, thin sections of liver were fixed in 10% neutral-buffered formalin during necropsy. In livers with visible gross masses, the boundary between mass and normal-appearing tissue was included in the fixed sections, to analyze the potential invasive properties of the mass. Once at the IVAC, samples were transferred to 70% ethanol, routinely processed and stained with H&E for histopathology. Lipid vacuolation was the primary outcome of interest, but additional morphologic changes were observed by the veterinary pathologist, including: hepatocyte hypertrophy, multinucleated hepatocytes, proliferative alterations (hepatocellular adenoma, nodular hyperplasia, mixed cell, clear cell, and eosinophillic foci), and non-proliferative alterations (cell infiltrates, oval cell and Kupffer cell hyperplasia, and inflammation). Morphologic changes were also scored on a 4-point scale based

on the percent of tissue affected: 1 = minimal (0-9%), 2 = mild (10-19%), 3 = moderate (20-49%), 4 = marked (>50%).

Serum Hormone Analyses

Hormone levels were measured in blood samples collected via closed chest cardiac exsanguination [65], on semi-fasted mice. Whole blood was allowed to clot, serum separated, and stored at -80^{o}C. On average, more than 650 μL of whole blood was collected from dams, 100 μL from PND10 pups, and 1565 μL from 10-month offspring. For dams and PND10 offspring, Millipore Mouse ELISA kits were used to quantify serum leptin (EZML-82K, sensitivity 0.05 ng/mL, 3.0% CV) and insulin (EZRMI-13K, sensitivity 0.2 ng/mL, 6.0% CV) levels. Due to low serum volume from PND10 offspring, only leptin was analyzed.

Serum from 10-month offspring was analyzed on a Luminex xMAP (ThermoFischer), at the MDRC Chemistry Lab. Six serum hormones were simultaneously measured via the Multiplex Mouse Adipokine Magnetic Bead Panel (MADKMAG-71K), with intra-assay %CV <10% and inter-assay %CV <20%. Assay sensitivity varied by hormone: leptin (4.2 pg/mL), insulin (13.0 pg/mL), resistin (1.1 pg/mL), IL-6 (2.3 pg/mL), PAI-1 (4.0 pg/mL), and TNFα (5.3 pg/mL). All hormone levels reported in this paper are the average of duplicate measures for each mouse.

Statistical Analyses

Values for all variables were inspected; values outside of a biologically plausible range were discarded. A probability plot of residuals was used to test the normality of each variable's distribution. Right-skewed variables were ln-transformed. The effects of diet and BPA on liver

TG levels, serum hormones, body and organ weights were analyzed by ANOVA, followed by Tukey's post-hoc analyses; this allowed comparison across all six perinatal exposure groups. Significance was pre-set at $p < 0.05$.

Previous studies have reported sexually dimorphic metabolic responses to prenatal exposures [66,67], so cross-sectional PND10 and 10-month offspring comparisons were sex-stratified. Results from the initial independent t-tests and ANOVAs suggested that experimental diets might have a larger effect than BPA on both dams and offspring. One-way ANOVAs were also run to compare all six perinatal exposure groups among dams, PND10 and 10-month offspring (Tables 2.2, S2.2, S2.3, S2.4, S2.5). We ran linear mixed models (LMM) to determine predictors of hepatic TGs, with perinatal diet and BPA exposure included as separate variables. This allowed us to examine the independent effects of experimental diets and BPA, and to examine the interaction of diet*BPA on metabolic outcomes.

'Cohort' was significantly correlated to many dam and 10-month offspring variables, so was added to these models as a random intercept. However, 'Cohort' did not improve effect sizes or model fit (by AIC) in PND10 models, and since no PND10 variable means differed by cohort, we decided to remove it from those models. Some PND10 mice had littermates included in the models, so 'Litter ID' was included as a random effect in PND10 models. The range in litter size (3-11pups) was large in this study. 'Litter size' was included in all PND10 and 10-month models, as an *a priori* factor that could influence offspring metabolic health. Other variables previously identified as covariates for offspring metabolic changes were tested in regression models. Final models were selected based on the model with the largest effect size on perinatal exposure variables: experimental diets and BPA. All analyses were conducted in SAS 9.4 (Cary, NC, USA).

RESULTS

Reproductive Outcomes

Days to delivery averaged 27.7 days across all dams (n=97), with no difference by perinatal exposure group. On average, seven pups were born per litter across all exposure groups; however, 2.7-fold more Control+BPA pups died than Control ($p = 0.0149$) (Table 2.2). Pup deaths within the first three days of life is not unusual, especially in large litters, so the number of surviving pups per litter on postnatal day 4 (PND4) was also assessed. Initial litter size impacts *in utero* nutrient availability, while the number of living pups per litter can affect lactation nutrient availability and maternal care. Living pup litter size was 1.5-fold greater in Control compared to Control+BPA litters ($p = 0.0075$), consistent with the higher death rate among Control+BPA pups. The proportion of male and female pups did not differ by perinatal exposure group, with 51% male pups on average. Although Control+BPA (58.4%) and Mediterranean (57.8%) litters contained a greater percent of A^{vy}/a offspring than Control (48.4%) litters, the differences were not significant. Pup deaths prior to PND4 may have occurred disproportionately in a particular sex or genotype group, but it was not possible to determine, since some dead offspring were not retrievable.

Metabolic Outcomes

Dams' Response to Experimental Diets

Dam body weight did not differ at initial exposure (Table S2.2). But pre-pregnancy weight change, during the first two weeks on the experimental diets, was greater in Western and Mediterranean diet groups (Figure S2.1). Dams on the Control and Control+BPA diets lost weight (-0.22 to -0.55g), while the other four groups gained weight (0.15 to 0.79g), especially

dams on Western ($p = 0.0006$), Western+BPA ($p = 0.0001$), and Mediterranean ($p = 0.0062$) diets. This exposure-derived weight gain led to greater mean pre-gestation body weights in Western (19.63g), Western+BPA (19.42g), and Mediterranean (19.66g) dams compared to Controls (18.55g), but the differences were not significant. Gestational weight gain did not differ between the six exposure groups, and although Western, Western+BPA, and Mediterranean dams remained heavier, on average (27.42g), than Control dams (25.72g), the differences were not significant at sacrifice, four days after pup weaning (Figure S2.2).

Relative liver weight in dams did not differ by diet nor BPA exposure; on average, dam livers composed 5.0-5.5% of their overall body weight. However, greater relative mWAT (3.2-3.3%) was collected from Western ($p = 0.0035$), and Western+BPA ($p = 0.0078$), compared to Control dams (2.4%). Exposure groups associated with greater pre-gestational body weight gain were the same as the groups that had higher relative mWAT at sacrifice.

Post-partum, semi-fasting serum insulin levels in dams on Mediterranean and Western diet trended lower (~1.5ng/mL) than Control and Control+BPA (~2.5ng/mL) serum insulin levels, but the differences were not significant. Serum leptin levels were consistent in dams across all dietary exposure groups, ranging from about 4.5-5.9 ng/mL.

Hepatic TGs did not differ by BPA exposure in dams. However, hepatic TGs were significantly higher in dams exposed to the Western ($p = 0.0077$), Western+BPA ($p = 0.0410$), Mediterranean ($p = 0.0001$), or Mediterranean+BPA ($p = 0.0365$) diets, compared to Control (Figure 2.3, Table S2.2). Control+BPA hepatic TGs trended higher than Control TGs, but hepatic TGs in Mediterranean+BPA and Western+BPA groups trended lower than their respective diets without BPA, suggestive of a potential interaction between these experimental diets and BPA.

PND10 Offspring's Response to Experimental Diets

PND10 offspring (n = 118) were exposed to experimental diets and BPA via maternal *in utero* transfer for at least three weeks and via mother's milk for 10 days. The experimental diets did not impact body weight or relative liver weight in PND10 females (n = 60) or males (n = 58). Control+BPA males had lower average body weight (4.74g) compared to Control males (5.78g, p = 0.0434) at PND10; addition of BPA to the Mediterranean and Western diets did not result in decreased body weight (Table S2.3). Body weight did not differ among PND10 females based on perinatal exposure group. At this early age, average body weight did not differ by offspring sex, with males averaging 5.53g and females averaging 5.68g (p = 0.4130). Despite the lower body weight in Control+BPA males, relative liver weight did not differ between Control+BPA and Control males, comprising 3% total body weight in both groups. There were no differences in relative liver weight among other male or any female perinatal exposure groups either (Table S2.3).

Serum leptin levels were consistently highest among both female and male PND10 offspring exposed to the Mediterranean diet. Female Mediterranean diet pups had average serum leptin levels 2.6-fold higher than Control females (p = 0.0011), while males exposed to the Mediterranean had levels 4.8-fold greater than Control males (p = 0.0284) (Table S2.3). Variation of leptin levels was greatest among Mediterranean offspring of both sexes, suggestive of inconsistency in hormonal response between individual Mediterranean mice. Serum leptin levels did not differ by Western diet or BPA exposure in female or male PND10 offspring.

Hepatic TG levels were highest among Mediterranean PND10 offspring of both sexes. Mediterranean males had 7.3-fold greater hepatic TG levels compared to Control males (p < 0.0001) (Table S2.3). Although not significant, hepatic TGs were 1.6-fold higher among

Mediterranean females compared to Controls. As observed in the PND10 serum leptin levels, variation in hepatic TG levels was largest among Mediterranean pups of both sexes at PND10, suggestive of considerable inconsistency in hepatic TG storage between individual Mediterranean mice (Figure 2.4A & B). Neither perinatal Western diet nor BPA exposure altered hepatic TG levels in either sex.

10-Month Offspring's Response to Experimental Diets

By 10-months of age (n = 138), offspring had not been exposed to the experimental diets or BPA for more than nine months, since weaning. Body weight at 10-months did not differ by perinatal exposure. As expected, male offspring (44.87g) weighed more than females (34.63g), on average, at 10-months (Table S2.4). Females perinatally exposed to Western (3.8%), Western+BPA (3.7%) and Mediterranean (3.8%) diets, had greater relative liver weights compared to Control females (3.1%, $p < 0.02$). Relative liver weight did not differ by perinatal exposure in 10-month males. However, males perinatally exposed to the Western+BPA diet had greater relative mWAT (4.1%) compared to Control males (3.4%, $p = 0.0235$). Relative mWAT weight did not differ by perinatal exposure in 10-month females.

Perinatal diet impacted serum leptin levels among 10-month males; Western+BPA serum leptin was 1.6-fold higher and Mediterranean was 1.5-fold higher than Control levels ($p < 0.02$) (Table S2.4). Western+BPA males also had 1.6-fold higher serum resistin levels compared to Control males ($p = 0.0046$). Among 10-month males, serum insulin and PAI-1 levels did not differ by perinatal exposure group. However, in 10-month females, serum PAI-1 levels were 1.6-fold greater in Mediterranean+BPA than Controls ($p = 0.0043$). No other serum hormones differed by perinatal exposure, among female offspring at 10-months.

Hepatic TGs were 2.4-fold higher in females perinatally exposed to the Mediterranean+BPA diet compared to Control females ($p = 0.0118$) (Table S2.4). Although all female experimental diet groups had hepatic TGs greater than Control, no other group differences were significant. Hepatic TGs did not differ by perinatal exposure group among male offspring at 10-months (Figure S2.4).

Comparison of Hepatic Steatosis Measures in 10-month Offspring

H&E vacuolation and OilRedO staining were highly correlated to hepatic TG levels, in 10-month offspring of both sexes (Table 2.3), indicating that hepatic TG quantification is an accurate proxy measure of hepatic lipid accumulation. The full range of scores, from 0 (negligible) to 4 (severe lipid accumulation), was observed in both male and female 10-month offspring (Figure 2.5).

Hepatocellular response to perinatal exposures was sexually dimorphic, with multiple morphologic lesions differing significantly among experimental diets in female 10-month offspring, but not males (Table S2.5). Hepatocellular vacuolation was 1.7-fold greater in Mediterranean+BPA females compared to Controls ($p = 0.0258$), consistent with the observed hepatic TG difference. Surprisingly, OilRedO score was 2.3-fold higher among Control females than Western+BPA females ($p = 0.0124$), suggestive of less lipid accumulation in Western+BPA exposed offspring. Perinatal exposure did not impact hepatic steatosis, as measured by vacuolation or OilRedO score, in male offspring at 10-month (Table S2.5).

Hepatic adenomas were observed in 11 male mice, spread across five of the experimental exposure groups, including Control (Table S2.5). No adenomas were found in any female mice, but proliferative lesions differed by perinatal exposure groups. Proliferative lesions were only

observed in Control+BPA and Mediterranean+BPA females. Non-proliferative lesions also differed by perinatal exposure group in 10-month females; Control females had more non-proliferative responses than Western, Western+BPA, and Mediterranean ($p < 0.02$).

Predictive Modeling of Liver Triglycerides

Dam Liver Triglycerides

Linear mixed models (LMM) were used to explore the contribution of perinatal experimental diets, perinatal BPA, and the interaction of these two concurrent exposures (Table 2.4). Both experimental diets were associated with increased dam hepatic TG levels; Western dams had 11.24 mg/g ($p = 0.0009$) and Mediterranean dams had 18.02 mg/g ($p = 0.0518$) higher TGs than Control dams. BPA did not affect dam hepatic TG levels ($p = 0.2279$), but the Mediterranean*BPA interaction term had 13.24 mg/g ($p = 0.0634$) lower TG levels than Mediterranean dams. Every one gram increase in dam body weight at sacrifice was associated with 1.43 mg/g ($p = 0.0116$) greater hepatic TGs. Other potential covariates did not contribute significantly to the model, including: dam gestational weight gain, dam serum leptin, or insulin levels.

PND10 Offspring Liver Triglycerides

Sex-stratified LMMs predicting Ln-transformed hepatic TGs in PND10 offspring included litter size as a covariate (Table 2.5). Neither perinatal experimental diets, BPA, nor their interaction terms significantly contributed to hepatic TG levels in female PND10 offspring. Western PND10 males had 5.9% ($p = 0.0543$) and Mediterranean males had 18.2% ($p < 0.0001$) greater TGs than Control males. Perinatal BPA exposure did not affect male PND10 hepatic TG levels, but the Mediterranean*BPA interaction term decreased TG levels by 12.7% ($p = 0.0150$)

compared to Mediterranean males. Of note, the same two maternal factors associated with dam hepatic TGs, pre-gestational weight change and post-partum body weight, impacted hepatic TGs in PND10 males. PND10 male hepatic TGs increased by 4.8% ($p = 0.0761$) for every one-gram increase in dam body weight at sacrifice, but decreased by 4.6% (n.s.) for every one-gram change in pre-gestational weight. Although not significant, dam post-partum weight and pre-gestational weight change improved model fit for PND10 females also (5.3% and -10.7%, respectively). Other potential covariates did not contribute significantly to the models, including all of the other PND10 variables (body weight, relative liver weight, and serum leptin levels) and dam variables (relative liver and mWAT weights, gestational weight gain, serum leptin, and insulin levels).

10-month Offspring Liver Triglycerides

Similar to PND10 models, sex-stratified LMMs predicting Ln-transformed hepatic TGs in 10-month offspring included litter size as a covariate (Table 2.6). Perinatal exposure to neither experimental diet nor BPA alone significantly contributed to female 10-month offspring hepatic TG levels. However, the Mediterranean*BPA interaction term had 15.2% ($p = 0.0309$) higher TG levels than Mediterranean females. Also in females, hepatic TGs were associated with a 24.6% ($p = 0.0547$) increase for every 1% increase in 10-month female relative mWAT weight. Although it did not contribute significantly to the model ($p = 0.4143$), presence of hepatic nodular hyperplasia improved model fit for 10-month female hepatic TGs. In 10-month males, neither perinatal diet nor BPA were significant predictors of Ln-transformed hepatic TGs. However, the best-fit model included the same covariates as the 10-month female model: 10-month relative mWAT (25.4% increase, $p = 0.1418$) and nodular hyperplasia (6.3% increase, p =

0.0265). No other factors measured in 10-month offspring or their mothers (dams) were predictive of hepatic TGs in 10-month offspring of either sex.

DISCUSSION

This longitudinal exposure study aimed to determine the life-course impact of perinatal experimental diets and BPA on offspring hepatic steatosis and metabolic health. Although dam hepatic TG levels differed by diet, alterations in metabolic outcomes were more pronounced than the effect on hepatic steatosis in offspring at PND10 and 10-months. Of note, the perinatal exposure that impacted offspring health outcomes differed by offspring age. At PND10, only the Mediterranean diet was associated with increased serum leptin (all offspring) and elevated hepatic TGs (males only); however, the large variation of these measures suggested considerable inter-individual differences in mouse response to the Mediterranean diet. In 10-month offspring, perinatal exposure to experimental diets+BPA was associated with sex-specific alterations in relative organ weights and serum hormone levels; this lasting impact of perinatal exposures, after >9 months on a Control diet supports the theory that offspring metabolic health can be developmentally programmed. Although not the original subjects of interest in this study, dam hepatic and metabolic responses to both experimental diets were marked and also predicted hepatic TG levels in PND10 offspring. Thus, dams may be an important group to phenotype in future murine models and epidemiological studies of perinatal exposures.

Reproductive Outcomes

We found no effect of perinatal diet on reproductive outcomes, including time to delivery, litter size, and percentage of male pups or A^{vy}/a pups per litter. A 2009 systematic

review of perinatal HFD exposure outcomes also reported no difference in litter size or offspring birth weight between HFD and Control groups [68]. However, a recent study in C57BL/6J mice reported that HFD (60% fat, mostly lard) consumption, independent of dam obesity, was associated with decreased ovarian function due to depleted primordial follicles, and impaired fertility with fewer litters and smaller litter sizes [69]. The discrepancy may result from differences in fat content of the HFDs. The experimental diets in our study contained 42% of calories from fat, mimicking human HFDs, which rarely contain more than 45% kcal from fat [70]. The 60% fat diet used in the recent C57BL/6J study [69] is common in murine models of diet-induced metabolic disease, but it may not be directly applicable to human health.

Perinatal BPA exposure is associated with decreases in uterine receptivity, implantation [71], and increases in offspring mortality [72]. Consistent with these findings, we observed greater Day 3 pup mortality in the Control+BPA offspring. However, the increased mortality did not occur in Western+BPA or Mediterranean+BPA groups, suggestive of a diet*BPA interaction. A potential mechanism for and implications of this interaction are examined later in this Discussion.

Dam Metabolic & Hepatic Outcomes

Irrespective of BPA content, dams consuming the Western or Mediterranean experimental diets had increased hepatic TGs compared to Control dams. Adult C57BL/6J mice are known to develop hepatocellular ballooning and hepatic simple steatosis after 7-8 weeks on a high fat diet [73–75]. However, the impact of HFDs on hepatic steatosis has not been reported in pregnant dams. While many studies use perinatal HFD exposure to investigate effects on offspring health [76], potential alterations in maternal health and physiology are rarely published.

In this study, dams were exposed to the experimental diets for an average of 8-9 weeks, so the observed hepatic steatosis aligned with the previously reported time to develop steatosis in non-pregnant adult mice.

Dams exposed to the Western, Western+BPA, or Mediterranean diets had greater pre-gestational weight gain and relative mWAT weight. Weight gain on HFDs has previously been attributed to gains in adiposity, not lean mass [74]. The increased relative mWAT weight, without increases in relative liver weight, among dams on the experimental diets suggests that increased adiposity contributed to the greater pre-gestational weight gain compared to Control dams. Gestational weight gain is associated with an increased risk of overweight in children (2-18 years) in many human birth cohort studies [77]. However, a large prospective cohort study in the Netherlands reported maternal pre-gestational obesity increased odds of childhood obesity, but excessive gestational weight gain had a more limited impact on children's outcomes [78]. This distinction may explain the lack of effect the differential pre-gestational weight gain had on mouse offspring; none of the study dams were obese prior to mate-pairing. The clarification that some, not all, dam weight measures may impact offspring health trajectory supports the need to better characterize the maternal environment and metabolism when studying the potential perinatal programming of offspring metabolic disease.

PND10 Metabolic & Hepatic Outcomes

Perinatal exposure to experimental diets in this study impacted hepatic TGs only in male PND10 offspring. This sexually dimorphic response has been previously observed in mice and rats [79,80], with male offspring consistently more likely to develop hepatic steatosis following perinatal HFD exposure than females. Among youth (2-19 years) accidental deaths in San Diego

County, males were also more likely to have hepatic steatosis (15%) than females (9%) [81], suggesting the sex-specific difference in rates of hepatic steatosis may translate to human populations.

Metabolic outcomes at PND10 were most pronounced in offspring perinatally exposed to the Mediterranean diet. Although maternal HFD during pregnancy has been associated with increased serum leptin in 12-week old offspring [82], we only observed this elevated leptin response among Mediterranean diet offspring; the Western diet had no effect. The difference in offspring age, 12-weeks vs. PND10, may contribute to the dissimilar effect of perinatal exposure on offspring leptin levels. Breast milk contains leptin and is thought to contribute to regulation of food intake in offspring prior to weaning [83–85], so the serum leptin levels in our PND10 offspring likely reflected variations in leptin from mother's milk, not endogenously produced leptin as seen in 12-week old offspring. Among adults, elevated leptin may be indicative of leptin resistance, failure to regulate food intake despite high circulating leptin levels, often observed in metabolic conditions (obesity, MetSyn, T2DM) [86]. However, the postnatal leptin surge that occurs in the first two weeks of life in rodents is necessary for establishing hypothalamic pathways that will control food intake [87] and for the maturation of other organs involved in energy homeostasis, including the kidney, pancreas, ovary, and thymus [88]. Thus, elevated leptin levels in PND10 offspring exposed to the Mediterranean diet likely have a greater impact on organ development than on life long metabolic health.

10-month Metabolic & Hepatic Outcomes

This study was designed to investigate the perinatal programming of hepatic steatosis. To strengthen potential findings, three measures of steatosis were included, quantification of hepatic

TGs, OilRedO staining of hepatic lipids, and analysis of hepatocellular vacuolation via histopathology. We observed hepatic steatosis among 10-month offspring, but it did not differ strongly by perinatal exposure group, suggesting it may have been normal, age-related steatosis. C57BL/6J mice are known to develop hepatic cysts and adenomas [89,90]; this age-related pathology has been previously observed in our viable yellow agouti colony, with 10.5% of non-agouti, *a/a* 10-month male Controls displaying neoplastic lesions and over 50% of 10-month females and males exhibiting simple steatosis [42]. The presence of hepatic adenomas in only 10-month males, not females, in our study is consistent with the higher background incidence of hepatic tumors in male C57BL/6J mice [91] .

Strikingly, histopathologic evaluation of hepatic tissue found Control offspring had more non-proliferative changes than offspring perinatally exposed to the experimental diets. This may suggest Control mice retained the ability to regenerate and repair injured tissue more at 10-months than did other groups. Thus, although age-related damage may be occurring, Control livers may still be able to respond to environmental stressors, returning to a healthier state, whereas livers perinatally exposed to experimental diets may not be able to repair as easily, and thus amass more tissue damage.

Perinatal exposure to diet+BPA combinations (Western+BPA and Mediterranean+BPA) had the greatest effect on 10-month offspring metabolic outcomes. This response differed from both dams and PND10 offspring, suggesting that various perinatal exposures may impact offspring health at different ages. HFD consumption has been associated with increased relative liver weight in adult animals [92], we observed this effect in 10-month female offspring. However, in our mice, they had not been exposed to the experimental diets since weaning, suggesting that perinatal HFD exposure can also impact relative liver weight across the life

course. Interestingly, the increased liver weight did not translate to greater hepatic TGs, so mass was not necessarily due to increased lipid accumulation. The borderline increase in body weight and significant increase in relative mWAT weight among Western+BPA males at 10-months suggests that these males accumulated more visceral adipose across their life, due to the combined exposure in early life. These Western+BPA males also had the highest serum leptin and resistin levels and the lowest serum PAI-1, suggesting that the greater mWAT mass was metabolically active.

Multifaceted Effects of the Experimental Diets & BPA

Effects of the six perinatal exposures were much more complex than our original hypotheses. The Western diet had the expected, adipogenic effect [17–19] in dams, but barely impacted offspring. Conversely, we expected our Mediterranean diet would protect against metabolic and hepatic alterations, but in dams it was generally indistinguishable from the adipogenic effect of the Western diet, and was the only diet to increase PND10 hepatic TGs. Individual components of the Mediterranean diet (fish oil, olive oil, and polyphenols) have been investigated, but a complete Mediterranean diet has never been created for mice. A previous comparison of two purified perinatal diets found that maternal diets high in saturated fat promoted offspring hyperphagia, but equivalent consumption of a fish oil diet did not [93]. Further, a recent study in rats, reported that adding fish oil to a HFD in dams during pregnancy, prevented insulin resistance in adult male offspring, independent of body weight [94]. In adult mice predisposed to hepatic steatosis, daily 2% DHA/EPA supplementation reduced hepatic TGs by almost 40% [95], further supporting the health benefits of a fish-oil enriched diet.

On the other hand, consumption of olive oil, the primary fat component in the Mediterranean diet, is associated with increased steatosis in mice [96,97]. In mice, olive oil decreases activity of carnitine palmitoyltransferase I (CPT1), the rate limiting step for mitochondrial fatty acid β-oxidation. This depression of CPT1 activity does not occur in humans consuming olive oil, thus may partially explain the differential outcome of the diet between the two species. In human studies, Mediterranean diet is associated with reversal of biopsy-confirmed NAFLD in adults, significantly reducing hepatic steatosis and improving insulin sensitivity, even without weight loss [26,98]. A proteomics analysis reported that tissue oxidation and atherosclerotic plaque formation was delayed in mice consuming olive oil, despite increased hepatic steatosis and insulin resistance; the authors attributed this paradoxical response to the differential regulation of 80 hepatic antioxidant enzymes, including heat shock proteins, superoxide dismutase, glutathione peroxidase 1, thioredoxin peroxidase 2, fatty acid binding protein, carboxylesterase, and apolipoprotein A1 [97]. Thus, the hepatic steatosis observed in mice consuming olive oil may be protective, as it does not appear to induce the detrimental metabolic alterations. This protective antioxidant effect may also explain why mice perinatally exposed to Mediterranean diet in this study did not exhibit altered hepatic or metabolic responses at 10-months.

In this entire study, perinatal BPA exposure on a Control diet (Control+BPA) only impacted pup Day 3 mortality and body weight in PND10 offspring; no effects were observed in dams or 10-month offspring. Perinatal low-dose BPA exposure has been associated with lower birth weight in murine models [99]; we observed this decreased body weight at PND10, but only among male offspring. Our findings support the previously reported detrimental effect of perinatal BPA exposure on reproductive outcomes [71,72,99], but suggest that maternal diet may

have a greater impact on offspring health trajectory, than BPA exposure. This is good news from a public health standpoint; in general, people have more control over the food they eat than over food and beverage packaging. Perinatal BPA exposure may not be sufficient to alter offspring health trajectory, an additional postnatal stressor or dietary challenge may be required. A theory of developmental "priming" by perinatal EDC exposure has been proposed, suggesting that exposure-induced changes in gene methylation may prime the loci for increased transcription in response to a later challenge [100]. Two mouse studies that examined perinatal BPA exposure followed by a postnatal HFD challenge reported greater hepatic TGs in offspring exposed to both the BPA and HFD [40,41], supportive of BPA's potential priming effect. Thus, perinatal low-dose BPA exposure may increase offspring susceptibility to hepatic steatosis, but postnatal diet may be required to trigger the pathologic response.

In contrast to the lack of metabolic or hepatic response to the Control+BPA diet, the diet*BPA combination diets, especially Western+BPA, significantly impacted organ weights and serum hormone levels in a sex-specific manner among 10-month offspring. In 10-month males, the predicted aggravation of Western diet by BPA was observed; the effect was not seen in 10-month females. This is consistent with previous reports of prenatal programming of metabolic disease by maternal HFD occurring in male offspring, without effect in females [19,101]. The Mediterranean+BPA diet had a distinctly different effect on mice at all ages. Among dams, Mediterranean+BPA exposure was similar to Western and Western+BPA effects, and associated with increased weight gain and hepatic TGs, but decreased serum insulin. Among PND10 offspring of both sexes, however, adding BPA to the Mediterranean diet seemed protective against adipogenic effects of Mediterranean.

Interestingly, perinatal BPA exposure was also protective, when combined with maternal over-nutrition in a sheep model of perinatal exposure also [102,103]. We recently reported that prenatal exposure to BPA protected overfed sheep offspring from the increased blood pressure and greater left ventricle area observed in overfed offspring not exposed to BPA [102]. Gene network analysis identified a reversal of overfeeding effects on gene expression, by prenatal BPA exposure, at *FABP4, A2M, APOD, HLA-C* [104], suggestive of greater fatty acid uptake in adipocytes, more cytokine transport, and greater circulating high-density lipoproteins. Multiple animal models now suggest a diet*BPA interaction, so future perinatal BPA exposure studies should include multiple background diets to elucidate this interaction. Human populations are simultaneously exposed to BPA and a variety of diets; improved understanding this interaction may be critical for interpretation of epidemiologic data on BPA exposure.

Challenges & Limitations

Assessing the impact of perinatal exposures across time in offspring can be challenging in the same mouse. In order to assess hepatic steatosis in offspring at multiple time points (PND10 and 10-months), an invasive biopsy procedure would have been required at PND10, to follow the same mouse to 10-months. Although liver tissue regenerates [105], we decided against this study design due to the added stress (metabolic and mental) that surgery induces. Instead we analyzed sex-matched littermates at PND10 and 10-months to approximate the effects over time. Non-invasive tissue samples could be collected at multiple time points from the same mouse to avoid the littermate challenge.

This study investigated phenotypic outcomes in dams, PND10, and 10-month offspring. However, subtle changes could have occurred due to perinatal exposures that would be apparent

at a molecular level. Perinatal exposure to both HFDs and BPA has been associated with alterations in epigenetic modifications and differential gene expression. Future analyses of nucleic acids may reveal changes in metabolic and/or hepatic regulation.

Study Innovation & Future Directions

Due to widespread interest in perinatal programming of NAFLD, human birth cohorts and animal models are simultaneously exploring the epidemiological factors and molecular pathways involved in altering offspring health trajectories. However, most studies focus on offspring outcomes and do not explore the impact perinatal exposures can have on the directly exposed mothers. Mothers are commonly considered the route of exposure for a developing fetus, but the predictive power of maternal phenotype is often overlooked. In this study, dam pre-gestational weight change and post-partum body weight impacted models predicting PND10 hepatic TGs. Detailed characterization of maternal metabolic phenotype during pregnancy in human birth cohorts could provide additional insight into factors affecting perinatal programming. Maternal adaptation to a diet or chemical exposure may be triggering the altered health trajectory in offspring, not the exposure itself. For instance, we know maternal pre-gestational weight increases risk of offspring metabolic dysregulation [8–11], maternal diet may be responsible for increased maternal pre-gestational weight, so the impacts attributed to the diet exposure, may actually result from the mom's diet-altered weight status.

CONCLUSION

In this study, we examined the potential role of perinatal diet and BPA, two common daily exposures, on developmental programming of NAFLD, assessed via hepatic TGs and associated metabolic outcomes. This is the first study, examining perinatal programming of NAFLD, to begin exposures prior to gestation, to provide diet and BPA exposures concurrently, and to investigate the potential interaction of BPA with multiple background diets. The differential response of dams, PND10 and 10-month offspring to the six experimental diets suggests that environmental exposures can exert dissimilar effects at different ages and life stages. The hepatic and metabolic effects of these perinatal diets differed not only by mouse age, but also by offspring sex and diet composition. The substantial impact of experimental diets on maternal phenotype and the subsequent effect of maternal phenotype on PND10 offspring hepatic TGs, suggests that detailed characterization of maternal factors would improve our understanding of developmental programming. To build on these insights, future studies should consider beginning perinatal exposures prior to gestation, continuing to explore diet*EDC interactions, including more detailed maternal phenotyping measures, increasing the number of offspring evaluations (between PND10 and 10-months), and incorporating molecular analyses with phenotypic outcomes.

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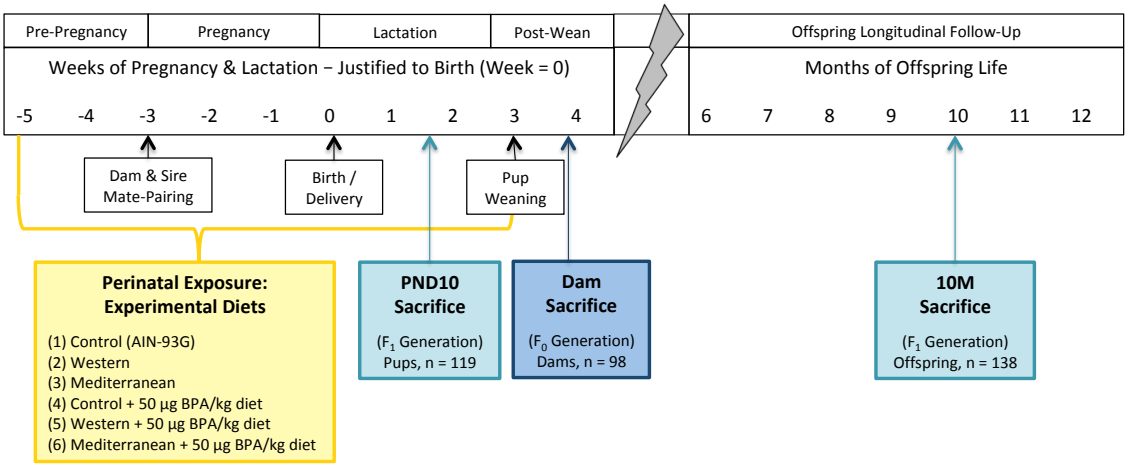
Figure 2.1 Experimental Design of Longitudinal Mouse Exposure Study



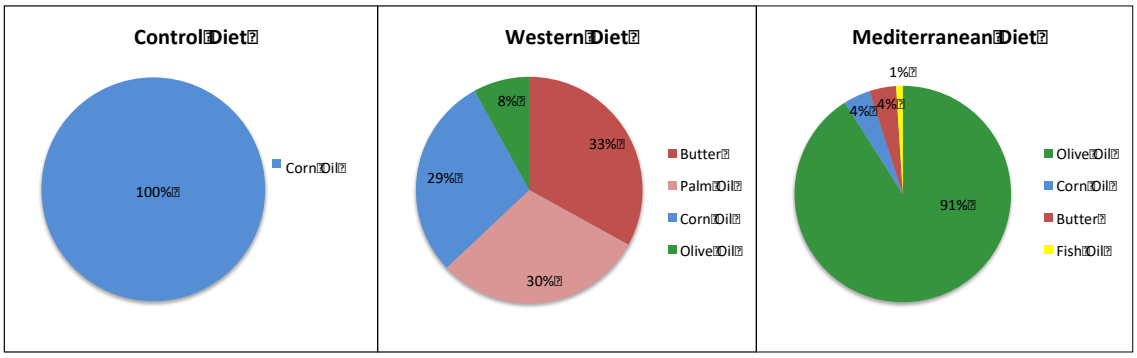
Wild type (*a/a*), virgin dams (8-10 weeks of age) were randomly assigned to one of six experimental diets. They were mate-paired two weeks later with young (7.5 week old), virile, A^{vy}/a males. Dams remained on their assigned experimental diet throughout pregnancy and lactation. Offspring were exposed to the diet *in utero* and via mother's milk. All offspring were weaned onto the Control diet at postnatal day 21 (PND21). Thus, 10M offspring had not been exposed to their perinatal experimental diet for over nine months at the time of sacrifice and tissue collection. Sacrifices were performed at three time points: (1) dams: 4 days post-weaning (PND25), (2) offspring: at PND10, and (3) offspring: at 10M.

Table 2.1 Composition of Experimental Diets: Mice Perinatal Exposures

Diet Ingredients	3 Experimental Mouse Diets		
	Control	Western	Mediterranean
MACRONUTRIENTS			
Calories (Kcal/g chow)	3.98	4.72	4.53
% Calories from Fat	16	40	42
PUFA : SFA : MUFA	1 : 0.2 : 0.5	1 : 1.9 : 1.6	1 : 1.3 : 5.6
Protein (casein) (g/100g chow)	20	19	19
Carbohydrate Content (g/100g chow)			
Cornstarch	40	14	23
Sucrose	10	25.5	9.2
Cellulose	5	2	8
VITAMINS & MINERALS			
Vitamin A (IU/kg chow)	4000	4000	8000
Vitamin C (mg/kg chow)	0	0	500
Vitamin D (IU/kg chow)	1000	400	1000
Vitamin E (IU/kg chow)	75	25	75
Folic Acid (mg/kg chow)	2	1	4
Sodium (mg/kg chow)	1039	7000	1039
Potassium (mg/kg chow)	3600	3600	8000
Magnesium (mg/kg chow)	513	513	850

The Control diet is equivalent to AIN-93G, except that corn oil replaced soybean oil as the source of fat. The experimental Mediterranean and Western diets were designed to reflect the nutrient content of human dietary patterns in Crete and the U.S., respectively. Nutrient content of the mouse diets were achieved on a per weight basis.

Figure 2.2 Lipid Composition of the Three Experimental Mouse Diets



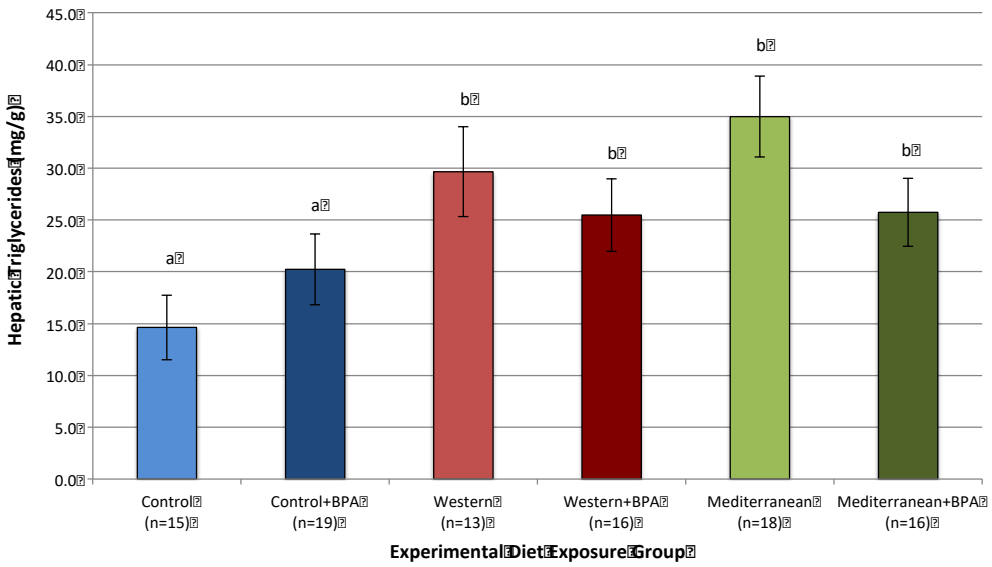
This figure illustrates the difference in lipid sources used to create each of the study's three experimental diets. Corn oil was the sole lipid source in the Control diet. Saturated fats composed >60% of the Western diet, contributed largely by butter and palm oil. In the Mediterranean diet, olive oil was the main source; fish oil was also added to reflect the higher n-3 PUFA content of human Mediterranean diets.

Table 2.2 Reproductive Outcomes by Perinatal Exposure Group

Reproductive Outcomes	Perinatal BPA Exposure	Experimental Diet: Mean (SD)			ANOVA p-value	Tukey's p-values*
		Control	Western	Mediterranean		
Delivery Time (days)	No	25.29 (10.00)	30.50 (12.04)	26.55 (9.23)	0.2502	n.s.
	Yes	24.62 (5.47)	30.67 (8.33)	28.56 (9.51)		
Litter Size (pups)	No	7.20 (1.15)	7.43 (1.95)	6.85 (2.37)	0.6141	n.s.
	Yes	6.52 (1.72)	6.53 (2.53)	7.38 (1.63)		
Dead Pups (pups / litter)	No	0.73 (0.88)	0.64 (0.84)	1.25 (1.71)	**0.0223**	Cont vs. CBPA = **0.0149**
	Yes	2.00 (1.76)	1.33 (2.23)	0.38 (0.81)		
Live Pups (pups / litter)	No	6.47 (0.99)	6.79 (1.76)	5.60 (2.60)	**0.0037**	Cont vs. CBPA = **0.0075**
	Yes	4.52 (2.66)	5.20 (2.04)	7.00 (1.59)		
Male Pups (% / litter)	No	47.71 (19.58)	51.90 (16.12)	53.02 (17.56)	0.8532	n.s.
	Yes	51.34 (23.20)	45.71 (20.90)	45.71 (20.90)		
A^{vy}/a Offspring (% / litter)	No	48.38 (17.10)	50.27 (17.28)	58.38 (17.80)	0.4245	n.s.
	Yes	57.77 (28.14)	45.94 (27.43)	49.44 (14.32)		

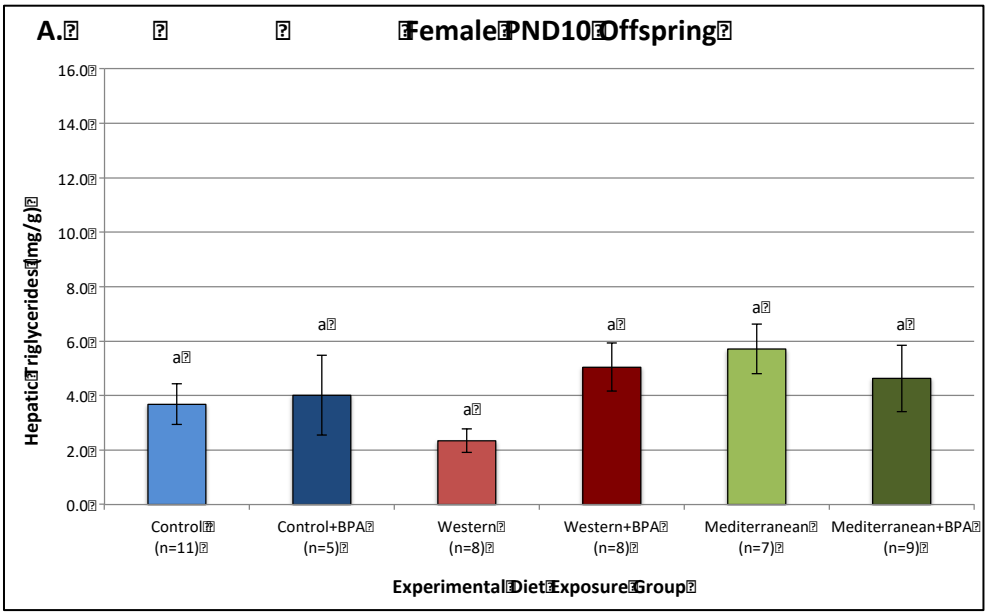
*Only significant ($p < 0.05$) or borderline significant ($p < 0.10$) comparisons are provided; all others are not significant ($p > 0.10$). The definition of the reproductive outcome variables presented in this table are as follows: 'Delivery Time' = the number of days from mate-pairing to delivery, 'Litter Size' = the number of pups initially delivered, 'Dead Pups' = the number of pups per litter that died within the first 3 days of life, 'Live Pups' = the number of pups per litter that were alive from day 3 onwards. Dam sample size varied slightly by experimental exposure group: Control = 15, Western = 14, Mediterranean = 20, Control+BPA = 21, Western+BPA = 15, Mediterranean+BPA = 16.

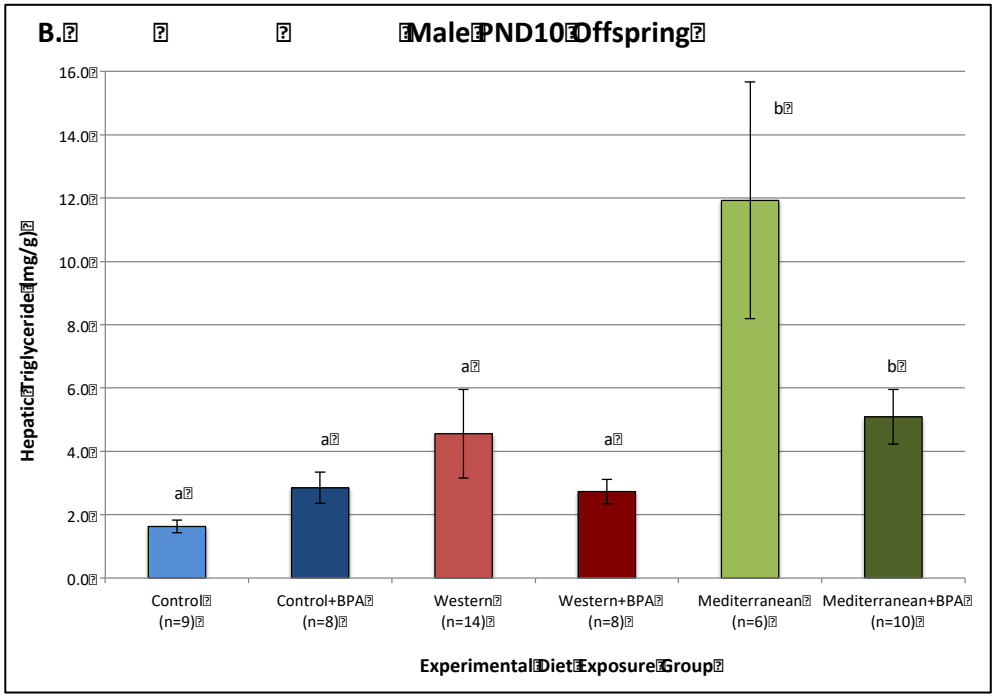
Figure 2.3 Dam Hepatic Triglyceride Levels, Four Days After Weaning Offspring (25 Days Post-Partum)



Average hepatic triglycerides (TGs) in dams (n=97), four days after weaning offspring, by experimental exposure group. [a] Denotes the average TGs of Control dams; groups that do not differ significantly from Control are also marked with 'a'. [b] Denotes exposure groups with average TGs that differ significantly ($p < 0.05$) from Control.

Figure 2.4 PND10 Offspring Hepatic Triglyceride Levels by Exposure Group



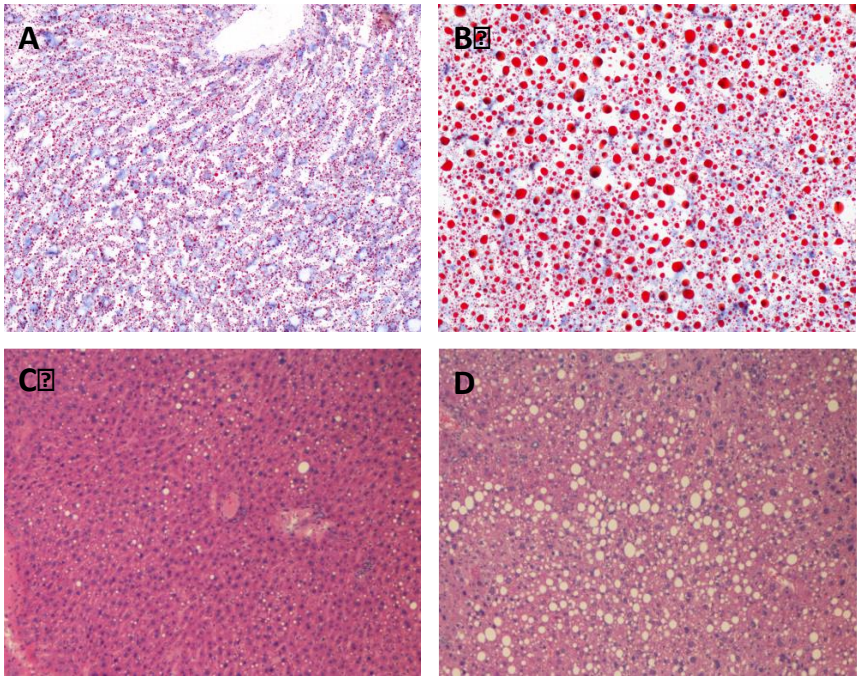


Average hepatic triglycerides (TGs) in PND10 offspring (n = 118), by experimental exposure group: Panel A = female PND10 offspring (n = 60), Panel B = male PND10 offspring (n = 58). [a] Denotes the average TGs of Control PND10 offspring; groups that do not differ significantly from Control are also marked with 'a'. [b] Denotes exposure groups with average TGs that differ significantly ($p < 0.05$) from Control.

Table 2.3 Correlation of Three Hepatic Steatosis Measures in 10-Month Offspring

	Pearson's Correlation	Hepatic TGs (mg/g)	OilRedO (score)	Vacuolation (score)
Female 10-month Offspring				
Hepatic TGs (mg/g)	Coefficient	1.000	0.4130	0.6189
	p-value	---	0.0006	<0.0001
OilRedO (score)	Coefficient	0.4130	1.000	0.6453
	p-value	0.0006	---	<0.0001
Vacuolation (score)	Coefficient	0.6189	0.6453	1.000
	p-value	<0.0001	<0.0001	---
Male 10-month Offspring				
Hepatic TGs (mg/g)	Coefficient	1.000	0.2758	0.4163
	p-value	---	0.0239	0.0005
OilRedO (score)	Coefficient	0.2758	1.000	0.5826
	p-value	0.0239	---	<0.0001
Vacuolation (score)	Coefficient	0.4163	0.5826	1.000
	p-value	0.0005	<0.0001	---

Figure 2.5 Histopathologic Measures of Hepatic Steatosis at 10-Months



Hepatic steatosis ranged from negligible to severe (>50% of tissue affected) in liver samples from the 10-month offspring. (A) OilRed0 staining, score 0: no visible lipid accumulation, (B) OilRedO staining, score 4: severe accumulation, (C) H&E staining, score 1: minimal (<9% tissue affected), (D) H&E staining, score 4: severe.

Table 2.4 Impact of Experimental Diet Exposure on Dam Hepatic Liver Triglyceride Levels 25 Days Post-Partum.

Experimental Diet	Model 1		Model 2		Model 3	
	Change in TGs	p-value	Change in TGs	p-value	Change in TGs	p-value
Western	15.49	**0.0058**	11.85	**0.0343**	11.24	**0.0009**
Mediterranean	19.98	**0.0002**	18.65	**0.0003**	18.02	**0.0518**
BPA	5.29	0.2918	6.37	0.1978	6.05	0.2279
Western*BPA	-9.71	0.1891	-8.76	0.2317	-8.63	0.2410
Mediterranean*BPA	-13.58	**0.0589**	-13.73	**0.0503**	-13.24	**0.0634**

Linear mixed effect models with 'Cohort' as a random effect were run to assess the impact of experimental diet components and their interaction on dam (n=97) hepatic TG levels, 25 days post-partum. Effect size p-values were **bolded** if significant ($p < 0.05$) or borderline significant ($p < 0.10$) to highlight the experimental diet components that contribute to model prediction of hepatic TG levels.

- Model 1: includes only the experimental diet variables, no additional covariates.
- Model 2: adjusted for dam body weight, 25 days post-partum.
- Model 3: additionally adjusted for dam pre-gestational body weight change.

Table 2.5 Impact of Perinatal Experimental Diet Exposure on Hepatic Liver Triglyceride Levels in PND10 Offspring, Sex-Stratified

Experimental Diet	Model 1		Model 2		Model 3	
	% Change TGs	p-value	% Change TGs	p-value	% Change TGs	p-value
Female PND10 Offspring						
Western	-1.7	0.6680	-3.60	0.3820	-3.53	0.3926
Mediterranean	6.7	0.1194	5.48	0.2034	6.14	0.1620
BPA	2.2	0.6113	2.31	0.5836	3.38	0.4377
Western*BPA	1.3	0.8214	2.71	0.6479	2.70	0.6489
Mediterranean*BPA	-7.3	0.2367	-6.47	0.2883	-8.02	0.2048
Male PND10 Offspring						
Western	6.80	**0.0265**	5.47	**0.0586**	5.92	**0.0543**
Mediterranean	17.75	**<0.0001**	17.43	**<0.0001**	18.16	**< 0.0001**
BPA	4.53	0.1679	4.84	0.1165	5.29	0.1061
Western*BPA	-6.73	0.1396	-5.78	0.1678	-6.19	0.1539
Mediterranean*BPA	-12.29	**0.0145**	-11.77	**0.0132**	-12.73	**0.0150**

Linear mixed effect models were run to assess the impact of perinatal experimental diet components and their interaction on Ln-transformed hepatic TG levels in PND10 offspring. Models were sex-stratified (female: n=48, male: n=55), and all included 'Litter ID' as a random effect. Effect size p-values were **bolded** if significant ($p < 0.05$) or borderline significant ($p < 0.10$) to highlight the experimental diet components that contribute to model prediction of hepatic TG levels.

- Model 1 adjusted for litter size.
- Model 2 additionally adjusted for dam body weight, 25 days post-partum.
- Model 3 additionally adjusted for dam pre-gestational body weight change.

Table 2.6 Impact of Perinatal Experimental Diet Exposure on Hepatic Liver Triglyceride Levels in 10-month Offspring, Sex-Stratified

Experimental Diet	Model 1		Model 2		Model 3	
	% Change TGs	p-value	% Change TGs	p-value	% Change TGs	p-value
Female 10-month Offspring						
Western	3.21	0.5067	4.08	0.3914	4.22	0.3765
Mediterranean	0.69	0.8881	0.64	0.8944	0.59	0.9022
BPA	-4.13	0.4263	-3.66	0.4716	-2.55	0.6281
Western*BPA	2.36	0.7378	1.74	0.8021	5.85	0.9341
Mediterranean*BPA	15.28	**0.0318**	14.71	**0.0355**	15.21	**0.0309**
Male 10-month Offspring						
Western	-6.70	0.1430	-6.86	0.1344	-5.00	0.2639
Mediterranean	-1.99	0.6466	-2.09	0.6318	-1.27	0.7635
BPA	2.39	0.6000	2.13	0.6415	2.32	0.5993
Western*BPA	1.95	0.7649	1.07	0.8703	1.08	0.8644
Mediterranean*BPA	-5.00	0.4396	-4.75	0.4632	-3.40	0.5878

Linear mixed effect models were run to assess the impact of perinatal experimental diet components and their interaction on Ln-transformed hepatic TG levels in 10-month offspring. Models were sex-stratified (female: n=65, male: n=67) and all included 'Cohort' as a random effect. Effect size p-values were **bolded** if significant ($p < 0.05$) or borderline significant ($p < 0.10$) to highlight the experimental diet components that contribute to model prediction of hepatic TG levels.

- Model 1 adjusted for litter size.
- Model 2 additionally adjusted for relative mWAT in 10-month offspring.
- Model 3 additionally adjusted for the presence of nodular hyperplasia in 10-month offspring.

Supplemental Information

Table S2.1 Complete Composition of Three Experimental Diets

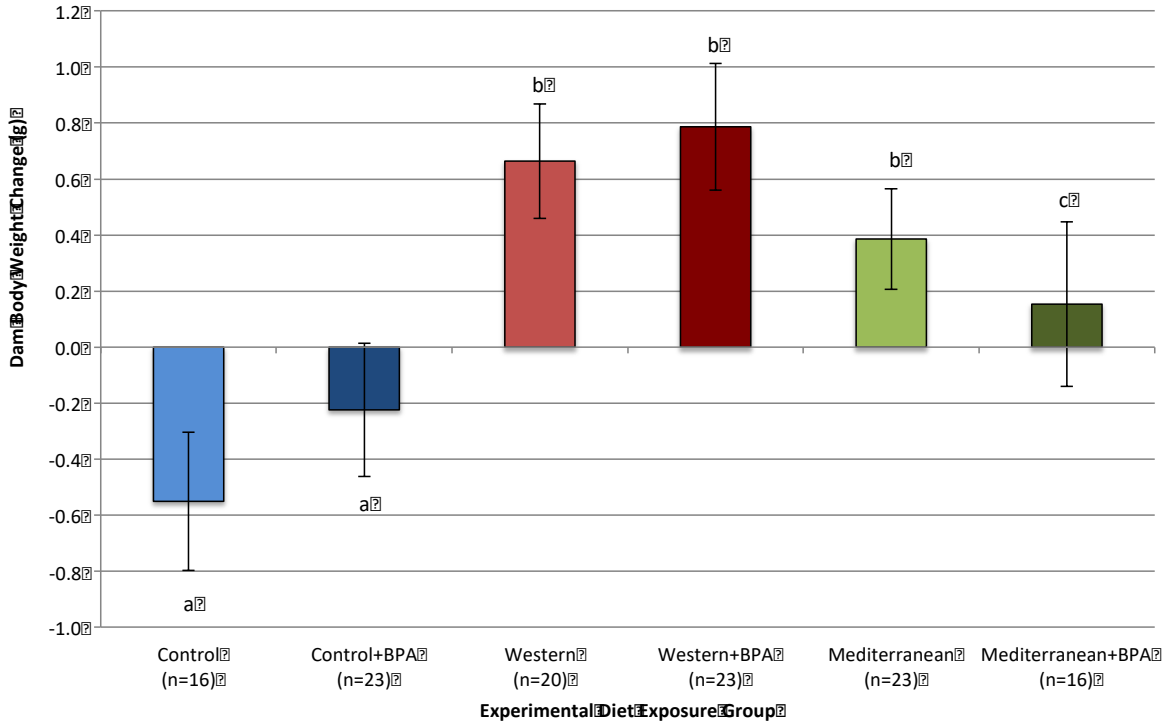
Ingredient	Experimental Exposure Diets		
	Control	**Western**	**Mediterranean**
Main Ingredients (g/100g food)			
Fat Source	7	**20.5***	**21.4**
Casein	20	19	19
Cornstarch	40	**14**	**23**
Dyestrose	13.2	13.2	13.2
Sucrose	10	**25.5**	**9.2**
Cellulose	5	**2**	**8**
Mineral Mix	3.5	**3.8**	**3.8**
Vitamin Mix	1	**1.1**	**1.1**
Composition of Fat Source (g/100g food)			
Corn Oil	100	**29**	**4**
Butter, anhydrous	0	**33**	**4**
Palm Oil	0	**30**	0
Olive Oil	0	**8**	**91**
Menheden (Fish) Oil	0	0	**1**
Vitamins (unit/kg food)			
Nicotinic acid (mg)	30	30	30
Pantothenate (mg)	15	15	15
Pyridoxine (mg)	6	6	6
Thiamin (mg)	5	5	5
Riboflavin (mg)	6	6	6
Folic acid (mg)	2	**1**	**4**
Biotin (mg)	0.2	0.2	0.2
Vitamin B12 (ug)	25	**10**	**10**
Vitamin K (ug)	900	900	900
Vitamin E (IU)	75	**25**	75
Vitamin A (IU)	4000	4000	**8000**
Vitamin D (IU)	1000	**400**	1000
Choline (mg)	1000	1000	1000
Vitamin C (mg)	0	0	**500**

Table S2.1 Complete Composition of Three Experimental Diets, Continued

Ingredient	Experimental Exposure Diets		
	Control	**Western**	**Mediterranean**
Minerals (unit/kg food)			
Calcium (mg)	5000	5000	5000
Phosphorus (mg)	3000	3000	3000
Magnesium (mg)	513	513	**850**
Sodium (mg)	1039	**7000**	1039
Potassium (mg)	3600	3600	**8000**
Chloride (mg)	1631	1631	1631
Sulfur (mg)	300	300	300
Iron (mg)	45	45	45
Zinc (mg)	38	38	38
Manganese (mg)	10	10	10
Copper (mg)	6	6	6
Iodine (mg)	0.2	0.2	0.2
Molybdenum (mg)	0.15	0.15	0.15
Selenium (mg)	0.15	0.15	0.15
Silicon (mg)	5	5	5
Chromium (mg)	1	1	1
Fluoride (mg)	1	1	1
Nickel (mg)	0.5	0.5	0.5
Boron (mg)	0.5	0.5	0.5
Lithium (mg)	0.1	0.1	0.1
Vanadium (mg)	0.1	0.1	0.1

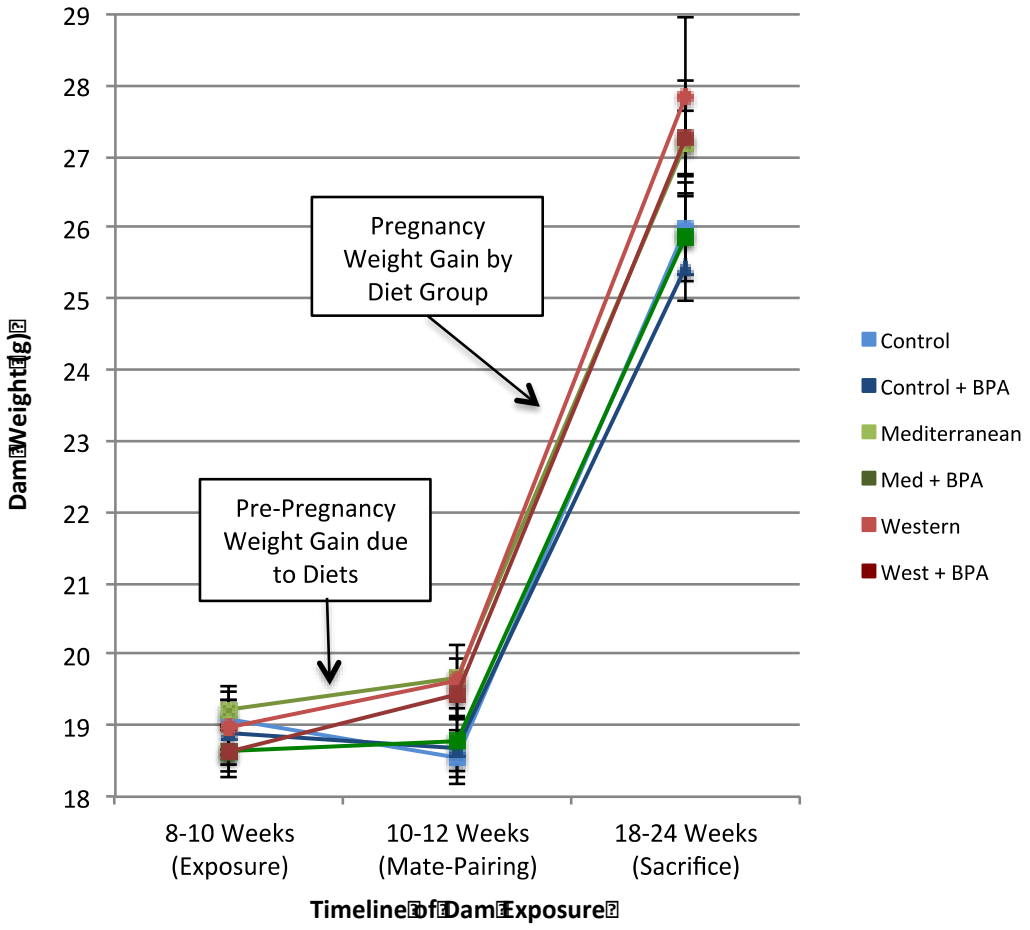
* **Bolded text** is used to emphasize nutrients in the Western and Mediterranean diet that differ significantly from the Control diet. These differences reflect nutrient differences in human Western and Mediterranean dietary intake patterns.

Figure S2.1 Pre-Gestational Body Weight Change in Dams by Exposure Group: First Two Weeks of Exposure



Dams were exposed to one of six experimental diets for two weeks prior to mate-pairing. Body weight changed as the dams adjusted to their new diets. This figure illustrates the average weight change by exposure group; error bars denote the SEM for each group. [a] Denotes the average TGs of Control PND10 offspring; groups that do not differ significantly from Control are also marked with 'a'. [b] Denotes exposure groups with average TGs that differ significantly ($p < 0.05$) from Control. [c] Denotes the exposure group with average TGs that differ from Control with borderline significance ($p < 0.10$).

Figure S2.2 Body Weight Change in Dams by Exposure Group: Initial Exposure to Offspring Weaning



Dam body weight was measured at three times: (1) start of exposure to experimental diets, at 8-10 weeks of age, (2) at mate-pairing, two weeks later, (3) and four days after their pups were weaned (PND25). Mean body weight of dams in each exposure group is plotted above with the SEM designated by the error brackets.

Table S2.2 Dam Metabolic Outcomes

Metabolic Outcomes in Dams	Perinatal BPA Exposure	Experimental Diet: Mean (SD)			ANOVA p-value	Tukey's p-values*
		Control	Western	Mediterranean		
Exposure Body Weight (grams)	No Yes	19.10 (1.15) 18.91 (1.72)	18.97 (1.95) 18.64 (2.53)	19.22 (2.37) 18.64 (1.63)	0.6141	n.s.
Mate-Pairing Body Weight (grams)	No Yes	18.55(0.88) 18.68 (1.76)	19.63 (0.84) 19.42 (2.23)	19.66 (1.71) 18.74 (0.81)	**0.0223**	
Post-Weaning Body Weight (grams)	No Yes	25.99 (2.54) 25.45 (2.13)	27.84 (4.10) 27.25 (2.54)	27.17 (1.91) 25.86 (2.46)	0.0960	Cont vs. West = 0.0728
Exposure Weight Change (grams)	No Yes	-0.55 (0.99) -0.22 (1.14)	0.66 (0.91) 0.79 (1.08)	0.39 (0.86) 0.15 (1.17)	**0.0004**	Cont vs. West = 0**.0006** Cont vs. WBPA = **0.0001** Cont vs Med = **0.0062** Cont vs. MBPA = 0.0560
Pregnancy Weight Change (grams)	No Yes	7.46 (1.92) 6.62 (3.01)	9.01 (3.35) 7.65 (2.49)	7.26 (1.43) 7.07 (1.99)	0.1420	Cont vs. West = 0.0903
Relative Liver Weight (liver weight / body weight)	No Yes	0.054 (0.007) 0.054 (0.007)	0.055 (0.007) 0.051 (0.008)	0.050 (0.006) 0.055 (0.009)	0.1446	Cont vs. Med = 0.0842
Relative Mesenteric Adipose Weight (MAT weight / body weight)	No Yes	0.025 (0.005) 0.026 (0.007)	0.033 (0.008) 0.032 (0.010)	0.028 (0.008) 0.025 (0.006)	**0.0065**	Cont vs. West = **0.0035** Cont vs. WBPA = **0.0078**
Serum Insulin Levels (ng/dL)	No Yes	2.64 (2.60) 2.41 (1.46)	1.33 (0.54) 1.59 (1.17)	1.69 (1.28) 1.59 (1.03)	0.1913	Cont vs. West = **0.0433** Cont vs. WBPA = 0.0730 Cont vs. MBPA = 0.0782
Serum Leptin Levels (ng/dL)	No Yes	5.16 (4.20) 5.93 (4.28)	4.54 (2.46) 5.76 (3.99)	5.63 (4.34) 5.59 (3.92)	0.9598	n.s.
Hepatic TG levels (mg/g)	No Yes	14.64 (12.11) 20.24 (15.00)	29.66 (15.66) 25.48 (13.98)	34.98 (16.60) 25.74 (13.17)	**0.0030**	Cont vs. West = **0.0077** Cont vs. WBPA = **0.0410** Cont vs. Med = **0.0001** Cont vs. MBPA = **0.0365**

*Only significant ($p < 0.05$) or borderline significant ($p < 0.10$) comparisons are shown; all others are not significant (n.s.).

Table S2.3 PND10 Offspring Metabolic Outcomes

Metabolic Outcomes	Perinatal BPA Exposure	Experimental Diet: Mean (SD)			ANOVA p-value	Tukey's p-values*
		Control	Western	Mediterranean		
PND10 Female Offspring						
PND10 Body Weight (grams)	No Yes	5.97 (0.54) 5.43 (1.14)	5.71 (0.75) 5.55 (0.90)	5.34 (0.95) 5.89 (1.17)	0.5371	Cont vs. Med = 0.0955
Relative Liver Weight (liver weight / body weight)	No Yes	0.030 (0.003) 0.029 (0.005)	0.029 (0.005) 0.032 (0.008)	0.030 (0.005) 0.027 (0.004)	0.4358	n.s.
Serum Leptin Levels (ng/dL)	No Yes	3.40 (2.13) 5.47 (4.38)	2.15 (1.60) 7.21 (6.93)	8.84 (6.77) 7.48 (6.42)	0.0668	Cont vs. Med = **0.0284**
Hepatic TG levels (mg/g)	No Yes	3.68 (2.50) 4.01 (3.26)	2.34 (1.23) 4.63 (3.45)	5.71 (2.42) 5.04 (2.63)	0.1859	n.s.
PND10 Male Offspring						
PND10 Body Weight (grams)	No Yes	5.78 (0.67) 4.74 (0.74)	5.71 (0.99) 5.98 (1.33)	5.59 (0.89) 5.44 (1.59)	0.2075	Cont vs. CBPA = **0.0434**
Relative Liver Weight (liver weight / body weight)	No Yes	0.026 (0.004) 0.026 (0.005)	0.027 (0.006) 0.029 (0.005)	0.031 (0.004) 0.026 (0.007)	0.3109	Cont vs. Med = 0.0691
Serum Leptin Levels (ng/dL)	No Yes	2.40 (3.23) 1.66 (1.80)	4.62 (5.65) 3.91 (2.71)	11.55 (7.82) 5.38 (5.23)	**0.0063**	Cont vs. Med = **0.0011**
Hepatic TG levels (mg/g)	No Yes	1.63 (0.60) 2.85 (1.38)	4.56 (5.26) 2.73 (1.07)	11.93 (9.17) 5.09 (2.71)	**0.0007**	Cont vs. Med **< 0.0001** Cont vs. MBPA = 0.0803

*Only significant ($p < 0.05$) or borderline significant ($p < 0.10$) comparisons are shown; all others are not significant (n.s.).

Table S2.4 10-Month Offspring Metabolic Outcomes

Metabolic Outcomes	Perinatal BPA Exposure	Experimental Diet: Mean (SD)			ANOVA p-value	Tukey's p-values*
		Control	Western	Mediterranean		
10-month Female Offspring						
Body Weight (grams)	No Yes	32.92 (8.02) 34.25 (6.94)	35.44 (8.23) 32.85 (5.97)	36.50 (7.48) 35.46 (4.48)	0.7765	n.s.
Relative Liver Weight (liver weight / body weight)	No Yes	0.031 (0.007) 0.033 (0.005)	0.038 (0.006) 0.037 (0.007)	0.038 (0.006) 0.034 (0.007)	**0.0356**	Cont vs. West = **0.0122**, Cont vs. WBPA = **0.0166,** Cont vs. Med = **0.0092**
Relative Mesenteric Adipose Weight (MAT weight / body weight)	No Yes	0.040 (0.014) 0.038 (0.008)	0.037 (0.011) 0.036 (0.008)	0.040 (0.013) 0.043 (0.015)	0.8011	n.s.
Serum Leptin (ng/dL)	No Yes	8.47 (4.33) 8.62 (3.21)	8.90 (5.55) 10.60 (5.20)	12.54 (6.26) 11.16 (4.31)	0.3579	n.s.
Serum Insulin (ng/dL)	No Yes	1.59 (1.58) 1.95 (3.16)	2.58 (3.95) 2.98 (2.67)	3.22 (2.35) 1.86 (0.88)	0.6845	n.s.
Serum Resistin (ng/dL)	No Yes	1.99 (9.63) 1.88 (0.55)	2.47 (1.29) 2.07 (0.72)	2.56 (1.09) 1.63 (0.42)	0.1663	n.s.
Serum PAI-1 (ng/dL)	No Yes	7.73 (1.91) 7.89 (2.55)	6.82 (4.20) 6.73 (2.71)	6.13 (2.55) 12.37 (5.59)	**0.0020**	Cont vs. MBPA = **0.0043**
Hepatic Triglycerides (mg/g)	No Yes	5.79 (4.30) 6.64 (8.72)	8.10 (7.11) 7.13 (7.72)	7.47 (8.16) 13.62 (5.45)	0.1228	Cont vs. MBPA = **0.0118**

Table S2.4 10-Month Offspring Metabolic Outcomes, continued

Metabolic Outcomes	Perinatal BPA Exposure	Experimental Diet: Mean (SD)			ANOVA p-value	Tukey's p-values*
		Control	Western	Mediterranean		
10-month Male Offspring						
Body Weight (grams)	No Yes	43.41 (5.49) 44.30 (2.40)	44.15 (4.89) 46.78 (4.16)	45.69 (5.53) 44.70 (4.77)	0.5703	Cont vs. WBPA = 0.0846
Relative Liver Weight (liver weight / body weight)	No Yes	0.036 (0.009) 0.039 (0.010)	0.041 (0.007) 0.039 (0.004)	0.043 (0.016) 0.038 (0.009)	0.6172	n.s.
Relative Mesenteric Adipose Weight (MAT weight / body weight)	No Yes	0.034 (0.006) 0.036 (0.010)	0.034 (0.010) 0.041 (0.007)	0.035 (0.008) 0.033 (0.007)	0.1575	Cont vs. WBPA = **0.0235**
Serum Leptin (ng/dL)	No Yes	10.02 (3.09) 11.93 (6.35)	12.74 (4.51) 16.13 (4.12)	14.63 (4.51) 13.24 (5.11)	**0.0448**	Cont vs. WBPA = **0.0021** Cont vs. Med = **0.0213** Cont vs. MBPA = 0.0965
Serum Insulin (ng/dL)	No Yes	5.02 (3.77) 5.20 (2.62)	5.48 (3.04) 6.23 (2.84)	6.11 (3.44) 5.91 (5.26)	0.9551	n.s.
Serum Resistin (ng/dL)	No Yes	1.23 (0.60) 1.25 (0.45)	1.64 (0.74) 2.00 (0.78)	1.57 (0.55) 1.38 (0.63)	**0.0496**	Cont vs. WBPA = **0.0046**
Serum PAI-1 (ng/dL)	No Yes	11.40 (6.98) 10.07 (6.01)	8.41 (3.48) 7.78 (4.40)	8.96 (4.74) 8.98 (2.69)	0.5564	Cont vs. WBPA = 0.0774
Hepatic Triglycerides (mg/g)	No Yes	9.38 (8.59) 13.52 (10.41)	4.85 (4.05) 7.61 (5.96)	10.66 (10.37) 6.39 (6.59)	0.1815	n.s.

*Only significant ($p < 0.05$) or borderline significant ($p < 0.10$) comparisons are shown; all others are not significant (n.s.).

Table S2.5 10-Month Offspring Hepatic Lesions Assessed via Histopathology

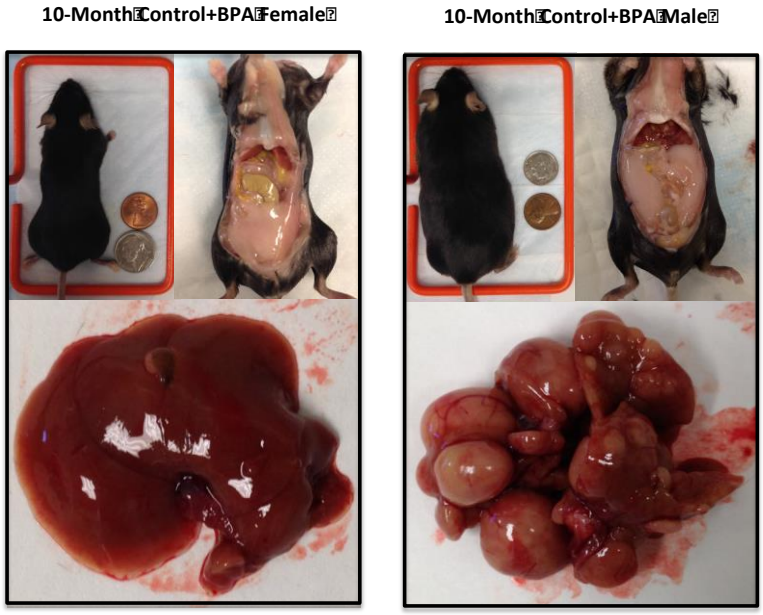
Hepatic Lesion	Perinatal BPA Exposure	Experimental Diet: Mean (SD)			ANOVA p-value	Tukey's p-values*
		Control	Western	Mediterranean		
10-month Female Offspring						
Continuous Variables: Mean (SD)						
Oil Red O	No Yes	2.10 (0.57) 1.67 (1.12)	1.58 (1.31) 0.91 (0.83)	1.91 (1.04) 2.25 (1.22)	0.0591	Cont vs. WBPA = **0.0124**
Hepatocellular Vacuolation	No Yes	1.50 (0.85) 1.56 (1.01)	1.25 (1.29) 1.18 (1.25)	1.55 (1.37) 2.58 (0.69)	**0.0424**	Cont vs. MBPA = **0.0258**
Hepatocellular Hypertrophy	No Yes	2.30 (1.70) 1.33 (1.22)	1.50 (1.38) 0.64 (1.03)	0.91 (1.30) 2.42 (1.73)	**0.0231**	Cont vs. WBPA = **0.0096** Cont vs. Med = **0.0291**
Proliferative	No Yes	0.00 (0.00) 0.44 (0.73)	0.00 (0.00) 0.00 (0.00	0.00 (0.00) 0.33 (0.49)	**0.0061**	Cont vs. CBPA = **0.0063** Cont vs. MBPA = **0.0263**
Non-Proliferative	No Yes	4.30 (4.11) 2.11 (2.57)	1.58 (2.15) 1.36 (1.21)	1.27 (1.10) 4.33 (3.63)	**0.0132**	Cont vs. West = **0.0217** Cont vs. WBPA = **0.0153** Cont vs. Med = **0.0126** Cont vs. BPA = 0.0819
Discrete Variables (Presence/Absence): Frequency (%)					Chi-squared p-value	
Adenoma	No Yes	0 0	0 0	0 0	n.s.	n.s.
Nodular Hyperplasia	No Yes	0/10 (0.00) 2/9 (22.22)	0/12 (0.00) 0/11 (0.00)	0/11 (0.00) 4/12 (33.33)	**0.0122**	n.s.
Oval Cell Hyperplasia	No Yes	5/11 (45.45) 3/11 (27.27)	1/12 (8.33) 1/11 (9.09)	1/12 (8.33) 8/12 (66.67)	**0.0094**	n.s.
Kupffer Cell Hyperplasia	No Yes	5/11 (45.45) 2/11 (18.18)	1/12 (8.33) 2/11 (18.18)	2/12 (16.67) 8/12 (66.67)	**0.0299**	n.s.
Cell Infiltrates	No Yes	8/11 (72.73) 7/11 (63.64)	9/12 (75.00) 9/11 (81.82)	9/12 (75.00) 11/12 (91.67)	0.7327	n.s.
Eosinophilic Foci	No Yes	0/11 (0.00) 1/11 (9.09)	0/12 (0.00) 0/11 (0.00)	0/12 (0.00) 0/12 (0.00)	0.3398	n.s.
Mixed Cell Foci	No Yes	0 0	0 0	0 0	n.s.	n.s.

Table S2.5 10-Month Offspring Hepatic Lesions Assessed via Histopathology, continued

Hepatic Lesion	Perinatal BPA Exposure	Experimental Diet: Mean (SD)			ANOVA p-value	Tukey's p-values*
		Control	Western	Mediterranean		
10-month Male Offspring						
Continuous Variables: Mean (SD)						
Oil Red O	No	1.67 (0.98)	1.40 (0.52)	1.83 (1.19)	0.7078	n.s.
	Yes	1.90 (1.20)	1.75 (1.06)	2.08 (0.79)		
Hepatocellular Vacuolation	No	1.50 (1.38)	1.40 (0.97)	1.80 (1.23)	0.9471	n.s.
	Yes	1.58 (1.00)	1.75 (0.75)	1.75 (1.14)		
Hepatocellular Hypertrophy	No	2.25 (1.22)	2.30 (1.42)	2.20 (1.55)	0.3538	Cont vs. WBPA = **0.0478**
	Yes	2.75 (1.29)	1.67 (1.07)	2.75 (1.36)		
Proliferative	No	0.83 (1.27)	0.50 (0.85)	1.10 (1.45)	0.5303	n.s.
	Yes	1.10 (1.45)	0.75 (1.22)	0.25 (0.62)		
Non-Proliferative	No	6.33 (5.00)	2.80 (3.65)	4.00 (4.82)	0.1704	Cont vs. WBPA = **0.0136** Cont vs. West = 0.0573
	Yes	4.60 (4.06)	1.92 (2.64)	5.00 (4.77)		
Discrete Variables (Presence/Absence): Frequency (%)					Chi-squared p-value	Tukey's p-values*
Adenoma	No	1/12 (8.33)	2/12 (16.67)	2/10 (20.00)	0.4076	n.s.
	Yes	3/10 (30.00)	3/12 (25.00)	0/12 (0.00)		
Nodular Hyperplasia	No	7/12 (58.33)	5/12 (41.67)	3/10 (30.00)	0.2804	n.s.
	Yes	5/10 (50.00)	3/12 (25.00)	2/12 (16.67)		
Oval Cell Hyperplasia	No	7/12 (58.33)	4/12 (33.33)	2/10 (20.00)	0.1349	n.s.
	Yes	5/11 (45.45)	6/12 (50.00)	1/12 (8.33)		
Kupffer Cell Hyperplasia	No	7/12 (58.33)	4/12 (33.33)	2/10 (20.00)	0.1826	n.s.
	Yes	6/11 (54.55)	6/12 (50.00)	2/12 (16.67)		
Cell Infiltrates	No	10/12 (83.33)	10/12 (83.33)	9/10 (90.00)	0.8363	n.s.
	Yes	8/11 (72.73)	10/12 (83.33)	10/12 (83.33)		
Eosinophilic Foci	No	0/12 (0.00)	1/12 (8.33)	0/10 (0.00)	0.2093	n.s.
	Yes	2/11 (18.18)	0/12 (0.00)	0/12 (0.00)		
Mixed Cell Foci	No	0/12 (0.00)	1/12 (8.33)	0/10 (0.00)	0.6153	n.s.
	Yes	0/11 (0.00)	1/12 (8.33)	1/12 (8.33)		

*Only significant ($p < 0.05$) or borderline significant ($p < 0.10$) comparisons are shown; all others are not significant (n.s.).

Figure S2.3 Variation in Body Size, Liver Health and Mesenteric Adiposity at 10-Months



A female and male mouse perinatally exposed to the Control+BPA diet had very different hepatic responses to the exposure. The female mouse (left) weighed only 25.1g, had a relative liver weight of 3.6%, relative MAT weight of 2.1%, and a dark red, healthy liver with only minimal vacuolation and hypertrophy. In contrast, the male mouse (right) weighed 47.4g, had a relative liver weight of 5.9%, relative MAT weight of 3.3%, and a yellowish-pink liver, riddled with masses and moderate to severe hyperplasia. This example is indicative of the range of responses observed within the same perinatal exposure group, across all study groups. So although many hepatic outcomes did not differ by perinatal exposure, there was a wide range of responses among the individual study animals.

REFERENCES

1. Loomba R, Sirlin CB, Schwimmer JB, Lavine JE. Advances in Pediatric Nonalcoholic Fatty Liver Disease. Hepatology. 2009;50: 1282–93.

2. Lindbäck SM, Gabbert C, Johnson BL, Smorodinsky E, Sirlin CB, Garcia N, et al. Pediatric Nonalcoholic Fatty Liver Disease: A Comprehensive Review. Adv Pediatr. 2010;57: 85–140.

3. Giorgio V, Prono F, Graziano F, Nobili V. Pediatric Non Alcoholic Fatty Liver Disease: Old and New Concepts on Development, Progression, Metabolic Insight and Potential Treatment Targets. BMC Pediatr. BMC Pediatrics; 2013;13: 40.

4. Lonardo A, Ballestri S, Marchesini G, Angulo P, Loria P. Nonalcoholic Fatty Liver Disease: A Precursor of the Metabolic Syndrome. Dig Liver Dis. Editrice Gastroenterologica Italiana; 2015;47: 181–190.

5. Angulo P. Obesity and Nonalcoholic Fatty Liver Disease. Nutr Rev. 2007;65: 57–63.

6. Sevastianos V a, Hadziyannis SJ. Nonalcoholic Fatty Liver Disease: From Clinical Recognition to Treatment. Expert Rev Gastroenterol Hepatol. 2008;2: 59–79.

7. Padmanabhan V, Cardoso RC, Puttabyatappa M. Developmental Programming , a Pathway to Disease. Endocrinology. 2016;157: 1328–1340.

8. Aviram A, Hod M, Yogev Y. Maternal Obesity: Implications for Pregnancy Outcome and Long-Term Risks: A Link to Maternal Nutrition. Int J Gynecol Obs. 2011;115: S6–S10.

9. Boney CM, Verma A, Tucker R, Vohr BR. Metabolic Syndrome in Childhood: Association With Birth Weight, Maternal Obesity, and Gestational Diabetes Mellitus. Pediatrics. 2005;115: e290–e296.

10. Catalano PM, Farrell K, Thomas A, Huston-Presley L, Mencin P, Mouzon SH De, et al. Perinatal Risk Factors for Childhood Obesity and Metabolic Dysregulation. Am J Clin Nutr. 2009;90: 1303–12.

11. Heerwagen MJR, Miller MR, Barbour LA, Friedman JE. Maternal Obesity and Fetal Metabolic Programming: A Fertile Epigenetic Soil. Am J Physiol Regul Integr Comp Physiol. 2010;299: R711–R722.

12. Sullivan EL, Smith MS, Grove KL. Perinatal Exposure to High-Fat Diet Programs Energy Balance , Metabolism and Behavior in Adulthood. Neuroendocrinology. 2011;93: 1–8.

13. Hajj N El, Schneider E, Lehnen H, Haaf T. Epigenetics and Life-Long Consequences of an Adverse Nutritional and Diabetic Intrauterine Environment. Reproduction. 2014;148: R111–R120.

14. Brumbaugh DE, Friedman JE. Developmental Origins of Nonalcoholic Fatty Liver Disease. Pediatr Res. 2014;75: 140–7.

15. Li M, Reynolds CM, Segovia SA, Gray C, Vickers MH. Developmental Programming of Nonalcoholic Fatty Liver Disease: The Effect of Early Life Nutrition on Susceptibility and Disease Severity in Later Life. Biomed Res Int. 2015; 1–12.

16. Wesolowski SR, Kasmi KCE, Jonscher KR, Friedman JE. Developmental Origins of NAFLD: A Womb with a Clue. Nat Rev Gastroenterol Hepatol. 2017;14: 81–96.

17. Pruis MGM, Lendvai A, Bloks VW, Zwier M V, Baller JFW, Bruin A De, et al. Maternal Western Diet Primes Non-Alcoholic Fatty Liver Disease in Adult Mouse Offspring. Acta Physiol. 2014;210: 215–227.

18. Kruse M, Seki Y, Vuguin PM, Du XQ, Fiallo A, Glenn AS, et al. High-Fat Intake During Pregnancy and Lactation Exacerbates High-Fat Diet-Induced Complications in Male Offspring in Mice. Endocrinology. 2013;154: 3565–3576.

19. Dahlhoff M, P S, Blutke A, Rozman J, Klingenspor M, Deutsch MJ, et al. Peri-Conceptional Obesogenic Exposure Induces Sex-Specific Programming of Disease Susceptibilities in Adult Mouse Offspring. Biochim Biophys Acta. 2014;1842: 304–317.

20. Mccurdy CE, Bishop JM, Williams SM, Grayson BE, Smith MS, Friedman JE, et al. Maternal High-Fat Diet Triggers Lipotoxicity in the Fetal Livers of Nonhuman Primates. J Clin Invest. 2009;119: 323–335.

21. Simino De Paula LA, de Fante T, Fontana MF, Borges FO, Torsoni MA, Milanski M, et al. Lipid Overload during Gestation and Lactation can Independently Alter Lipid Homeostasis in Offspring and Promote Metabolic Impairment after New Challenge to High-Fat Diet. Nutr Metab (Lond). Nutrition & Metabolism; 2017;14: 1–15.

22. Oddy WH, Herbison CE, Jacoby P, Ambrosini GL, O'Sullivan T a, Ayonrinde OT, et al. The Western Dietary Pattern is Prospectively Associated with Nonalcoholic Fatty Liver Disease in Adolescence. Am J Gastroenterol. 2013; 1–8.

23. Mollard RC, Senechal M, Macintosh AC, Hay J, Wicklow BA, Wittmeier KDM, et al. Dietary Determinants of Hepatic Steatosis and Visceral Adiposity in Overweight and Obese Youth at Risk of Type 2 Diabetes. Am J Clin Nutr. 2014;99: 804–812.

24. Ryan M, Itsiopoulos C, Thodis T, Ward G, Trost N, Hofferberth S, et al. The Mediterranean Diet Improves Hepatic Steatosis and Insulin Sensitivity in Individuals with Non-Alcoholic Fatty Liver Disease. J Hepatol. 2013;59: 138–143.

25. Kontogianni MD, Tileli N, Margariti A, Georgoulis M, Deutsch M, Tiniakos D, et al. Adherence to the Mediterranean Diet is Associated with the Severity of Non-Alcoholic Fatty Liver Disease. Clin Nutr. 2014;33: 678–683.

26. Baratta F, Pastori D, Polimeni L, Bucci T, Ceci F, Calabrese C, et al. Adherence to Mediterranean Diet and Non-Alcoholic Fatty Liver Disease: Effect on Insulin Resistance. Am J Gastroenterol. Nature Publishing Group; 2017;112: 1832–1839.

27. Zelber-Sagi S, Salomone F, Mlynarsky L. The Mediterranean Dietary Pattern as the Diet of Choice for Non-Alcoholic Fatty Liver Disease: Evidence and Plausible Mechanisms. Liver Int. 2017;37: 936–949.

28. Heindel JJ, Blumberg B, Cave M, Machtinger R, Mantovani A, Mendez MA, et al. Metabolism Disrupting Chemicals and Metabolic Disorders. Reprod Toxicol. Elsevier Inc.; 2017;68: 3–33.

29. Arciello M, Gori M, Maggio R, Barbaro B, Tarocchi M, Galli A, et al. Environmental Pollution: A Tangible Risk for NAFLD Pathogenesis. Int J Mol Sci. 2013;14: 22052–22066.

30. Foulds CE, Treviño LS, York B, Walker CL. Endocrine-Disrupting Chemicals and Fatty Liver Disease. Nat Rev Endocrinol. Nature Publishing Group; 2017;13: 445–457.

31. Polyzos SA, Kountouras J, Deretzi G, Zavos C, S. Mantzoros C. The Emerging Role of Endocrine Disruptors in Pathogenesis of Insulin Resistance: A Concept Implicating Nonalcoholic Fatty Liver Disease. Curr Mol Med. 2012;12: 68–82.

32. Environmental PA. Risk Management for Bisphenol A (BPA). In: Assessing and Managing Chemicals under TSCA.

33. Schonfelder G, Wittfoht W, Hopp H, Talsness C, Paul M, Chahoud I. Parent Bisphenol A Accumulation in the Human Maternal-Fetal-Placental Unit. Env Heal Perpsect. 2002;110: A703–A707.

34. Nahar MS, Liao C, Kannan K, Harris C, Dolinoy DC. *In Utero* Bisphenol A Concentration, Metabolism, and Global DNA Methylation Across Matched Placenta, Kidney, and Liver in the Human Fetus. Chemosphere. 2015;124: 54–60.

35. Nahar MS, Liao C, Kannan K, Dolinoy DC. Fetal Liver Bisphenol A Concentrations and Biotransformation Gene Expression Reveal Variable Exposure and Altered Capacity for Metabolism in Humans. 2013;27: 116–123.

36. Ke Z-H, Pan J-X, Jin L-Y, Xu H-Y, Yu T-T, Ullah K, et al. Bisphenol A Exposure May Induce Hepatic Lipid Accumulation via Reprogramming the DNA Methylation Patterns of Genes Involved in Lipid Metabolism. Sci Rep. Nature Publishing Group; 2016;6: e31331.

37. Shimpi PC, More VR, Paranjpe M, Donepudi AC, Goodrich JM, Dolinoy DC, et al. Hepatic Lipid Accumulation and *Nrf2* Expression Following Perinatal and Peripubertal Exposure to Bisphenol A in a Mouse Model of Nonalcoholic Liver Disease. Environ Health Perspect. 2017;125: 1–10.

38. Jirtle RL, Skinner MK. Environmental Epigenomics and Disease Susceptibility. Nat Rev Genet. 2007;8: 253–62.

39. Faulk C, Dolinoy DC. Timing is Everything: The When and How of Environmentally Induced Changes in the Epigenome of Animals. Epigenetics. 2011;6: 791–797.

40. Strakovsky RS, Wang H, Engeseth NJ, Flaws JA, Helferich WG, Pan Y, et al. Developmental Bisphenol A (BPA) Exposure Leads to Sex-Specific Modification of Hepatic Gene Expression and Epigenome at Birth that may Exacerbate High-Fat Diet-Induced Hepatic Steatosis. Toxicol Appl Pharmacol. Elsevier Inc.; 2015;284: 101–112.

41. Wei J, Sun X, Chen Y, Li Y, Song L, Zhou Z, et al. Perinatal Exposure to Bisphenol A Exacerbates Nonalcoholic Steatohepatitis-Like Phenotype in Male Rat Offspring Fed on a High-Fat Diet. J Endocrinol. 2014;222: 313–325.

42. Weinhouse C, Anderson O, Bergin I, Vandenbergh D, Gyekis J, Dingman M, et al. Dose-Dependent Incidence of Hepatic Tumors in Adult Mice following Perinatal Exposure to Bisphenol A. Env Heal Perpsect. 2014;122(5): 485-491.

43. Waterland RA, Jirtle RL. Transposable Elements: Targets for Early Nutritional Effects on Epigenetic Gene Regulation. Mol Cell Biol. 2003;23: 5293–5300.

44. Institute for Lab Animal Research (ILAR). Guide for the Care and Use of Laboratory Animals. National Academy of Sciences. 2011.

45. Nowland MH, Lebowsky R. ULAM Guidelines and SOPs for Mice. In: University of Michigan, Institutional Animal Care and Use Committee (IACUC). 2016. https://wiki.med.umich.edu/display/ULAMGSOP/Table+.

46. Morgan H, Suterhland H, Martin D, Whitelaw E. Epigenetic Inheritance at the Agouti Locus in the Mouse. Nat Genet. 1999;23: 314–318.

47. Dolinoy DC. The Agouti Mouse Model: An Epigenetic Biosensor for Nutritional and Environmental Alterations on the Fetal Epigenome. Nutr Rev. 2008;66: S7–S11.

48. Dolinoy DC, Weinhouse C, Jones TR, Rozek LS, Jirtle RL. Variable Histone Modifications at A(vy) Metastable Epiallele. Epigenetics. 2010;5: 637–644.

49. Duhl D, Vrieling H, Miller K, Wolff G, Barsh G. Neomorphic Agouti Mutations in Obese Yellow Mice. Nat Genet. 1994;8: 59–65.

50. Miltenberger R, Mynatt R, Wilkinson J, Woychick R. The Role of the Agouti Gene in the Yellow Obese Syndrome. J Nutr. 1997;127: S1902–S1907.

51. Dolinoy DC, Huang D, Jirtle RL. Maternal Nutrient Supplementation Counteracts Bisphenol A-Induced DNA Hypomethylation in Early Development. Proc Natl Acad

Sci. 2007;104: 13056–61.

52. Caligioni C. Assessing Reproductive Status/Stages in Mice. Curr Protoc Neurosci. 2009;Appendix 4: 1–11.

53. Flurkey K, Currer J, Harrison D. Mouse Models in Aging Research. The Mouse in Biomedical Research, 2nd Edition New York; Elsevier, Volume 3: 2007. pp. 637–672.

54. Reeves PG. Components of the AIN-93 Diets as Improvements in the AIN-76A Diet. J Nutr. 1997;123: S838–S841.

55. Reeves PG, Nielsen FH, Fahey GC. Committee Report AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet. J Nutr. 1993;123: 1939–1951.

56. Dolinoy DC, Weidman JR, Waterland R a., Jirtle RL. Maternal Genistein Alters Coat Color and Protects Avy Mouse Offspring from Obesity by Modifying the Fetal Epigenome. Environ Health Perspect. 2006;114: 567–572.

57. Guerrero-Bosagna CM, Sabat P, Valdovinos FS, Valladares LE, Clark SJ. Epigenetic and Phenotypic Changes Result from a Continuous Pre and Post Natal Dietary Exposure to Phytoestrogens in an Experimental Population of Mice. BMC Physiol. 2008;8: 17.

58. Kafatos AG, Verhagen H, Moschandreas J, Apostolaki I, van Westerop JJM. Mediterranean Diet of Crete: Foods and Nutrient Content. J Am Diet Assoc. 2000;1000: 1487–1493.

59. Trichopoulou A, Toupadaki N, Tzonou A, Katsouyanni K, Manousos O, Kada E, et al. The Macronutrient Composition of the Greek Diet: Estimates Derived from Six Case-Control Studies. Eur J Clin Nutr. 1993;47: 549–558.

60. Trichopoulou A, Katsouyanni K, Gnardellis C. The Traditional Greek Diet. Eur J Clin Nutr. 1993;47: S76–S81.

61. Block G, Dresser CM, Hartman AM, Carroll MD. Nutrient Sources in the American Diet: Quantitative Data from the NHANES II survey. Vitamins and minerals. Am J Epidemiol. 1985;122: 13–26.

62. Block G, Dresser CM, Hartman AM, Carroll MD. Nutrient Sources in the American Diet: Quantitative Data from the NHANES II Survey: Macronutrients and Fats. Am J Epidemiol. 1985;122: 27–40.

63. Anderson OS, Nahar MS, Faulk C, Jones TR, Liao C, Kannan K, et al. Epigenetic Responses Following Maternal Dietary Exposure to Physiologically Relevant Levels of Bisphenol A. Environ Mol Mutagen. 2012;342: 334–342.

64. Crosson SM, Khan A, Printen J, Pessin JE, Saltiel AR. PTG Gene Deletion Causes Impaired Glycogen Synthesis and Developmental Insulin Resistance. J Clin Invest. 2003;111: 1423–1432.

65. Hoff J. Methods of Blood Collection in the Mouse. Lab Anim (NY). 2000;29: 47–53.

66. Anderson OS, Peterson KE, Sanchez BN, Zhang Z, Mancuso P, Dolinoy DC. Perinatal bisphenol A exposure promotes hyperactivity, lean body composition, and hormonal responses across the murine life course. FASEB J. 2013;27(4):1784-1792.

67. Oben JA, Mouralidarane A, Samuelsson A, Matthews PJ, Morgan ML, Mckee C, et al. Maternal Obesity during Pregnancy and Lactation Programs the Development of Offspring Non-Alcoholic Fatty Liver Disease in Mice. J Hepatol. European Association for the Study of the Liver; 2010;52: 913–920.

68. Rasmussen KM, Yaktine AL. Weight Gain during Pregnancy: Reexamining the Guidelines. National Academy of Sciences. 2009.

69. Skaznik-Wikiel ME, Swindle DC, Allshouse AA, Polotsky AJ, McManaman JL. High-Fat Diet Causes Subfertility and Compromised Ovarian Function Independent of Obesity in Mice. Obstet Gynecol Surv. 2016;71: 532–533.

70. Warden CH, Fisler JS. Comparisons of Diets Used in Animal Models of High Fat Feeding. Cell Metab. 2008;7: 277–279.

71. Peretz J, Vrooman L, Ricke WA, Hunt PA, Ehrlich S, Hauser R, et al. Bisphenol A and Reproductive Health: Update of Experimental and Human Evidence, 2007-2013. Env Heal Perpsect. 2014;122: 775–786.

72. Cabaton NJ, Wadia PR, Rubin BS, Zalko D, Schaeberle CM, Askenase MH, et al. Perinatal Exposure to Environmentally Relevant Levels of Bisphenol A Decreases Fertility and Fecundity in CD-1 Mice. Environ Health Perspect. 2011;119: 547–552.

73. Fengler VHI, Macheiner T, Kessler SM, Czepukojc B, Gemperlein K, Muller R, et al. Susceptibility of Different Mouse Wild Type Strains to Develop Diet-Induced NAFLD / AFLD-Associated Liver Disease. PLoS One. 2016;11: 1–21.

74. Lohr K, Pachl F, Moghaddas Gholami A, Geillinger KE, Daniel H, Kuster B, et al. Reduced Mitochondrial Mass and Function Add to Age-Related Susceptibility toward Diet-Induced Fatty Liver in C57BL/6J Mice. Physiol Rep. 2016;4: 1–17.

75. Waller-Evans H, Hue C, Fearnside J, Rothwell AR, Lockstone HE, Caldérari S, et al. Nutrigenomics of High Fat Diet Induced Obesity in Mice Suggests Relationships between Susceptibility to Fatty Liver Disease and the Proteasome. PLoS One. 2013;8: 1–12.

76. Williams L, Seki Y, Vuguin PM, Charron MJ. Animal Models of *In Utero* Exposure to a

High Fat Diet: A Review. Biochim Biophys Acta. Elsevier B.V.; 2014;1842: 507–519.

77. Lau EY, Liu J, Archer E, McDonald SM, Liu J. Maternal Weight Gain in Pregnancy and Risk of Obesity among Offspring: A Systematic Review. J Obes. Hindawi Publishing Corporation; 2014;2014: 1–16.

78. Gaillard R, Durmuş B, Hofman A, MacKenbach JP, Steegers EAP, Jaddoe VWV. Risk Factors and Outcomes of Maternal Obesity and Excessive Weight Gain during Pregnancy. Obesity. 2013;21: 1046–1055.

79. Burgueño AL, Cabrerizo R, Gonzales Mansilla N, Sookoian S, Pirola CJ. Maternal High-Fat Intake during Pregnancy Programs Metabolic-Syndrome-Related Phenotypes through Liver Mitochondrial DNA Copy Number and Transcriptional Activity of Liver PPARGC1A. J Nutr Biochem. Elsevier Inc.; 2013;24: 6–13.

80. Strakovsky R, Zhang X, Zhou D, Pan Y. The Regulation of Hepatic PON1 by a Maternal High-Fat Diet is Gender Specific and may Occur through Promoter Histone Modifications in Neonatal Rats. J Nutr Biochem. 2014;25: 170–176.

81. Schwimmer JB, Deutsch R, Kahen T, Lavine JE, Stanley C, Behling C. Prevalence of Fatty Liver in Children and Adolescents. Pediatrics. 2006;118: 1388–93.

82. Griffiths PS, Walton C, Samsell L, Perez MK, Piedimonte G. Maternal High-Fat Hypercaloric Diet during Pregnancy Results in Persistent Metabolic and Respiratory Abnormalities in Offspring. Pediatr Res. 2016;79: 278–286.

83. Casabiell X, Pineiro V, Tome MA, Peino R, Dieguez C, Casanueva FF. Presence of Leptin in Colostrum and/or Breast Milk from Lactating Mothers: A Potential Role in the Regulation of Neonatal Food Intake. J Clin Endocrinol Metab. 1997;82: 4270–4273.

84. Sánchez J, Oliver P, Miralles O, Ceresi E, Picó C, Palou A. Leptin Orally Supplied to Neonate Rats is Directly Uptaken by the Immature Stomach and may Regulate Short-Term Feeding. Endocrinology. 2005;146: 2575–2582.

85. Nozhenko Y, Asnani-Kishnani M, Rodríguez AM, Palou A. Milk Leptin Surge and Biological Rhythms of Leptin and Other Regulatory Proteins in Breastmilk. PLoS One. 2015;10: 1–17.

86. Myers MG, Cowley MA, Münzberg H. Mechanisms of Leptin Action and Leptin Resistance. Annu Rev Physiol. 2008;70: 537–556.

87. Bouret S. Minireview: Leptin and Development of Hypothalamic Feeding Circuits. Endocrinology. 2004;145: 2621–2626.

88. Attig L, Larcher T, Gertler A, Abdennebi-Najar L, Djiane J. Postnatal Leptin is Necessary for Maturation of Numerous Organs in Newborn Rats. Organogenesis.

2011;7: 88–94.

89. Pettan-Brewer C, M. Treuting P. Practical Pathology of Aging Mice. Pathobiol Aging Age-related Dis. 2011;1: 1–16.

90. Snyder JM, Ward JM, Treuting PM. Cause-of-Death Analysis in Rodent Aging Studies. Vet Pathol. 2016;53: 233–243.

91. Park EJ, Lee JH, Yu GY, He G, Ali SR, Holzer RG, et al. Dietary and Genetic Obesity Promote Liver Inflammation and Tumorigenesis by Enhancing IL-6 and TNF Expression. Cell. 2010;140: 197–208.

92. Milagro FI, Campión J, Martíez JA. Weight Gain Induced by High-Fat Feeding Involves Increased Liver Oxidative Stress. Obesity. 2006;14: 1118–1123.

93. Nakashima Y. Fish-Oil High-Fat Diet Intake of Dams after Day 5 of Pregnancy and during Lactation Guards Against Excessive Fat Consumption of their Weaning Pups. J Nutr Sci Vitaminol (Tokyo). 2008;54: 46–53.

94. Albert BB, Vickers MH, Gray C, Reynolds CM, Segovia SA, Derraik JGB, et al. Fish Oil Supplementation to Rats Fed High-Fat Diet during Pregnancy Prevents Development of Impaired Insulin Sensitivity in Male Adult Offspring. Sci Rep. 2017;7: 1–11.

95. Xu P, Wang H, Kayoumu A, Wang M, Huang W, Liu G. Diet rich in Docosahexaenoic Acid / Eicosapentaenoic Acid Robustly Ameliorates Hepatic Steatosis and Insulin Resistance in Seipin Deficient Lipodystrophy Mice. Nutr Metab (Lond). Nutrition & Metabolism; 2015;12: 1–10.

96. Ferramosca A. Modulation of Hepatic Steatosis by Dietary Fatty Acids. World J Gastroenterol. 2014;20: 1746.

97. Arbones-Mainar JM, Ross K, Rucklidge GJ, Reid M, Duncan G, Arthur JR, et al. Extra Virgin Olive Oils Increase Hepatic Fat Accumulation and Hepatic Antioxidant Protein Levels in APOE -/- Mice. J Proteome Res. 2007;6: 4041–4054.

98. Ryan M, Itsiopouls C, Thodis T, Ward G, Trost N, Hofferberth S. The Mediterranean Diet Improves Hepatic Steatosis and Insulin Sensitivity in Individuals with Non-Alcoholic Fatty Liver Disease. J Hepatol. 2013;59: 138–143.

99. Ferguson S, Law C, Abshire J. Developmental Treatment with Bisphenol A or Ethinyl Estradiol Causes Few Alterations on Early Preweaning Measures. Toxicol Sci. 2011;124: 149–160.

100. Walker CL. Minireview: Epigenomic Plasticity and Vulnerability to EDC Exposures. Mol Endocrinol. 2016;30: 848–855.

101. Burgueño AL, Cabrerizo R, Gonzales Mansilla N, Sookoian S, Pirola CJ. Maternal High-

Fat Intake during Pregnancy Programs Metabolic-Syndrome-Related Phenotypes through Liver Mitochondrial DNA Copy Number and Transcriptional Activity of Liver PPARGC1A. J Nutr Biochem. Elsevier Inc.; 2013;24: 6–13.

102. Mohankumar SMJ, Rajendran TD, Vyas AK, Hoang V, Asirvatham-Jeyaraj N, Veiga-Lopez A, et al. Effects of Prenatal Bisphenol-A Exposure and Postnatal Overfeeding on Cardiovascular Function in Female Sheep. J Dev Orig Health Dis. 2017;8: 65–74.

103. Koneva LA, Vyas AK, Mceachin RC, Puttabyatappa M, Wang H-S, Sartor M, et al. Developmental Programming: Interaction Between Prenatal BPA and Postnatal Overfeeding on Cardiac Tissue Gene Expression in Female Sheep. Environ Mol Mutagen. 2017;58: 4–18.

104. Koneva LA, Vyas AK, Mceachin RC, Puttabyatappa M, Wang H-S, Sartor MA, et al. Developmental Programming: Interaction Between Prenatal BPA and Postnatal Overfeeding on Cardiac Tissue Gene Expression in Female Sheep. Environ Mol Mutagen. 2017;58: 4–18.

105. Michalopoulos GK. Liver Regeneration after Partial Hepatectomy: Critical Analysis of Mechanistic Dilemmas. Am J Pathol. American Society for Investigative Pathology; 2010;176: 2–13.

CHAPTER 3

Perinatal High Fat Diets and Bisphenol A Exposure Differentially Alters Maternal Metabolic Parameters that Affect Metabolic Health and Redox Markers in Postnatal Offspring

ABSTRACT

Metabolic diseases can be developmentally programmed, with exposures during perinatal development altering life long disease risk. Perinatal exposure to bisphenol A (BPA), a ubiquitous high production volume chemical used in polycarbonate plastics and epoxy resins, has been associated with perinatal metabolic programming and alterations in tissue oxidation. Maternal high fat diet (HFD) during pregnancy is known to alter offspring metabolic health, but the potential for HFD to modify perinatal BPA exposure has not been examined. This study investigated whether perinatal exposure to BPA and HFDs altered offspring metabolic health at postnatal day 10 (PND10) and 10-months, and whether these alterations were accompanied by changes in the hepatic redox environment or markers of lipid peroxidation. Dams were randomized to one of six experimental diets from pre-gestation through lactation; a Control diet, one of two HFDs, or each of these diets with 50 μg BPA/kg diet added. Offspring were weaned onto the Control diet and followed to postnatal day 10 (PND10) or 10-months of age. Perinatal

exposure to HFD increased body weight and relative mWAT weight in dams and 10-month offspring; serum leptin levels were elevated in HFD-exposed offspring at PND10 and 10-months. This impact of perinatal HFD extended to hepatic lipid peroxidation, with elevated 8-isoprostane-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) levels observed in offspring perinatally exposed to HFDs. Hepatic S-glutathionylation (S-glut), protein-bound glutathione, was highly variable but decreased in HFD-exposed dams and 10-month female offspring. Glutathione and cysteine redox potentials (E_hGSH and E_hCys) were not impacted by perinatal HFD, but perinatal BPA exposure was associated with more oxidizing E_hGSH in PND10 females. Perinatal BPA did not have the anticipated detrimental effect on offspring metabolic health or hepatic lipid peroxidation. In this study, a human-relevant dose of BPA did not perinatally program alterations in metabolic health, hepatic redox environment, or lipid peroxidation; the minimal impact only occurred at PND10, while offspring were still exposed to BPA. Perinatal HFD did have programming effects, consistently altering metabolic health and hepatic 8-iso-$PGF_{2\alpha}$ at PND10 and 10-months, but there was no evidence of diet-modification of BPA effects. Hepatic redox potentials at 10-months did not differ by perinatal exposure, suggesting the redox environment was not perinatally programmed, but likely remains sensitive to ongoing changes in physiologic and postprandial states, and environmental stressors.

INTRODUCTION

Prevalence of chronic metabolic diseases is increasing worldwide. Metabolic disease risk can be elevated due to perinatal exposures [1–3]. This process, known as developmental programming, proposes that *in utero* exposures can alter lifelong offspring health trajectory [4,5]. Perinatal exposures to bisphenol A (BPA) and to a maternal high fat

diet (HFD) have both been associated with increased risk of offspring metabolic disease. Perinatal BPA exposure has been associated with increased body weight, adiposity, impaired glucose homeostasis and insulin resistance in offspring [6,7]. Maternal HFD during pregnancy has been correlated with altered birth weight, growth trajectory, neuroendocrine function, and energy homeostasis [8–10]. Metabolic disease has been associated with a classical definition of oxidative stress [11,12], whereby an imbalanced redox environment leads to a preponderance of oxidizing equivalents. However, these classical oxidative stress biomarkers only provide a qualitative characterization, whereas intracellular redox potentials (E_h) are a more sensitive gauge of overall redox environment [13–16]. Under normal, healthy conditions, the post-translational modifications of protein thiol groups into reduced and oxidized states occurs in stable non-equilibrium [14,17,18]. As this homeostatic balance shifts towards a more oxidized environment, protein thiol groups oxidize, which can alter protein function, thus disrupting cell signaling and redox sensing [14,18]. Perinatal programming of E_h has not been investigated.

Perinatal exposure to bisphenol A (BPA) is associated with altered developmental programming of metabolic disease [6,19–23] and with increased classic measures of oxidation in adult mice [24–27]. Free BPA can induce reactive oxygen species (ROS) via enzymatic (peroxidase/H_2O_2, CYP450/NADPH) and non-enzymatic (HOCl/-OCL, CO_2/peroxynitrite) radical generation [28–31]. Levels of other radical species (peroxides, superoxides, and hydroxyl radicals) are often observed following BPA exposure, likely due to ROS acting on intracellular glutathione or NADPH [29,30]. However these measures do not give insight into the redox environment; the impact of BPA exposure on redox parameters has never been examined. BPA has been shown to cross the placenta and is

measureable in breast milk in both human and murine models [32–35], making it an good model exposure to study for potential impact on perinatal programming of tissue oxidation. BPA is a synthetic, high-production volume chemical, used in a wide range of consumer products including the polycarbonate plastics and epoxy resins used in food packaging [36,37]. Therefore, a substantial portion of human BPA exposure occurs via ingestion. These combined factors suggest that maternal ingestion of a human-relevant dose of BPA during the perinatal period would be an ideal way to test whether developmental programming of hepatic redox imbalance and lipid oxidation can occur.

Maternal diet during pregnancy has also been linked to altered risk of metabolic disease in offspring [8,9,38]. Among adult rodents and humans, Western-style HFDs have been associated with increased classical oxidative stress measures across a variety of tissue types, including liver [39–44]. Western HFDs contain high total and saturated fat, high sugar / low fiber, high salt, and low antioxidant content [45–48]. On the other hand, a Mediterranean-style HFD has been clinically recommended to prevent, slow or reverse progression of metabolic disease [49], due to the associations of Mediterranean diet consumption with improved insulin sensitivity, reduction in hepatic steatosis, plasma lipids and oxidative stress in adults [50–54]. A Mediterranean HFD contains a comparable percent of total fat as Western HFDs, but monounsaturated fat from olive oil is the main component not saturated fat; the diet is also characterized by high fiber, low salt, and high micronutrient content [55–57]. In human adults, adherence to a Mediterranean diet has been associated with decreased oxidative stress, measured by an increased GSH/GSSG ratio [58]. However, a complete Mediterranean diet has not been investigated in animal models,

so the potentially disparate effect of the two HFDs, on the perinatal programming of offspring oxidative outcomes, has not yet been examined.

To date, studies analyzing the association between metabolic health and oxidative response or examining the response to BPA or HFD exposures have used the classical measures of oxidative stress mentioned above. Unfortunately, these measures are usually qualitative, characterizing the downstream responses to elevated ROS, without shedding light on the redox environment. To address this gap, direct quantification of the redox environment based on changes in the GSH/GSSG and Cys/CySS redox couples has been proposed [14–16,59]. Intracellular GSH is considered the most reliable indicator of changes in intracellular redox state because of its abundance and direct involvement in cellular redox biochemistry [60]. Measuring thiol concentrations is critical to understanding the redox environment, but levels of these thiols fluctuate considerably over time [61,62]. Calculating E_h, from intracellular thiol concentrations, provides a useful measure of steady state perturbations, which can trigger altered intracellular signaling, changes in protein activity, or transcriptional regulation [14–16]. The redox environment is tightly controlled to maintain homeostasis. Rapid response of thiols to oxidative insults is expected, but due to their short turnover time, they quickly maintain stable non-equilibrium, which would suggest that redox environment could not be perinatally programmed. A recent study examining perinatal exposure to phthalates used E_h to examine embryonic alterations following perinatal exposure [62], so this study will build on that precedent.

A common measure of lipid peroxidation, F_2-isoprostane, 8-iso-prostaglandin $F_{2\alpha}$, (8-iso-$PGF_{2\alpha}$), has been used to examine oxidative outcomes during pregnancy and birth [63–65]. Since prenatal programming was not expected to alter the redox environment, 8-

iso-$PGF_{2\alpha}$ will also be measured in this study, to determine if this lipid metabolism marker can be impacted by perinatal exposures. Urinary and plasma 8-iso-$PGF_{2\alpha}$ have been widely used in human epidemiologic studies [11,66]. Despite its widespread use as a marker of lipid peroxidation, and its identification by the NIEHS Biomarkers of Oxidative Stress Study (BOSS) as an ideal marker [67–69], 8-iso-$PGF_{2\alpha}$ has inaccurately been assumed to be exclusively associated with increased reactive oxygen species (ROS) because they are often positively correlated, but the direct connection is rarely measured. The half-life of serum 8-iso-$PGF_{2\alpha}$ is 4-6 minutes in rodents, suggesting that elevated ROS levels would need to be maintained for extended periods if the 8-iso-$PGF_{2\alpha}$ were actually formed predominantly from the non-enzymatic free radical initiated peroxidation of arachidonic acid [59,70]. Recent findings suggest that 8-iso-$PGF_{2\alpha}$ may also result from prolonged inflammation, catalyzed by the ROS-independent prostaglandin-endoperoxidase synthetase (PES) [59,70]. This inflammation-induced mechanism for lipid peroxidation/lipid metabolism is most likely to remain relevant over the longer-term evaluation periods used in this longitudinal study design.

Since prenatal programming has become a concern in the rising prevalence of metabolic diseases, it is timely to examine if alterations in redox environment occurred, indicating a potential programming of lifelong redox response. To examine whether offspring hepatic redox environment or lipid peroxidation could be perinatally programmed by maternal diet or BPA, we designed a perinatal exposure study with longitudinal offspring follow-up. BPA, a daily exposure for many U.S. women, shares an oral route of exposure with maternal diet, so it was incorporated into diet pellets, to examine the impact of various diets (Control, Mediterranean, and Western) with and without

simultaneous BPA exposure. Hepatic redox environment was assessed by redox potentials (*Eh*GSH, *Eh*Cys) and S-glutathionylation (S-glut), while hepatic lipid peroxidation was measured by 8-isoprostane-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) to determine if redox parameters and inflammation-induced lipid peroxidation responded similarly to perinatal exposures. This is the first study to examine the potential for perinatal programming of redox parameters by two common developmental exposures, BPA and maternal diet. Examining oxidative responses in dams and offspring at two time points, postnatal day 10 (PND10) and 10-months, provided further insight into the impact perinatal exposures can have across generations and in offspring across time. This study aimed to explore whether perinatal BPA and HFD exposures could: affect the hepatic redox environment of offspring postnally, impact hepatic lipid peroxidation in offspring across time, and if changes in redox environment or lipid peroxidation were associated with alterations in offspring metabolic health.

METHODS

Experimental Design

This study used mice from a viable yellow agouti (A^{vy}) mouse colony, maintained for over 250 generations. Mating of agouti males (A^{vy}/a) with wild type females (a/a) maintains forced heterozygosity of the A^{vy} allele. Agouti mice have been extensively characterized previously [71,72]. Briefly, litter composition approximates 50% A^{vy}/a and 50% a/a offspring; A^{vy}/a mice, heterozygous for the A^{vy} allele, display a range of coat colors from yellow, mottled, to pseudoagouti and develop obesity and diabetes in adulthood. Coat color and disease severity vary based on epigenetic marks in the cryptic promoter of the Agouti gene. Wild type a/a mice

have black coats and remain lean throughout life [73–76]. This study only followed *a/a* offspring to avoid potential confounding from adult-onset obesity and diabetes in A^{vy}/a mice. Per the Institute for Laboratory Animal Research (ILAR) guidelines [77], mice were treated humanely, provided enrichment in their polycarbonate-free cages, and given *ad libitum* access to food and water 24 hours a day, in a climate-controlled room with a 12-hour light-dark cycle. The study protocol was approved by the University of Michigan's University Committee on Use and Care of Animals (UCUCA) [78].

Virgin, 8-10 week old, *a/a* dams were randomly assigned to one of six experimental diets: Control, Control + 50 μg BPA/kg diet, Mediterranean, Mediterranean + 50 μg BPA/kg, Western, or Western + 50 μg BPA/kg diet. Dams were mate-paired with young, virile A^{vy}/a males (average: 7.5 weeks old) and remained on their assigned experimental diet throughout pregnancy and lactation. At postnatal day 21 (PND21), all offspring were weaned onto the Control diet with TBHQ (Figure 3.1). Offspring were followed to two static time points of analysis. To standardize measurements and normalize diurnal fluctuations, all sacrifices were conducted in the afternoon (2-5pm) and 10-month females were sacrificed while in estrus, determined by vaginal cytology [79].

To assess the potential for perinatal exposures to program alterations in hepatic E_h and lipid peroxidation in adult offspring, one male and one female *a/a* pup per litter were followed to 10-months of age. Agouti mice at 10-months are analogous to middle aged humans [80], providing insight into adult offspring health prior to aging-related declines. All other *a/a* pups were sacrificed at postnatal day (PND10). In rodents, serum leptin levels surge at PND10 [81]; this surge has been associated with hypothalamic programming, that may alter satiety, food intake, and energy homeostasis for the offspring's lifespan [82,83]. Thus, alterations in leptin

levels at PND10 could impact offspring metabolic health into adulthood. PND10 data provides insight at an early time point, when young offspring are still exposed to the experimental diets via mother's milk, and provides a comparison for metabolic outcomes among 10-month offspring.

Experimental Diet Composition

The Control diet was a modification of the standard AIN93G mouse diet [84,85]. Corn oil replaced soybean oil in the Control diet to prevent potential developmental programming by phytoestrogens in soybean oil (the standard fat source in AIN93G), [86,87]. The Western and Mediterranean experimental diets differed from Control in their lipid, carbohydrate, vitamin, and mineral compositions, reflecting distinctions in human dietary patterns. Casein was the sole source of protein and was consistently provided in all three experimental diets. The Western diet, based upon NHANES II dietary intake data [47,48], simulated U.S. dietary intake with high saturated fat, sugar, and sodium but low fiber and antioxidant content. In contrast, the Mediterranean diet was based on the traditional Cretan diet, which includes high fruit, vegetable, and nut intake [56,57,88]. Mouse diets were designed on a nutrient / kg weight basis at the University of Michigan and were manufactured by Harlan Teklad (Madison, WI). Additional information on diet formulation has been previously reported (Chapter 2).

A human-relevant, oral dose of BPA was incorporated into the pelleted mouse diets. A previous study, using mice from this same agouti colony, reported that 50 μg BPA/kg diet produced an average 2.02 ng BPA/g liver [89]; this falls within the range of BPA levels assessed in human fetal liver samples (< LOQ to 96.8 ng BPA/g liver) [35]. BPA, supplied by the National Toxicology Program (NTP, Durham, NC), was mixed into sucrose in glass containers to

create a 0.1% BPA/sucrose mixture. Harlan Teklad, incorporated this BPA mixture into three of the six experimental diets at 0.05 g/kg, creating in the Control+BPA, Mediterranean+BPA, and Western+BPA diets.

To avoid masking the impact of perinatal BPA and HFD on tissue E_h and lipid peroxidation, TBHQ was removed from all six perinatal experimental diets. The preservative tert-butylhydroquinone (TBHQ) has potent antioxidant properties that prevent fatty acid rancidity in chow, thereby increasing shelf life [84,85]. TBHQ potently activates nuclear factor erythroid factor 2 (Nrf2), a redox sensitive transcription factor that binds to antioxidant response elements (ARE) in genes involved in responding to oxidative injury and inflammation [90]. Nrf2 activation by TBHQ has been associated with preserving microvascular endothelial function [91], attenuating hypertension and inflammatory response in the paraventricular nucleus [92], and decreasing hepatic ischemia-reperfusion injury [93], in murine models. This dissertation was designed to investigate the lifelong impact of perinatal exposure to BPA and/or HFD, so the exposures ended and TBHQ removal was no longer necessary once offspring transitioned out of the perinatal period.

Body Weight and Tissue Collection

Dam body weight was recorded at three times: initial exposure (pre-gestation), mate-pairing (2 weeks after exposure), and sacrifice (4 days post-weaning). Dam pre-gestational weight change (initial exposure to mate-pairing) and gestational weight gain (mate-pairing to sacrifice) were calculated from the three dam body weight measures. PND10 and 10-month offspring body weights were recorded at sacrifice only. Liver and mesenteric white adipose tissue (mWAT) were recorded for dams and 10-month offspring. At PND10, mice have

negligible mWAT, so only liver weights were recorded. Percent liver and mWAT weights were calculated by: absolute organ weight / total body weight * 100%. All body and organ weights were measured using a SLF103 balance (Fisher Scientific), to the hundredths digit.

During necropsy, the liver was dissected and separated by lobe; all analyses were conducted on left lobe tissue. A 5-10mg liver aliquot was placed into 300 μL of HPLC preservation buffer (5% perchloric acid, 0.2M boric acid, and 10μM γ-glutamylglutamate [γ-EE]) [94], to preserve the *in vivo* oxidation state of the hepatic tissue and to prevent further tissue oxidation during storage. Sample aliquots were flash frozen in liquid nitrogen and stored at -80^{o}C until HPLC processing.

Hepatic Redox Environment via HPLC

Reverse-phase HPLC analysis was used to quantify hepatic concentration of soluble thiols and protein-bound glutathione, as previously described [94]. Supelcosil LC-NH_2 column (Sigma-Aldrich, St. Louis, MO) was used to measure thiol concentrations on a Waters 2695 Alliance Separations Module. HPLC mobile phases were composed of (A) 80% HPLC grade methanol (Fisher Scientific) and 20% ddiH_20, and (B) 62.5% methanol, 12.5% glacial acetic acid (Fisher Scientific), and 214 mg/mL sodium acetate trihydrate (Sigma-Aldrich) in ddiH_20 with a gradient flow rate of 1.0 mL/min. Peak visualization, via fluorescence detection at 335 and 518 nm, was performed using a Waters 2474 fluorescence detector. Waters Empower software (Waters, Milford, MA) processed and compiled the peak visualization data.

The area under the curve (AUC) was resolved for each peak to quantify soluble thiol concentrations in each sample. AUC was normalized to the sample-specific internal standard, cellular volume and protein concentration, assessed via bicinchoninic acid assay (BCA assay,

Thermo Fisher) adapted for a microplate reader [95,96]. The soluble thiols measured in this study, reduced glutathione (GSH), oxidized glutathione disulfide (GSSG), reduced cysteine (Cys), and oxidized (CysS), were reported as intracellular concentrations (μM). E_h for GSSG:GSH and CysS:Cys were calculated for each redox couple using the Nernst equation. At pH 7.4, the E_0 = -264 mV and RT/nF = 30, so: $E_h = -264 + 30*\log([GSSG]/[GSH]^2)$ and $E_h = -264 + 30*\log([CysS/Cys]^2)$.

Hepatic S-glutathionylation (S-glut), a measure of protein-bound GSH, was quantified via further processing of the protein pellet remaining from the first derivation [94], used to quantify redox couples above. Briefly, a chloroform:methanol extraction was used to cleave the disulfide bonds formed between hepatic proteins and GSH; thus freeing GSH into solution. The solution was derivatized via 150 μL of 2X HPLC buffer and 150 μL of 15.4 mg/mL dithiolthreitol (DTT, Sigma-Aldrich). Dansyl chloride was added to 300 μL of aqueous layer from the derivatized sample. Samples were analyzed via the same reverse-phase HPLC method described above. GSH peaks in this second derivative were normalized to internal standard and protein concentrations, and reported as nmol/mg liver tissue.

Hepatic Lipid Peroxidation via 8-iso-PGF$_{2\alpha}$

During necropsy, a 15-20mg liver aliquot was immersed in a microcentrifuge tube filled with 0.005% BHT, to prevent oxidation during storage. Serum samples were stored at -80^{o}C until all samples could be sent to Cayman Chemical for testing at once. Samples were transferred to Cayman Chemical (Ann Arbor, MI, USA) on dry ice and 8-iso-PGF$_{2\alpha}$ analyses were conducted via enzyme immunoassay (EIA) kit (No. 516351), with a sensitivity of 2.7 pg/mL and

an intra-assay %CV of 9.5%. Hepatic 8-iso-PGF$_{2\alpha}$ measures were adjusted by total protein content of the liver, thus adjusting for sample-specific cellular concentration.

Statistical Analyses

All values were examined for biological plausibility; statistical outliers, defined as values 1.5 times the interquartile range, were removed prior to further analyses. Variable distribution was examined for normality via a Q-Q probability plot of the residuals. E_hGSH and E_hCys were normally distributed, but thiol concentrations (GSH, GSSG, Cys, CysS, S-glut), and 8-iso-PGF$_{2\alpha}$ distributions were right skewed (Table S1). Skewed variables were ln-transformed for univariate analyses. The impact of perinatal BPA and HFD on hepatic 8-iso-PGF$_{2\alpha}$ and redox parameters were analyzed by one-way ANOVA, with Tukey's post hoc analyses, to compare means from all six perinatal exposure groups. ANOVAs were conducted cross-sectionally, comparing differences between group means among mice of each age: dams, PND10, and 10-months.

Univariate analyses were conducted via generalized linear models between continuous predictor variables (i.e. dam and offspring metabolic parameters) and tissue oxidation measures. To examine whether the two measures of hepatic oxidative response (8-iso-PGF$_{2\alpha}$ and redox parameters) were correlated, Kendall's tau correlation matrices were computed. Sexually dimorphic responses to perinatal exposures occurred, so cross-sectional comparisons were sex-stratified for PND10 and 10-month offspring. Significance was pre-determined at $p < 0.05$. Borderline significance ($p < 0.10$) and suggestive significance ($p < 0.20$) were also highlighted to assess biological patterns that occurred, but may not have reached statistical significance. All analyses were conducted in SAS 9.4 (Cary, NC, USA).

RESULTS

Metabolic Characteristics Altered by Perinatal Exposures

Perinatal exposure to HFDs, but not BPA, affected the metabolic health of dams and offspring. HFD exposure was consistently associated with increased body weight, relative mWAT weight, and altered serum hormone levels (Table 3.1) in mice at all three time points. Among dams, body weight and relative mWAT weight were higher in the Mediterranean, Western, and Western+BPA groups, while relative liver weight was decreased by these diets. This supports the theory that greater postpartum maternal body weight is due to increased adiposity, not gain in lean mass. Although leptin levels usually increase with gains in adipose tissue, no difference was observed in this study; serum leptin levels did not differ by exposure group. However, consistent with decreased intracellular fuel needs in individuals on hyper caloric diets, postpartum serum insulin levels were lower in dams on the HFDs than Control. Gestational BPA exposure did not impact dam metabolic health.

Perinatal HFD exposure had a minimal impact on body weight and relative liver weight in offspring at PND10. Unlike dams, where HFDs were associated with increased body weight, at PND10, body weight tended to be lower among HFD-exposed pups compared to Control offspring. PND10 serum leptin levels were affected by perinatal exposure to HFDs, especially the Mediterranean diet. PND10 females perinatally exposed to Mediterranean diet had 2.6-fold higher leptin levels, while 4.8-fold higher levels were observed in males. High variation among the leptin measures in Mediterranean offspring supports tremendous inter-individual variation, suggesting individual mice responded differently to the same diet. Although not significant, the other HFDs tended to have greater serum leptin levels also in PND10 offspring of both sexes. Since the PND10 leptin surge impacts neural circuitry in the hypothalamus, the increased leptin

levels following perinatal HFD exposure may alter offspring metabolic health across their lifespan. The only PND10 metabolic outcome impacted by perinatal BPA exposure was male body weight; Control+BPA males weighed 82% less than Control males, consistent with previous reports of low birth weight following perinatal BPA exposure. However, the lack of more widespread alterations based on perinatal BPA suggests that the 50 μg BPA/kg diet dose does not offspring metabolic health, in this mouse model.

Perinatal exposure to HFDs, but not BPA, was associated with altered metabolic health effects in adult offspring, at 10-months. Mice at 10-months had not been exposed to the experimental diets for over 9 months, which suggests altered health outcomes at this age may be the result of perinatal programming. Despite the elevated serum leptin levels observed at PND10 among HFD-exposed offspring, body weight and mWAT weight were not systematically elevated at 10-months as would be expected if energy homeostasis had been altered by the increased leptin surge. The only perinatal effect on 10-month weights was observed in males perinatally exposed to Western+BPA diet, which had an 8% greater body weight and 22% higher mWAT weight compared to Controls. This is consistent with previous reports of metabolic programming occurring in male offspring, but not females. Of note, perinatal HFD exposure was associated with elevated serum leptin levels in offspring at 10-months, just as it was at PND10. This tended to be true of serum insulin also, with non-significantly greater insulin levels observed in 10-month offspring perinatally exposed to HFDs. Although these findings were not statistically consistent at 10-months, the biologic trend of altered metabolic outcomes occurring subsequent to perinatal HFD exposure suggests that maternal diet during pregnancy may impact offspring health into adulthood.

Hepatic Soluble Thiol Concentrations

Rapidly changing soluble thiol concentrations play an important role in many cell activities, like intracellular signaling, enzyme activity, and transcription factor binding. The selective reduction and oxidation of protein thiols for these cellular processes means thiol levels vary considerably. This large variation is apparent in the thiols measured in this study (Figure 3.2), making them challenging to use them as reliable markers of the overall redox environment. This natural variation in thiol concentrations may also be masking differences in hepatic concentrations based on perinatal exposure group.

Hepatic Redox Potentials

Evaluating E_h, instead of variable soluble thiols, provides value as a quantitative measure of perturbations to the steady state redox environment (Table 3.2, Figure 3.3). Hepatic E_hGSH was consistently about -230 mV in PND10 and 10-month offspring perinatally exposed to the Control diet: -233.1 $\pm$ 8.0 mV in PND10 females, -229.6 $\pm$ 9.5 mV in PND10 males, -229.4 $\pm$ 7.4 mV in 10-month females, and -229.3 $\pm$ 7.8 mV in 10-month males. This E_hGSH falls between highly reduced cellular proliferation conditions (-240 to -250 mV) and more oxidized differentiation conditions (-200 to -220 mV) that have been determined from a large variety of different species, cells and tissues. This suggests Control offspring livers are healthy and in a natural state of tissue repair and regeneration (Figure S3.1). Hepatic E_hGSH and E_hCys did not differ by perinatal exposure among dams or 10-month offspring. However, in offspring at PND10 perinatal exposures did impact hepatic (E_h).

Among PND10 females, perinatal BPA exposure was associated with decreased E_hGSH: Control+BPA (-216.7 $\pm$ 23.2 mV), Mediterranean+BPA (-208.7 $\pm$ 36.7 mV), and Western+BPA

(-220.8 ± 22.1 mV). These levels suggest differentiating redox conditions. At this young age, differentiating conditions are not entirely unexpected since the pups are undergoing rapid growth and development at PND10. However, the consistent reduction in E_hGSH, towards a more oxidized environment, only among BPA-exposed offspring, supports the theory that the BPA exposure may trigger steady state perturbations that result in more hepatic tissue repair. Of note, perinatal BPA exposure did not have the same oxidizing effect in PND10 males. Perinatal Mediterranean diet exposure was associated with a decreased E_hGSH (-216.8 ± 8.8 mV) in males.

These oxidizing shifts in E_hGSH were aligned with metabolic health alterations in male, but not female, PND10 offspring. In PND10 females, E_hGSH was altered by perinatal BPA exposure, but serum leptin levels differed by perinatal HFD exposure, not BPA. However, in PND10 males, perinatal Mediterranean diet exposure was associated with a more oxidized E_hGSH and increased relative liver weight and serum leptin levels. Since these measures were collected cross-sectionally, they are not evidence of causation; the association does suggest that the Mediterranean diet was able to affect both the redox environment and metabolic outcomes. These impacts no longer exist in 10-month offspring, though, so the Mediterranean diet may be exerting a direct effect at PND10 that does not continue into adulthood via perinatal programming.

Hepatic Protein S-glutathionylation

Hepatic S-glut represents a measure of GSH bound to intracellular proteins. Among dams and 10-month females, HFDs were associated with decreased S-glut levels. S-glut is thought to protect protein thiols from irreversible oxidation, thus maintaining their ability to respond to

alterations in redox environment. Thus, decreased S-glut levels could impair a tissue's redox responsiveness, which in turn could alter intracellular signaling and regulatory functions. The decreased S-glut observed in 10-month offspring may be an age-related decline in the ability to maintain redox homeostasis.

Hepatic 8-iso-PGF$_{2\alpha}$ Levels

Under inflammatory conditions, prostaglandin endoperoxide synthase can synthesize 8-iso-PGF$_{2\alpha}$, resulting in elevated levels. Hepatic 8-iso-PGF$_{2\alpha}$ varied by perinatal diet in mice at all time points. Among dams, those exposed to the Western+BPA diet had 1.46-fold greater 8-iso-PGF$_{2\alpha}$ levels compared to Controls. Perinatal HFD exposure altered 8-iso-PGF$_{2\alpha}$ in offspring at both PND10 and 10-months. In PND10 females, Mediterranean diet pups had 1.35-fold higher 8-iso-PGF$_{2\alpha}$ than Controls; levels were 1.59-fold higher in Western diet pups and 1.57-fold higher in Western+BPA pups. Female 10-month offspring also had elevated 8-iso-PGF$_{2\alpha}$ levels, if they were perinatally exposed to a HFD, with 1.26- to 1.42-fold greater 8-iso-PGF$_{2\alpha}$ than Controls. In contrast, perinatal exposure to the Mediterranean and Mediterranean+BPA diets were associated with decreased 8-iso-PGF$_{2\alpha}$ levels (0.66- and 0.70-fold, respectively) in PND10 males. But among 10-month males, perinatal HFDs were again associated with increased 8-iso-PGF$_{2\alpha}$ levels. Perinatal BPA exposure only affected 8-iso-PGF$_{2\alpha}$ levels in 10-month males, who had 1.41-fold greater levels than Controls. Thus, the metabolic alterations observed in offspring, following perinatal HFD exposure, were mirrored by increased hepatic 8-iso-PGF$_{2\alpha}$ levels in HFD exposed offspring. It is often posited that metabolic dysfunction is accompanied by increased systemic inflammation. The findings in this study are consistent with the theory.

Sex-Specific Effects in Metabolic and Oxidative Outcomes

At PND10, mean hepatic E_hGSH and S-glut differ between female and male offspring (Table 3.4). PND10 males have a suggestively more reduced E_hGSH (-226.8 mV) than females (-222.4 mV), but alterations in signaling pathways have not been observed in E_h changes of less than 15 mV. So although E_hGSH differs significantly by PND10 sex, the difference is unlikely to have a biological impact. On the other hand, PND10 females had a significantly greater S-glut, with 250.7 $\pm$ 392.5 nmol/mg liver tissue, compared to males (66.7 $\pm$ 245.4 nmol/mg). But the variance of S-glut within each sex exceeds the group mean, suggesting this measure is highly variable at PND10, and the sex difference may be an artifact of measurement timing, not a biologically relevant difference. At 10-months, S-glut levels were higher in males (1.1 nmol/mg) than females (0.9 nmol/mg). Hepatic 8-iso-PGF$_{2\alpha}$ levels did not differ by offspring sex at PND10 or 10-months.

At PND10, there were few differences in metabolic parameters by offspring sex, but by 10-months, every parameter differed (Table 3.4). At PND10, relative liver weight was greater in females (2.95%) than in males (2.73%), but body weight and serum leptin did not differ by sex. At 10-months, every metabolic outcome differed by sex; males had greater body weight, percent liver weight, serum leptin, and serum insulin levels, while females had a greater percent mWAT weight than males. Thus, sex-specific results were more apparent and biologically relevant in metabolic outcomes than in hepatic redox parameters or lipid peroxidation.

DISCUSSION

A major objective of this study was to investigate the potential to alter perinatal programming of the hepatic redox environment through dietary intervention and

environmental (chemical) stimuli. In offspring at both PND10 and 10-months post partum, hepatic 8-iso-PGF$_{2\alpha}$ levels, a biomarker of lipid peroxidation, were more impacted by perinatal exposure than were redox parameters, suggestive of a greater susceptibility to perinatal programming in 8-iso-PGF$_{2\alpha}$. Multiple dam metabolic and oxidative parameters were observed to impact oxidative responses in offspring, emphasizing the critical impact the maternal physiologic states could have on developing offspring.

The differential response of hepatic 8-iso-PGF$_{2\alpha}$ levels and redox parameters to perinatal maternal diet and BPA exposure, suggests that the redox environment itself is not altered long term by perinatal exposure, but that mechanisms underlying the regulation of 8-iso-PGF$_{2\alpha}$ levels may be altered. Isoprostanes are prostaglandin-like molecules, produced via free-radical induced auto-oxidation of arachidonic acid, in a cyclooxygenase (COX) free process or through a free radical-independent prostaglandin-endoperoxidase synthetase pathway [97,98]. Formation of 8-iso-PGF$_{2\alpha}$ is rate-limited by the presence of molecular oxygen and free radicals in the tissue [99]; however, dietary intake may also alter 8-iso-PGF$_{2\alpha}$ levels. Although urinary 8-iso-PGF$_{2\alpha}$ levels were not affected by a 2-day, very low fat diet (5%) [100] or the consumption of a fast food meal [101], levels increased following elevated consumption of conjugated linoleic acid (CLA) via diet [102] or supplementation [103,104]. CLA is a metabolic precursor to arachidonic acid; thus, the increased 8-iso-PGF$_{2\alpha}$ levels likely reflect an abundance of the parent molecule, arachidonic acid. Despite the reported association between elevated 8-iso-PGF$_{2\alpha}$ levels and metabolic diseases [105–110], 8-iso-PGF$_{2\alpha}$ has a short half life: 16 minutes in humans [111,112], and 4 minutes in rodents, with 80% elimination by four hours [113]. Thus, continuous production of 8-iso-PGF$_{2\alpha}$ due to an excess of dietary precursors or molecular oxygen and free radicals would

be necessary to maintain the association between 8-iso-$PGF_{2\alpha}$ and chronic metabolic disease over time. Studies reporting the link between metabolic disease and 8-iso-$PGF_{2\alpha}$ do not control for dietary intake and since ROS production is not chronically sustained, elevated 8-iso-$PGF_{2\alpha}$ over extended periods is likely due to synthesis via inflammation-induced PES enzymes.

Although free radicals and reactive oxygen species alter the intracellular redox environment, driving the formation of more oxidizing equivalents, the redox state of thiols is tightly controlled by numerous enzymes. For instance, GSH biosynthesis is determined by availability of amino acid precursors [114,115] and γ-glutamate cysteine ligase (GCLc) activity [14,114], while GSH conjugate degradation is regulated by γ-glutamyl transpeptidase and GSSG reduction is regulated by glutathione disulfide reductase [116,117]. Redox couples and their respective redox potentials play a critical role in intracellular signaling and redox sensing [15,18], so are carefully regulated to maintain cellular homeostasis [14,17,18]. Numerous studies conducted in monocultured cells and tissues from a variety of different species have shown that shifts in redox potential as small as +/- 15 mV are sufficient to change cellular functions from proliferation to differentiation to apoptosis (Figure S1). Even though measurements in tissues containing multiple cell types may exhibit smaller E_h changes, it is likely that redox shifts within individual cell types can be significantly greater. Evidence of this homeostatic control was also observed in human plasma, where the antioxidant capacity of the GSH:GSSG redox pair was retained through age 45, despite linear oxidation of Cys:CysS with age [118]. The 10-month old mice in this study are equivalent to human middle age, so their GSH:GSSG antioxidant capacity may still be tightly controlled, accounting for the minimal impact of perinatal exposures on

E_hGSH. Further supporting the independent signaling role of each redox pair (e.g. GSH:GSSG, Cys:CysS), evidence suggests redox pairs are individually affected by oxidative changes to the intracellular environment [16,119,120]. In this study, the independent response was observed in the different impact of perinatal exposures, dam metabolic and oxidative responses on offspring E_hGSH and E_hCys. At 1.6-2.4 minutes in human plasma [121,122], the half-life of GSH is even shorter than that of 8-iso-$PGF_{2\alpha}$, making GSH even more acutely responsive to environmental alterations, such as postprandial state, xenobiotic stress, and inflammatory cascades. This rapid turnover and tight homeostatic control of redox parameters results in a rapid return to baseline, ensuring that intracellular signaling continues.

Protein S-glut is a reversible post-translational modification that protects cysteine residues from irreversible oxidation and may also play a critical role in relaying redox signals [123–125]. Preventing oxidation of protein thiols protects their ability to respond to changes in redox environment, preserving the capacity for intracellular signaling, appropriate protein function, and transcriptional regulation [123–126]. The decreased S-glut levels observed in dams and 10-month females perinatally exposed to the HFDs in this study, suggest that the hepatic GSH reserve might be depleted in these animals. The lack of differential impact of perinatal HFD on 10-month males suggests a potential sexually dimorphic response; however, a sex-specific analysis did not find a biologically relevant difference in S-glut levels among 10-month females and males. Offspring at 10-months had not been exposed to the perinatal diets for more than nine months, suggesting that differences observed among 10-month female S-glut levels might be perinatally programmed. This was the only measure of the redox environment impacted at 10-months;

it warrants additional investigation to confirm these findings. HFD exposure has been linked to elevations in classical oxidative stress markers [40–43] and was associated with increased inflammation-induced lipid peroxidation, thus it is possible that perinatal exposure to a HFD could perinatally program post-translational modifications in hepatic tissue. However, in this situation, programming would be expected to protect against the increased stress presented by the HFD. Instead, the depressed S-glut levels observed in this study could reflect an increased lifelong GSH demand, required to maintain redox homeostasis in a regularly stressed tissue. Since S-glut levels in PND10 offspring varied drastically, no pattern of sex-specific or perinatal exposure-specific differences could be identified. The lack of impact on PND10 S-glut by perinatal exposures further suggests that the exposures do not alter S-glut regulation directly.

Perinatal HFD exposure altered oxidative responses, especially hepatic 8-iso-$PGF_{2\alpha}$, in offspring at PND10 and 10-months. Postprandial oxidative stress can result from sustained hyperglycemia or hyperlipidemia [127] and may contribute to the differences in hepatic 8-iso-$PGF_{2\alpha}$ observed in this study according to perinatal HFD exposure. Dietary studies in human adults have reported increased reactive oxygen species following intake of glucose [128], cream [129], and a high fat, high carbohydrate meal [130]. Few studies have examined the impact of maternal diet during pregnancy on offspring oxidative response, but a study in C57BL/6J mice reported that dam HFD intake during gestation was associated with increases in serum 8-OHdG and hepatic 3-nitrotyrosine levels in offspring at 8 weeks [131]. This is consistent with our findings that the Western and Mediterranean HFD increased hepatic 8-iso-$PGF_{2\alpha}$ in offspring at PND10 and 10-months. Perinatal HFD exposure had a greater impact on hepatic 8-iso-$PGF_{2\alpha}$ than redox parameters in offspring

at PND10 or 10-months. Although perinatal BPA has been linked to altered offspring metabolic health [6,19–23], and to increased oxidative outcomes in adult animals [24–27], no studies had investigated the potential to impact offspring redox environment. The results of this study suggest that perinatal exposure to a human-relevant dose of BPA may not induce perinatal programming of oxidative responses later in life.

Sexually dimorphic responses to perinatal HFD and BPA occurred in both metabolic and oxidative offspring outcomes, but the differences were only biologically relevant in the metabolic outcomes among 10-month offspring. In murine models, maternal HFD during pregnancy has previously been associated with sex-specific effects in offspring metabolic health [132–134]. The differential effect of estrogen sulfotransferase (EST) by sex has been associated with altered risk of type 2 diabetes; in females EST ablation improves insulin sensitivity, while decreasing hepatic lipogenesis and gluconeogenesis, while in males EST ablation was linked to decreased islet β-cell mass and glucose-activated insulin secretion, aggravating the diabetic phenotype [135]. EST sulfonates and deactivates estrogens, thus regulating estrogen homeostasis. Induction of EST can occur by Nrf2 activation following hepatic ischemia-reperfusion injuries; in females this induction inhibits estrogen activity, whereas in males EST ablation exacerbated the injury [136]. Based on these sexually dimorphic effects of EST activity, differences in redox parameters and 8-iso-$PGF_{2\alpha}$ could be expected. However, no biologically relevant differences were observed in this study, which suggests these results were estrogen-independent.

In this study, dam metabolic parameters were consistently associated with oxidative response in PND10 and 10-month offspring (Figure 3.4), although the exact changes differed by age and offspring sex (Tables 3.4, 3.5, 3.6). The predictive dam metabolic

parameters: gestational weight gain, dam body weight, percent liver weight, and serum insulin, have all previously been associated with metabolic alterations in offspring. Maternal pre-pregnancy obesity and gestational weight gain have been linked to increased risk of childhood obesity and metabolic comorbidities [137–140], but few studies have examined their effect on offspring oxidative responses. A human birth cohort study reported maternal pre-pregnancy obesity was associated with increased oxidative outcomes in newborn umbilical cord plasma, assessed by malondialdehyde and nitric oxide levels [141]. These results are supported by our study findings; dam body weight was consistently associated with oxidizing changes in dams, PND10, and 10-month offspring in this study.

Dam oxidative parameters (8-iso-$PGF_{2\alpha}$ and redox parameters) were also associated with oxidative response in offspring at both PND10 and 10-months. Together with the impact of dam metabolic parameters, this suggests that the maternal physiologic environment during pregnancy may be as important if not more important than other perinatal exposures (like diet and BPA exposure). Interestingly, despite the fact that dams were directly exposed to the six experimental diet groups for a minimum of 8 weeks, the exposures had a negligible impact on dam 8-iso-$PGF_{2\alpha}$ and redox parameters. Thus, the impact of dam oxidative parameters on offspring oxidative response does not appear to be a 'translation' of perinatal exposure effect through the dam, but rather a physiologic response to the dam's redox state.

Given the high global rates of metabolic disease, this study examined the relevant question of whether oxidative response could be perinatally programmed, thus providing a potential mechanism for the perinatal programming of metabolic diseases. Although two

different markers of oxidative response, hepatic 8-iso-$PGF_{2\alpha}$ and GSH-based redox parameters, were measured in this study, further insight could be provided by assessing additional markers of oxidative response, including enzyme levels, antioxidant capacity, and levels of reactive oxygen and nitrogen species. Although BPA did not have a strong impact on offspring oxidative response, in this study, other common chemical exposures might induce perinatal programming. Offspring oxidative response was only investigated in hepatic tissue, in this study; investigation into the potential for oxidative programming in other tissues is warranted. Despite the need to examine these additional avenues before complete understanding of perinatal effects on oxidative response, the experimental design of this study has multiple strengths. *In vivo* study of oxidative response is critical due to the sensitivity of redox parameters to minute physiologic changes and exocrine cell signaling, which cannot be captured by *in vitro* models. Examining offspring at multiple ages allows assessment of changes in response over time, and provides the opportunity to introduce post-natal interventions, like diet or chemical challenges.

Study limitations include the oral dosing of BPA, the single static time point used to assess perinatal programming, and the use of a non-specific biomarker of lipid peroxidation, 8-iso-$PGF_{2\alpha}$. The ingested route of BPA exposure was chosen to mimic human BPA exposure, which commonly occurs via food packaging. A trade-off of designing a human-relevant exposure study is that the exact quantity of BPA ingested by each animal is not known, mice ate *ad libitum.* So although mice in three experimental diet groups had access to 50 μg BPA/kg diet, their BPA body burden may have differed considerably. This may partially account for the lack of altered health effects observed following perinatal BPA exposure in this study. To better assess the potential for perinatal programming,

intermediate time points assessing metabolic, redox, and lipid peroxidation outcomes would have provided additional insight. Other life periods, such as peripuberty, have been identified as sensitive to environmental exposures; measuring offspring health at peripuberty and other times prior to 10-months might have illuminated trends or demonstrated a consistent impact across time, both of which would be instructive for translation to human health outcomes.

Lastly, although 8-iso-$PGF_{2\alpha}$ has been widely used in human epidemiology studies as a marker of classic oxidative stress, the potential for 8-iso-$PGF_{2\alpha}$ to be synthesized via two distinct pathways makes mechanistic interpretation difficult. This lack of biomarker specificity occurs because 8-iso-$PGF_{2\alpha}$ can be produced via non-enzymatic oxidation of arachidonic acid by ROS or via inflammation-induced PES enzymes. However, the time course for these two mechanisms differs; ROS generation is a rapid response, whereas inflammation-induced changes can occur over a more sustained time frame. In this study, since 8-iso-$PGF_{2\alpha}$ levels remained high at 10-months, the biomarker likely reflects the prolonged inflammatory response associated with altered metabolic health. Despite these limitations, this study advanced understanding of perinatal programming.

In conclusion, perinatal exposure to HFDs was associated with increased hepatic 8-iso-$PGF_{2\alpha}$ in offspring at PND10 and 10-months, but with no changes in redox parameters. Exposure to the HFDs had a greater impact than perinatal BPA exposure, supporting the importance of maternal diet during pregnancy. Although direct exposure to the HFDs and BPA had a negligible effect on dams, dam metabolic and oxidative parameters were associated with offspring hepatic 8-iso-$PGF_{2\alpha}$ and redox parameters. Associations were sex-specific and age-dependent. Given the inconsistent impact of perinatal exposures on

offspring redox parameters, the role of postnatal diet and chemical exposures may have a greater effect on redox imbalance. Comparing the influence of perinatal and postnatal exposures on metabolic disease risk is a critical next step in understanding the link between oxidative responses early in life and metabolic disease development.

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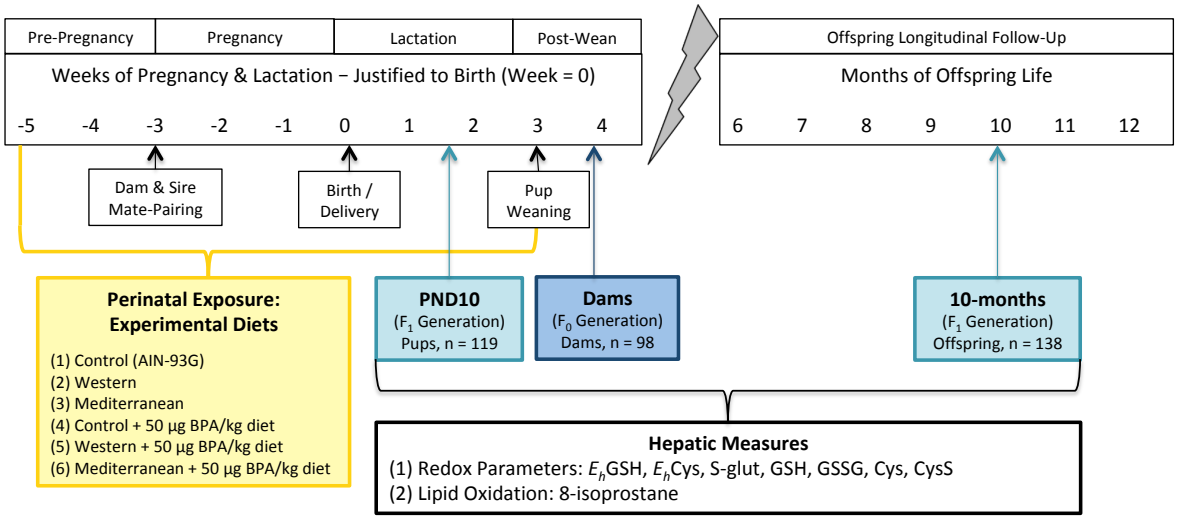
Figure 3.1 Experimental Design of Mouse Perinatal Exposure Study



Primiparous, wild type (*a/a*) dams were randomly assigned to one of six experimental diets at 8-10 weeks of age. The antioxidant preservative, TBHQ, was removed from all six experimental diets. Two weeks later, these dams were mate-paired with virile, young (7.5 week old), A^{vy}/a males. Dams had *ad libitum* access to their assigned experimental diet from pre-gestation through lactation. At postnatal day 21 (PND21), all offspring were weaned onto the Control diet with TBHQ. Mice were sacrificed for examination of hepatic oxidation measures at three time points: (1) dams at PND25: 4 days after weaning, (2) offspring at PND10: still exposed to mother's milk, and (3) offspring at 10-months: not exposed to the experimental diets for nine months.

Table 3.1 Metabolic Characteristics of the Mouse Population

Mouse Exposure Group	*N*	Body Weight			% Liver Weight			% mWAT Weight			Serum Leptin			Serum Insulin		
		Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*
Dams																
ALL GROUPS				**0.096**[d]			**0.145**[e]			**0.007**[b]			0.960			**0.191**[e]
Control	15	25.99	2.54	--	5.43	0.70	--	2.47	0.53	--	5.16	4.20	--	2.64	2.60	--
Con+BPA	19	25.45	2.13	0.572	5.43	0.69	0.994	2.59	0.68	0.628	5.93	4.28	0.599	2.41	1.46	0.697
Mediterranean	18	27.17	1.91	0.219	4.98	0.01	**0.084**[d]	2.84	0.75	**0.148**[e]	5.63	4.34	0.746	1.69	1.28	**0.111**[e]
Med+BPA	16	25.86	2.46	0.899	5.52	0.90	0.715	2.53	0.64	0.806	5.59	3.92	0.771	1.59	1.03	**0.078**[d]
Western	14	27.84	4.10	**0.073**[d]	5.49	0.72	0.812	3.29	0.81	**0.004**[b]	4.54	2.46	0.699	1.33	0.54	**0.043**[c]
West+BPA	15	27.25	3.16	0.212	5.06	0.83	**0.177**[e]	3.20	0.98	**0.008**[b]	5.76	3.99	0.684	1.59	1.17	**0.073**[d]
Female PND10 Offspring																
ALL GROUPS				0.537			0.436			--			**0.067**[d]			--
Control	13	5.97	0.54	--	2.99	0.34	--	--	--	--	3.40	2.13	--	--	--	--
Con+BPA	6	5.43	1.14	0.231	2.92	0.53	0.798	--	--	--	5.47	4.38	0.492	--	--	--
Mediterranean	11	5.34	0.95	**0.096**[d]	3.00	0.53	0.963	--	--	--	8.84	6.77	**0.028**[c]	--	--	--
Med+BPA	11	5.89	1.17	0.831	2.68	0.44	**0.155**[e]	--	--	--	7.48	6.42	**0.104**[e]	--	--	--
Western	10	5.71	0.75	0.499	2.92	0.51	0.747	--	--	--	2.15	1.60	0.622	--	--	--
West+BPA	9	5.55	0.90	0.293	3.18	0.75	0.398	--	--	--	7.21	6.93	**0.138**[e]	--	--	--
Male PND10 Offspring																
ALL GROUPS				0.208			0.319			--			**0.006**[b]			--
Control	9	5.78	0.67	--	2.59	0.44	--	--	--	--	2.40	3.23	.	--	--	--
Con+BPA	10	4.74	0.74	**0.043**[c]	2.63	0.52	0.873	--	--	--	1.66	1.80	0.754	--	--	--
Mediterranean	6	5.59	0.89	0.744	3.13	0.45	**0.069**[d]	--	--	--	11.55	7.82	**0.001**[a]	--	--	--
Med+BPA	11	5.44	1.59	0.495	2.56	0.71	0.915	--	--	--	5.38	5.23	0.209	--	--	--
Western	14	5.71	0.99	0.874	2.73	0.56	0.543	--	--	--	4.62	5.65	0.319	--	--	--
West+BPA	8	5.98	1.33	0.713	2.92	0.45	0.220	--	--	--	3.91	2.71	0.541	--	--	--

Table 3.1 Metabolic Characteristics of the Mouse Population, Continued

Mouse Exposure Group	*N*	Body Weight			% Liver Weight			% mWAT Weight			Serum Leptin			Serum Insulin		
		Mean	SD	p-value	Mean	SD	p-value	Mean	SD	p-value	Mean	SD	p-value	Mean	SD	p-value
Female 10-month Offspring																
ALL GROUPS				0.777			**0.036**[c]			0.801			0.358			0.685
Control	10	32.92	8.02	--	3.07	0.71	--	4.03	1.36	--	8.47	4.33	--	1.59	1.58	--
Con+BPA	9	34.25	6.94	0.679	3.25	0.47	0.534	3.83	0.83	0.717	8.62	3.21	0.947	1.95	3.16	0.767
Mediterranean	11	36.50	7.48	0.243	3.80	0.57	**0.009**[b]	4.01	1.26	0.970	12.54	6.26	**0.071**[d]	3.22	2.35	**0.176**[e]
Med+BPA	12	35.46	4.48	0.395	3.39	0.66	0.237	4.25	1.54	0.671	11.16	4.31	0.220	1.86	0.88	0.816
Western	12	35.44	8.23	0.399	3.76	0.57	**0.012**[c]	3.72	1.09	0.538	8.90	5.55	0.842	2.58	3.95	0.388
West+BPA	11	32.85	5.97	0.983	3.74	0.69	**0.017**[c]	3.59	0.80	0.397	10.60	5.20	0.340	2.98	2.67	0.247
Male 10-month Offspring																
ALL GROUPS				0.570			0.617			**0.158**[e]			**0.045**[c]			0.955
Control	12	43.41	5.49	--	3.60	0.95	--	3.37	0.58	--	10.02	3.09	--	5.02	3.77	--
Con+BPA	10	44.30	2.40	0.659	3.90	1.05	0.489	3.56	1.00	0.570	11.93	6.35	0.344	5.20	2.62	0.906
Mediterranean	12	45.69	5.53	0.239	4.32	1.62	**0.087**[d]	3.52	0.77	0.647	14.63	4.51	**0.021**[c]	6.11	3.44	0.475
Med+BPA	12	44.70	4.77	0.503	3.79	0.90	0.650	3.32	0.72	0.882	13.24	5.11	**0.097**[d]	5.91	5.26	0.553
Western	10	44.15	4.89	0.712	4.08	0.68	0.267	3.39	0.97	0.964	12.74	4.51	**0.179**[e]	5.48	3.04	0.768
West+BPA	12	46.78	4.16	**0.085**[d]	3.88	0.37	0.495	4.12	0.73	**0.024**[c]	16.13	4.12	**0.002**[b]	6.23	2.84	0.417

One-way ANOVAs compared group means of metabolic characteristics by perinatal exposure group. The p-value in the 'All Groups' rows represents the significance of the ANOVA comparing all six exposures. Subsequent p-values represent the significance of each exposure group mean compared to the Control group mean, assessed via Tukey's post-hoc analysis.

To highlight trends of potential biological importance, a wide range of p-values were annotated in this Table: Significance was defined as: [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$. Borderline significant p-values were defined as: [d] $p< 0.10$. Suggestive p-values were defined as: [e] $p<0.20$.

Table 3.2 Comparison of Hepatic Lipid Peroxidation and Redox Potentials by Perinatal Exposure Group

Perinatal Exposure	*N*	8-iso-$PGF_2\alpha$ (ρg/mg)			E_hGSH (mV)			E_hCys (mV)			S-glut (nmol/mg)		
		Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*
Dams													
All Groups				0.2582			0.9835			0.6218			0.4142
Control	15	156.81	88.61	--	-223.56	10.57	--	-151.16	32.29	--	38.95	43.00	--
Con+BPA	19	170.51	70.62	0.6734	-221.37	11.62	0.6204	-144.08	18.04	0.4640	27.62	26.45	0.2359
Mediterranean	18	171.06	54.38	0.6611	-222.68	14.56	0.8458	-157.85	36.66	0.5063	22.03	18.96	**0.0815**[d]
Med+BPA	16	170.66	87.38	0.6783	-222.85	10.12	0.8776	-148.00	18.09	0.7503	32.14	28.85	0.4922
Western	14	158.43	74.43	0.9633	-223.52	12.48	0.9942	-159.53	24.44	0.4331	22.12	18.89	**0.1027**[e]
West+BPA	16	228.74	145.54	**0.0333**[a]	-220.61	16.07	0.5275	-151.63	29.08	0.9623	21.31	22.38	**0.0820**[d]
PND10 Females													
All Groups				**0.0049**[b]			**0.1315**[e]			0.7337			0.6756
Control	13	193.78	96.64	--	-233.07	7.99	--	-209.68	34.55	--	118.84	238.13	--
Con+BPA	6	196.35	102.69	0.9554	-216.67	23.24	**0.1200**[e]	-184.52	46.22	**0.1998**[e]	447.61	532.13	**0.1112**[e]
Mediterranean	11	261.82	100.61	**0.0782**[d]	-225.54	10.15	0.3859	-203.55	44.88	0.7046	279.54	350.47	0.3750
Med+BPA	11	181.76	85.89	0.7522	-208.68	36.72	**0.0065**[b]	-215.18	38.43	0.7341	194.00	262.84	0.6609
Western	10	307.67	88.29	**0.0050**[b]	-225.03	14.85	0.3674	-212.35	36.13	0.8724	256.57	380.35	0.4465
West+BPA	9	303.27	80.76	**0.0085**[b]	-220.82	22.13	**0.1848**[e]	-206.28	38.14	0.8423	315.26	612.36	0.2791
PND10 Males													
All Groups				**0.1270**[e]			0.2901			0.9552			0.8044
Control	9	287.53	75.17	--	-229.60	9.45	--	-202.96	45.16	--	110.44	297.73	--
Con+BPA	10	252.24	113.59	0.4712	-223.68	20.99	0.3388	-213.49	34.86	0.5900	16.85	23.49	0.4367
Mediterranean	6	190.67	78.73	**0.0883**[d]	-216.78	8.78	**0.0722**[d]	-195.64	43.11	0.7422	3.69	2.17	0.4359
Med+BPA	11	202.39	56.16	**0.0792**[d]	-230.01	10.80	0.9465	-207.77	43.92	0.8014	22.08	45.27	0.4627
Western	14	258.28	150.04	0.5204	-226.59	10.56	0.6009	-205.63	37.16	0.8836	132.92	432.95	0.8412
West+BPA	8	322.59	96.84	0.4983	-231.65	11.57	0.7535	-197.73	44.46	0.7998	72.61	111.15	0.7649

Table 3.2 Comparison of Hepatic Lipid Peroxidation and Redox Potentials by Perinatal Exposure Group, Continued

10-month Females												
All Groups				0.3584			0.9048			0.5521			0.3141
Control	10	147.86	48.26	--	-229.38	7.40	--	-141.30	22.53	--	1.14	0.72	--
Con+BPA	9	159.22	34.08	0.7488	-227.34	8.05	0.4769	-147.94	18.67	0.4951	0.95	0.41	0.2714
Mediterranean	11	206.86	113.39	**0.0842**[d]	-229.52	4.13	0.9609	-145.32	17.45	0.6585	0.82	0.13	**0.0739**[d]
Med+BPA	12	210.07	88.87	**0.0637**[d]	-229.64	6.77	0.9216	-135.47	10.98	0.5110	0.98	0.23	0.3450
Western	12	186.97	80.96	0.2395	-230.44	3.73	0.7195	-145.10	22.87	0.7084	0.86	0.22	**0.1208**[e]
West+BPA	11	192.32	55.65	**0.1908**[e]	-230.45	3.68	0.7169	-152.88	22.74	0.2369	0.75	0.13	**0.0368**[c]
10-month Males												
All Groups				**0.0495**[c]			0.9375			0.9079			0.7477
Control	10	161.48	41.91	--	-229.25	7.77	--	-152.56	27.09	--	1.22	0.52	--
Con+BPA	8	226.99	104.77	**0.0349**[c]	-229.62	8.48	0.9365	-148.29	33.21	0.7536	0.98	0.31	0.2057
Mediterranean	10	173.03	57.61	0.6913	-228.33	9.22	0.8373	-152.60	29.00	0.9975	1.07	0.34	0.4008
Med+BPA	10	233.90	99.21	**0.0151**[c]	-230.37	5.59	0.8066	-139.99	21.09	0.3573	1.21	0.16	0.9774
Western	8	218.20	54.13	**0.0666**[d]	-225.60	16.81	0.4412	-143.53	26.46	0.4910	1.15	0.23	0.7110
West+BPA	7	171.46	45.57	0.7314	-229.95	8.90	0.8866	-143.43	25.09	0.5223	1.21	0.16	0.9953

The effect of perinatal HFD and BPA on lipid oxidation or redox parameters was analyzed via one-way ANOVA, with Tukey's post-hoc analysis. Means and standard deviations (SDs) presented in this table represent average and distribution by perinatal exposure group. The 'All Groups' p-values represent the values for the overall ANOVA for each mouse age and sex group. Additional p-values represent the comparison between the mean of each perinatal exposure group and the Control group mean, as analyzed by Tukey's post hoc analyses. To highlight trends of potential biological importance, a wide range of p-values were annotated in this Table: Significance was defined as: [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$. Borderline significant p-values were defined as: [d] $p< 0.10$. Suggestive p-values were defined as: [e] $p<0.20$.

Figure 3.2 Hepatic Intracellular Thiol Concentrations in Dams and Offspring

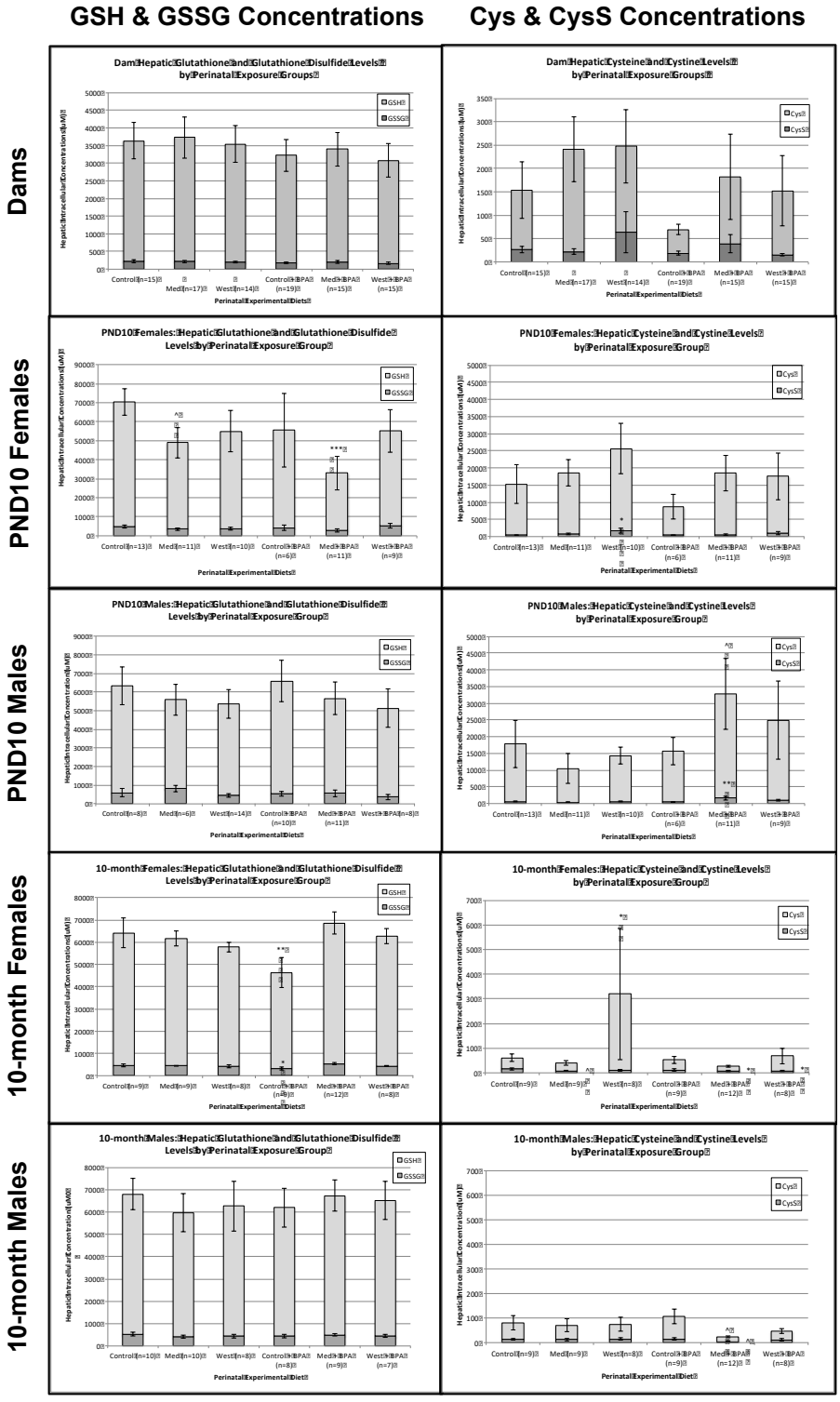


Figure 3.3 Hepatic Glutathione and Cysteine Redox Potentials in Dams and Offspring

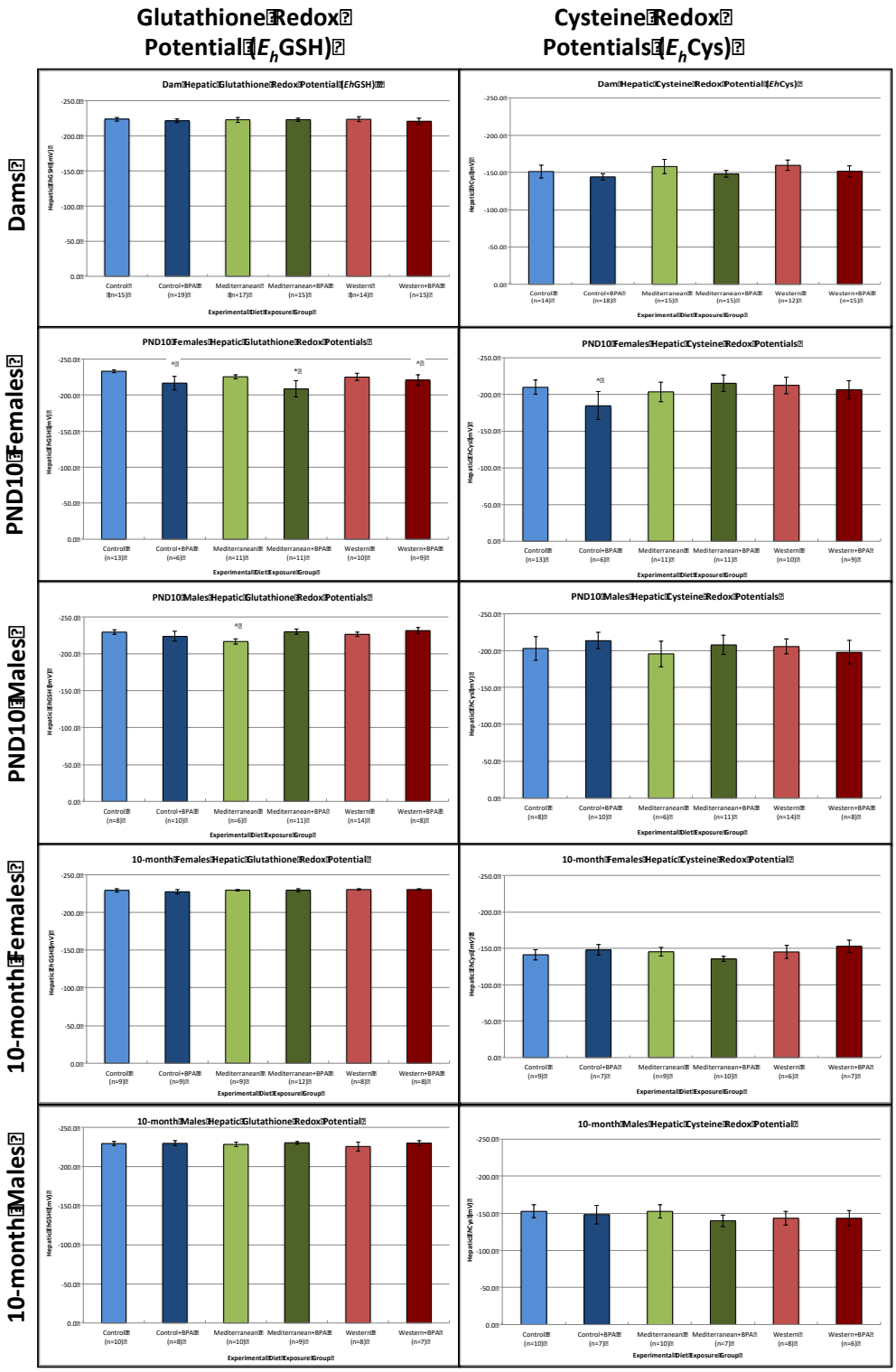
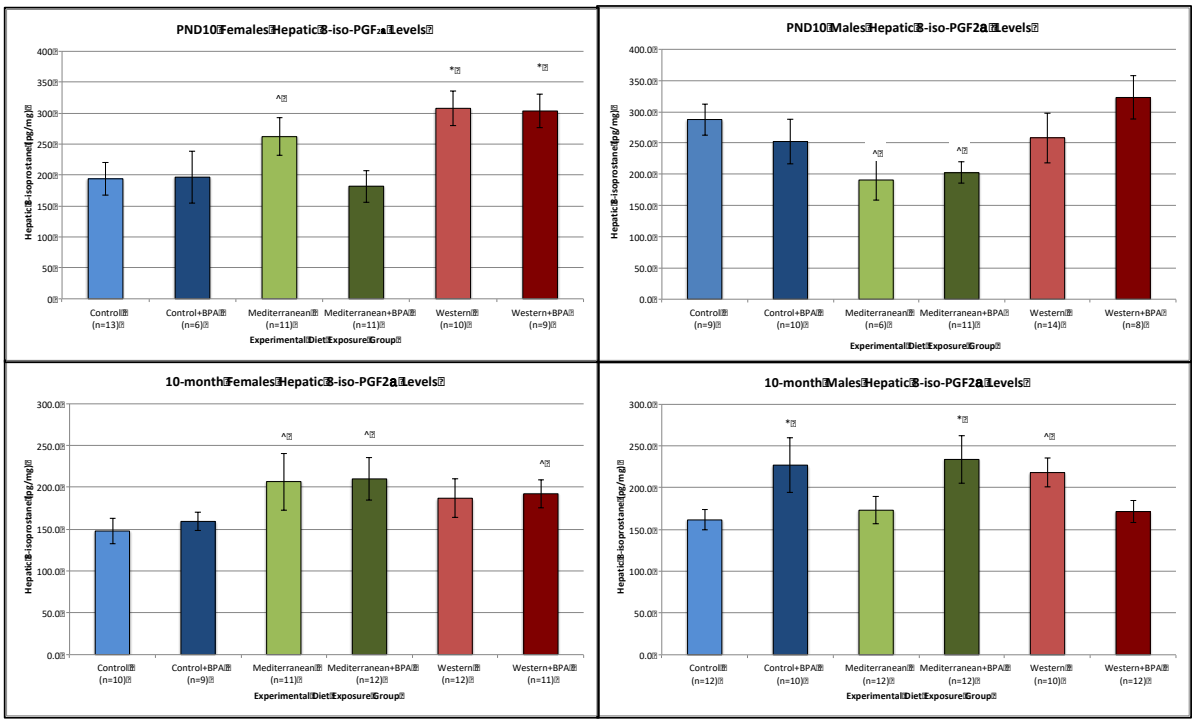


Figure 3.4 Hepatic 8-iso-PGF$_{2\alpha}$ Levels in Offspring by Perinatal Exposure Group



Perinatal exposure to HFDs impacted offspring hepatic 8-iso-PGF$_{2\alpha}$ levels at both PND10 and 10-months. This bar chart illustrates differences between group means by perinatal exposure group; error bars represent the standard error of the mean (SEM) for each group. Average hepatic 8-iso-PGF$_{2\alpha}$ values that differ from the Control mean are marked: * $p < 0.05$, ^ $p < 0.10$.

Table 3.3 Summary of Findings: Impact of Perinatal Exposures on Hepatic 8-iso-$PGF_{2\alpha}$ and Redox Parameters in Offspring at PND10 and 10-months

Hepatic Oxidation Measures	Perinatal Exposure Groups compared to Sex-Specific Controls									
	CBPA	Med	MBPA	West	WBPA	CBPA	Med	MBPA	West	WBPA
	PND10 Females					PND10 Males				
8-iso	--	↑	--	↑↑↑	↑↑↑	--	↓↓	↓↓	--	--
Eh GSH	↑	--	↑↑↑	--	↑	--	↑↑	--	--	--
GSH	--	↓	↓↓↓	--	--	--	--	--	--	--
GSSG	--	--	↓↓	--	--	--	--	--	--	--
Eh Cys	↓	--	--	--	--	--	--	--	--	--
Cys	--	--	--	--	--	--	--	↑	--	--
CysS	--	--	--	↑↑↑	--	--	--	↑↑↑	--	--
S-glut	--	--	--	--	--	--	--	↑	--	--
	10-month Females					10-month Males				
8-iso	--	↑↑	↑↑	--	↑	↑↑↑	--	↑↑↑	↑↑	--
Eh GSH	--	--	--	--	--	--	--	--	--	--
GSH	↓↓↓	--	--	--	--	--	--	--	--	--
GSSG	↓↓	--	--	--	--	--	--	--	--	--
Eh Cys	--	--	--	--	--	--	--	--	--	--
Cys	--	--	--	↑↑	--	--	--	↓	--	--
CysS	--	↓	↓↓	--	↓↓	--	--	↓	--	--
S-glut	--	↓↓	--	↓	↓↓↓	--	--	--	--	--

Data from one-way ANOVAs and Tukey's post-hoc analyses are summarized here.

- Group mean was higher among the designated exposure group, compared to the Control group: ↑↑↑ = $p < 0.05$, ↑↑ = $p < 0.10$, ↑ = $p < 0.20$.
- Group mean was lower in the designated exposure group, than in the Control group: ↓↓↓ = $p < 0.05$, ↓↓ = $p < 0.10$, ↓ = $p < 0.20$.

Table 3.4 Differences in Hepatic Lipid Peroxidation and Redox Potentials by Offspring Sex

PND10 Offspring							
Variable		Female (n=60)		Male (n=58)		T-test p-value*	F-test p-value**
Category	Name	Mean	SD	Mean	SD		
Lipid Oxidation	Liver 8-iso-$PGF_2\alpha$	239.70	103.00	253.10	109.50	0.4966	0.6404
Redox Parameters	Liver E_hGSH	-222.40	21.72	-226.80	13.09	**0.1815**[e]	**0.0002**[a]
	Liver E_hCys	-207.00	38.53	-204.90	39.50	0.7719	0.8492
	Liver S-glut	250.70	392.50	66.72	245.40	**0.0040**[b]	**0.0007**[a]
Metabolic Parameters	Body Weight	5.68	0.90	5.53	1.12	0.4151	**0.0943**[d]
	Percent Liver Weight	2.95	0.52	2.73	0.55	**0.0284**[c]	0.6109
	Serum Leptin	5.93	5.66	4.59	5.32	0.2072	0.6560
10-month Offspring							
Variable		Female (n=65)		Male (n=68)		T-test p-value*	F-test p-value**
Category	Name	Mean	SD	Mean	SD		
Lipid Oxidation	Liver 8-iso-$PGF_2\alpha$	185.60	77.25	196.00	74.49	0.4312	0.7673
Redox Parameters	Liver E_hGSH	-229.40	5.85	-228.90	9.54	0.7073	**0.0005**[a]
	Liver E_hCys	-144.00	18.94	-147.50	26.36	0.4581	**0.0256**[c]
	Liver S-glut	0.93	0.38	1.14	0.38	**0.0043**[b]	0.9236
Metabolic Parameters	Body Weight	34.63	6.81	44.87	4.67	**0.0001**[a]	**0.0026**[b]
	Percent Liver Weight	3.52	0.66	3.92	1.00	**0.0066**[b]	**0.0010**[b]
	Percent mWAT Weight	3.91	1.17	3.55	0.82	**0.0439**[c]	**0.0042**[b]
	Serum Leptin	10.05	4.98	13.14	4.91	**0.0006**[a]	0.9195
	Serum Insulin	2.37	2.62	5.67	3.54	**0.0001**[a]	**0.0180**[c]

*Independent t-tests were used to compare variable means between offspring sexes.
** Equality of Variance was assessed via F-test, where the null hypothesis is no difference in variance between sexes.
To highlight trends of potential biological importance, a wide range of p-values were annotated in this Table: Significance was defined as: [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$. Borderline significant p-values were defined as: [d] $p< 0.10$. Suggestive p-values were defined as: [e] $p<0.20$.

Table 3.5 Non-parametric Correlations between Hepatic Lipid Peroxidation and Redox Potentials in Dams and Offspring

Measure	Kendall's Tau	8-iso-$PGF_{2\alpha}$	E_h GSH	E_h Cys	S-glut	8-iso-$PGF_{2\alpha}$	E_h GSH	E_h Cys	S-glut
		Dams (n=97)							
8-iso-$PGF_{2\alpha}$	Coefficient	1.0000	0.0313	0.0885	0.1425				
	p-value	n/a	0.6565	0.2250	0.0419				
E_h GSH	Coefficient	0.0313	1.0000	0.4712	-0.2939				
	p-value	0.6565	n/a	**<0.0001[a]**	**<0.0001[a]**				
E_h Cys	Coefficient	0.0885	0.4712	1.0000	-0.0167				
	p-value	0.2250	**<0.0001[a]**	n/a	0.8154				
S-glut	Coefficient	0.1425	-0.2939	-0.0167	1.0000				
	p-value	0.0419	**<0.0001[a]**	0.8154	n/a				
		PND10 Females (n=60)				**PND10 Males (n=58)**			
8-iso-$PGF_{2\alpha}$	Coefficient	1.0000	0.0961	-0.0418	0.1300	1.0000	0.0790	0.1378	0.0013
	p-value	n/a	0.2783	0.6370	0.1612	n/a	0.3857	**0.1299[e]**	0.9887
E_h GSH	Coefficient	0.0961	1.0000	0.0147	0.1286	0.0790	1.0000	0.2594	-0.3338
	p-value	0.2783	n/a	0.8683	0.1656	0.3857	n/a	**0.0044[b]**	**0.0003[a]**
E_h Cys	Coefficient	-0.0418	0.0147	1.0000	0.4168	0.1378	0.2594	1.0000	-0.3701
	p-value	0.6370	0.8683	n/a	**<0.0001[a]**	**0.1299[e]**	**0.0044[b]**	n/a	**<0.0001[a]**
S-glut	Coefficient	0.1300	0.1286	0.4168	1.0000	0.0013	-0.3338	-0.3701	1.0000
	p-value	0.1612	0.1656	**<0.0001[a]**	n/a	0.9887	**0.0003[a]**	**<0.0001[a]**	n/a
		10-month Females (n=65)				**10-month Males (n=68)**			
8-iso-$PGF_{2\alpha}$	Coefficient	1.0000	-0.0370	0.1631	0.0093	1.0000	0.0564	0.2134	0.0472
	p-value	n/a	0.6897	**0.1020[e]**	0.9662	n/a	0.6911	**0.1453[e]**	0.6700
E_h GSH	Coefficient	-0.0370	1.0000	-0.0904	-0.1327	0.0564	1.0000	-0.0109	0.1614
	p-value	0.6897	n/a	0.3646	0.1527	0.6911	n/a	0.9416	**0.1057[e]**
E_h Cys	Coefficient	0.1631	-0.0904	1.0000	0.1791	0.2134	-0.0109	1.0000	-0.1436
	p-value	**0.1020[e]**	0.3646	n/a	**0.0726[d]**	**0.1453[e]**	0.9416	n/a	**0.1499[e]**
S-glut	Coefficient	0.0093	-0.1327	0.1791	1.0000	0.0472	0.1614	-0.1436	1.0000
	p-value	0.9662	0.1527	**0.0726[d]**	n/a	0.6700	**0.1057[e]**	**0.1499[e]**	n/a

Kendall's Tau, a non-parametric, rank-based correlation method was used due to the non-normal distributions of hepatic 8-iso and S-glut dams and offspring. To highlight trends of potential biological importance, a wide range of p-values were annotated in this Table: Significance was defined as: [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$. Borderline significant p-values were defined as: [d] $p< 0.10$. Suggestive p-values were defined as: [e] $p<0.20$.

Table 3.6 Dam Univariate Analysis of Variables Impacting Hepatic Lipid Peroxidation and Redox Potentials

Variable	Ln-(8-iso-PGF$_2\alpha$)		E_hGSH		E_hCys		Ln-(S-glut)	
	Effect Estimate	p-value	Effect Estimate	p-value	Effect Estimate	p-value	Effect Estimate	p-value
Mediterranean Diet	0.108	0.3934	-0.425	0.8902	-5.592	0.4110	-0.144	0.7198
Western Diet	0.181	**0.1723**[e]	0.320	0.9198	-7.963	0.2598	-0.370	0.3778
BPA	0.151	**0.1538**[e]	1.604	0.5303	8.382	**0.1399**[e]	-0.086	0.7980
Litter Size (# of pups)	-0.111	0.2888	-0.104	0.3118	-0.102	0.3409	0.152	**0.0989**[d]
Time to Delivery (days)	-0.151	**0.1464**[e]	-0.068	0.5132	-0.130	0.2235	0.014	0.4402
Percent Male Pups	0.003	0.9790	0.072	0.4840	0.037	0.7268	0.007	0.4518
Percent *Avy/a* Pups	-0.054	0.6074	-0.154	**0.1341**[e]	-0.080	0.4537	0.000	0.9666
Dam Body Weight @ Exposure	0.071	0.4915	0.191	**0.0622**[d]	0.175	**0.0999**[d]	0.091	0.3697
Dam Pre-Gestational Weight Change	0.161	**0.1183**[e]	-0.064	0.5377	-0.004	0.9718	0.153	0.3121
Dam Body Weight @ Mate-Pairing	0.177	**0.0869**[d]	0.144	**0.1609**[e]	0.169	**0.1122**[e]	0.151	0.1238
Dam Pregnancy Weight Gain	-0.215	**0.0378**[c]	-0.053	0.6070	-0.032	0.7659	0.013	0.8373
Dam Body Weight @ Sacrifice	-0.084	0.4211	0.040	0.7010	0.071	0.5050	0.067	0.2647
Dam Percent Liver Weight	-0.268	**0.0090**[b]	-0.242	**0.0173**[c]	-0.091	0.3960	52.91	**0.0171**[c]
Dam Percent mWAT Weight	0.061	0.5600	0.073	0.4768	0.129	0.2266	9.35	0.6330
Dam Serum Insulin	-0.295	**0.0093**[b]	0.100	0.3869	0.015	0.9005	-0.133	0.3108
Dam Serum Leptin	-0.089	0.4114	-0.040	0.7104	0.044	0.6934	0.081	**0.0754**[d]

Single linear regression models were performed to assess the univariate association between potential predictive variables and outcomes measures of lipid oxidation or redox parameters. To highlight trends of potential biological importance, a wide range of p-values were annotated in this Table: Significance was defined as: [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$. Borderline significant p-values were defined as: [d] $p< 0.10$. Suggestive p-values were defined as: [e] $p<0.20$.

Table 3.7 PND10 Offspring Sex-Stratified Univariate Analysis of Variables Impacting Hepatic Lipid Peroxidation and Redox Potentials

Variable	Ln-(8-iso-$PGF_{2}\alpha$)		E_hGSH		E_hCys		Ln-(S-glut)	
	Effect Estimate	p-value	Effect Estimate	p-value	Effect Estimate	p-value	Effect Estimate	p-value
FEMALE PND10 OFFSPRING								
Mediterranean Diet	0.106	0.5334	10.784	**0.1168**[e]	-7.626	0.5352	0.573	0.4584
Western Diet	0.549	**0.0027**[b]	4.858	0.4914	-7.736	0.5437	0.185	0.8148
BPA	-0.156	0.3073	13.542	**0.0154**[c]	3.461	0.7335	0.193	0.7592
Litter Size (# of pups)	-0.021	0.8753	-0.265	**0.0411**[c]	0.018	0.8891	0.020	0.9305
Time to Delivery (days)	0.069	0.6027	0.055	0.6752	-0.232	**0.0739**[d]	-0.070	**0.0414**[c]
PND10 Body Weight @ Sacrifice	-0.041	0.7584	-0.381	**0.0027**[b]	-0.081	0.5400	-0.660	**0.0695**[d]
PND10 Percent Liver Weight	0.072	0.5851	-0.051	0.7000	0.183	**0.1628**[e]	70.43	0.2467
PND10 Serum Leptin	0.083	0.5551	-0.208	**0.1344**[e]	-0.171	0.2217	-0.093	**0.0987**[d]
Dam Body Weight @ Exposure	0.278	**0.0318**[c]	-0.027	0.8376	-0.210	**0.1079**[e]	-0.203	0.3553
Dam Pre-Gestational Weight Change	-0.122	0.3536	-0.146	0.2646	0.016	0.9056	-0.410	**0.1145**[e]
Dam Body Weight @ Mate-Pairing	0.067	0.6134	-0.028	0.8334	-0.365	**0.0041**[b]	-0.331	**0.0049**[b]
Dam Pregnancy Weight Gain	-0.018	0.8895	-0.024	0.8549	-0.286	**0.0267**[c]	-0.440	**0.0021**[b]
Dam Body Weight @ Sacrifice	0.075	0.5706	-0.086	0.5126	-0.318	**0.0132**[c]	-0.388	**0.0003**[a]
Dam Percent Liver Weight	-0.220	**0.0916**[d]	0.014	0.9160	-0.054	0.6824	-49.97	0.2761
Dam Percent mWAT Weight	0.177	**0.1767**[e]	0.060	0.6506	-0.184	**0.1586**[e]	-90.48	**0.0552**[d]
Dam Serum Insulin	-0.263	**0.0929**[d]	-0.041	0.7954	0.089	0.5762	0.171	0.6563
Dam Serum Leptin	-0.115	0.4080	-0.039	0.7821	-0.237	**0.0845**[d]	-0.208	**0.0157**[c]
Dam Liver 8-iso-$PGF_{2}\alpha$	0.344	**0.0072**[b]	0.156	0.2346	0.113	0.3881	0.006	**0.0254**[c]
Dam Liver E_hGSH (mV)	0.132	0.3131	-0.069	0.6013	-0.177	**0.1764**[e]	-0.003	0.9181
Dam Liver E_hCys (mV)	0.054	0.6911	-0.073	0.5900	-0.248	**0.0632**[d]	-0.026	**0.0415**[c]

Table 3.7 PND10 Offspring Sex-Stratified Univariate Analysis of Variables Impacting Hepatic Lipid Oxidation and Redox Parameters, Continued

MALE PND10 OFFSPRING								
Mediterranean Diet	-0.264	**0.0880**[d]	0.973	0.829	5.321	0.697	-0.234	0.7013
Western Diet	0.004	0.978	-2.113	0.618	6.052	0.637	0.289	0.6085
BPA	0.032	0.793	-2.931	0.403	-4.251	0.688	0.300	0.5242
Litter Size (# of pups)	-0.154	0.247	-0.283	**0.0330**[c]	-0.108	0.425	-0.151	0.3068
Time to Delivery (days)	-0.225	**0.0890**[d]	-0.112	0.409	-0.172	0.201	0.019	0.4291
PND10 Body Weight @ Sacrifice	-0.105	0.434	-0.031	0.820	-0.011	0.934	0.215	0.3098
PND10 Percent Liver Weight	0.007	0.957	0.257	**0.0535**[d]	0.233	**0.0808**[d]	-52.44	0.2129
PND10 Serum Leptin	-0.201	**0.1412**[e]	0.049	0.721	0.052	0.708	-0.023	0.6233
Dam Body Weight @ Exposure	0.226	**0.0879**[d]	0.164	0.223	-0.055	0.686	-0.002	0.9858
Dam Pre-Gestational Weight Change	-0.403	**0.0017**[b]	0.048	0.723	-0.026	0.847	0.119	0.5991
Dam Body Weight @ Mate-Pairing	-0.045	0.736	0.283	**0.0328**[c]	-0.006	0.965	0.017	0.8804
Dam Pregnancy Weight Gain	-0.314	**0.0162**[c]	-0.472	**0.0002**[a]	-0.149	0.269	0.002	0.9732
Dam Body Weight @ Sacrifice	-0.291	**0.0268**[c]	-0.339	**0.0100**[c]	-0.173	**0.1969**[e]	0.012	0.8603
Dam Percent Liver Weight	-0.178	**0.1810**[e]	-0.110	0.416	-0.137	0.308	-8.674	0.7350
Dam Percent mWAT Weight	-0.105	0.431	-0.155	0.251	0.027	0.841	12.858	0.6541
Dam Serum Insulin	0.169	0.290	-0.048	0.768	-0.157	0.335	0.167	0.5128
Dam Serum Leptin	0.042	0.766	-0.046	0.750	-0.072	0.617	0.012	0.8536
Dam Liver 8-iso-$PGF_2\alpha$	0.371	**0.0041**[b]	0.080	0.553	0.170	0.206	-0.004	0.1364
Dam Liver E_hGSH (mV)	0.108	0.419	0.149	0.268	0.194	**0.1487**[e]	0.008	0.7628
Dam Liver E_hCys (mV)	0.068	0.630	-0.104	0.464	-0.080	0.574	0.004	0.6899

Single linear regression models were performed to assess the univariate association between potential predictive variables and outcomes measures of lipid oxidation or redox parameters. To highlight trends of potential biological importance, a wide range of p-values were annotated in this Table: Significance was defined as: [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$. Borderline significant p-values were defined as: [d] $p< 0.10$. Suggestive p-values were defined as: [e] $p<0.20$.

Table 3.8 10-month Offspring Sex-Stratified Univariate Analysis of Variables Impacting Hepatic Lipid Peroxidation and Redox Potentials

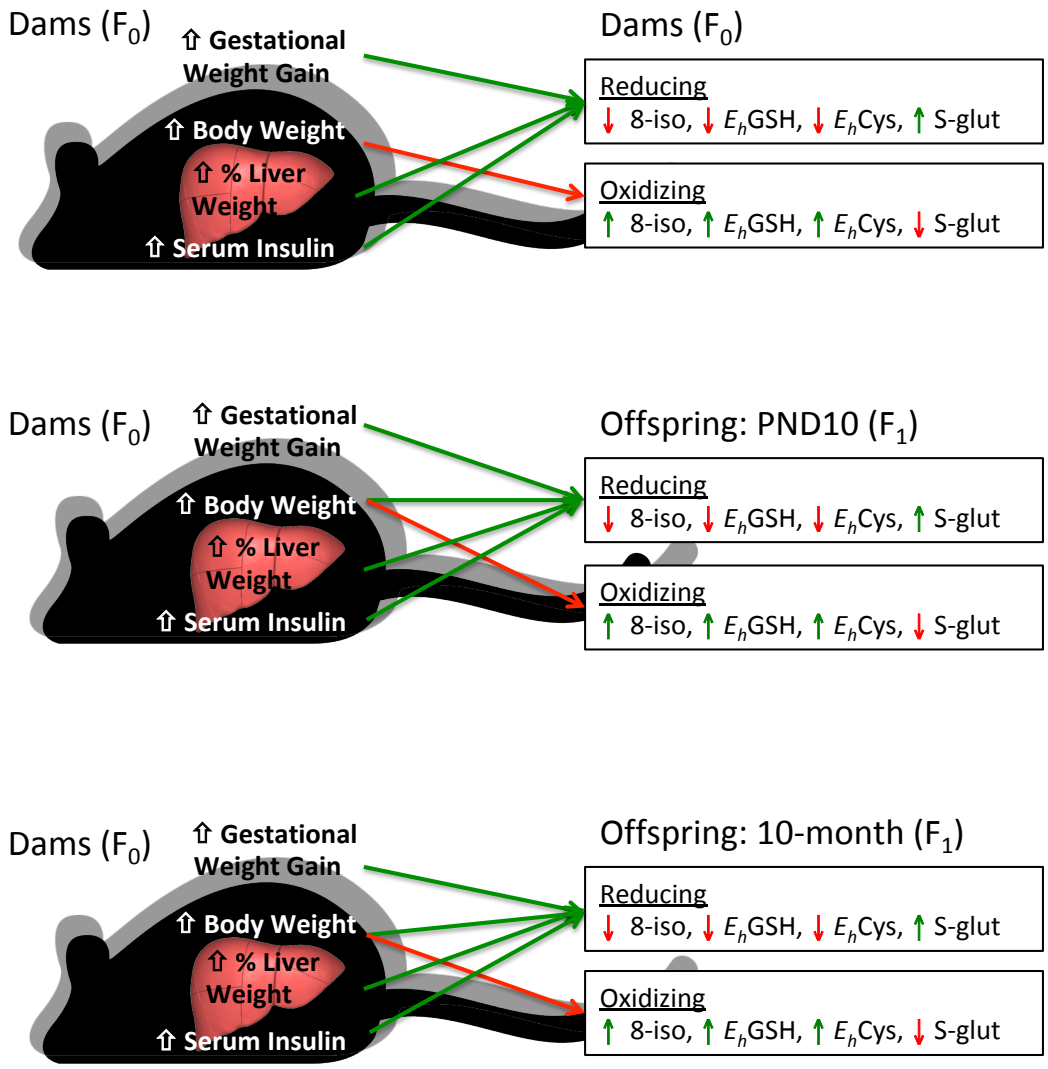
Variable	Ln-(8-iso-$PGF_2\alpha$)		E_hGSH		E_hCys		Ln-(S-glut)	
	Effect Estimate	p-value	Effect Estimate	p-value	Effect Estimate	p-value	Effect Estimate	p-value
FEMALE 10-month OFFSPRING								
Mediterranean Diet	0.250	**0.0246**[c]	-1.232	0.518	4.070	0.531	0.007	0.9565
Western Diet	0.191	**0.0842**[d]	-2.087	0.308	-5.082	0.477	-0.126	0.3933
BPA	0.073	0.419	0.604	0.706	-0.427	0.939	0.026	0.8192
Litter Size (# of pups)	-0.147	0.242	-0.069	0.615	0.179	0.224	0.005	0.8876
Time to Delivery (days)	-0.127	0.315	-0.069	0.616	0.013	0.928	-0.002	0.8107
10M Body Weight @ Sacrifice	0.001	0.995	-0.199	**0.1459**[e]	0.209	**0.1537**[e]	0.014	**0.1087**[e]
10M Percent Liver Weight	0.121	0.338	0.085	0.538	-0.142	0.335	-5.862	0.4982
10M Percent mWAT Weight	-0.059	0.639	-0.274	**0.0427**[c]	0.016	0.912	2.183	0.6511
10M Serum Leptin	0.087	0.501	-0.218	**0.1173**[e]	-0.008	0.956	0.000	0.4549
10M Serum Insulin	-0.031	0.810	-0.198	**0.1546**[e]	-0.149	0.323	0.000	0.9595
10M Serum Resistin	0.106	0.414	-0.042	0.767	-0.180	0.232	0.000	0.3798
Dam Body Weight @ Exposure	0.007	0.955	0.243	**0.0741**[d]	-0.036	0.807	0.062	**0.1575**[e]
Dam Pre-Gestational Weight Change	0.180	**0.1512**[e]	0.006	0.965	-0.007	0.961	-0.019	0.7319
Dam Body Weight @ Mate-Pairing	0.066	0.602	0.212	**0.1199**[e]	-0.072	0.626	0.012	0.6322
Dam Pregnancy Weight Gain	-0.241	**0.0534**[c]	-0.138	0.314	0.017	0.910	0.012	0.7118
Dam Body Weight @ Sacrifice	-0.112	0.375	0.037	0.791	-0.010	0.944	0.024	0.3443
Dam Percent Liver Weight	-0.100	0.429	-0.054	0.697	0.182	0.216	9.055	0.2427
Dam Percent mWAT Weight	0.118	0.350	-0.196	**0.1510**[e]	0.025	0.868	3.195	0.6712
Dam Serum Insulin	0.101	0.455	-0.221	**0.1320**[e]	0.192	0.218	0.068	**0.0640**[d]
Dam Serum Leptin	0.136	0.287	-0.010	0.942	-0.095	0.529	-0.013	0.4195
Dam Liver 8-iso-$PGF_2\alpha$	-0.053	0.679	0.055	0.691	-0.014	0.924	-0.001	0.3367
Dam Liver E_hGSH (mV)	0.223	**0.0768**[d]	0.137	0.323	-0.090	0.548	-0.002	0.6785
Dam Liver E_hCys (mV)	0.179	**0.1646**[e]	0.171	0.226	-0.152	0.320	-0.001	0.6855

Table 3.8 10-month Offspring Sex-Stratified Univariate Analysis of Variables Impacting Hepatic Lipid Peroxidation and Redox Potentials, Continued

MALE 10-month OFFSPRING								
Mediterranean Diet	0.058	0.569	0.118	0.971	3.395	0.713	0.019	0.8648
Western Diet	0.039	0.706	1.788	0.600	7.314	0.453	0.110	0.3546
BPA	0.114	**0.1666**[e]	-2.120	0.430	6.062	0.438	0.005	0.9557
Litter Size (# of pups)	0.143	0.244	-0.207	**0.1403**[e]	0.127	0.391	-0.024	0.3861
Time to Delivery (days)	-0.205	**0.0933**[d]	-0.237	**0.0908**[d]	0.062	0.675	0.008	0.2183
10M Body Weight @ Sacrifice	-0.070	0.573	-0.026	0.855	-0.043	0.771	0.001	0.9124
10M Percent Liver Weight	0.036	0.768	0.030	0.833	0.104	0.482	-1.372	0.7533
10M Percent mWAT Weight	0.122	0.323	-0.142	0.316	-0.182	0.215	1.157	0.8371
10M Serum Leptin	-0.054	0.664	-0.067	0.640	0.030	0.843	-0.000	0.7130
10M Serum Insulin	-0.057	0.645	0.082	0.569	0.000	0.998	-0.000	0.6430
10M Serum Resistin	-0.112	0.366	0.159	0.264	-0.014	0.926	-0.000	0.6040
Dam Body Weight @ Exposure	-0.030	0.810	0.161	0.255	-0.293	**0.0432**[c]	0.030	0.2545
Dam Pre-Gestational Weight Change	-0.075	0.544	0.132	0.353	0.144	0.329	-0.003	0.9531
Dam Body Weight @ Mate-Pairing	-0.084	0.497	0.248	**0.0761**[d]	-0.190	**0.1957**[e]	0.005	0.8020
Dam Pregnancy Weight Gain	0.067	0.587	-0.094	0.509	0.007	0.962	0.048	**0.0280**[c]
Dam Body Weight @ Sacrifice	0.011	0.927	0.091	0.523	-0.135	0.360	0.054	**0.0045**[b]
Dam Percent Liver Weight	-0.016	0.895	-0.175	0.214	-0.009	0.952	12.166	**0.0966**[d]
Dam Percent mWAT Weight	-0.008	0.949	0.203	**0.1495**[e]	-0.157	0.286	7.329	**0.1549**[e]
Dam Serum Insulin	0.058	0.665	-0.283	**0.0571**[d]	0.238	**0.1244**[e]	0.037	0.2003
Dam Serum Leptin	0.067	0.597	0.205	**0.1573**[e]	0.014	0.926	-0.007	0.5937
Dam Liver 8-iso-$PGF_2\alpha$	-0.217	**0.0819**[d]	-0.018	0.901	-0.216	**0.1549**[e]	-0.000	**0.1661**[e]
Dam Liver E_hGSH (mV)	0.172	**0.1640**[e]	0.294	**0.0342**[c]	-0.045	0.760	0.003	0.4654
Dam Liver E_hCys (mV)	0.047	0.710	0.310	**0.0300**[c]	-0.189	0.213	0.000	0.8766

Single linear regression models were performed to assess the univariate association between potential predictive variables and outcomes measures of lipid oxidation or redox parameters. To highlight trends of potential biological importance, a wide range of p-values were annotated in this Table: Significance was defined as: [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$. Borderline significant p-values were defined as: [d] $p< 0.10$. Suggestive p-values were defined as: [e] $p<0.20$.

Figure 3.υ Associations between Dam Metabolic Parameters and Oxidative Outcomes in Dams, PND10, and 10-month Offspring



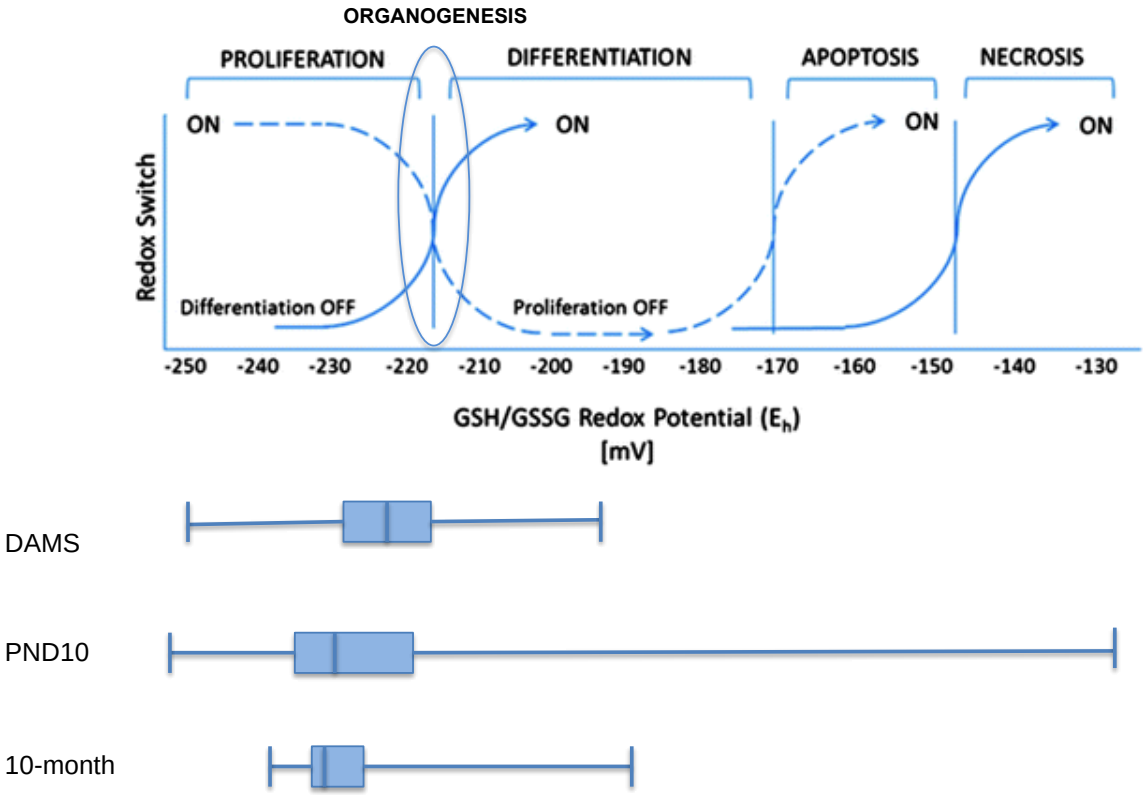
Multiple dam metabolic parameters had consistent effects on oxidative outcomes in mice across generation and offspring age. Oxidative outcomes are grouped by their general impact on the redox environment: reducing or oxidizing. Arrows between the dam diagram (left column) and oxidative outcome boxes represent general trends; it does not imply that every oxidative response in the box is associated with the metabolic parameter, just that the impact of the metabolic parameter corresponds to one or more of the outcomes in the box. For example, greater gestational weight gain (GWG) is associated with decreased 8-iso in dams, PND10 males, and 10-month females; GWG is also associated with decreased E_hGSH in PND10 males, decreased E_hCys in PND10 females, and increased s-glut in 10-month males, all of which result in a reducing redox environment. In PND10 and 10-month offspring, dam body weight is associated with both reducing and oxidizing outcomes.

Supplemental Information

Table S3.1 Distributions of Hepatic Lipid Oxidation and Redox Parameters in Dams and Offspring

Hepatic Measure	*N*	Mean	SD	Min.	25%	50%	75%	Max.
Dam Levels								
8-iso (pg/mg)	95	176.77	91.73	8.71	123.71	155.23	201.15	575.04
E_hGSH (mV)	95	-222.39	12.43	-250.58	-228.49	-223.94	-217.58	-192.51
GSH (uM)	96	3229.60	1987.46	292.88	1793.76	3112.16	4464.68	7742.00
GSSG (uM)	95	197.33	118.98	14.49	96.80	203.88	284.29	562.73
E_hCys (mV)	90	-151.49	26.77	-225.15	-165.37	-144.91	-133.68	-107.16
Cys (uM)	95	140.54	265.78	0.00	18.56	38.56	132.59	1508.23
CysS (uM)	95	29.95	72.78	0.00	6.24	15.04	28.03	639.35
S-glut (nmol/mg)	97	27.31	27.49	0.32	3.11	25.12	43.78	162.17
PND10 Offspring Levels								
8-iso (pg/mg)	118	246.27	105.99	14.06	171.92	239.85	308.09	652.75
E_hGSH (mV)	117	-224.56	18.10	-254.44	-235.68	-229.07	-219.10	-114.13
GSH (uM)	117	5068.36	3051.27	63.31	2126.61	5645.79	7706.09	9911.53
GSSG (uM)	117	468.24	390.03	12.90	104.68	410.62	678.86	2020.42
E_hCys (mV)	117	-205.96	38.85	-269.88	-234.42	-220.36	-171.21	-125.56
Cys (uM)	117	1795.93	2040.61	25.35	141.31	1405.59	2221.24	9741.66
CysS (uM)	117	80.54	112.59	0.46	26.75	41.43	79.42	675.86
S-glut (nmol/mg)	111	157.89	338.05	0.38	3.60	6.25	68.79	1833.28
10-month Offspring Levels								
8-iso (pg/mg)	133	190.96	75.74	84.04	139.36	175.25	214.32	510.79
E_hGSH (mV)	107	-229.15	7.83	-239.62	-233.80	-231.55	-227.36	-188.76
GSH (uM)	107	5780.99	2010.15	512.50	4957.41	6120.13	6976.98	9576.17
GSSG (uM)	107	452.86	188.78	45.63	380.26	465.75	556.42	1109.45
E_hCys (mV)	96	-145.72	22.90	-213.41	-156.04	-140.13	-129.28	-105.45
Cys (uM)	107	66.99	214.78	3.43	14.49	26.05	46.09	2176.87
CysS (uM)	107	10.89	12.11	0.00	3.17	7.23	14.43	67.11
S-glut (nmol/mg)	109	1.03	0.39	0.10	0.80	0.96	1.21	2.89

Figure S3.1 Glutathione Redox Potentials of this Mouse Study Population



This figure is modified from Hansen & Harris, 2012 [94]. The three boxplots at the bottom: dams, PND10, and 10-months, represent the distribution of glutathione redox potentials (E_hGSH). The box represents the interquartile range (IQR) of each age group's E_hGSH, the line in the box represents the median, and the whiskers represent the minimum and maximum. This illustrates the median and IQR are similar across all ages.

REFERENCES

1. Brumbaugh DE, Friedman JE. Developmental Origins of Nonalcoholic Fatty Liver Disease. Pediatr Res. 2014;75: 140–7.

2. McMillen IC, Robinson JS. Developmental Origins of the Metabolic Syndrome: Prediction, Plasticity, and Programming. Physiol Rev. 2005;85: 571–633.

3. Rinaudo P, Wang E. Fetal Programming and Metabolic Syndrome. Annu Rev Physiol. 2012;74: 107–130.

4. Barker DJ. The Fetal and Infant Origins of Disease. Eur J Clin Invest. 1995;25: 457–63.

5. Barker DJ. The Developmental Origins of Chronic Adult Disease. J Am Coll Nutr. 2004;23: S588–S595.

6. Alonso-Magdalena P, Quesada I, Nadal A. Prenatal Exposure to BPA and Offspring Outcomes: The Diabesogenic Behavior of BPA. Dose Response. 2015; 1–8.

7. Valentino R, D'Esposito V, Ariemma F, Cimmino I, Beguinot F, Formisano P. Bisphenol A Environmental Exposure and the Detrimental Effects on Human Metabolic Health: Is it Necessary to Revise the Risk Assessment in Vulnerable Populations? J Endocrinol Invest. Springer International Publishing; 2016;39: 259–263.

8. El Hajj N, Schneider E, Lehnen H, Haaf T. Epigenetics and Life-Long Consequences of an Adverse Nutritional and Diabetic Intrauterine Environment. Reproduction. 2014;148: R111–R120.

9. Sullivan EL, Smith MS, Grove KL. Perinatal Exposure to High-Fat Diet Programs Energy Balance, Metabolism and Behavior in Adulthood. Neuroendocrinology. 2011;93: 1–8.

10. Lee H-S. Impact of Maternal Diet on the Epigenome during In Utero Life and the Developmental Programming of Diseases in Childhood and Adulthood. Nutrients. 2015;7: 9492–9507.

11. Manna P, Jain SK. Obesity, Oxidative Stress, Adipose Tissue Dysfunction, and the Associated Health Risks: Causes and Therapeutic Strategies. Metab Syndr Relat Disord. 2015;13: 423–444.

12. Rani V, Deep G, Singh RK, Palle K, Yadav UC. Oxidative Stress and Metabolic Disorders: Pathogenesis and Therapeutic Strategies. Life Sci. 2016;148: 183–193.

13. Hansen JM, Harris C. Glutathione during Embryonic Development. Biochimica et Biophysica Acta. 2015;1850: 1527–1542.

14. Hansen JM, Harris C. Redox Control of Teratogenesis. Reprod Toxicol. 2013;35: 165–

179.

15. Jones DP. Redox sensing: Orthogonal Control in Cell Cycle and Apoptosis signaling. J Intern Med. 2011;268: 432–448.

16. Jones DP. Redefining Oxidative Stress. Antioxid Redox Signal. 2006;8: 1865–1879.

17. Ying J, Clavreul N, Sethuraman M, Adachi T, Cohen RA. Thiol Oxidation in Signaling and Response to Stress: Detection and Quantification of Physiological and Pathophysiological Thiol Modifications. Free Radic Biol Med. 2007;43: 1099–108.

18. Go Y-M, Jones DP. Thiol/Disulfide Redox States in Signaling and Sensing. Crit Rev Biochem Mol Biol. 2013;48: 173–81.

19. Bae S, Lim YH, Lee YA, Shin CH, Oh SY, Hong YC. Maternal Urinary Bisphenol A Concentration during Midterm Pregnancy and Children's Blood Pressure at Age 4. Hypertension. 2017;69: 367–374.

20. Debenedictis B, Guan H, Yang K. Prenatal Exposure to Bisphenol A Disrupts Mouse Fetal Liver Maturation in a Sex-Specific Manner. J Cell Biochem. 2016;117: 344–350.

21. Ryan KK, Haller AM, Sorrell JE, Woods SC, Jandacek RJ, Seeley RJ. Perinatal Exposure to Bisphenol-A and the Development of Metabolic Syndrome in CD-1 Mice. Endocrinology. 2010;151: 2603–2612.

22. van Esterik J, Dollé M, Lamoree M, van Leeuwen S, Hamers T, Legler J, et al. Programming of Metabolic Effects in C57BL/6JxFVB Mice by Exposure to Bisphenol A during Gestation and Lactation. Toxicology. 2014;321: 40–52.

23. Liu J, Yu P, Qian W, Li Y, Zhao J, Huan F, et al. Perinatal Bisphenol A Exposure and Adult Glucose Homeostasis: Identifying Critical Windows of Exposure. PLoS One. 2013;8: e64143.

24. Gassman NR. Induction of Oxidative Stress by Bisphenol A and Its Pleiotropic Effects. Environ Mol Mutagen. 2017;58: 60–71.

25. Bindhumol V, Chitra KC, Mathur PP. Bisphenol A Induces Reactive Oxygen Species Generation in the Liver of Male Rats. Toxicology. 2003;188: 117–124.

26. Hassan ZK, Elobeid MA, Virk P, Omer SA, ElAmin M, Daghestani MH, et al. Bisphenol A Induces Hepatotoxicity through Oxidative Stress in Rat Model. Oxid Med Cell Longev. 2012; 1–6.

27. Kazemi S, Mousavi SN, Aghapour F, Rezaee B, Sadeghi F, Moghadamnia AA. Induction Effect of Bisphenol A on Gene Expression Involving Hepatic Oxidative Stress in Rat. Oxid Med Cell Longev. 2016; 1–5.

28. Atkinson A, Roy D. In Vitro Conversion of Environmental Estrogenic Chemical

Bisphenol A to DNA Binding Metabolite(s). Biochem Biophys Res Commun. 1995;210: 424–433.

29. Babu S, Uppu S, Claville MO, Uppu R. Prooxidant Actions of Bisphenol A (BPA) Phenoxyl Radicals: Implications to BPA Related Oxidative Stress and Toxicity. Toxicol Mech Methods. 2013;23: 273–280.

30. Sakuma S, Nakanishi M, Morinaga K, Fujitake M, Wada S, Fujimoto Y. Bisphenol A 3,4-quinone Induces the Conversion of Xanthine Dehydrogenase into Oxidase *In Vitro*. Food Chem Toxicol. 2010;48: 2217–2222.

31. Yoshida M, Ono H, Mori Y, Chuda Y, Onishi K. Oxidation of Bisphenol A and Related Compounds. Biosci Biotechnol Biochem. 2001; 1444–1446.

32. Balakrishnan B, Henare K, Thorstensen EB, Ponnampalam AP, Mitchell MD. Transfer of Bisphenol A across the Human Placenta. Am J Obstet Gynecol. 2010;202: 393.e1-393.e7.

33. Schönfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I. Parent Bisphenol A Accumulation in the Human Maternal – Fetal – Placental Unit. Env Heal Perpsect. 2002;110: A703–A707.

34. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons W V. Human Exposure to Bisphenol A (BPA). Reprod Toxicol. 2007;24: 139–177.

35. Nahar MS, Liao C, Kannan K, Dolinoy DC. Fetal Liver Bisphenol A Concentrations and Biotransformation Gene Expression Reveal Variable Exposure and Altered Capacity for Metabolism in Humans. 2012;00: 1–8.

36. Schecter A, Malik N, Haffner D, Smith S, Harris TR, Paepke O, et al. Bisphenol A (BPA) in U.S. Food. Env Sci Technol. 2010;44: 9425–9430.

37. Rudel RA, Gray JM, Engel CL, Rawsthorne TW, Dodson RE, Ackerman JM, et al. Food Packaging and Bisphenol A and Bis(2-Ethyhexyl) Phthalate Exposure: Findings from a Dietary Intervention. Env Heal Perpsect. 2011;119: 914–920.

38. Barker P. Fetal Origins of Coronary Heart Disease. Br Med J. 1995;311: 171–174.

39. Milagro FI, Campión J, Martíez JA. Weight Gain Induced by High-Fat Feeding Involves Increased Liver Oxidative Stress. Obesity. 2006;14: 1118–1123.

40. Ballal K, Wilson CR, Harmancey R, Taegtmeyer H. Obesogenic High Fat Western Diet Induces Oxidative Stress and Apoptosis in Rat Heart. Mol Cell Biochem. 2013;18: 15.

41. Erdelyi I, Levenkova N, Lin EY, Pinto JT, Lipkin M, Quimby FW, et al. Western-Style Diets Induce Oxidative Stress and Dysregulate Immune Responses in the Colon in a Mouse Model of Sporadic Colon Cancer. J Nutr. 2009;139: 2072–2078.

42. Morrison CD, Pistell PJ, Ingram DK, Johnson WD, Liu Y, Fernandez-Kim SO, et al. High Fat Diet Increases Hippocampal Oxidative Stress and Cognitive Impairment in Aged Mice: Implications for Decreased Nrf2 Signaling. J Neurochem. 2010;114: 1581–1589.

43. Sverdlov AL, Elezaby A, Behring JB, Bachschmid MM, Luptak I, Tu VH, et al. High Fat, High Sucrose Diet Causes Cardiac Mitochondrial Dysfunction due in Part to Oxidative Post-Translational Modification of Mitochondrial Complex II. J Mol Cell Cardiol. 2015; 165–173.

44. McCarthy CG, Farney TM, Canale RE, Dessoulavy ME, Bloomer RJ. High-Fat Feeding, but not Strenuous Exercise, Increases Blood Oxidative Stress in Trained Men. Appl Physiol Nutr Metab. 2013;38: 33–41.

45. Buettner R, Scholmerich J, Bollheimer LC. High-fat diets: Modeling the Metabolic Disorders of Human Obesity in Rodents. Obesity. 2007;15: 798–808.

46. Warden CH, Fisler JS. Comparisons of Diets Used in Animal Models of High Fat Feeding. Cell Metab. 2008;7: 277–279.

47. Block G, Dresser CM, Hartman AM, Carroll MD. Nutrient Sources in the American Diet: Quantitative Data From the NHANES II Survey: Macronutrients and Fats. Am J Epidemiol. 1985;122: 27–40.

48. Block G, Dresser CM, Hartman AM, Carroll MD. Nutrient Sources in the American Diet: Quantitative Data from the NHANES II Survey. Vitamins and minerals. Am J Epidemiol. 1985;122: 13–26.

49. Zelber-Sagi S, Salomone F, Mlynarsky L. The Mediterranean Dietary Pattern as the Diet of Choice for Non-Alcoholic Fatty Liver Disease: Evidence and Plausible Mechanisms. Liver Int. 2017;37: 936–949.

50. Depner CM, Philbrick KA, Jump DB. Docosahexaenoic Acid Attenuates Hepatic Inflammation, Oxidative Stress, and Fibrosis without Decreasing Hepatosteatosis in a Ldlr-/- Mouse Model of Western Diet-Induced Nonalcoholic Steatohepatitis. J Nutr. 2013;143: 315–323.

51. Zern TL, Wood RJ, Greene C, West KL, Liu Y, Aggarwal D, et al. Grape Polyphenols Exert a Cardioprotective Effect in Pre- and Postmenopausal Women by Lowering Plasma Lipids and Reducing Oxidative Stress. J Nutr. 2005;135: 1911–1917.

52. Kontogianni MD, Tileli N, Margariti A, Georgoulis M, Deutsch M, Tiniakos D, et al. Adherence to the Mediterranean Diet is Associated with the Severity of Non-Alcoholic Fatty Liver Disease. Clin Nutr. 2014;33: 678–683.

53. Baratta F, Pastori D, Polimeni L, Bucci T, Ceci F, Calabrese C, et al. Adherence to Mediterranean Diet and Non-Alcoholic Fatty Liver Disease: Effect on Insulin

Resistance. Am J Gastroenterol. Nature Publishing Group; 2017;112: 1832–1839.

54. Ryan MC, Itsiopoulos C, Thodis T, Ward G, Trost N, Hofferberth S, et al. The Mediterranean Diet Improves Hepatic Steatosis and Insulin Sensitivity in Individuals with Non-Alcoholic Fatty Liver Disease. J Hepatol. 2013;59: 138–143.

55. Kafatos AG, Verhagen H, Moschandreas J, Apostolaki I, van Westerop JJM. Mediterranean Diet of Crete: Foods and Nutrient Content. Journal of the American Dietetic Association. 2000. pp. 1487–1493.

56. Trichopoulou A, Katsouyanni K, Gnardellis C. The Traditional Greek Diet. Eur J Clin Nutr. 1993;47: S76–S81.

57. Trichopoulou A, Toupadaki N, Tzonou A, Katsouyanni K, Manousos O, Kada E, et al. The Macronutrient Composition of the Greek Diet: Estimates Derived from Six Case-Control Studies. Eur J Clin Nutr. 1993;47: 549–558.

58. Dai J, Jones DP, Goldberg J, Ziegler TR, Bostick RM, Wilson PW, et al. Association Between Adherence to the Mediterranean Diet and Oxidative Stress. Am J Clin Nutr. 2011;88: 1364–1370.

59. van't Erve TJ, Kadiiska MB, London SJ, Mason RP. Classifying Oxidative Stress by F2-Isoprostane Levels across Human Diseases: A Meta-Analysis. Redox Biol. Elsevier B.V.; 2017;12: 582–599.

60. Schafer FQ, Buettner GR. Redox Environment of the Cell as Viewed Through the Redox State of the Glutathione Disulfide / Glutathione Couple. Free Radic Biol Med. 2001;30: 1191–1212.

61. Hansen JM. Oxidative Stress as a Mechanism of Teratogenesis. Birth Defects Res Part C - Embryo Today Rev. 2006;78: 293–307.

62. Sant KE, Dolinoy DC, Jilek JL, Shay BJ, Harris C. Mono-2-Ethylhexyl Phthalate (MEHP) Alters Histiotrophic Nutrition Pathways and Epigenetic Processes in the Developing Conceptus. J Nutr Biochem. 2016;27: 211–218.

63. Ferguson KK, Cantonwine DE, Rivera-González LO, Loch-Caruso R, Mukherjee B, Anzalota Del Toro L V, et al. Urinary Phthalate Metabolite Associations with Biomarkers of Inflammation and Oxidative Stress across Pregnancy in Puerto Rico. Environ Sci Technol. 2014;48: 7018–7025.

64. Watkins DJ, Ferguson KK, Anzalota Del Toro L V., Alshawabkeh AN, Cordero JF, Meeker JD. Associations between Urinary Phenol and Paraben Concentrations and Markers of Oxidative Stress and Inflammation among Pregnant Women in Puerto Rico. Int J Hyg Environ Health. 2015;218: 212–219.

65. Holland N, Huen K, Tran V, Street K, Nguyen B, Bradman A, et al. Urinary Phthalate

Metabolites and Biomarkers of Oxidative Stress in a Mexican-American Cohort: Variability in Early and Late Pregnancy toxics. Toxics. 2016;4: 1–18.

66. Crujeiras AB, Diaz-Lagares A, Carreira MC, Amil M, Casanueva FF. Oxidative Stress Associated to Dysfunctional Adipose Tissue: A Potential Link between Obesity, Type 2 Diabetes Mellitus and Breast Cancer. Free Radic Res. 2013;47: 243–256.

67. Kadiiska MB, Gladen BC, Baird DD, Germolec D, Graham LB, Hensley K, et al. Biomarkers of Oxidative Stress Study II. Are Oxidation Products of Lipids, Proteins, and DNA Markers of CCl4 Poisoning? Free Radic Biol Med. 2005;38: 698–710.

68. Kadiiska MB, Gladen BC, Baird DD, Graham LB, Parker CE, Ames BN, et al. Biomarkers of Oxidative Stress Study III. Effects of the Nonsteroidal Anti-Inflammatory Agents Indomethacin and Meclofenamic Acid on Measurements of Oxidative Products of Lipids in CCl4 Poisoning. Free Radic Biol Med. 2005;38: 711–718.

69. Neier K, Marchlewicz EH, Dolinoy DC, Padmanabhan V. Assessing Human Health Risk to Endocrine Disrupting Chemicals: A Focus on Prenatal Exposures and Oxidative Stress. Endocr Disruptors. 2015;3: e1069916.

70. van't Erve T, Lih FB, Kadiiska MB, Deterding LJ, Eling TE, Mason RP. Reinterpreting the Best Biomarker of Oxidative Stress: The 8-iso-PGF2a/PGF2a Ratio Distinguishes Chemical from Enzymatic Lipid Peroxidation. Free Radic Biol Med. 2016;83: 245–251.

71. Duhl D, Vrieling H, Miller K, Wolff G, Barsh G. Neomorphic Agouti Mutations in Obese Yellow Mice. Nat Genet. 1994;8: 59–65.

72. Dolinoy DC. The Agouti Mouse Model: An Epigenetic Biosensor for Nutritional and Environmental Alterations on the Fetal Epigenome. Nutr Rev. 2008;66: S7–S11.

73. Morgan H, Suterhland H, Martin D, Whitelaw E. Epigenetic Inheritance at the Agouti Locus in the Mouse. Nat Genet. 1999;23: 314–318.

74. Dolinoy DC, Weinhouse C, Jones TR, Rozek LS, Jirtle RL. Variable Histone Modifications at A(vy) Metastable Epiallele. Epigenetics. 2010;5: 637–644.

75. Miltenberger R, Mynatt R, Wilkinson J, Woychick R. The Role of the Agouti Gene in the Yellow Obese Syndrome. J Nutr. 1997;127: S1902–S1907.

76. Dolinoy DC, Huang D, Jirtle RL. Maternal Nutrient Supplementation Counteracts Bisphenol A-Induced DNA Hypomethylation in Early Development. Proc Natl Acad Sci. 2007;104: 13056–13061.

77. Institute for Lab Animal Research (ILAR). Guide for the Care and Use of Laboratory Animals [Internet]. National Academy of Sciences. 2011.

78. Nowland MH, Lebowsky R. ULAM Guidelines and SOPs for Mice. In: University of Michigan, Institutional Animal Care and Use Committee (IACUC). 2016 p. https://wiki.med.umich.edu/display/ULAMGSOP/Table+.

79. Caligioni C. Assessing Reproductive Status/Stages in Mice. Curr Protoc Neurosci. 2009;Appendix 4: 1–11.

80. Flurkey K, Currer J, Harrison D. Mouse Models in Aging Research. The Mouse in Biomedical Research, 2nd Edition New York; Elsevier, Volume 3: 2007. pp. 637–672.

81. Delahaye F, Breton C, Risold PY, Enache M, Dutriez-Casteloot I, Laborie C, et al. Maternal Perinatal Undernutrition Drastically Reduces Postnatal Leptin Surge and Affects the Development of Arcuate Nucleus Proopiomelanocortin Neurons in Neonatal Male Rat Pups. Endocrinology. 2008;149: 470–475.

82. Bouret SG. Nutritional Programming of Hypothalamic Development: Critical Periods and Windows of Opportunity. Int J Obes Suppl. 2012;2: S19–S24. d

83. Reynolds CM, Gray C, Li M, Segovia SA, Vickers MH. Early Life Nutrition and Energy Balance Disorders in Offspring in Later Life. Nutrients. 2015;7: 8090–8111.

84. Reeves PG. Components of the AIN-93 Diets as Improvements in the AIN-76A Diet. J Nutr. 1997;123: S838–S841.

85. Reeves PG, Nielsen FH, Fahey GC. Committee Report AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet. J Nutr. 1993;123: 1939–1951.

86. Dolinoy DC, Weidman JR, Waterland R a., Jirtle RL. Maternal Genistein Alters Coat Color and Protects Avy Mouse Offspring from Obesity by Modifying the Fetal Epigenome. Environ Health Perspect. 2006;114: 567–572.

87. Guerrero-Bosagna CM, Sabat P, Valdovinos FS, Valladares LE, Clark SJ. Epigenetic and Phenotypic Changes Result from a Continuous Pre and Postnatal Dietary Exposure to Phytoestrogens in an Experimental Population of Mice. BMC Physiol. 2008;8: 17.

88. Kafatos AG, Verhagen H, Moschandreas J, Apostolaki I, van Westerop JJM. Mediterranean Diet of Crete: Foods and Nutrient Content. J Am Diet Assoc. 2000;1000: 1487–1493.

89. Anderson OS, Nahar MS, Faulk C, Jones TR, Liao C, Kannan K, et al. Epigenetic Responses Following Maternal Dietary Exposure to Physiologically Relevant Levels of Bisphenol A. Environ Mol Mutagen. 2012;53: 334–342.

90. Yamamoto XM, Kensler TW, Motohashi H. The Keap1-Nrf2 System: A Thiol-Based Sensor-Effector Apparatus for Maintaining Redox Homeostasis. Physiol Rev.

2018;98: 1169–1203.

91. Wang C, Luo Z, Carter G, Wellstein A, Jose PA, Tomlinson J, et al. Nrf2 Prevents Hypertension, Increased Adma, Microvascular Oxidative Stress and Dysfunction in Mice With Two Weeks of Angiotensin II Infusion. Am J Physiol Regul Integr Comp Physiol. 2018;314: 399–406.

92. Bai J, Yu XJ, Liu KL, Wang FF, Jing GX, Li HB, et al. Central Administration of tert-butylhydroquinone Attenuates Hypertension via Regulating Nrf2 Signaling in the Hypothalamic Paraventricular Nucleus of Hypertensive Rats. Toxicol Appl Pharmacol. Elsevier; 2017;333: 100–109.

93. Zeng XP, Li XJ, Zhang QY, Liu QW, Li L, Xiong Y, et al. Tert-Butylhydroquinone Protects Liver Against Ischemia/Reperfusion Injury in Rats Through Nrf2-Activating Anti-Oxidative Activity. Transplant Proc. 2017;49: 366–372.

94. Harris C, Hansen J. Oxidative Stress, Thiols, and Redox Profiles. CH21. Methods in Molecular Biology - Developmental Toxicology: Methods and Protocols. 2012. p. 889: 325-346.

95. Harris C, Shuster DZ, Roman Gomez R, Sant KE, Reed MS, Pohl J, et al. Inhibition of Glutathione Biosynthesis Alters Compartmental Redox Status and the Thiol Proteome in Organogenesis-Stage Rat Conceptuses. Free Radic Biol Med. 2013;63: 325–37.

96. Kirlin W, Cai J, Thompson S, Diaz D, Kavanagh T, Jones D. Glutathione Redox Potential in Response to Differentiation and Enzyme Inducers. Free Radic Biol Med. 1999;27: 1208–1218.

97. Montuschi P, Barnes PJ, Roberts II LJ. Isoprostanes: Markers and Mediators of Oxidative Stress. FASEB J. 2004;18: 1791–1800.

98. Morrow JD. The Isoprostanes: their Quantification as an Index of Oxidant Stress Status *In Vivo*. Drug Metab Rev. 2000;32: 377–385.

99. Basu S, Helmersson J. Factors Regulating Isoprostane Formation *In Vivo.* Antioxid Redox Signal. 2005;7: 221–235.

100. Richelle M, Turini ME, Guidoux R, Tavazzi I, Métairon S, Fay LB. Urinary Isoprostane Excretion is not Confounded by the Lipid Content of the Diet. FEBS Lett. 1999;459: 259–262.

101. Gopaul NK, Halliwell B, Änggård EE. Measurement of Plasma F2-Isoprostanes as an Index of Lipid Peroxidation does not Appear to be Confounded by Diet. Free Radic Res. 2000;33: 115–127.

102. Turpeinen A, Basu S, Mutanen M. A High Linoleic Acid Diet Increases Oxidative Stress

In Vivo and Affects Nitric Oxide Metabolism in Humans. Prostaglandins, Leukot Essent Fat Acids. 1998;59: 229–233.

103. Basu S, Riserus U, Turpeinen A, Vessby B. Conjugated Linoleic Acid Induces Lipid Peroxidation in Men with Abodominal Obesity. Clin Sci. 2000;99: 511–516.

104. Basu S, Smedman A, Vessby B. Conjugated Linoleic Acid Induces Lipid Peroxidation in Humans. FEBS Lett. 2000;468: 33–36.

105. Konishi M, Iwasa M, Araki J, Kobayashi Y, Katsuki A, Sumida Y, et al. Increased Lipid Peroxidation in Patients with Non-Alcoholic Fatty Liver Disease and Chronic Hepatitis C as Measured by the Plasma Level of 8-Isoprostane. J Gastroenterol Hepatol. 2006;21: 1821–1825.

106. Hansel B, Giral P, Nobecourt E, Chantepie S, Bruckert E, Chapman MJ, et al. Metabolic Syndrome is Associated with Elevated Oxidative Stress and Dysfunctional Dense High-Density Lipoprotein Particles Displaying Impaired Antioxidative Activity. J Clin Endocrinol Metab. 2004;89: 4963–4971.

107. Vassalle C, Botto N, MG A, Berti S, Biagini A. Evidence for Enhanced 8-isoprostane Plasma Levels, as Index of Oxidative Stress *In Vivo*, in Patients with Coronary Artery Disease. Coron Artery Dis. 2003;14: 213–218.

108. Kaviarasan S, Muniandy S, Qvist R, Ismail IS. F2-Isoprostanes as Novel Biomarkers for Type 2 Diabetes: A Review. J Clin Biochem Nutr. 2009;45: 1–8.

109. Rho YH, Chung CP, Oeser A, Solus JF, Gebretsadik T, Shintani A, et al. Interaction between Oxidative Stress and HDL Cholesterol is Associated with Severity of Coronary Artery Calcification in Rheumatoid Arthritis. October. 2010;62: 1473–1480.

110. Mueller T, Dieplinger B, Gegenhuber A, Haidinger D, Schmid N, Roth N, et al. Serum Total 8-iso-prostaglandin F2α: A New and Independent Predictor of Peripheral Arterial Disease. J Vasc Surg. 2004;40: 768–773.

111. Chiabrando C, Valagussa A, Rivalta C, Durand T, Guy A, Zuccato E, et al. Identification and Measurement of Endogenous Beta-Oxidation Metabolites of 8-epi-Prostaglandin F2alpha. J Biol Chem. 1999;274: 1313–9.

112. Roberts LJ, Moore KP, Zackert WE, Oates JA, Morrow JD. Identification of the Major Urinary Metabolite of the F2alpha-isoprostane 8-Iso-prostaglandin F2alpha in Humans. J Biol Chem. 1996;271: 20617–20620.

113. Basu S. Metabolism of 8-iso-prostaglandin F2alpha. FEBS Lett. 1998;428: 32–36.

114. Griffith OW. Biologic and Pharmacologic Regulation of Mammalian Glutathione Synthesis. Free Radic Biol Med. 1999;27: 922–935.

115. Harris C, Jilek JL, Sant KE, Pohl J, Reed M, Hansen JM. Amino Acid Starvation Induced by Protease Inhibition Produces Differential Alterations in Redox Status and the Thiol Proteome in Organogenesis-Stage Rat Embryos and Visceral Yolk Sacs. J Nutr Biochem. 2015;26: 1589–1598.

116. Wendel A, Heinle H, Silbernagl S. The Degradation of Glutathione Derivatives in the Rat Kidney. Curr Probl Clin Biochem. 1977;8: 73–84.

117. Speisky H, Shackel N, Varghese G, Wade D, Israel Y. Role of Hepatic γ-Glutamyltransferase in the Degradation of Circulating Glutathione: Studies in the Intact Guinea Pig Perfused Liver. Hepatology. 1990;11: 843–849.

118. Jones DP, Mody VC, Carlson JL, Lynn MJ, Sternberg P. Redox Analysis of Human Plasma Allows Separation of Pro-Oxidant Events of Aging from Decline in Antioxidant Defenses. Free Radic Biol Med. 2002;33: 1290–1300.

119. Nkabyo Y, Ziegler T, Gu L, Watson W, Jones D. Glutathione and Thioredoxin Redox during Differentiation in Human Colon Epithelial (Caco-2) Cells. Am J Physiol Gastrointest Liver Physiol. 2002;283: G1352-1359.

120. Hansen J, Zhang H, Jones D. Differential Oxidation of Thioredoxin-1, Thioredoxin-2, and Glutathione by Metal Ions. Free Radic Biol Med. 2006;40: 138–145.

121. Wendel A, Cikryt P. The Level and Half-Life of Glutathione in Human Plasma. FEBS Lett. 1980;120: 209–211.

122. Bianchi G, Bugianesi E, Ronchi M, Fabbri A, Zoli M, Marchesini G. Glutathione Kinetics in Normal Man and in Patients with Liver Cirrhosis. J Hepatol. 1997;26: 606–613.

123. Gallogly MM, Mieyal JJ. Mechanisms of Reversible Protein Glutathionylation in Redox Signaling and Oxidative Stress. Curr Opin Pharmacol. 2007;7: 381–391.

124. Mieyal JJ, Chock PB. Posttranslational Modification of Cysteine in Redox Signaling and Oxidative Stress: Focus on S-Glutathionylation. Antioxid Redox Signal. 2012;16: 471–475.

125. Sabens Liedhegner EA, Gao X-H, Mieyal JJ. Mechanisms of Altered Redox Regulation in Neurodegenerative Diseases—Focus on S-Glutathionylation. Antioxid Redox Signal. 2012;16: 543–566.

126. Mailloux RJ, Treberg JR. Protein S-glutathionlyation Links Energy Metabolism to Redox Signaling in Mitochondria. Redox Biol. Elsevier; 2016;8: 110–118.

127. Sies H, Stahl W, Sevanian A. Nutritional, Dietary and Postprandial Oxidative Stress. J Nutr. 2005;135: 969–972.

128. Mohanty P, Hamouda W, Garg R, Aljada A, Ghanim H, Dandona P. Glucose Challenge

Stimulates Reactive Oxygen Species (ROS) Generation by Leucocytes. J Clin Endocrinol Metab. 2000;85: 2970–2973.

129. Mohanty P, Ghanim H, Hamouda W, Aljada A, Garg R, Dandona P. Both Lipid and Protein Intakes Stimulate Increased Generation of Reactive Oxygen Species by Polymorphonuclear Leukocytes and Mononuclear Cells. Am J Clin Nutr. 2002;75: 767–772.

130. Aljada A, Mohanty P, Ghanim H, Abdo T, Tripathy D, Chaudhuri A, et al. Increase in Intranuclear Nuclear Factor kappaB and Decrease in Inhibitor kappaB in Mononuclear Cells after a Mixed Meal: Evidence for a Proinflammatory Effect. Am J Clin Nutr. 2004;79: 682–690.

131. Alfaradhi MZ, Fernandez-twinn DS, Martin-gronert MS, Musial B, Fowden A, Ozanne SE. Oxidative Stress and Altered Lipid Homeostasis in the Programming of Offspring Fatty Liver by Maternal Obesity. Am J Physiol Regul Integr Comp Physiol. 2014;307: 26–34.

132. Bostick B, Habibi J, DeMarco VG, Jia G, Domeier TL, Lambert MD, et al. Mineralocorticoid Receptor Blockade Prevents Western Diet-Induced Diastolic Dysfunction in Female Mice. Am J Physiol - Hear Circ Physiol. 2015;308: H1126–H1135.

133. Chaudhari HN, Kim SW, Yun JW. Gender-Dimorphic Regulation of Antioxidant Proteins in Response to High-Fat Diet and Sex Steroid Hormones in Rats. Free Radic Res. 2014;48: 587–598.

134. Albert BB, Vickers MH, Gray C, Reynolds CM, Segovia SA, Derraik JGB, et al. Fish Oil Supplementation to Rats Fed High-Fat Diet during Pregnancy Prevents Development of Impaired Insulin Sensitivity in Male Adult Offspring. Sci Rep. 2017;7: 1–11.

135. Gao J, He J, Shi X, Stefanovic-Racic M, Xu M, O'Doherty RM, et al. Sex-Specific Effect of Estrogen Sulfotransferase on Mouse Models of Type 2 Diabetes. Diabetes. 2012;61: 1543–1551.

136. Guo Y, Hu B, Huang H, Tsung A, Gaikwad NW, Xu M, et al. Estrogen Sulfotransferase Is an Oxidative Stress-responsive Gene That Gender-specifically Affects Liver Ischemia / Reperfusion Injury. J Biol Chem. 2015;290: 14754–14764.

137. Gaillard R, Durmuş B, Hofman A, MacKenbach JP, Steegers EAP, Jaddoe VWV. Risk Factors and Outcomes of Maternal Obesity and Excessive Weight Gain during Pregnancy. Obesity. 2013;21: 1046–1055.

138. Lau EY, Liu J, Archer E, McDonald SM, Liu J. Maternal Weight Gain in Pregnancy and Risk of Obesity among Offspring: A Systematic Review. J Obes. 2014;2014: 1–16.

139. Hemond J, Robbins RB, Young PC. The Effects of Maternal Obesity on Neonates,

Infants, Children, Adolescents, and Adults. Clin Obstet Gynecol. 2016;59: 216–227.

140. Poston L, Harthoorn LF, Van Der Beek EM, ILSI Europe Workshop. Obesity in Pregnancy: Implications for the Mother and Lifelong Health of the Child. A Consensus Statement. Pediatr Res. 2011;69: 175–180.

141. Gallardo JM, Gomez-Lopez J, Juarez-Sanchez F, Medina-Bravo P, Contreras-Ramos A, Galicia-Esquivel M, et al. Maternal Obesity Increases Oxidative Stress in the Newborn. Obesity. 2015;23: 1650–1654.

CHAPTER 4

Trimester-Specific Influences of Prenatal Bisphenol A and Mediterranean Diet on Metabolic Risk Score and Serum Lipid Oxidation in Human Adolescents

ABSTRACT

Prenatal programming of metabolic syndrome (MetSyn) may contribute to the growing prevalence of MetSyn worldwide. Gestational diet and exposure to bisphenol A (BPA) are associated with altered MetSyn risk in children. To examine the prenatal programming impact of maternal BPA exposure and the potential protective effect adherence to a Mediterranean diet (MDS) might have on MetSyn risk in youth, we re-enrolled 250 maternal-child dyads from a longitudinal human birth cohort, Early Life Exposure in Mexico to ENvironmental Toxicants (ELEMENT). Predictive models analyzed the impact of pregnancy-average and trimester-specific maternal urinary BPA levels, MDS, and the BPA*MDS interaction on metabolic risk score (MRS) and serum lipid oxidation (8-isoprostane, 8-iso) levels in peripubertal youth. Pubertal status, urinary BPA, diet, physical activity and other youth characteristics were included in linear regression analyses to examine the potential for postnatal alteration of prenatal programming effects. In models of all youth, maternal pregnancy average and second trimester (T2) urinary BPA were associated with suggestive ($p<0.20$) decreases in youth MRS (-0.36 and -0.29, respectively); while the pregnancy average BPA*MDS interaction was associated with a

suggestive increase in MRS (0.065, $p<0.20$). These associations were driven by boys; no relationship was observed among exposures and MRS in girls. Maternal MDS did not impact youth MRS. Among all youth, maternal pregnancy average and T2 urinary BPA was associated with increased serum 8-iso levels (16.8%, $p<0.20$, and 20.1%, $p<0.10$, respectively). In this case, the associations, between prenatal exposures and youth 8-iso, were driven by girls. Maternal pregnancy average and T2 MDS impacted youth serum 8-iso, but in opposite directions by sex; MDS was associated with increased 8-iso in girls, but decreased 8-iso in boys. Pregnancy average and T2 maternal BPA were suggestive predictors of both youth health outcomes (MRS and serum 8-iso), supportive of the second trimester as a critical window in the prenatal programming of MetSyn. Although sex-specific differences were observed, maternal MDS only impacted youth 8-iso, while the BPA*MDS interaction contributed more to youth MRS. The contribution of youth BPA exposure, MDS, vigorous physical activity levels, and pubertal status to predictive models of youth MRS and 8-iso supports the theory that healthy lifestyle behaviors during peripuberty may alter the effect of prenatal programming. Thus, a child's health trajectory at birth may be modified later in life.

INTRODUCTION

Prenatal programming of metabolic syndrome (MetSyn) has been reported in experimental and epidemiologic studies [1–6]. The maternal metabolic environment during pregnancy, including maternal obesity [7,8], under nutrition [9,10], and glucocorticoid levels [10,11] has been linked to an increased risk of offspring MetSyn. Perinatal exposure to the endocrine disrupting chemical (EDC), bisphenol A (BPA) has also been associated with increased MetSyn risk in offspring [12–15]. Youth MetSyn development is known to be

impacted by lifestyle behaviors, such as diet, leisure-time physical activity, and parental health literacy [16,17], but prenatal programming suggests that disease risk can also be affected before birth. This amplified risk of MetSyn at birth can increase lifelong morbidity, thus altering the child's health trajectory. Pediatric MetSyn is associated with greater odds of obesity and poor cardiovascular health in childhood [18,19], and with a 2- to 3-fold greater risk of MetSyn, Type 2 Diabetes (T2DM), and atherosclerosis in adulthood [20]. Improved understanding of environmental factors capable of prenatally programming MetSyn is warranted.

Prenatal BPA exposure has been associated with alterations in early life health outcomes, such as decreased birth weight, increased birth length [21], increased blood pressure at 4 years [22]. Animal models of perinatal BPA exposure report increased MetSyn development in adulthood [23–25], suggestive of the potential for this prenatal programming in humans, also. The ability of prenatal BPA to alter offspring metabolism is concerning, because urinary BPA levels are measurable in >95% of pregnant mothers in the U.S. [26,27]. BPA crosses the placenta [28,29], and reduced fetal expression of BPA-specific biotransformation enzymes (e.g. *UGT2B15, SULT1A1,* and *STS*) [30], suggests an impaired fetal ability to metabolize BPA. BPA is present in food and beverage packaging in the form of epoxy resins and polycarbonate plastics [31–33]. Thus, studying the interaction of BPA exposure and maternal diet during pregnancy will more accurately model concurrent real-world exposures, rather than investigating BPA or diet independently.

Prenatal programming research often focuses on exposures that increase disease risk, such as BPA, but a healthy maternal diet may exert a protective effect on offspring, conferring resilience against disease later in life. A Mediterranean-style diet is clinically recommended to prevent and reverse progression of metabolic diseases in adults [34]. Mediterranean diet

consumption has been associated with better glycemic control [35], improved lipid profiles [36], and a decreased risk of metabolic diseases [37–40]. A meta-analysis reported Mediterranean diet adherence in adults was protective against many MetSyn factors, including waist circumference, systolic and diastolic blood pressure, circulating HDL-C, triglycerides, and glucose [41]. Despite the overwhelming metabolic health benefits attributed to Mediterranean diet adherence in adults, the affect of perinatal Mediterranean diet exposure on offspring MetSyn has not been examined. Maternal Mediterranean diet consumption during pregnancy is associated with protection against low birth weight (LBW) and low placental weight [42]. LBW has been associated with greater risk of coronary heart disease [43] and insulin resistance [44] in adulthood. If maternal Mediterranean diet intake can reduce the incidence of LBW, intake may also be associated with decreased metabolic disease later in life. This link, between maternal consumption of a Mediterranean diet during pregnancy and offspring metabolic health, has not been investigated.

Diagnosing MetSyn in children is challenging, due to developmental changes in anthropometric variables, blood pressure, and serum lipids as children grow [45]. Age-specific diagnostic criteria are recommended to define pediatric MetSyn [46]. Pubertal development compounds the challenge of diagnosing MetSyn in youth. Pubertal changes include sex-specific fat accumulation and distribution, a ~30% decrease in insulin sensitivity, with a concurrent increase in insulin secretion [45,47,48]. We used both a Metabolic Risk Score [49] and a measure of lipid oxidation, serum 8-isoprostane [50], to analyze MetSyn in this study population. Oxidative stress, defined as an imbalance of oxidizing and reducing molecules, has been implicated as an underlying risk factor in the pathogenesis of many chronic metabolic diseases, including MetSyn [51,52].

Among U.S. youth, 12-19 years old, MetSyn prevalence is greatest among Hispanic boys (12.9%) and girls (8.2%), compared to non-Hispanic white and black children [53,54]. The reason for this disproportionate risk is not well understood. To address the lack of evidence in human studies supporting the theory that MetSyn can be prenatally programmed by maternal diet and EDC exposure, this study examines the effect of prenatal BPA exposure and Mediterranean diet on metabolic risk and serum lipid oxidation levels in Hispanic youth. To assess whether current, peripubertal exposures and health characteristics could impact the prenatal programming, youth BPA, diet, blood pressure, serum hormone, lipid, and lipid oxidation levels, physical activity, and pubertal status data were collected from adolescents participating in the Early Life Exposures in Mexico to ENvironmental Toxicants (ELEMENT) human birth cohort in Mexico City. A secondary analysis investigated the potential effect modification of prenatal BPA on adolescent metabolic health by maternal Mediterranean diet adherence during pregnancy.

METHODS

Study Population

The human birth cohort, Early Life Exposures in Mexico to ENvironmental Toxicants (ELEMENT) is built on a 23-year collaboration with the Instituto Nacional de Salud Publica (*INSP*) in Mexico. The 250 mother-child dyads included in this study were originally recruited from hospitals serving low-to-moderate income populations from 1997–2005 [55–59]. Most mothers (n=236) were recruited during their first trimester (T1) prenatal visit; maternal height, weight, urine samples, and questionnaires on food intake and demographic data were collected at each trimester of pregnancy. The additional 14 mothers were recruited at the time of delivery, so pregnancy values are not available.

The children have been continuously followed from birth through childhood to adolescence. At the adolescent visit, conducted in 2010-2012, experienced study personnel collected biological samples, anthropometry, physical activity and food frequency questionnaires (Figure 4.1). Youth re-recruitment for this study included 132 girls and 118 boys; MRS was calculated for 99% of boys and girls. Serum 8-isoprostane was measured in 97% of boys and 96% of girls, dependent on serum sample volume. All participants provided informed consent prior to enrollment; the study protocols were approved by the research and ethics committees of *INSP* and the University of Michigan.

Urinary Bisphenol A (BPA) Analysis

Spot urine samples from mothers at Trimester 1 (n=119), Trimester 2 (n=200), and Trimester 3 (n=225) prenatal clinic visits were analyzed for total (free + glucuronidated) BPA at NSF International (Ann Arbor, MI, USA) via isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) [55]. The NSF protocol was developed based on the Centers for Disease Control and Prevention (CDC) Laboratory Procedure Manuals (method no. 6301.01; revised: April 13, 2009); thus measurements correspond to urinary BPA levels measured in NHANES [60]. Specific gravity (SG) was measured with a digital refractometer (ATAGO, Company Ltd, Tokyo, Japan) in all urine samples to adjust for variability in urine output and concentration between study participants. Youth urinary BPA levels (n = 242) were assessed at NSF International by the same ID-LC-MS/MS method as maternal urinary samples. We have previously published additional details of the BPA analysis method [55].

Mediterranean Diet Score (MDS) Adherence

Mothers completed an interviewer-administered food frequency questionnaire (FFQ) at their prenatal clinic visits in all three trimesters (n=227, 234, 235, respectively). The FFQ used for this study was a 116 item, semi-quantitative FFQ, validated among 134 women in Mexico City [61], based on the 131-item Harvard FFQ [62]. Study youth (n=250) completed FFQs, similar to the FFQs their mothers completed during pregnancy [63]. Youth MDS was calculated based on these FFQs, and was added to predictive models to adjust for youth's current diet.

Foods were compiled into food groups prior to coding into the MDS. Mediterranean diet scoring was first proposed by Trichopoulou [64–66]. This original MDS uses scoring criteria from 0-9 points (lowest to highest adherence). One point is given for above-median consumption of the five beneficial food categories: fish, legumes, fruits & nuts, vegetables, and cereal (assumed to be whole grain), and for below-median consumption of the three detrimental food categories: meat, poultry, and dairy (assumed to be whole fat). Individuals received an additional point if their daily alcohol consumption was within a moderate range (2-25g/day for women) [64].

Multiple variations on the Trichopoulou MDS have been proposed by other authors, including adding categories for high-fat convenience foods, desserts, and sugar sweetened beverages [67]. We applied these variations, but the additions did not improve the MDS effect size or model fit, assessed by Akaike Information Criterion (AIC). A 2011 comparison [165] of 10 Mediterranean diet indices, including the Trichopoulou MDS and the aforementioned alterations, found that the Trichopoulou MDS had the highest correlation (r=0.84) with the core factors determining 'adherence to the Mediterranean diet'. Since Trichopoulou MDS best characterized core Med diet factors and our models did not improve when adding additional food groups, we used the Trichopoulou MDS to assess maternal Med diet adherence, in this study

[67]. The only variation we made was to remove the alcohol consumption category, since alcohol intake was negligible amongst both pregnant women and adolescents; thus the MDS in this study ranges from 0-8 points (Figure S4.1, Table S4.1).

Youth Serum Metabolic Markers

Fasting glucose, triglycerides (TG) and HDL-C were measured with a biochemical analyzer (Cobas Mira Plus, Roche Diagnostics, Basel, Switzerland) on-site, in Mexico City. Following venous blood collection, serum aliquots were frozen at -80^{o}C and shipped from INSP in Mexico City to the Michigan Diabetes Research Center (MDRC) Chemistry Lab for analysis. Serum leptin was measured via RIA (Millipore), IGF-1 by chemiluminescence immunoassay (Immulite 1000). Youth metabolic health was assessed via a Metabolic Risk Score (MRS), previously validated for diagnosing MetSyn in youth [49]. Sex-specific z-scores, based on this sample, were calculated for four adolescent metabolism variables: waist circumference, fasting glucose, fasting lipids (TG/HDL-C), and average blood pressure ((SBP+DBP)/2). The sex-specific MRSs, calculated by averaging the four z-scores, were normalized to zero.

Youth Serum Lipid Oxidation

Quantitation of serum 8-isoprostane was performed to assess oxidative stress in study adolescents. Serum samples were stored at -80^{o}C with 0.005% BHT to prevent oxidation during storage, then transferred on dry ice to Cayman Chemical (Ann Arbor, MI, USA). The 8-isoprostane analyses were conducted via a competitive enzyme immunoassay (EIA) kit (No. 516351), with sensitivity 2.7 pg/mL and intra-assay %CV 9.5%. The NIEHS initiative, Biomarkers of Oxidative Stress Study (BOSS) determined that F_2-isoprostanes were readily

quantifiable markers of lipid peroxidation that relate to disease-relevant measures [68,69]. Other recent reviews of oxidative stress measures deemed 8-isoprostane to be a highly accurate measure of lipid peroxidation [70–72].

Model Covariates

Maternal Characteristics

Trimester-specific and post-partum maternal height and weight were measured on a professional scale (PAME, Puebla) to the nearest 0.1cm and 0.1kg, respectively. Maternal Trimester 1 (T1) BMI and gestational weight gain were calculated from these measures. Pre-pregnancy BMI and gestational weight gain have been positively associated with infant birth weight [73], macrosomia, large for gestational age (LGA) [74], and child obesity [75–78]. Thus, both of these measures were examined as potential confounders in generalized linear models (GLM).

At baseline, mothers completed questionnaires on maternal education, length of time in Mexico City, and smoking history. In this cohort, maternal education is used as a proxy for socio-economic status; the two factors are highly correlated, but maternal education data was collected on more mothers. Study personnel recorded maternal age-at-pregnancy, parturition status, and delivery type (vaginal or Cesarian section).

Youth Characteristics

Peripubertal youth returned to the clinic for a single follow-up visit at 8-14 years of age. Youth height (to 0.1cm) and weight (to 0.1kg) were measured on a professional scale (PAME, Puebla). Waist circumference was measured to the nearest 1mm in a consistent location, guided

by sex-specific diagrams of measuring tape placement. All anthropometric data were obtained after adolescents removed clothing and shoes, remaining in their undergarments and a hospital gown. Seated blood pressure measurements were taken in duplicate and the average systolic blood pressure (SBP) and diastolic blood pressure (DBP) levels were used in analyses. Fasting, venous blood samples were collected from adolescents and analyzed for serum hormone levels (leptin, IGF-1).

Youth in this study were peripubertal. A dichotomous puberty variable (Yes/No) was added to predictive models of youth MRS and 8-iso. The variable was a composite of physician-assessed Tanner stages, recorded for all youth (n=250) during the study clinic visit. Tanner staging is a five-point scale of physical pubertal development based on pubic hair (boys and girls), breast (girls), and genital (boys) development [56,58,79].

Advancement through Tanner stages during pubertal development is associated with the decreased insulin sensitivity of adolescence [80]. Sex-specific changes in circulating leptin levels also occur at puberty. Pre-pubertal boys and girls have similar leptin levels that increase with age; at puberty, leptin levels rise in girls and fall in boys, mirrored by sex-stratified changes in fat mass [81]. Insulin-like growth factor-1 (IGF1) levels also increase at puberty, triggered by increased gonadal sex steroid levels [82].

Physical activity has been associated with decreased risk of MetSyn and insulin resistance in youth [83]. At the adolescent visit of this study, youth completed a self-reported physical activity questionnaire, previously validated in Mexican youth [84,85]. Responses were coded into vigorous (e.g. swimming, running, soccer) or moderate activities (e.g. walking, cleaning) and cumulative measures were created by adding the hour per week spent in each type of activity.

Statistical Analysis

Normality of all variables was investigated via Q-Q plots of residuals; right-skewed variables were Ln-transformed, including urinary BPA and serum 8-isoprostane. The distributions of the prenatal exposures (urinary BPA and MDS) were explored and intraclass correlation coefficients (ICCs) were calculated to compare exposure measures across trimesters, T1 vs. T2, T1 vs. T3, T2 vs. T3. Independent t-tests were performed on all study variables to determine which variables differed by youth sex.

Generalized linear models (GLMs) were constructed to predict the two youth health outcomes of interest, (1) MRS and (2) serum 8-iso. To examine if prenatal timing of environmental exposures alters metabolic programming in offspring, trimester-specific models were run. For example, these models examined whether maternal Trimester 1 urinary BPA, MDS, and BPA*MDS impacted adolescent MRS and allowed comparison to the impact of Trimester 3 BPA, MDS, BPA*MDS on MRS. Pregnancy averages of urinary BPA and MDS were calculated for each mother, and models using these average exposures were also estimated. For instance, this model examined whether average maternal urinary BPA, MDS, and BPA*MDS across pregnancy altered adolescent MRS. Analyzing pregnancy average values may improve confidence in the associations observed between the prenatal exposures and youth metabolic health outcomes; potential variability inherent in spot urine samples is balanced by averaging values across pregnancy. Significant results in pregnancy average models, but not trimester-specific models, could also suggest that timing of the exposures (BPA & MDS) is less critical than the magnitude of exposure across pregnancy. Finally, due to previous studies identifying sexually dimorphic effects of BPA, sex-stratified models were run, in addition to

models including all adolescents. Predictive models did not differ if the 14 youth whose mother's were recruited at delivery were excluded, thus all 250 mother-child dyads were included in the modeling process.

Unadjusted models included the main prenatal exposure variables: maternal trimester-specific urinary BPA, MDS, and BPA*MDS interaction, for both youth MRS and serum 8-iso outcomes. The BPA*MDS interaction term was added to all models to determine if maternal MDS modified the effect of prenatal BPA exposure on youth MRS or serum 8-isoprostane. Maternal education, youth puberty status, youth BPA and MDS were added to subsequent models as *a priori* covariates. Additional maternal and youth characteristics were investigated as potential model covariates, including: maternal trimester-1 BMI, maternal pregnancy weight gain, youth serum leptin and IGF-1, youth vigorous and moderate physical activity. In this study, predictive variables were sex specific; maternal T1 BMI contributed significantly in models for adolescent girls, while vigorous physical activity of adolescent boys significantly impacted models.

Statistical significance was determined *a priori* at $p < 0.05$. Borderline significance ($p < 0.10$) and suggestive significance ($p < 0.20$) were also used to assess data trends in this study. All analyses were conducted in SAS 9.4 (Cary, NC, USA).

RESULTS

Study Population

Mother's age at recruitment into the ELEMENT cohort ranged from 14 – 44 years, averaging 26.8 years. A high school education was attained by 86% of the mothers. Maternal BMI at the T1 clinic visit averaged 25.9 kg/m^2, and mothers gained an average of 2.6 kg during

pregnancy. Female adolescents comprised 52.8% of youth in the cohort. Only 34.8% of female youth had begun puberty, but 49.2% of males had entered puberty according to physician-assessed Tanner staging (Table 4.1).

Few variables differed significantly by youth sex. Maternal T1 urinary BPA was slightly greater among girls (1.60 ng/mL) than boys (1.46 ng/mL), but was only borderline significant ($p = 0.059$). BPA did not differ significantly in T2, T3, or youth urinary samples. Maternal adherence to the MDS did not differ by sex, at any trimester, nor did any other maternal covariates. Adolescent health outcomes of interest (MRS and serum 8-isoprostane) did not differ by youth sex, but other youth health measures did. Systolic blood pressure was greater in boys than girls ($p = 0.016$), while serum triglycerides ($p < 0.001$), serum leptin ($p < 0.001$), and serum IGF-1 levels ($p < 0.001$) were higher in girls compared to boys (Table S4.2).

Maternal Exposures - Comparison Across Trimesters

Median, specific-gravity adjusted, BPA measurements ranged from 1.14-1.48 ng/mL in maternal urine, but levels did not differ by trimester (Table 4.3). In all trimesters, the distribution of maternal urinary BPA was highly variable, illustrated by the large ICCs (Table S4.3, Figure S4.2A). The median MDS adherence of mothers did not differ across trimesters (Table 4.3). Although the ICCs comparing trimester 1 MDS to trimesters 2 and 3 were low (-0.03 & 0.11, respectively), the higher ICC comparing trimesters 2 and 3 (0.44, $p < 0.05$) suggests that maternal diets fluctuated more between T1 and T2, then stabilized in the second two trimesters of pregnancy (Table S4.3, Figure S4.2B).

Adolescent Metabolic Risk Score & Serum 8-Isoprostane Levels

Metabolic Risk Score (MRS) was normally distributed amongst the Mexican youth in this study. Median MRS amongst all youth was -0.02, with a range from -1.65 to 2.00. Since MRS was calculated as a sex-specific z-score, there were no differences by sex. Adolescent serum 8-isoprostane levels were non-normal; a few children had high serum levels, right-skewing the data. Median 8-isoprostane measurements did not differ by youth sex; both boys and girls had extreme high values (Figure S4.2).

Adolescent MRS differed by youth age at re-enrollment, pubertal status, and serum leptin levels (Table 4.2). Among all youth, age was directly associated with increased MRS ($p = 0.036$); this relationship was driven by boys ($p = 0.044$), not girls ($p = 0.427$). Similarly, youth who had entered puberty had significantly greater MRS ($p = 0.000$) than pre-pubertal youth; this was true amongst both girls ($p = 0.001$) and boys ($p = 0.046$). Higher serum leptin levels are associated with greater MRS in all youth ($p = 0.000$). Adolescent MRS did not differ by maternal age at pregnancy, educational attainment, and Trimester 1 BMI. Serum 8-isoprostane levels in youth did not differ by any maternal or adolescent factors (Table S4.4).

Predictive Modeling of Adolescent Metabolic Risk Score

Among all youth, average maternal urinary BPA and the BPA*MDS interactions across pregnancy trimesters tended to predict MRS. Average, Ln-transformed urinary BPA was associated with a suggestive decrease in MRS (-0.359, $p = 0.132$), while the BPA*MDS interaction associated with a suggestive increase in MRS (0.065, $p = 0.191$). Average BPA in T2 was also associated with a suggestive decrease in MRS (-0.287, $p = 0.183$) among all youth. The relationships did not exist between T1 or T3 BPA and youth MRS. Maternal MDS adherence during pregnancy did not impact youth metabolic risk score in any adjusted models (Table 4.4).

Among male youth, the average maternal urinary BPA and the BPA*MDS interaction across pregnancy did not predict youth MRS, but suggestive changes were observed in trimester-specific models, adjusted for *a priori* and predictor covariates. T2 maternal urinary BPA was associated with a suggestive decrease in MRS (-0.63, p = 0.147) among males. The BPA*MDS interaction had a differential effect on male youth, depending on the trimester. Trimester 1 BPA*MDS was associated with a suggestive decrease in male MRS (-0.11, p = 0.188); but T2 BPA*MDS was associated with a suggestive increase in MRS (0.14, p = 0.140) in male youth. Neither prenatal BPA, MDS, nor their interaction (BPA*MDS) significantly predicted MRS in adjusted models for female youth (Table 4.4).

Pubertal status and T1 maternal BMI were both positively associated with increased MRS in all youth across all trimesters and the pregnancy average model. In models including all youth, adolescent MDS and vigorous physical activity were negatively associated with MRS. Interestingly, these predictive covariates varied by sex. Adolescent pubertal status and T1 maternal BMI were significant in female youth; both were associated with increased risk of MRS in girls across all three trimesters. Youth MDS and vigorous physical activity were significant predictors in male models, but were negatively associated with MRS (Table 4.4).

Predictive Modeling of Adolescent Serum 8-isoprostane

Maternal average Ln-BPA and T2 Ln-BPA were suggestive of associations with serum 8-isoprostane in all youth. In adjusted models for all youth, maternal average Ln-BPA was associated with a suggestive 16.8% (p = 0.190) increase in youth serum 8-isoprostane; T2 Ln-BPA was associated with a borderline 20.1% (p = 0.085) increase. Of note, these are the same time periods (pregnancy average and T2) that were predictive of youth MRS; thus, T2 may be a

perinatal period that is particularly sensitive to BPA exposure. However, the direction of association differs by youth outcome. Greater maternal BPA exposure is associated with higher 8-isoprostane levels in youth; in contrast, maternal BPA exposure is associated with lower youth MRS. Neither maternal MDS nor the interaction of BPA*MDS were predictive of 8-isoprostane in models including both boys and girls (Table 4.5).

Among male youth, only T1 maternal Ln-BPA was a suggestive predictor of serum 8-isoprostane, with T1 Ln-BPA associated with a suggestive 39.4% increase ($p = 0.102$). Maternal MDS, pregnancy average and T2, was negatively associated with male 8-isoprostane. Maternal average MDS was associated with a significant 10.2% decrease ($p = 0.008$), and T2 MDS with a suggestive 5.2% decrease ($p = 0.180$) in male 8-isoprostane levels. The interaction of BPA*MDS did not predict 8-isoprostane levels in male youths (Table 4.5).

In female youth, pregnancy average and T2 Ln-BPA were suggestive predictors of serum 8-isoprostane. Pregnancy average Ln-BPA was associated with a suggestive 19.7% increase ($p = 0.182$), while T2 Ln-BPA was associated with a borderline 24.0% increase ($p = 0.067$) in female 8-isoprostane. Maternal MDS was positively associated with 8-isoprostane levels in female youth. Pregnancy average MDS was associated with a suggestive 4.2% increase ($p = 0.123$), T2 MDS with a significant 6.5% increase ($p = 0.020$), and T3 MDS with a suggestive 2.9% increase ($p = 0.189$) in female 8-isoprostane. Of note, this association between maternal MDS and youth 8-isoprostane is in the opposite direction in male and female youth; these opposing, sex-specific relationships are a likely reason no association is observed in models that combine boys and girls. T2 BPA*MDS was associated with a borderline 4.4% decrease ($p = 0.093$) in 8-isoprostane among girls. The interaction is not a significant predictor at any other time point (Table 4.5).

In the adjusted model of T2 exposures predicting serum 8-isoprostane in all youth, youth urinary BPA negatively associated with serum 8-isoprostane. BPA measured in youth urine did not significantly contribute to any other models. Including youth MDS improved pregnancy average, T1, and T2 models of male 8-isoprostane, but had no impact in models predicting female 8-isoprostane levels. Youth vigorous physical activity improved prediction of 8-isoprostane in males only, in pregnancy average and T3 models. These findings suggest that peripubertal exposures (e.g. BPA) and behavior (e.g. diet and physical activity) can impact the relationship between prenatal exposures and peripubertal serum 8-isoprostane. Of note, pubertal status had no effect on models of youth serum 8-isoprostane, supportive of a peripuberty-independent variation in lipid oxidation. Pubertal status was still included in these models as an *a priori* covariate, due to the innumerable physiologic changes occurring during this developmental transition.

DISCUSSION

This is the first human study to accomplish a long-term follow up of prenatal BPA and dietary exposures, which not only explores the EDC-diet interaction, but also examines the impact on multiple measures of metabolic health during the peripubertal transition. We examined the potential impact of prenatal BPA and maternal dietary intake on peripubertal metabolic risk and serum lipid oxidation (Figure 4.2). Maternal urinary T2 BPA was associated with a suggestive decrease in MRS among boys only. Similarly, when male and female youth were combined, pregnancy average and T2 BPA were suggestive of decreases in youth MRS. On the other hand, pregnancy average and T2 maternal urinary BPA were associated with suggestive increases in serum 8-isoprostane among girls and all youth combined. Interestingly, maternal

MDS contributed to pregnancy average and T2 models of 8-isoprostane in both boys and girls, but the direction of association differed. The interaction of maternal BPA*MDS was associated with suggestive increases in MRS in boys and all youth combined, but was associated with a borderline decrease in serum 8-isoprostane in girls. Youth characteristics, such as urinary BPA, MDS, vigorous physical activity, and pubertal status, improved models of youth MRS and 8-isoprostane, supporting the theory that prenatal programming may be altered by later life exposures and behavior. The peripubertal transition may be a developmental period that is particularly sensitive to this potential reprogramming.

This is the first report of prenatal BPA impacting peripubertal serum lipid peroxidation in humans. In another pregnancy cohort, one interquartile range increase in urinary BPA was associated with a 8.79% ($p = 0.02$) increase in urinary 8-isoprostane in the mothers [87]. A recent study reported that prenatal BPA exposure was associated with increased levels of a different marker of oxidative stress, 3-nitrotyrosine levels, in cord blood samples [86], but did not follow children after birth. Precedence for perinatal BPA exposure leading to later life oxidative stress has been reported in murine models, where perinatal BPA was correlated with higher oxidative stress markers into puberty [88] and adulthood [89], even when BPA exposure stopped at weaning. The brief half life of 8-isoprostane, 16 minutes in humans [90], suggests that the 8-isoprostane measured in peripubertal youth cannot be the same 8-isoprostane generated prenatally. Thus, the positive association between prenatal BPA exposure and youth serum 8-isoprostane levels may be due to confounding by postnatal characteristics. In this study, the association between prenatal exposures and youth serum 8-isoprostane was impacted by concurrent youth exposures and behavior, suggestive of a significant impact of postnatal confounders.

The minimal impact of prenatal BPA on peripubertal MRS was unexpected. Studies in human children [91,92] and adults [93,94] have reported increased urinary BPA levels are correlated with increased waist circumference and impaired insulin sensitivity. In murine models, prenatal low-dose BPA exposure has been associated with a detrimental effect on male offspring, including increased body weight, liver weight, abdominal fat mass, serum insulin and leptin accompanied by decreased glucose tolerance [23]. On the other hand, the CHAMACOS birth cohort reported BPA levels at 9 years were associated with increased adiposity at that age, but prenatal BPA exposure was inversely correlated to 9 year old adiposity [95]. These conflicting effects support the theory that the timing and life-stage of exposure to BPA are critical in determining the health impact [58,96]. Despite our original hypothesis that prenatal BPA would be associated with increased peripubertal MRS, a recent study investigating the impact of prenatal and concurrent BPA exposure on adolescent lipid profile (total cholesterol, triglycerides, HDL-C) in this ELEMENT cohort also found no BPA effect [56]. Trimester 3 BPA levels were not correlated to other metabolic outcomes in ELEMENT youth, including: serum hormones, pubertal status [57], BMI z-score, and skinfold thickness measures [59]. These variable findings in the ELEMENT and CHAMACOS birth cohorts warrant additional investigation into the potential of prenatal BPA exposure on offspring metabolic risk in late childhood.

Maternal, specific-gravity adjusted urinary BPA levels in this study were within the range of previously reported maternal urinary BPA levels (0.5 – 3.5 ng/mL) [97–99]; the ICC also correspond to previously reported ICC for BPA measures across trimesters of pregnancy (0.19-0.32) [98]. Although spot urine collections have been criticized as an inaccurate method of measuring BPA exposure, due to the BPA's short half-life, maternal urinary BPA was consistent

across trimesters when considering the population as a whole. Previous investigation into BPA levels within the ELEMENT cohort found no difference in urine samples collected in morning compared to afternoon; BPA levels also did not differ by child age, 8-10 vs. 11-13yrs [55]. Despite consistent average BPA levels in all three trimesters, predictive effects on youth 8-iso were trimester-specific in LMMs. Thus, timing of BPA exposure may be more important than the level of BPA exposure when determining the potential impact on offspring oxidation status.

Across the study population of ELEMENT mothers, average maternal MDS was consistent across all three trimesters. Maternal adherence to a Mediterranean diet during pregnancy altered lipid oxidation in both boys and girls, but had an opposite direction of effect. Surprisingly, maternal MDS during pregnancy did not impact MRS in youth of either sex. The interaction term of trimester-specific BPA*MDS had a minimal contribution to predictive models of youth MRS or 8-iso. However, among boys only, including youth MDS as a covariate in predictive models of MRS and 8-iso improved model fit. This again suggests that postnatal confounders can alter the health trajectory of youth after the initial period of prenatal programming.

Longitudinal, observational studies have many strengths, built into their study design. Repeated maternal urinary BPA and Med diet adherence measures allowed comparison of exposures across all three trimesters of pregnancy. The trimester-specific measures allowed evaluation of critical windows of susceptibility to each exposure; this proved insightful, since Trimester 1 and 2 BPA exerted a greater effect, while only Trimester 3 MDS was predictive of youth health outcomes. All predictive models were adjusted for youth BPA and MDS to account for current exposures. The suggestive contribution of youth MDS, especially among boys, to youth metabolic health outcomes, supports the theory that postnatal diet may alter youth health

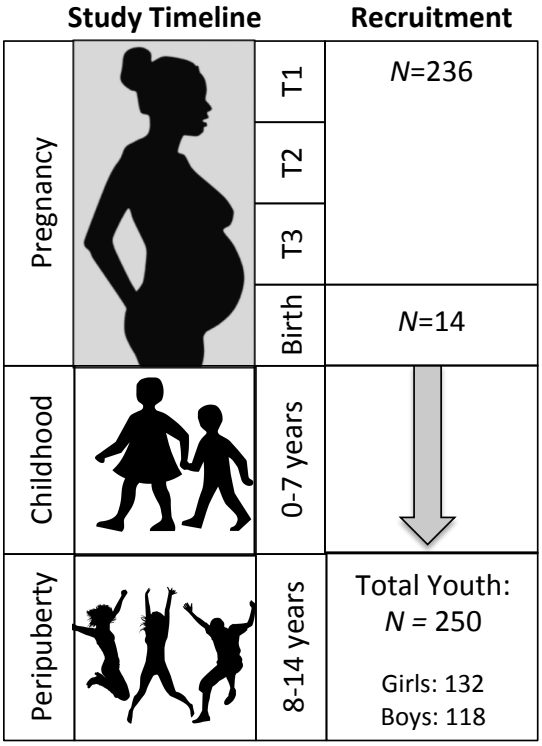
risk. Thus, although prenatal programming may alter health trajectory, this study suggests the peripubertal period may provide a chance to alter that trajectory again. Examining peripubertal health effects in both girls and boys allowed sex-specific comparisons. BPA is known to have sexually-dimorphic effects on offspring, so sex-stratified analyses allowed an investigation into potential differences in metabolic programming.

Limitations of this study include the spot sample urine collection for BPA measurement and the cross-sectional measure of health outcomes. Urinary BPA concentrations vary by recent exposure; thus, a single urine sample may not be representative of a subject's regular BPA level. However, averaging BPA measures across the three trimesters of pregnancy may have reduced this potential inaccuracy. The fact that pregnancy average models often reflected the suggestive results in T2 models, supports the validity of these findings. Health outcome measures at a single time point in adolescence cannot provide insights into possible alterations metabolic health over time. Comparison of offspring metabolic health measures from birth through puberty would be more instructive for determining if prenatal exposures impact offspring health at specific times, or if trajectory of MRS and/or serum 8-iso changes over time based on prenatal BPA exposure.

Contradictory trends in youth metabolic health suggest the causes, prevention, and treatment strategies are complex. Understanding the contribution that the *in utero* environment plays on youth metabolic health is critical to improve future interventions. Results from this study suggest a sexual-dimorphism in the impact of both prenatal BPA and diet exposures.. The significant impact of puberty on MRS reinforces the knowledge that the pubertal transition is a sensitive period for potential metabolic disease development. This sensitive life stage may be a second chance to "reprogram" future health risks. Children whose metabolic disease

susceptibility was increased due to perinatal exposures, may be able to lower their risk through conscientious health decisions during puberty.

Figure 4.1. Study Participant Recruitment and Follow-Up Timeline



This study followed 250 mother-child dyads. Most (n=236) of the mothers were recruited in Trimester 1 of pregnancy, but 14 mothers were recruited at delivery. Thus, prenatal exposure measures (urinary BPA and diet intake) were not measured in these 14 mothers. Youth were re-recruited during the peripubertal transition (8-14 years). Adolescent metabolic health was assessed via Metabolic Risk Score and serum 8-isoprostane, among 132 girls and 118 boys.

Table 4.1 Characteristics of the ELEMENT Study Population (n=250)

Maternal Characteristics		
Variable	**N**	**%**
Education (years)		
≤ 9	89	35.6
10 – 12	126	50.4
> 12	35	14.0
Delivery Type		
Vaginal Birth	149	59.6
C-section	99	39.6
Missing Data	2	
Trimester 1 BMI (kg/m^2)		
<18.5	5	2.0
≥18.5 to <25.0	100	40.0
≥25.0 to <30.0	83	33.2
≥30.0 to <35.0	29	11.6
≥ 35.0	6	2.4
Missing Data	27	10.8

Adolescent Characteristics		
Variable	**N**	**%**
Sex		
Female	132	52.8
Male	118	47.2
Pubertal Status: Females		
Pre-Pubertal	86	65.2
Pubertal	46	34.8
Pubertal Status: Males		
Pre-Pubertal	60	50.8
Pubertal	58	49.2
Adolescent BMI (kg/ m^2)		
<18.5	118	47.2
≥18.5 to <25.0	114	45.6
≥25.0 to <30.0	15	6.0
≥30.0 to <35.0	3	1.2
≥ 35.0	0	0.0

Maternal Characteristics				
Variable	**N**	**Mean**	**SD**	**(Range)**
Age at Recruitment (years)	250	26.82	5.63	(14.00 – 44.00)
Breastfeeding (weeks)	250	8.10	5.86	(0.00 – 30.00)
Trimester 1 BMI (kg/m^2)	223	25.85	3.95	(17.26 – 40.48)
Pregnancy Weight Gain (kg)	215	2.59	3.75	(-10.00 – 18.70)
Adolescent Characteristics				
Variable	**N**	**Mean**	**SD**	**(Range)**
Age at Clinic Visit (years)	250	10.32	1.67	(8.10 – 14.70)
Adolescent BMI (kg/m^2)	250	19.38	3.60	(13.33 – 33.38)
Serum Leptin (ng/mL)	248	11.25	8.98	(1.40 – 62.20)
Serum IGF-1 (ng/mL)	248	257.07	104.54	(92.90 – 606.00)

Table 4.2 Distribution of Adolescent Metabolic Risk Score (-2 to 2) across background characteristics of ELEMENT mother-child dyads.

Variable	All Youth (n=250)				Girls (n=132)				Boys (n=118)			
	N	Mean	SD	*P**	*N*	Mean	SD	*P**	*N*	Mean	SD	*P**
Maternal Characteristics												
Age at Pregnancy												
15-24 years	101	0.005	0.625	0.648	51	0.020	0.594	0.667	50	-0.011	0.661	0.881
25-34 years	117	-0.026	0.595		60	-0.044	0.546		57	-0.007	0.646	
35-44 years	30	0.092	0.692		20	0.088	0.764		10	0.099	0.556	
Education												
< 10 years	88	0.035	0.645	0.735	50	0.034	0.611	0.847	38	0.036	0.696	0.619
10-12 years	125	-0.030	0.596		64	-0.009	0.578		61	-0.051	0.617	
> 12 years	35	0.025	0.638		17	-0.058	0.670		18	0.103	0.616	
Trimester 1 BMI												
< 18	1	-0.525	.	0.578	0	.	.	0.461	1	-0.525	.	0.352
18-24.9	103	-0.052	0.634		48	-0.124	0.537		55	0.012	0.707	
25 - 29.9	84	0.016	0.632		51	0.099	0.639		33	-0.113	0.608	
30 - 34.9	25	-0.002	0.551		15	0.047	0.612		10	-0.076	0.464	
≥ 35	8	0.287	0.867		3	-0.066	1.397		5	0.499	0.437	
Gestational Weight Gain												
< 6 kg	48	0.029	0.639	0.622	28	0.063	0.649	**0.039**	20	-0.020	0.638	0.170
6 - 8.5 kg	66	-0.008	0.629		34	-0.195	0.541		32	0.191	0.662	
>8.5 - 11 kg	55	-0.105	0.549		28	-0.096	0.598		27	-0.114	0.504	
>11 kg	52	0.050	0.714		27	0.264	0.626		25	-0.181	0.743	

Table 4.2 Distribution of Adolescent Metabolic Risk Score (-2 to 2) across background characteristics of ELEMENT mother-child dyads, Continued

Variable	All Youth (n=250)				Girls (n=132)				Boys (n=118)			
	N	Mean	SD	*P**	*N*	Mean	SD	*P**	*N*	Mean	SD	*P**
Youth Characteristics												
Age at Re-enrollment												
8-10	172	-0.050	0.651	**0.036**	93	-0.021	0.661	0.427	79	-0.083	0.641	**0.044**
11-12	54	0.039	0.528		25	-0.024	0.408		29	0.093	0.616	
13-14	22	0.303	0.464		13	0.208	0.392		9	0.440	0.546	
Pubertal Status**												
Prepubertal	144	-0.116	0.617	**0.000**	85	-0.115	0.632	**0.001**	59	-0.117	0.601	**0.046**
Pubertal	104	0.162	0.584		46	0.215	0.469		58	0.120	0.663	
Serum Leptin												
<5	62	-0.512	0.411	**0.000**	18	-0.700	0.329	**0.000**	44	-0.435	0.419	**0.000**
5 - 8	64	-0.135	0.495		30	-0.255	0.388		34	-0.030	0.557	
>8 - 15	60	0.129	0.519		39	0.014	0.509		21	0.343	0.478	
> 15	62	0.529	0.527		44	0.450	0.503		18	0.722	0.546	
Physical Activity (METS)												
< 17	62	-0.021	0.723	0.828	35	-0.060	0.676	0.583	27	0.030	0.789	0.993
17 - 26	62	-0.045	0.596		38	-0.060	0.623		24	-0.022	0.563	
>26 - 40	58	0.054	0.628		27	0.122	0.606		31	-0.005	0.651	
> 40	66	0.018	0.525		31	0.039	0.465		35	-0.001	0.579	

*p-values represent significance from the Wald test for linear trend, which uses an ordinal indicator entered into a model as a continuous variable. For binary variables, p-value is a measure of significance from an Independent *t*-test.
** Sex-specific prepubertal (≤1) and pubertal (>1) status was determined by physician-assessed Tanner staging for boys (genital or pubic hair development) and girls (breast or pubic hair development).

Table 4.3 Distribution of Exposure Variables from Prenatal and Adolescent Exposure Periods

Urinary BPA (ng/mL), specific-gravity adjusted								
Analyte	***N***	**GM**	**GSD**	**Min.**	**25%**	**50%**	**75%**	**Max.**
Maternal Measures								
Pregnancy Average	229	1.13	2.11	0.00	0.70	1.08	1.80	9.16
Trimester 1	198	1.57	2.22	0.23	0.87	1.48	2.76	23.40
Trimester 2	200	1.66	2.14	0.24	1.04	1.46	2.50	30.29
Trimester 3	225	1.28	1.99	0.31	0.80	1.14	1.83	23.38
Youth Measures								
8-14 Years	242	1.63	2.16	0.40	0.92	1.40	2.57	27.67
Mediterranean Diet Score (0-8)								
Analyte	***N***	**Mean**	**SD**	**Min.**	**25%**	**50%**	**75%**	**Max.**
Maternal Measures								
Pregnancy Average	236	4.71	1.15	1.67	4.00	4.67	5.33	7.67
Trimester 1	227	4.68	1.54	2	4	5	6	8
Trimester 2	234	4.65	1.46	1	4	5	6	8
Trimester 3	235	4.77	1.55	0	4	5	6	8
Youth Measures								
8-14 Years	250	3.76	1.47	0	3	4	5	8

Table 4.4 Percent Change in Youth Metabolic Risk Score per 1-Unit Urinary Ln-BPA Increase

Pregnancy Timing	Adolescent Metabolic Risk Score											
	Model 1: Unadjusted				Model 2: *A priori* Covariates				Model 3: Predictor Covariates			
	N	BPA % Δ	MDS % Δ	BPA*MDS % Δ	N	BPA % Δ	MDS % Δ	BPA*MDS % Δ	N	BPA % Δ	MDS % Δ	BPA*MDS % Δ
All Youth												
Average	225	**-0.376[b]**	0.044	0.066	218	-0.259	0.043	0.041	206	**-0.359[c]**	0.041	**0.065[c]**
Trimester 1	196	-0.101	0.000	0.009	189	-0.086	0.000	0.006	185	-0.095	0.001	0.016
Trimester 2	197	**-0.324[c]**	-0.013	**0.066[c]**	190	**-0.324[c]**	-0.007	**0.062[c]**	182	**-0.287[c]**	0.007	0.055
Trimester 3	222	-0.139	0.032	0.016	215	-0.066	0.031	0.00	206	-0.138	0.025	0.015
Girls												
Average	119	-0.262	**0.071[c]**	0.038	116	-0.181	0.043	0.025	109	-0.215	0.047	0.029
Trimester 1	106	-0.186	0.011	0.032	103	-0.215	-0.005	0.043	103	-0.221	0.006	0.045
Trimester 2	107	-0.113	0.047	0.012	104	-0.262	0.007	0.041	98	-0.179	0.033	0.016
Trimester 3	115	0.278	0.060	-0.058	112	0.391	0.044	**-0.080[c]**	107	0.337	0.042	-0.078
Boys												
Average	106	**-0.599[c]**	0.013	**0.118[c]**	102	-0.400	0.047	0.066	97	-0.581	0.038	0.109
Trimester 1	90	0.174	-0.012	-0.059	86	0.436	0.024	**-0.132[c]**	85	0.373	0.023	**-0.109[c]**
Trimester 2	90	-0.043	-0.002	**0.017[c]**	86	**-0.728[b]**	-0.025	**0.152[b]**	84	**-0.629[c]**	-0.015	**0.136[c]**
Trimester 3	107	**-0.422[c]**	0.006	0.070	103	-0.286	0.032	0.040	99	-0.382	0.024	0.057

Significance: [a]p < 0.05, [b]p < 0.10, [c]p < 0.20

Model 1: Adolescent serum 8-isoprostane ~ Maternal, trimester-specific urinary, specific-gravity adjusted BPA

Model 2: additionally adjusted for: maternal education (grade), youth pubertal status (Yes/No), adolescent urinary BPA and Mediterranean diet score adherence (0-8).

Model 3: Model 2 + additionally adjusted for maternal T1 BMI (kg/m^2), and adolescent vigorous physical activity (hours/week).

Table 4.5 Percent Change in Youth Serum 8-isoprostane per 1-Unit Urinary Ln-BPA Increase

Pregnancy Timing	Adolescent Serum 8-isoprostane											
	Model 1: Unadjusted				Model 2: *A priori* Covariates				Model 3: Predictor Covariates			
	N	BPA % Δ	MDS % Δ	BPA*MDS % Δ	N	BPA % Δ	MDS % Δ	BPA*MDS % Δ	N	BPA % Δ	MDS % Δ	BPA*MDS % Δ
All Youth												
Average	219	**0.192**[c]	0.000	**-0.035**[c]	212	0.157	-0.018	-0.025	200	**0.168**[c]	-0.011	-0.028
Trimester 1	192	**0.162**[c]	-0.002	-0.024	185	0.079	-0.019	-0.004	184	0.080	-0.019	-0.005
Trimester 2	186	**0.114**[c]	0.011	-0.009	185	**0.203**[b]	0.009	-0.025	177	**0.201**[b]	0.011	-0.023
Trimester 3	216	0.054	0.010	0.001	209	0.078	-0.001	-0.001	200	0.077	0.004	-0.001
Girls												
Average	115	**0.212**[c]	**0.038**[c]	**-0.043**[c]	112	**0.218**[c]	**0.034**[c]	**-0.044**[c]	105	**0.197**[c]	**0.042**[c]	-0.037
Trimester 1	103	-0.040	0.005	0.008	100	-0.089	0.004	0.019	100	-0.090	0.002	0.019
Trimester 2	103	**0.239**[b]	**0.066**[a]	**-0.048**[b]	100	**0.237**[b]	**0.064**[a]	**-0.046**[b]	94	**0.240**[b]	**0.065**[a]	**-0.044**[b]
Trimester 3	111	0.205	**0.034**[b]	-0.034	108	0.186	0.024	-0.027	103	0.172	**0.029**[c]	-0.023
Boys												
Average	104	0.249	**-0.055**[c]	-0.043	100	0.126	**-0.103**[a]	-0.008	95	0.150	**-0.102**[a]	-0.016
Trimester 1	89	**0.544**[a]	-0.011	**-0.092**[a]	85	**0.381**[c]	-0.041	-0.052	84	**0.394**[c]	-0.041	-0.055
Trimester 2	89	0.084	-0.027	0.009	85	0.228	**-0.056**[c]	-0.011	83	0.218	**-0.052**[c]	-0.007
Trimester 3	105	-0.089[c]	-0.014	0.038	101	-0.034	-0.033	0.029	97	-0.020	-0.033	0.029

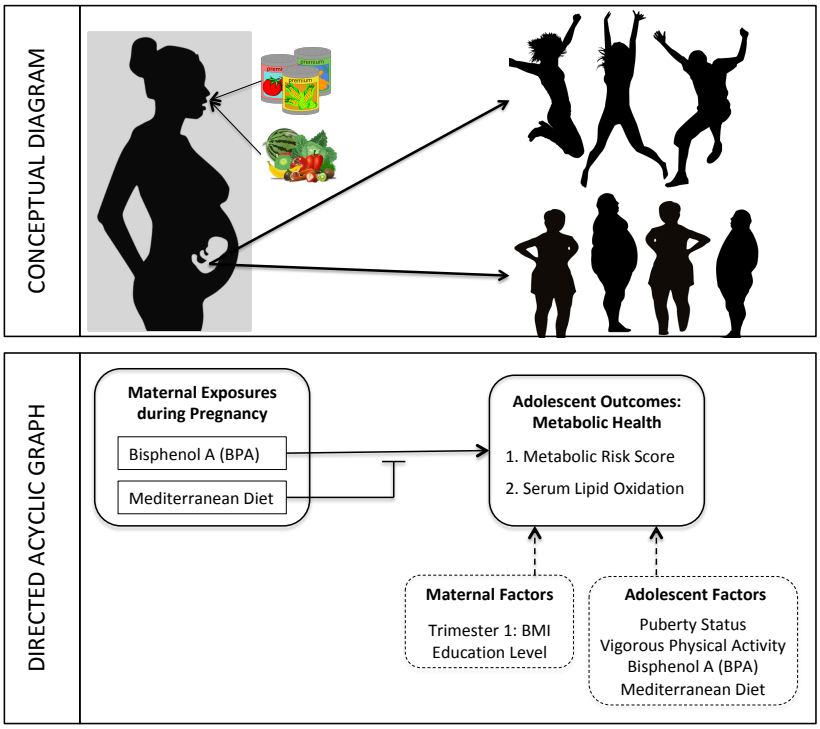
Significance: [a]p < 0.05, [b]p < 0.10, [c]p < 0.20

Model 1: Adolescent serum 8-isoprostane ~ Maternal, trimester-specific urinary, specific-gravity adjusted BPA

Model 2: additionally adjusted for: maternal education (grade), youth pubertal status (Yes/No), adolescent urinary BPA and Mediterranean diet score adherence (0-8).

Model 3: Model 2 + additionally adjusted for maternal T1 BMI (kg/m^2), and adolescent vigorous physical activity (hours/week).

Figure 4.2 Impact of Prenatal BPA and Mediterranean Diet Exposure on Adolescent Metabolic Health



This analysis investigated the potential for prenatal programming of adolescent metabolic health within the ELEMENT human birth cohort. Maternal exposure to BPA and adherence to a Mediterranean diet (MDS) during pregnancy significantly altered adolescent serum lipid oxidation, but not Metabolic Risk Score. In addition to diet's direct effect, maternal MDS also modified the effect of prenatal BPA on lipid oxidation. Maternal T1 BMI, youth puberty status and vigorous physical activity also impacted adolescent metabolic health

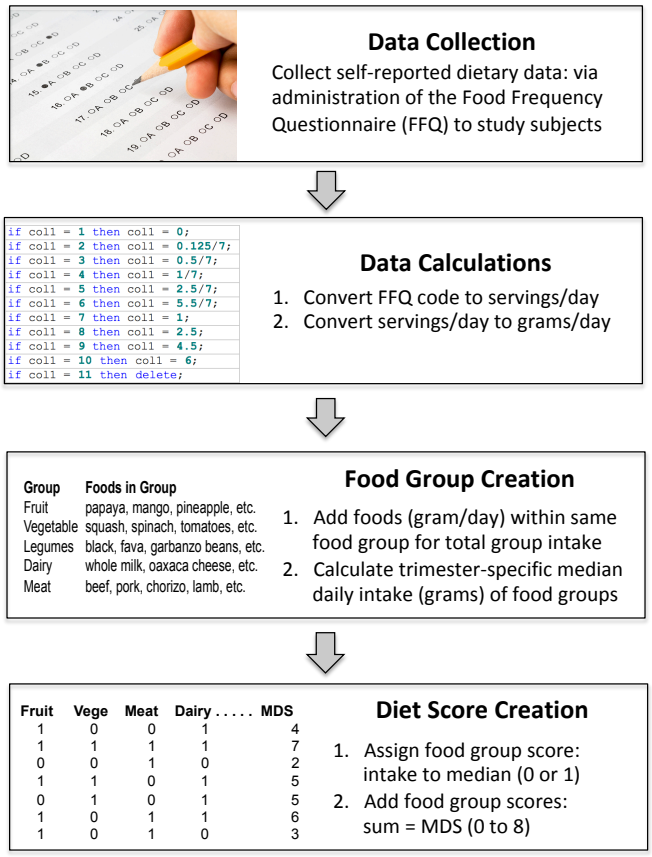
Supplemental Information

Table S4.1 Mediterranean Diet Score Calculations

Food Group	Median Daily Intake (grams)			Scoring	
	T1	T2	T3	> Median	< Median
Beneficial Foods					
Fish	44.36	44.36	41.04	1	0
Legumes	131.50	135.07	135.07	1	0
Fruits & Nuts	1214.80	1350.19	1074.34	1	0
Vegetables	799.66	722.37	667.86	1	0
Whole Grains	12.95	15.27	12.95	1	0
Detrimental Foods					
Meat	218.41	205.96	204.52	0	1
Poultry	78.57	78.57	78.57	0	1
Dairy	1102.68	1280.71	1268.00	0	1

To compute the trimester-specific Mediterranean Diet Score (MDS) for each ELEMENT mother in this study, the median daily intake (grams) was calculated for each of the eight food groups: fish, legumes, fruits & nuts, vegetables, whole grains, meat, poultry, and dairy. For beneficial foods, above median consumption was scored as '1'; below median intake of detrimental foods was scored '1'. Thus, MDSs ranged from 0-8 depending on adherence to the diet; greater adherence received a higher score, while poor adherence warranted a lower score.

Figure S4.1 Mediterranean Diet Score Calculation Workflow



Self-reported dietary data was collected via food frequency questionnaire (FFQ). Mothers completed an FFQ at a clinic visit during each trimester of pregnancy. FFQ responses corresponded to food intake in servings/day quantities. Intake of each food was then converted from servings/day to grams/day. Gram/day intake, of foods within each food group, was summed (e.g. apples, bananas, oranges, etc. were added to create a total Fruit intake value). Median gram/day quantities were computed for each of the eight food groups. Consumption of each food group was compared to the group median and scored (0 or 1) based on whether the food was beneficial or detrimental. Scores for all eight food groups were summed to create the Mediterranean Diet Score (MDS). MDS values ranged from 0 to 8.

Table S4.2 Sex-Stratification of Prenatal Exposures, Youth Health Outcomes, and Study Covariates

	Males				Females				Between[a] Sex p-value
	N	Median	IQR	Range	*N*	Median	IQR	Range	
Exposures									
T1_BPA_grav (ng/mL)	92	1.46	0.81 - 2.13	0.23 - 10.47	107	1.60	0.89 - 3.10	0.40 - 23.40	**0.059***
T2_BPA_grav (ng/mL)	91	1.33	0.94 - 2.00	0.24 - 12.21	109	1.57	1.11 - 2.67	0.57 - 17.60	0.111
T3_BPA_grav (ng/mL)	108	1.00	0.72 - 1.66	0.31 - 12.81	117	1.36	0.92 - 2.14	0.47 - 23.38	0.111
P20_BPA_grav (ng/mL)	113	1.37	0.90 - 2.43	0.40 - 27.67	129	1.46	0.94 - 2.81	0.42 - 20.44	0.545
T1_MDS (score: 0-8)	107	4.00	3.00 - 5.00	0.00 - 8.00	120	4.00	2.00 - 5.00	1.00 - 8.00	0.642
T2_MDS (score: 0-8)	109	4.00	3.00 - 5.00	1.00 - 8.00	125	4.00	3.00 - 5.00	0.00 - 8.00	0.412
T3_MDS (score: 0-8)	110	4.00	3.00 - 5.00	0.00 - 7.00	125	4.00	3.00 - 5.00	0.00 - 8.00	0.909
P20_MDS (score: 0-8)	118	4.00	3.00 - 5.00	1.00 - 7.00	132	4.00	3.00 - 5.00	0.00 - 8.00	0.277
Health Outcomes									
Serum 8-isoprostane (pg/mL)	115	478.91	365.46 - 614.06	202.80 - 1364.78	127	469.26	391.21 - 571.98	203.96 - 1044.43	0.552
Metabolic Risk Score (-2 to 2)	117	-0.01	-0.41 to 0.34	-1.67 to 1.99	131	-0.03	-0.44 to 0.44	-1.29 to 1.47	0.997
Maternal Covariates									
Highest Education (years)	118	12.00	9.00 - 12.00	3.00 - 20.00	132	11.00	9.00 - 12.00	2.00 - 21.00	0.256
Age at Pregnancy (years)	118	26.00	23.00 - 30.00	16.00 - 44.00	132	26.50	23.00 - 31.00	14.00 - 40.00	0.650
Pre-Pregnancy BMI (kg/m^2)	105	24.69	22.82 - 28.34	17.26 - 38.45	118	25.98	23.61 - 27.92	18.00 - 40.48	0.270
Pregnancy Weight Gain (kg)	102	3.00	0.98 - 5.00	-10.00 to 13.50	113	2.20	0.35 - 4.00	-9.90 to 18.70	0.611

Table S4.2 Sex-Stratification of Prenatal Exposures, Youth Health Outcomes, and Study Covariates, Continued

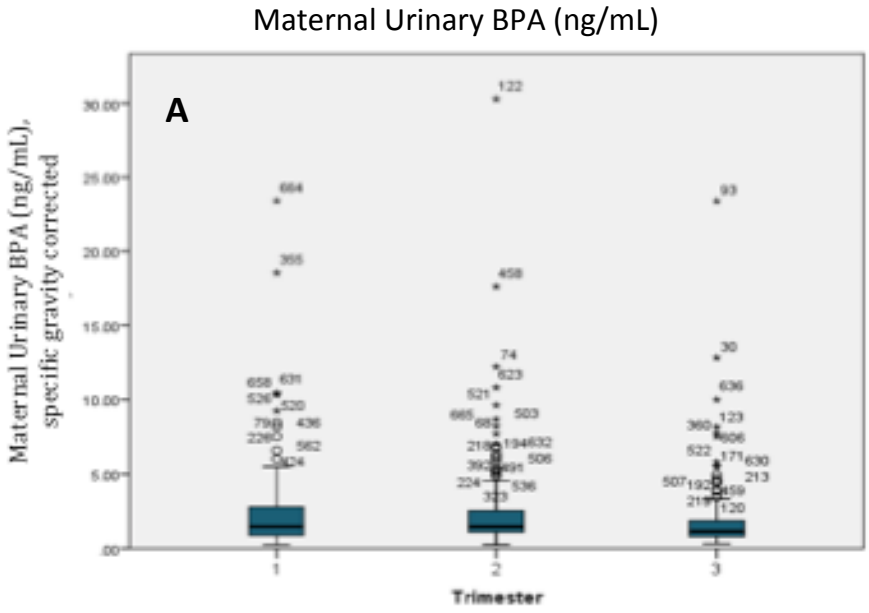
	Males				Females				Between[a] Sex p-value
	N	Median	IQR	Range	*N*	Median	IQR	Range	
Adolescent Covariates									
Age (years)	118	9.95	8.98 - 11.73	8.10 - 14.40	132	9.95	8.80 - 11.88	8.10 - 14.70	0.820
BMI (kg/m^2)	118	18.78	16.79 - 20.87	14.06 - 31.44	132	19.01	16.64 - 22.18	13.33 - 33.38	0.180
Waist Circumference (cm)	118	68.4	62.19 - 76.49	50.10 - 95.60	132	69.93	64.43 - 79.13	50.50 - 111.00	0.123
Systolic Blood Pressure (mmHg)	118	104.5	96.75 - 111.00	79.00 - 127.00	132	101	91.00 - 110.00	79.00 - 123.00	**0.016****
Diastolic Blood Pressure (mmHg)	118	67	61.00 - 71.00	49.00 - 83.00	132	67	60.00 - 70.00	44.00 - 82.00	0.847
Serum Glucose (mg/dL)	117	88	83.00 - 93.50	49.00 - 63.00	131	86	80.00 - 91.00	58.00 - 146.00	0.185
Serum HDL (mg/dL)	117	60	51.50 - 67.00	33.00 - 98.00	131	57	50.00 - 64.00	31.00 - 104.00	0.118
Serum Triglycerides (mg/dL)	117	70	53.00 - 90.50	21.00 - 245.00	131	81	65.00 - 121.00	35.00 - 269.00	**0.000****
Serum Leptin (ng/mL)	117	6.5	3.60 - 10.80	1.40 - 34.20	131	10.7	6.30 - 18.50	2.40 - 62.20	**0.000****
Serum IGF-1	117	206	165.50 - 270.00	92.90 - 568.00	131	250	198.00 - 359.00	102.00 - 606.00	**0.000****

[a] Independent t-tests were used to compare each variable by youth sex.
P-values that reflect significant differences by sex are bolded: * $p < 0.10$, ** $p < 0.05$

Table S4.3 Comparison of Prenatal Exposures (BPA & MDS) Across Trimesters

Intraclass Correlation Coefficient (ICC) between Trimesters									
	Trimester 1 (T1)			Trimester 2 (T2)			Trimester 3 (T3)		
	N	Coefficient	(95% CI)	N	Coefficient	(95% CI)	N	Coefficient	(95% CI)
Urinary BPA (ng/mL), SG-adjusted	183	0.24	(0.10-0.37)	196	0.32	(0.19-0.44)	196	0.12	(-0.02-0.26)
MDS	210	0.11	(-0.03-0.24)	233	0.44	(0.33-0.54)	211	-0.03	(-0.17-0.10)

Figure S4.2 Trimester-Specific Distributions of Prenatal Exposures: Urinary BPA Levels and Mediterranean Diet Score



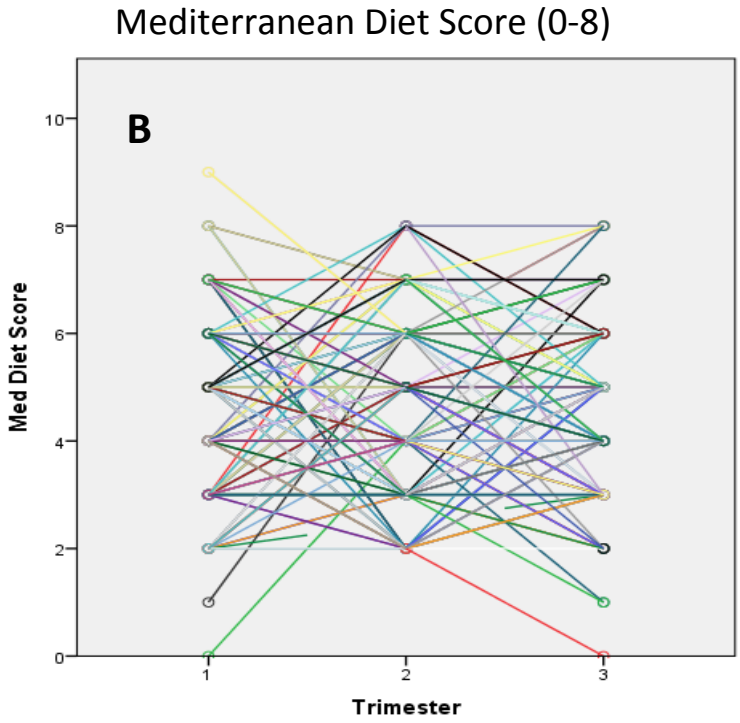


Table S4.4 Distribution of Adolescent Serum 8-isoprostane (pg/mL) across background characteristics of ELEMENT mother-child dyads.

Variable	All Youth (n=250)				Girls (n=132)				Boys (n=118)			
	N	Mean	SD	*P**	*N*	Mean	SD	*P**	*N*	Mean	SD	*P**
Maternal Characteristics												
Age at Pregnancy												
15-24 years	98	523.7	188.4	0.236	48	500.5	151.3	0.798	50	546.1	217.4	0.191
25-34 years	114	492.4	172.7		59	499.3	167.1		55	484.9	179.7	
35-44 years	30	468.9	140.8		20	473.8	152.2		10	459.1	121.9	
Education												
< 10 years	85	506.1	198.7	0.514	48	494.4	174.8	0.989	37	521.4	227.5	0.376
10-12 years	123	508.4	169.7		63	497.7	153.6		60	519.6	185.8	
> 12 years	34	469.8	135.7		16	492.1	128.6		18	450.1	142.3	
Trimester 1 BMI												
< 18	1	444.7	.	0.987	0	.	.	0.937	1	444.7	.	0.976
18-24.9	100	503.6	198.4		47	496.3	159.2		53	510.1	229.0	
25 - 29.9	81	509.4	170.5		48	506.1	174.8		33	514.3	166.6	
30 - 34.9	25	489.7	135.0		15	490.3	125.0		10	488.9	155.7	
$\geq$ 35	8	472.5	166.6		3	501.1	200.8		5	455.4	165.6	
Gestational Weight Gain												
< 6 kg	48	495.4	159.0	0.534	28	486.7	125.5	0.677	20	507.7	199.6	0.176
6 - 8.5 kg	63	517.1	180.9		32	479.9	157.4		31	555.6	197.6	
>8.5 - 11 kg	53	524.5	219.2		27	528.8	203.0		26	520.1	239.0	
>11 kg	51	469.4	145.5		26	508.4	154.7		25	428.8	125.8	

Table S4.4 Distribution of Adolescent Serum 8-isoprostane (pg/mL) across background characteristics of ELEMENT mother-child dyads, Continued

Variable	All Youth (n=250)				Girls (n=132)				Boys (n=118)			
	N	Mean	SD	*P**	*N*	Mean	SD	*P**	*N*	Mean	SD	*P**
Youth Characteristics												
Age at Re-enrollment												
8-10	168	501.3	181.4	0.312	90	498.6	157.9	0.277	78	504.4	206.2	0.794
11-12	53	523.8	167.9		25	517.1	165.5		28	529.7	172.9	
13-14	21	454.5	149.0		12	429.7	137.3		9	487.5	165.7	
Pubertal Status**												
Prepubertal	141	503.2	191.9	0.913	84	500.3	165.5	0.651	57	507.4	226.7	0.921
Pubertal	100	500.7	152.4		43	486.8	143.7		58	511.1	159.0	
Serum Leptin												
<5	59	483.7	169.5	0.284	17	475.7	183.2	0.411	42	486.9	165.8	0.641
5 - 8	64	534.8	200.9		30	524.1	144.3		34	544.3	242.0	
>8 - 15	60	480.5	166.1		39	466.9	143.7		21	505.6	202.8	
> 15	59	507.4	162.1		41	510.7	169.4		18	499.7	148.2	
Physical Activity (METS)												
< 17	60	518.8	167.2	0.742	34	548.6	176.4	0.150	26	479.8	148.6	0.726
17 - 26	60	494.1	198.9		36	482.5	161.4		24	511.6	247.5	
>26 - 40	57	510.2	181.9		26	476.4	131.4		31	538.7	213.4	
> 40	65	487.1	158.3		31	469.3	146.5		34	503.3	168.9	

*p-values represent significance from the Wald test for linear trend, which uses an ordinal indicator entered into a model as a continuous variable. For binary variables, p-value is a measure of significance from an Independent *t*-test.
** Sex-specific prepubertal (≤1) and pubertal (>1) status was determined by physician-assessed Tanner staging for boys (genital or pubic hair development) and girls (breast or pubic hair development).

REFERENCES

1. Reynolds CM, Gray C, Li M, Segovia SA, Vickers MH. Early Life Nutrition and Energy Balance Disorders in Offspring in Later Life. Nutrients. 2015;7: 8090–8111.

2. Rinaudo P, Wang E. Fetal Programming and Metabolic Syndrome. Annu Rev Physiol. 2012;74: 107–130.

3. Padmanabhan V, Cardoso RC, Puttabyatappa M. Developmental Programming , a Pathway to Disease. Endocrinology. 2016;157: 1328–1340.

4. Smith CJ, Ryckman KK. Epigenetic and Developmental Influences on the Risk of Obesity, Diabetes, and Metabolic Syndrome. Diabetes, Metab Syndr Obes Targets Ther. 2015;8: 295–302.

5. Miranda-Lora AL, Vilchis-Gil J, Molina-Dıaz M, Flores-Huerta S, Klunder-Klunder M. Heritability, Parental Transmission and Environment Correlation of Pediatric-Onset Type 2 Diabetes Mellitus and Metabolic Syndrome-Related Traits. Diabetes Res Clin Pract. 2017;126: 151–159.

6. McMillen IC, Robinson JS. Developmental Origins of the Metabolic Syndrome: Prediction, Plasticity, and Programming. Physiol Rev. 2005;85: 571–633.

7. Heerwagen MJR, Miller MR, Barbour LA, Friedman JE. Maternal Obesity and Fetal Metabolic Programming: A Fertile Epigenetic Soil. Am J Physiol Regul Integr Comp Physiol. 2010;299: R711–R722.

8. Zambrano E, Ibanez C, Martinez-Samoya PM, Lomas-Soria C, Durand-Carbajal M, Rodriguez-Gonzalez GL. Maternal Obesity: Lifelong Metabolic Outcomes for Offspring from Poor Developmental Trajectories During the Perinatal Period. Arch Med Res. 2016;47: 1–12.

9. Lakshmy R. Metabolic Syndrome: Role of Maternal Undernutrition and Fetal Programming. Rev Endocr Metab Disord. 2013;14: 229–240.

10. Correia-Branco A, Keating E, Martel F. Maternal Undernutrition and Fetal Developmental Programming of Obesity: The Glucocorticoid Connection. Reprod Sci. 2015;22: 138–145.

11. Seckl JR, Holmes MC. Mechanisms of Disease: Glucocorticoids, Their Placental Metabolism and Fetal “Programming” of Adult Pathophysiology. Nat Clin Pr Endocrinol Metab. 2007;3: 479–488.

12. Heindel JJ, Blumberg B, Cave M, Machtinger R, Mantovani A, Mendez MA, et al. Metabolism Disrupting Chemicals and Metabolic Disorders. Reprod Toxicol. Elsevier Inc.; 2017;68: 3–33.

13. Grandjean P, Barouki R, Bellinger DC, Casteleyn L, Chadwick LH, Cordier S, et al. Life-Long Implications of Developmental Exposure to Environmental Stressors : New

Perspectives. Endocrinology. 2015;156: 3408–3415.

14. Perera F, Herbstman J. Prenatal Environmental Exposures, Epigenetics, and Disease. Reprod Toxicol. Elsevier Inc.; 2011;31: 363–373.

15. Stel J, Legler J. The Role of Epigenetics in the Latent Effects of Early Life Exposure to Obesogenic Endocrine Disrupting Chemicals. Endocrinology. 2015;156: 3466–3472.

16. Dehghan M, Akhtar-Danesh N, Merchant AT. Childhood Obesity, Prevalence and Prevention. Nutr J. 2005;4: 1–8.

17. Ebbeling CB, Pawlak DB, Ludwig DS. Childhood Obesity: Public-Health Crisis, Common Sense Cure. Lancet. 2002;360: 473–482.

18. Ford ES, Ajani UA, Mokdad AH. The Metabolic Syndrome and Concentrations of C-Reactive Protein. Diabetes Care. 2005;28: 878–881.

19. Lee AM, Gurka MJ, Deboer MD. Correlation of Metabolic Syndrome Severity with Cardiovascular Health Markers in Adolescents. Metabolism. Elsevier Inc.; 2017;69: 87–95.

20. Morrison JA, Friedman LA, Wang P, Glueck CJ. Metabolic Syndrome in Childhood Predicts Adult Metabolic Syndrome and Type 2 Diabetes Mellitus 25 to 30 Years Later. J Pediatr. 2008;152: 201–206.

21. Veiga-Lopez A, Kannan K, Liao C, Ye W, Domino SE, Padmanabhan V. Gender-Specific Effects on Gestational Length and Birth Weight by Early Pregnancy BPA Exposure. J Clin Endocrinol Metab. 2015;100: E1394–E1403.

22. Bae S, Lim YH, Lee YA, Shin CH, Oh SY, Hong YC. Maternal Urinary Bisphenol A Concentration during Midterm Pregnancy and Children's Blood Pressure at Age 4. Hypertension. 2017;69: 367–374.

23. Angle BM, Phuong Do R, Ponzi D, Stahlhut RW, Drury BE, Nagel SC, et al. Metabolic Disruption in Male Mice due to Fetal Exposure to Low but Not High Doses of Bisphenol A (BPA): Evidence for Effects on Body Weight, Food Intake, Adipocytes, Leptin, Adiponectin, Insulin and Glucose Regulation. Reprod Toxicol. Elsevier Inc.; 2013;42: 256–268.

24. Ryan KK, Haller AM, Sorrell JE, Woods SC, Jandacek RJ, Seeley RJ. Perinatal exposure to bisphenol-A and the development of metabolic syndrome in CD-1 mice. Endocrinology. 2010;151: 2603–2612.

25. Veiga-lopez A, Moeller J, Sreedharan R, Singer K, Lumeng XC, Ye W, et al. Developmental Programming: Interaction between Prenatal BPA Exposure and Postnatal Adiposity on Metabolic Variables in Female Sheep. Am J Physiol Endocrinol Metab. 2016;310: 238–247.

26. Padmanabhan V, Siefert K, Ransom S, Johnson T, Pinkerton J, Anderson L, et al. Maternal Bisphenol-A Levels at Delivery: A Looming Problem? J Perinatol. 2008;28:

258–263.

27. Woodruff TJ, Zota AR, Schwartz JM. Environmental Chemicals in Pregnant Women in the United States: NHANES 2003–2004. Environ Health Perspect. 2011;119: 878–885.

28. Nahar MS, Liao C, Kannan K, Harris C, Dolinoy DC. In Utero Bisphenol A Concentration, Metabolism, and Global DNA Methylation across Matched Placenta, Kidney, and Liver in the Human Fetus. Chemosphere. 2015;124: 54–60.

29. Balakrishnan B, Henare K, Thorstensen EB, Ponnampalam AP, Mitchell MD. Transfer of Bisphenol A across the Human Placenta. Am J Obstet Gynecol. Elsevier Inc.; 2010;202: 393.e1-393.e7.

30. Nahar MS, Liao C, Kannan K, Dolinoy DC. Fetal Liver Bisphenol A Concentrations and Biotransformation Gene Expression Reveal Variable Exposure and Altered Capacity for Metabolism in Humans. 2013;27: 116–123.

31. Rudel RA, Gray JM, Engel CL, Rawsthorne TW, Dodson RE, Ackerman JM, et al. Food Packaging and Bisphenol A and Bis(2-Ethyhexyl) Phthalate Exposure: Findings from a Dietary Intervention. Env Heal Perpsect. 2011;119: 914–920.

32. Schecter A, Malik N, Haffner D, Smith S, Harris TR, Paepke O, et al. Bisphenol A (BPA) in U.S. Food. Env Sci Technol. 2010;44: 9425–9430.

33. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons W V. Human Exposure to Bisphenol A (BPA). Reprod Toxicol. 2007;24: 139–177.

34. García-Fernández E, Rico-Cabanas L, Estruch R, Estruch R, Estruch R, Bach-Faig A. Mediterranean Diet and Cardiodiabesity: A Review. Nutrients. 2014;6: 3474–3500.

35. Esposito K, Maiorino MI, Bellastella G, Chiodini P, Panagiotakos D, Giugliano D. A Journey into a Mediterranean Diet and Type 2 Diabetes: A Systematic Review with Meta-Analyses. BMJ Open. 2015;5: e008222.

36. Salas-Salvado J, Garcia-Arellano A, Estruch R, Marquez-Sandoval F, Corella D, Fiol M, et al. Components of the Mediterranean-Type Food Pattern and Serum Inflammatory Markers among Patients at High Risk for Cardiovascular Disease. Eur J Clin Nutr. 2008;62: 651–659.

37. de la Iglesia R, Loria-Kohen V, Zulet MA, Martinez JA, Reglero G, de Molina AR. Dietary Strategies Implicated in the Prevention and Treatment of Metabolic Syndrome. Int J Mol Sci. 2016;17: 1–21.

38. Salas-Salvado J, Guasch-Ferre M, Lee C-H, Estruch R, Clish CB, Ros E. Protective Effects of the Mediterranean Diet on Type 2 Diabetes and Metabolic Syndrome. J Nutr. 2016;146: 920S–927S.

39. Koloverou E, Esposito K, Giugliano D, Panagiotakos D. The Dffect of Mediterranean Diet on the Development of Type 2 Diabetes Mellitus: A Meta-Analysis of 10 Prospective Studies and 136,846 Participants. Metabolism. Elsevier Inc.; 2014;63: 903–911.

40. Estruch R, Ros E, Salas-Salvado J, Covas M-I, Corella D, Aros F, et al. Primary Prevention of Cardiovascular Disease with a Mediterranean Diet. N Engl J Med. 2013;368: 1279–1290.

41. Kastorini C-M, Milionis HJ, Esposito K, Giugliano D, Goudevenos JA, Panagiotakos DB. The Effect of Mediterranean Diet on Metabolic Syndrome and its Components: A Meta-Analysis of 50 Studies and 534,906 Individuals. J Am Coll Cardiol. Elsevier Inc.; 2011;57: 1299–1313.

42. Timmermans S, Steegers-Theunissen RP, Vujkovic M, den Breeijen H, Russcher H, Lindemans J, et al. The Mediterranean Diet and Fetal Size Parameters: The Generation R Study. Br J Nutr. 2012;108: 1399–1409.

43. Barker P. Fetal Origins of Coronary Heart Disease. Br Med J. 1995;311: 1–4.

44. Phillips DI, Barker DJ, Fall CH, Seckl JR, Whorwood CB, Wood PJ, et al. Elevated Plasma Cortisol Concentrations: A Link between Low Birth Weight and the Insulin Resistance Syndrome? J Clin Endocrinol Metab. 1998;83: 757–760.

45. Zimmet P, Alberti KGM, Kaufman F, Tajima N, Silink M, Arslanian S, et al. The Metabolic Syndrome in Children and Adolescents – An IDF Consensus Report. Pediatr Diabetes. 2007;8: 299–306.

46. Zimmet P, Alberti G, Kaufman F, Tajima N, Silink M, Arslanian S, et al. The Metabolic Syndrome in Children and Adolescents. Lancet. 2007;369: 2059–2061.

47. Bloch CA, Clemons P, Sperling MA. Puberty Decreases Insulin Sensitivity. J Pediatr. 1987;110: 481–487.

48. Caprio S, Plewe G, Diamond MP, Simonson DC, Boulware SD, Sherwin RS, et al. Increased Insulin Secretion in Puberty: A Compensatory Response to Reductions in Insulin Sensitivity. J Pediatr. 1989;114: 963–967.

49. Viitasalo A, Lakka TA, Laaksonen DE, Savonen K, Hanna-Maaria Lakka, Hassinen M, et al. Validation of Metabolic Syndrome Score by Confirmatory Factor Analysis in Children and Adults and Prediction of Cardiometabolic Outcomes in Adults. Diabetologia. 2014;57: 940–949.

50. Araki S, Dobashi K, Yamamoto Y, Asayama K, Kusuhara K. Increased Plasma Isoprostane is Associated with Visceral Fat, High Molecular Weight Adiponectin, and Metabolic Complications in Obese Children. Eur J Pediatr. 2010;169: 965–970.

51. Roberts CK, Sindhu KK. Oxidative Stress and Metabolic Syndrome. Life Sci. Elsevier Inc.; 2009;84: 705–712.

52. Ando K, Fujita T. Metabolic Syndrome and Oxidative Stress. Free Radic Biol Med. Elsevier B.V.; 2009;47: 213–218.

53. Gurka MJ, Ice CL, Sun SS, Deboer MD. A Confirmatory Factor Analysis of the Metabolic Syndrome in Adolescents: An Examination of Sex and Racial/Ethnic Differences.

Cardiovasc Diabetol. 2012; 128–137.

54. Walker S, Gurka M, Oliver M, Johns D, DeBoer M. Racial/Ethnic Discrepancies in the Metabolic Syndrome Begin in Childhood and Persist after Adjustment for Environmental Factors. Nutr Metab Cardiovasc Dis. 2012;22: 141–148.

55. Lewis RC, Meeker JD, Peterson KE, Lee JM, Pace GG, Cantoral A, et al. Predictors of Urinary Bisphenol A and Phthalate Metabolite Concentrations in Mexican Children. Chemosphere. Elsevier Ltd; 2013;93: 2390–2398.

56. Perng W, Watkins DJ, Cantoral A, Mercado-garcía A, Meeker JD, Téllez-rojo MM, et al. Exposure to Phthalates is Associated with Lipid Profile in Peripubertal Mexican Youth. Environ Res. Elsevier Inc.; 2017;154: 311–317.

57. Watkins DJ, Téllez-Rojo MM, Ferguson KK, Lee JM, Solano-Gonzalez M, Blank-Goldenberg C, et al. In Utero and Peripubertal Exposure to Phthalates and BPA in Relation to Remale Sexual Maturation. Environ Res. Elsevier; 2014;134: 233–241.

58. Watkins DJ, Peterson KE, Ferguson KK, Mercado-García A, Tamayo M, Cantoral A, et al. Relating Phthalate and BPA Exposure to Metabolism in Peripubescence: The Role of Exposure Timing, Sex, and Puberty. J Clin Endocrinol Metab. 2016;101: 79–88.

59. Yang TC, Peterson KE, Meeker JD, Sánchez BN, Zhang Z, Cantoral A, et al. Bisphenol A and Phthalates In Utero and in Childhood: Association with child BMI z-score and Adiposity. Environ Res. 2017;156: 326–333.

60. Calafat A, Ye X, Wong L, Reidy J, Needham L. Exposure of the U.S. Population to Bisphenol A and 4-tertiary-octylphenol: 2003-2004. Environ Health Perspect. 2008;116: 39–44.

61. Hernandez-Avila M, Romieu I, Parra S, Hernandez-Avila J, Madrigal H, Willett W. Validity and Reproducibility of a Food Frequency Questionnaire to Assess Dietary Intake of Women Living in Mexico City. Salud Publica Mex. 1998;40: 133–140.

62. Hu FB, Rimm E, Smith-warner SA, Feskanich D, Stampfer MJ, Ascherio A, et al. Reproducibility and Validity of Dietary Patterns Assessed with a Food-Frequency Questionnaire. Am J Clin Nutr. 1999;69: 243–249.

63. Rodríguez-Ramírez S, Mundo-Rosas V, Jimenez-Aguilar A, Shamah-Levy T. Methodology for the Analysis of Dietary Data from the Mexican National Health and Nutrition Survey 2006. Salud Publica Mex. 2009;51: S523–S529.

64. Costacou T, Bamia C, Ferrari P, Riboli E, Trichopoulos D, Trichopoulou A. Tracing the Mediterranean Diet through Principal Components and Cluster Analyses in the Greek Population. Eur J Clin Nutr. 2003;57: 1378–85.

65. Trichopoulou A, Toupadaki N, Tzonou A, Katsouyanni K, Manousos O, Kada E, et al. The Macronutrient Composition of the Greek Diet: Estimates Derived from Six Case-Control Studies. Eur J Clin Nutr. 1993;47: 549–558.

66. Trichopoulou A, Katsouyanni K, Gnardellis C. The Traditional Greek Diet. Eur J Clin Nutr. 1993;47: S76–S81.

67. Mila-Villarroel R, Bach-Faig A, Puig J, Puchal A, Farran A, Serra-Majem L, et al. Comparison and Evaluation of the Reliability of Indexes of Adherence to the Mediterranean Diet. Public Health Nutr. 2011;14: 2338–2345.

68. Kadiiska MB, Gladen BC, Baird DD, Germolec D, Graham LB, Hensley K, et al. Biomarkers of Oxidative Stress Study II. Are Oxidation Products of Lipids, Proteins, and DNA Markers of CCl4 Poisoning? Free Radic Biol Med. 2005;38: 698–710.

69. Kadiiska MB, Gladen BC, Baird DD, Graham LB, Parker CE, Ames BN, et al. Biomarkers of Oxidative Stress Study III. Effects of the Nonsteroidal Anti-Inflammatory Agents Indomethacin and Meclofenamic Acid on Measurements of Oxidative Products of Lipids in CCl4 Poisoning. Free Radic Biol Med. 2005;38: 711–718.

70. Milatovic D, Montine TJ, Aschner M. Measurement of Isoprostanes as Markers of Oxidative Stress. Methods Mol Biol. 2012;758: 195–204.

71. van 't Erve T, Lih FB, Kadiiska MB, Deterding LJ, Eling TE, Mason RP. Reinterpreting the Best Biomarker of Oxidative Stress: The 8-iso-PGF2a/PGF2a Ratio Distinguishes Chemical from Enzymatic Lipid Peroxidation. Free Radic Biol Med. 2016;83: 245–251.

72. Basu S. The Enigma of In Vivo Oxidative Stress Assessment: Isoprostanes as an Emerging Target. Scandanavian J Food Nutr. 2007;51: 48–61.

73. Frederick IO, Williams MA, Sales AE, Martin DP, Killien M. Pre-Pregnancy Body Mass Index, Gestational Weight Gain, and Other Maternal Characteristics in Relation to Infant Birth Weight. Matern Child Heal J. 2008;12: 557–567.

74. Li N, Liu E, Guo J, Pan L, Li B, Wang P, et al. Maternal Prepregnancy Body Mass Index and Gestational Weight Gain on Pregnancy Outcomes. PLoS One. 2013;8: e82310.

75. Oken E. Maternal and Child Obesity: The Causal Link. Obstet Gynecol Clin North Am. 2009;36: 361–377.

76. Oken E, Taveras EM, Kleinman KP, Rich-Edwards JW, Gillman MW. Gestational Weight Gain and Child Adiposity at Age 3 Years. Am J Obstet Gynecol. 2007;196: 322.e1-322.e8.

77. Fraser A, Tilling K, Macdonald-Wallis C, Sattar N, Brion M-J, Benfield L, et al. Association of maternal weight gain in pregnancy with offspring obesity and metabolic and vascular traits in childhood. Circulation. 2010;121: 2557–2564.

78. Stamnes Køpp UM, Dahl-Jørgensen K, Stigum H, Frost Andersen L, Næss O, Nystad W. The associations between maternal pre-pregnancy body mass index or gestational weight change during pregnancy and body mass index of the child at 3 years of age. Int J Obes. 2012;36: 1325–1331.

79. Chavarro JE, Watkins DJ, Afeiche MC, Zhang Z, Sánchez BN, Cantonwine D, et al.

Validity of Self-Assessed Sexual Maturation Against Physician Assessments and Hormone Levels. J Pediatr. Elsevier Inc.; 2017; Epub ahead of print.

80. Travers S, Jeffers B, Block C, Hill J, Eckel R. Gender and Tanner Stage Differences in Body Composition and Insulin Sensitivity in Early Pubertal Children. J Clin Endocrinol Metab. 1995;80: 172–178.

81. Ahmed ML, Ong KENKL, Morrell DJ, Cox LES, Drayer N, Perry L, et al. Longitudinal Study of Leptin Concentrations during Puberty: Sex Differences and Relationship to Changes in Body Composition. J Clin Endocrinol Metab. 1999;84: 899–905.

82. Mauras N, Rogol AD, Haymond MW, Veldhuis JD. Sex Steroids, Growth Hormone, Insulin-Like Growth Factor-1: Neuroendocrine and Metabolic Regulation in Puberty. Horm Res. 1996;45: 74–80.

83. Guinhouya BC, Samouda H, Zitouni D, Vilhelm C, Hubert H. Evidence of the Influence of Physical Activity on the Metabolic Syndrome and/or on Insulin Resistance in Pediatric Populations: A Systematic Review. Int J Pediatr Obes. 2011;6: 361–388.

84. Hernandez-Prado B, Gortmaker SL, Larid N, Colditz G, Parra-Cabrera, Peterson KE. Validez y Reproducibilidad de un Cuestionario de Actividad e Inactividad Física para Escolares de la Ciudad de México. Salud Publica Mex. 2000;42: 315–323.

85. Morales-Ruan M del C, Hernandez-Prado B, Gomez-Acosta LM, Shamah-Levy T, Cuevas-Nasu L. Obesity, overweight, screen time and physical activity in Mexican adolescents. Salud Publica Mex. 2009;51: S613-20.

86. Veiga-Lopez A, Pennathur S, Kannan K, Patisaul HB, Dolinoy DC, Zeng L, et al. Impact of Gestational Bisphenol A on Oxidative Stress and Free Fatty Acids: Human Association and Interspecies Animal Testing Studies. Endocrinology. 2015;156: 911–22.

87. Ferguson KK, Cantonwine DE, Mcelrath TF, Mukherjee B, Meeker JD. Repeated Measures Analysis of Associations between Urinary Bisphenol-A Concentrations and Biomarkers of Inflammation and Oxidative Stress in Pregnancy. Reprod Toxicol. 2016;66: 93–98.

88. Kabuto H, Amakawa M, Shishibori T. Exposure to Bisphenol A during Embryonic/Fetal Life and Infancy Increases Oxidative Injury and Causes Underdevelopment of the Brain and Testis in Mice. Life Sci. 2004;74: 2931–40.

89. Song S, Zhang L, Zhang H, Wei W, Jia L. Perinatal BPA Exposure Induces Hyperglycemia, Oxidative Stress and Decreased Adiponectin Production in Later Life of Male Rat Offspring. Int J Env Res Public Heal. 2014;11: 3728–3742.

90. Kaviarasan S, Muniandy S, Qvist R, Ismail IS. F2-Isoprostanes as Novel Biomarkers for Type 2 Diabetes: A Review. J Clin Biochem Nutr. 2009;45: 1–8.

91. Bhandari R, Xiao J, Shankar A. Urinary Bisphenol A and Obesity in US Children. Am J Epidemiol. 2013;18: 1–8.

92. Eng DS, Lee JM, Gebremariam A, Meeker JD, Peterson K, Padmanabhan V. Bisphenol A and Chronic Disease Risk Factors in US Children. Pediatrics. 2013;132: e637–e645.

93. Wang T, Li M, Chen B, Xu M, Xu Y, Huang Y, et al. Urinary Bisphenol A (BPA) Concentration Associates with Obesity and Insulin Resistance. J Clin Endocrinol Metab. 2012;97: 223–227.

94. Carwile JL, Michels KB. Urinary Bisphenol A and Obesity: NHANES 2003-2006. Environ Res. Elsevier; 2011;111: 825–30.

95. Harley KG, Schall RA, Chevrier J, Tyler K, Aguirre H, Bradman A, et al. Prenatal and Postnatal Bisphenol A Exposure and Body Mass Index in Childhood in the CHAMACOS Cohort. Env Heal Perpsect. 2013;121: 514–520.

96. Faulk C, Dolinoy DC. Timing is Everything: The When and How of Environmentally Induced Changes in the Epigenome of Animals. Epigenetics. 2011;6: 791–797.

97. Braun JM, Lanphear BP, Calafat AM, Deria S, Khoury J, Howe CJ, et al. Early-Life Bisphenol A Exposure and Child Body Mass Index: A Prospective Cohort Study. Env Heal Perpsect. 2014;122: 1239–1245.

98. Cantonwine DE, Meeker JD, Ferguson KK, Mukherjee B, Hauser R, Mcelrath TF. Urinary Concentrations of Bisphenol A and Phthalate Metabolites Measured during Pregnancy and Risk of Preeclampsia. Env Heal Perpsect. 2016;124: 1651–1655.

99. Chavarro JE, Mínguez-alarcón L, Chiu Y, Gaskins AJ, Souter I, Williams PL, et al. Soy Intake Modifies the Relation Between Urinary Bisphenol A Concentrations and Pregnancy Outcomes Among Women Undergoing Assisted Reproduction. J Clin Endocrinol Metab. 2016;101: 1082–1090.

100. Lee AM, Gurka MJ, Deboer MD. Trends in Metabolic Syndrome Severity and Lifestyle Factors Among Adolescents. Pediatrics. 2016;137: e20153177.

CHAPTER 5

Discussion

Significance of Research Findings

Global disease burden from chronic metabolic diseases, like NAFLD, continues to rise. NAFLD is now the most common liver disease amongst children and adults in the U.S., with incidence rising concomitantly with obesity. In the U.S., recommendations to prevent and decrease prevalence of childhood obesity have traditionally focused on altering personal behaviors, (e.g. poor diet quality, hypercaloric intake, physical inactivity) and more recently on altering the socio-political environment (e.g. safe access to healthy food, places to exercise, affordable healthcare, healthy default options) [1–3]. Despite initial publication more than 50 years ago, the influence of the *in utero* environment on offspring metabolic health has still not been incorporated into medical or policy recommendations. First identified following World War II, via natural experiments in free-living human populations (e.g. the Seige of Leningrad [4] and the Dutch Hunger Winter [5,6]), the potential for maternal exposures to affect offspring metabolic health was later codified into the Barker Hypothesis [7]. Over the past decade, popular media has raised awareness regarding the potential for perinatal exposures to increase susceptibility to chronic diseases later in life. However, an incomplete understanding of the

mechanism underlying this perinatal programming and a lack of clearly/proven beneficial interventions has hindered adoption of recommendations.

Over the past decade, BPA has become a substance of concern [8–13], garnering increased media and societal attention. Concern regarding the potential health effects of BPA stem from studies reporting obesogenic [14–16], diabetogenic [17], reproductive [18] effects, adipogenic proliferation [19] and oxidative stress [20]. Conflicting results, such as lean phenotype [21,22] vs. obesogenic agent, have muddled overarching conclusions about BPA's potential threat to human health [23,24]. Despite acknowledging that infants and children are exposed to the highest BPA levels, the FDA's 2008 report concluded that health risks associated with BPA exposure were only of negligible to some concern [25]. However, Health Canada's determination of BPA as a "dangerous substance" [26,27] led to the voluntary removal of BPA from many infant products in the U.S., including infant formula packaging and baby bottles [28,29]. In the U.S., no government regulations on safe consumption levels have been passed since 1982, when the NTP determined the lowest adverse effect level (LOAEL) of BPA to be 50 mg BPA/kg body weight/day [30]. In 2014 the FDA re-reviewed BPA literature and again concluded that it is a chemical of minimal concern [31]. Without a commitment to discontinue the manufacture and use of BPA in consumer products, complete elimination of exposure is challenging on an individual level.

BPA was detectable in the plasma of all pregnant women (0.5-22.3 ng/mL) assessed in Southeast Michigan in 2006 [32]. Documentation of BPA crossing the placenta [33,34] and transferring in breast milk [35], combined with lower levels of BPA metabolizing enzymes in fetal livers [36] emphasize potential increased risk of BPA exposure during fetal and infant development. To address mothers' challenge to avoid BPA during pregnancy, this dissertation

aimed to investigate maternal diet as a potential method for mitigating the detrimental developmental effects previously reported from perinatal BPA exposure. If maternal diet could help prevent the detrimental health effects, the unavoidable exposure might be of less concern. Thereby providing an inexpensive, generally accessible 'treatment' to the general public, for a difficult-to-avoid exposure.

The main goal of this dissertation was to examine if perinatal BPA exposure could perinatally program NAFLD in offspring and whether this relationship could be modified by maternal pregnancy diet. This research revealed that perinatal HFD had a greater impact on hepatic steatosis in mouse offspring at PND10 and 10M than did perinatal BPA exposure (Figure 5.1, Figure 5.2). Interestingly, perinatal HFD was predictive of PND10 hepatic 8-isoprostane also, but by 10M, perinatal BPA exposure was the main predictor of hepatic 8-isoprostane. When analogous outcomes were investigated in the human birth cohort, ELEMENT, a similar trend was observed. Prenatal BPA was a significant contributor in models predicting adolescent serum 8-isoprostane, with trimester specific impact of maternal MDS adherence. These findings were more pronounced in male youth, compared to females. However, the opposite trend was observed when predicting youth Metabolic Risk score; then T2 BPA was predictive of risk only in female youth, not males. Again, trimester specific maternal MDS adherence improved models, supporting a critical role for the inclusion of diet as a co-exposure in future toxiciology studies, especially investigating chemicals that share the main route of exposure to the body as food: ingestion.

The impact of perinatal BPA exposure on hepatic steatosis was not as pronounced as we expected. During necropsy, 26.6% of all 10M offspring were observed to have some level of hepatic masses, visible to the naked eye, of those with gross masses, 26.5% of the offspring were

in the Control group. Hepatic histology results for nodular hyperplasia, as read by a certified doctor of veterinary medicine, followed a similar trend; 15.6% of all 10M offspring presented with moderate to severe hyperplasia, 20% of these were Control mice. The steatotic effect of our Control diet was unexpected. To avoid the potential epigenetic programming effect of phytoestrogens in soybean oil [37], the default lipid source in standard mouse chow, we substituted corn oil Corn oil consumption in rats and mice has previously been reported to induce hepatic steatosis [38,39]. Thus, our Control lipid may have been more steatotic than either experimental HFD for the study mice. This would help to explain the high prevalence of hepatic hyperplasia in all exposure groups, including offspring perinatally exposed to the Con and CBPA diets.

Perinatal BPA exposure had a greater impact on offspring 8-iso than on the metabolic outcomes: liver triglycerides or MRS in mice and humans, respectively. Diet did not modify the effect of BPA on 8-iso, liver TGs, or MRS in mice or humans. However, perinatal HFD exposures in mice were independently significant predictors of metabolic parameters and redox potentials in dams and PND10 offspring. This suggests that perinatal diet may exert a larger impact on offspring lifelong health than perinatal BPA exposure. Another recent study also reported no effect of 50 μg BPA/kg/day on hepatic or serum triglyceride levels compared to Controls, in adult male Wistar rats exposed for 35 weeks [40]. Some researchers have questioned if the concern over BPA exposure is overblown [23], the findings of this mouse study imply that efforts focused on improving maternal diet may be more critical than eliminating BPA exposure during pregnancy.

Most toxicology studies investigating the health effects of BPA exposure do not account for the diet consumed during exposure. The liver is the main site of xenobiotic and nutrient

metabolism; thus the liver is an ideal tissue in which to examine the potential interaction between EDC exposure and dietary intake. The wide variation in human dietary consumption patterns and differing response to diet between mouse and rat strains may explain some of the previously reported conflicting study results. This project examined the potential importance of BPA and diet interactions during pregnancy by providing three different background diets to mouse moms (dams) and by rating adherence to a Med diet in ELEMENT mothers. Results from this project suggest that maternal pregnancy diet did *not* modify of the relationship between BPA and offspring health outcomes in either mice or humans. In fact, perinatal diet altered offspring metabolic health and oxidation status more than perinatal BPA exposure.

In order to scientifically isolate the impact of exposures during the perinatal period, offspring were not exposed to BPA or HFDs after weaning. This study design differs from many other studies that use a later life challenge following perinatal exposure [45–47], to determine if exposed offspring react differently to stressors compared to their non-exposed colony-mates. This experimental difference in timing (concurrent vs. delayed HFD exposure) may underscore the dissimilarity in findings in this thesis from previous studies. Metabolic and redox homeostasis are basic cellular functions required for species survival. Both outcomes have many, overlapping regulatory pathways to help ensure homeostasis is maintained; if an environmental exposure or stressor affects a usual metabolic pathway, another, redundant pathway can be upregulated. Without these compensatory mechanisms, mammals would not have survived and adapted to as many habitats and climate alterations as we have. A distinct difference in our evolutionary environment and the modern world is the frequency with which environmental stressors occur today. Humans and most mammals evolved to respond to one or two stressors at a time with a significant recovery time between stressors when the body was able to return to

metabolic and redox homeostasis. The constant bombardment of chemical, dietary, and physical stressors we face on a daily basis now likely overwhelms our system, mimicking a state of repeated and often overlapping challenges (like the HFD challenge following perinatal BPA exposure). Oxidative stress and metabolic disturbances may be unfairly blamed for the development of chronic diseases, when they are actually the body's natural, physiologic attempt to return to homeostasis in a world where the natural system cannot keep pace.

Study Strengths and Limitation

This dissertation had many strengths that set it apart from traditional environmental health sciences research. Cross-species comparisons were built into the study design of both the mouse and human studies, which increased translatability of our findings (Table 5.1). Murine and human birth cohort studies are often conducted by separate research teams with different measurement or analysis methods. Planning to correlate both studies from the beginning allowed us to select a lipid peroxidation measure, 8-isoprostane, which could be used on both mouse and human biological specimens. Creating the Mediterranean and Western HFDs for mice based on human dietary consumption patterns was a further attempt to make the mouse and human studies comparable.

A unique component of this mouse study was the addition of maternal diet as a potential modifier of the perinatal BPA exposure. Traditional toxicology studies take one of two forms: (1) dose-range finding studies, or (2) physiology-based pharmacokinetic (PBPK) studies. Dose-range finding studies aim to identify the level of toxicant at which adverse health effects occur; PBPK studies aim to understand how the toxicant works once inside the body. While it is critical to understand the level of toxic effects and modes of absorption, digestion, metabolism and

excretion for individual toxicants, these studies rarely account for dietary intake. People eat every day, so understanding nutrient toxicant interactions will be critical for realistic PBPK models. For lipophilic compounds, like BPA, the quantity and type of dietary fat consumed may alter the amount of BPA required to elicit a toxic response. Quantity and type of dietary fat likely also impacts the absorption and metabolism of BPA. This study was one of the first to begin investigating these critical nutrient-toxicant interactions.

However, during the experimental study design phase of planning this thesis project, considerations and concessions were made in order to make this study as translatable to human cohort studies as possible. For example, we examined both the directly exposed dams and the indirectly exposed pups, thus mimicking a human birth cohort study design. Instead of altering one dietary component at a time, as is often done in mouse studies, we developed new mouse diets to match the macronutrient and vitamin content to human Western and Mediterranean HFDs as closely as possible. The dose of BPA falls within the average human exposure range. Physiologic measures commonly collected in human birth cohorts, like maternal pre-pregnancy weight, weight gain during pregnancy, early life child weight were recorded in this study also.

Although findings from the longitudinal mouse exposure study did not translate exactly to findings in the human birth cohort, mice provide several experimental benefits. They have a shorter lifespan, thus making longitudinal studies more time-efficient; they also have 100% dietary compliance, removing the variability and uncertainty found in human diet and toxicology studies. It is also easier to study tissues of interest, e.g. liver and adipose, in mice, because all tissues are available following IACUC-approved sacrifice; whereas, in human birth cohorts, these same tissues would require invasive biopsy sampling. Although protein-coding regions of

mice and human genomes are 85% genetically homologous, there are species-specific differences not only in sequence but in regulatory control [43,44].

However, results from the ELEMENT cohort did not find Med diet adherence to be a strong predictor of offspring MRS or serum 8-iso. A different type of dietary analysis in human birth cohorts might reveal an increased importance of diet on youth health outcomes. We also only examined health outcomes during the pubertal transition, at which time many changes in metabolic and oxidative health are naturally occurring, which may have masked effects of both prenatal BPA and/or diet. Investigations into the impact of maternal pregnancy diet on prepubertal children or young adults after the pubertal transition may yield a greater magnitude of effect.

Unexpected results in my study findings highlight the importance of carefully thinking through physiological differences between species. Although we share the vast majority of our genome with other mammals, like mice, we evolved to inhabit different ecological niches (thus evolved with different ideal diets and environmental conditions). These evolutionary adaptations can lead to differences in metabolism of certain nutrients, like mono-unsaturated fatty acids (MUFA). The unexpected detrimental effect of perinatal Med diet exposure observed in the perinatal mouse exposure study may result from a species-specific difference in metabolizing enzymes. Mice are not as efficient at metabolizing monounsaturated fatty acids (MUFAs) as humans [41,42]. Oleic acid, the main lipid component of olive oil, comprised 89% of the humanized Med diet we developed for the mice. The increase in hepatic liver triglycerides observed in both male and female mice perinatally exposed to Med HFD may be the result of their inability to metabolize the lipid, not because of a broader hepatotoxic effect that would translate to human pathology in NAFLD.

Sample sizes in mouse exposure subgroups posed a limitation for some statistical comparisons. Although group sample sizes in the longitudinal mouse exposure study was based on power calculations from previous longitudinal phenotyping experiments on agouti mice in the Dolinoy Lab, larger sample sizes per group would have improved the ability to identify significant correlations. Since prenatal programming is known to have sexually dimorphic effects, many analyses were sex-stratified; further stratification by perinatal exposure group left group totals at 10-12 mice. Future studies would benefit from increasing the number of mice in each subgroup to improve power. An additional challenge of these studies is the redundancy of metabolic pathways. Metabolism on a cellular level is comprised of very ancient, largely conserved pathways; metabolism is a basis, evolutionarily necessary function, so nature created multiple backup paths. This means that observing phenotypic changes in mice or even basic physiologic measures (like liver triglycerides or 8-isoprostane quantification) may not accurately portray the scale of metabolic disruption on a cellular level, if another redundant pathway is able to adapt to include the inactive pathway.

Future Directions and Applications

Further Research Directions

Although epigenetic modifications have been identified as a mechanism of developmental programming, the data has not yet become clinically useful, nor applied to human health risk assessments. Current challenges hampering the application of epigenetic data include (1) many studies evaluate a single type of epigenetic modification (e.g. DNA methylation), but integration of all epigenetic changes will be necessary to fully understand the relevance to human health outcomes [48,49]. (2) Variability between tissues, species, study paradigms can be

considerable, so epigenetic data should be confirmed across multiple types of studies to ensure findings from experimental models translate to human physiologic responses and health outcomes of interest [50]. (3) Inconsistencies in epigenetic response to environmental exposure between experimental animal model strain, sex, life stage, and inter-individual variation are well-documented, thus studies will need to cover a range of these factors to make data useful [49]. (4) Much of the current epigenetic data is associative, with studies reporting a correlation between an exposure or disease state and an alteration in an epigenetic mark. Designing studies to assess causality of the would strengthen support for the connection between epigenetic modifications and pathogenesis of disease; for example, "pharmacologically blocking a specific epigenetic modification to eliminate or decrease the detrimental health.

Over the past decade, the integral role of the intestinal microbiome in obesity and its related metabolic conditions has been elucidated; NAFLD is no exception [59–61]. High fat, high fructose foods have been implicated in gut microbiome alterations and breakdown of the intestinal barrier, resulting in metabolic endotoxemia and low-grade systemic inflammation [62–66]. These diet-induced alterations are associated with increased risk of obesity and NAFLD. Animal models suggest the intestinal microbiome can contribute to NAFLD pathogenesis in multiple ways, by impacting energy homeostasis [67–71], choline metabolism [72,73], and the endocannabinoid system [74,75]. Regulation of energy homeostasis is altered due to increased carbohydrate fermentation into short chain fatty acids (SCFAs), which stimulates hepatic *de novo* lipogenesis (DNL), resulting in greater hepatic triglyceride accumulation (simple steatosis) [67–71]. Choline is required to synthesize very-low-density lipoproteins (VLDLs), which are the transport vesicles responsible for lipid export from the liver [72,73]. Intestinal endocannabinoids help regulate inflammation (inflammatory signaling cascades) and maintain integrity of the gut

barrier [74,75]. Administration of probiotics (mainly Lactobacilius and Bifidobacterium) have demonstrated beneficial impacts in animal models of NAFLD, improving hepatic steatosis, insulin resistance & sensitivity, hepatic inflammation [76–80]. A recent meta-analyses found that pre-biotic and probiotic consumption were associated with decreased BMI, body weight, and fat mass, suggesting this may be an additional therapeutic avenue for metabolic diseases, including NAFLD [81,82]. More work is needed to identify optimal probiotic strains for therapeutic use in NAFLD, but results are encouraging and suggest addition of probiotic therapy to diet and exercise interventions could enhance NAFLD treatment.

In addition to impacting the development of NAFLD, intestinal microbiota are sensitive to ingested environmental exposures, including. A recent study examined the impact of 10-weeks of oral BPA exposure on the intestinal microbiome of adult male CD-1 mice. Microbial species diversity decreased in BPA-exposed mice, similar to the mice exposed to a HFD, with increased growth of *Proteobacteria, Helicobacteraceae,* and reduced populations of *Firmicutes* and *Clostridia;* these patterns are representative of microbial dysbiosis and have been associated with obesity and T2DM [84]. Although this study was small in sample size (only 4 mice per exposure group), the effects were significant and warrant additional investigation. In another study, perinatal exposure to 50ug/kg body weight/day of C3H/HeN mice was associated with greater *E. coli* colony formation, and decreased *Bifidobacertium spp.* and *Clostridium* species, in the *Firmicutes* phylum among offspring at postnatal day 45 [85]. These alterations in intestinal microbiome preceded insulin resistance, which was not observed until PND160 and M1 macrophage inflammation, not observed until PND170 [85], supportive of microbiome dysbiosis as an early marker of later metabolic alterations.

Public Health Relevance

The rise in U.S. NAFLD prevalence poses a challenge due to the potential increase in young people affected by serious co-morbidities, decreased quality of life, and increased life-long medical bills. Studies are just beginning to explore the healthcare and quality of life costs associated with NAFLD [53]. Health-related quality of life is lower amongst NAFLD patients than those with other liver conditions, such as alcoholic liver disease, autoimmune or viral hepatitis (HBV & HCV), and cholestatic liver disease [55,56]. NAFLD patients had impairments in general health, physical functioning, bodily pain, vitality, role performance, and mental health; detriments were more pronounced in female patients, compared to males [56]. Physical health component scores (PCS) decreased with NAFLD severity; patients with cirrhosis had the lowest PCS, followed by non-alcoholic steatoheptitis patients, while those with simple steatosis had the least physical impairment. Interestingly, the mental component score did not differ by disease severity [57]. Compared to the general population, NAFLD patients have decreased lifespan largely attributable to cardiovascular disease and hepatocellular carcinoma [51].

The growing economic burden of NAFLD in the U.S. is apparent in a study of Medicare beneficiaries. The study found that in a five-year period, the number of outpatient visits doubled and expenses increased by 38%, although Medicare payments only increased by 12%, leaving NAFLD patients responsible for an increasing percentage of their growing health care costs. A cost of care model analyzing the projected health care costs by major practice category, estimated that per-capita costs of liver-related conditions would increase from 2% in 2000 to 11% in 2050, due to aging of the Baby Boomer generation [54]. NAFLD is associated with many cardio-metabolic comorbidities, which also contributed to the overall increase in per capita healthcare costs due to aging: heart & vascular conditions (44%), kidney disorders (55%),

gastrointestinal conditions (21%), endocrine conditions (20%) [54]. The studies above only account for medical costs associated with adult NAFLD, but growing childhood NAFLD rates are concerning due to the long period of medical dependence and thus much larger lifetime healthcare expense.

The need for liver transplants has been dropping in the U.S. due to decreases in hepatitis C, alcoholic fatty liver, autoimmune conditions, primary biliary cholangitis, and primary sclerosing cholangitis, transplant need due to NAFLD-induced cirrhosis is rising and is likely under-diagnosed [52]. Within the last 10 years, the supply of healthy livers available for donor transplant has decreased. This supply and demand discrepancy led to trials of "non-ideal" donor options. Unfortunately, hepatic steatosis is now present in 37-51% of donated livers; this is associated with a 3.7-fold increased risk of poor initial graft function and elevated risk of graft failure, decreasing chances of the recipient surviving [52].

Expanding the public health relevance of NAFLD to the global arena, rates of NAFLD are rapidly increasing in East Asian countries. The greatest population growth is predicted to occur in this region over the next decade and with some of the highest rates of NAFLD increase globally [58], the East Asian region is at risk for epidemic proportions of NAFLD. Improved understanding of the early, reversible stages of NALFD (steatosis and steatohepatitis) will support the creation of better non-invasive biomarkers that can help clinically diagnose NAFLD earlier in the disease course. Deeper characterization of the molecular pathways involved in these early stages will hopefully lead to new therapies that assist in the reversal or prevention of disease progression.

Table 5.1 Cross-Species Comparisons: Strengths and Limitations

	Species Comparison	
	Mice	**Humans**
Strengths	▪ Genetically identical background ▪ Controlled laboratory environment ▪ Short lifespan facilitates longitudinal study ▪ Access to internal tissue samples ▪ BPA & diet exposure during perinatal period only ▪ Creation of humanized diets for mice ▪ Discrete, consistent level of exposures	▪ Repeated maternal exposure measures in each trimester of pregnancy ▪ Species of interest – insights could be translated for clinical use
Limitations	▪ Lipid metabolism differs from humans ▪ NAFLD disease progression also differs	▪ Genetic heterogeneity ▪ BPA & diet exposures continue after prenatal period ▪ Exposure levels vary over time and between individuals ▪ Limited to bioavailable tissue specimens
Combined Benefits	▪ Ability to compare genetically identical background to heterogeneity inherent in human populations – helps to identify role of environment on health outcomes ▪ Limiting exposure to perinatal period in mice clarifies potential fetal programming outcomes, which may be more difficult to discern in humans with continuous exposure ▪ Measurement of BPA & diet during all three trimesters of pregnancy provides insight into trimester-specific effects that may occur with different prenatal exposures	

Figure 5.1 Translation of Study Findings from Longitudinal Mouse Study to Human Birth Cohort

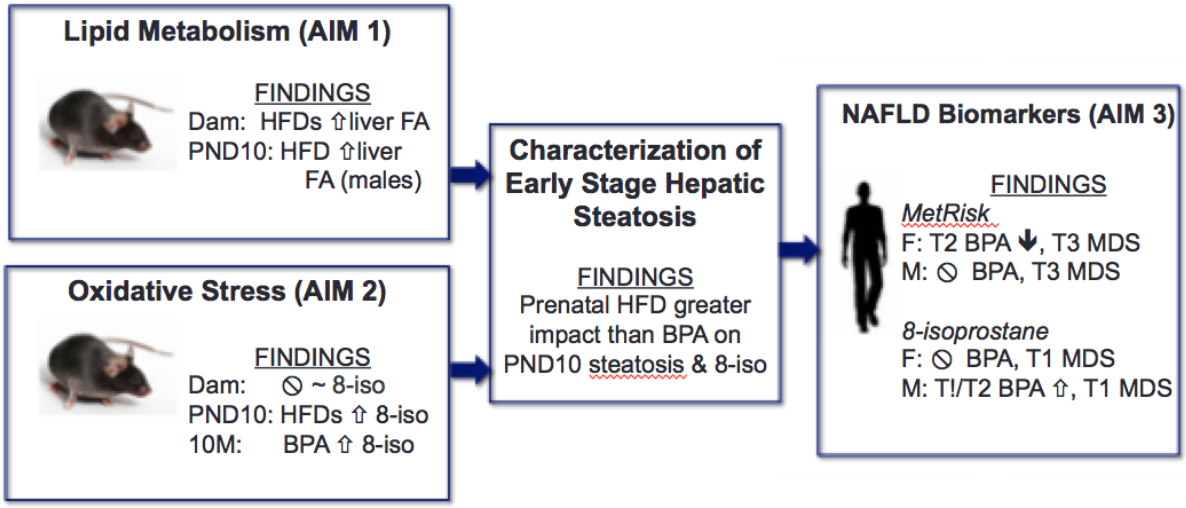
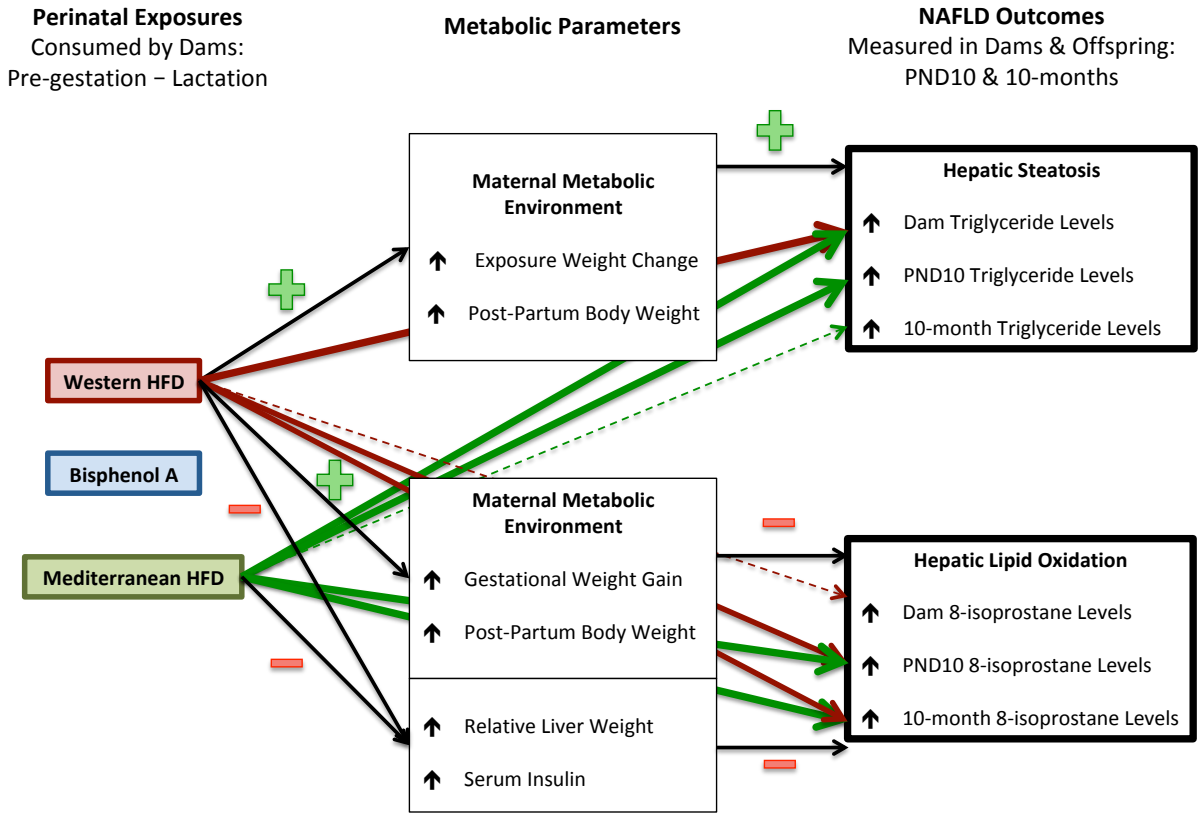


Figure 5.2 Summary of Dissertation Findings



REFERENCES

1. Institute of Medicine. Progress in Preventing Childhood Obesity: Focus on Communities. Food and Nutrition Board, National Academies Press, Washington, DC. 2005.

2. Koplan JP, Liverman CT, Kraak VI. Preventing Childhood Obesity: Health in the Balance. Institute of Medicine, National Academies Press, Washington, DC. 2005.

3. McGuire S. Early Childhood Obesity Prevention Policies. Institute of Medicine, National Academies Press, Washington, DC. 2011.

4. Antonov A. Children born during the siege of Leningrad in 1942. J Pediatr. 1947;30: 250–259.

5. Smith CA. The Effect of Wartime Starvation in Holland upon Pregnancy and its Product. Am J Obstet Gynecol. 1947;53: 599–608.

6. Stein Z, Susser M, Saenger G, Marolla F. Famine and Human Development: The Dutch Hunger Winter of 1944-1945. 1975.

7. Barker DJ. The Fetal and Infant Origins of Disease. Eur J Clin Invest. 1995;25: 457–463.

8. Rubin BS, Murray MK, Damassa DA, King JC, Soto AM. Perinatal Exposure to Low Doses of Bisphenol A Affects Body Weight, Patterns of Estrous Cyclicity, and Plasma LH Levels. Env Heal Perpsect. 2001;109: 675–680.

9. Palanza P, Howdeshell KL, Parmigiani S, vom Saal FS. Exposure to a Low Dose of Bisphenol A during Fetal Life or in Adulthood Alters Maternal Behavior in Mice. Environ Health Perspect. 2002;110: 415–422.

10. Schönfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I. Parent Bisphenol A Accumulation in the Human Maternal-Fetal-Placental Unit. Env Heal Perpsect. 2002;110: A703–A707.

11. Rubin BS, Lenkowski JR, Schaeberle CM, Vandenberg LN, Ronsheim PM, Soto AM. Evidence of Altered Brain Sexual Differentiation in Mice Exposed Perinatally to Low Environmentally Relevant Levels of Bisphenol A. Endocrinology. 2006;147: 3681–3691.

12. Vom Saal FS, Hughes C. An Extensive New Literature Concerning Low-Dose Effects of Bisphenol A Shows the Need for a New Risk Assessment. Env Heal Perpsect. 2005;113: 926–933.

13. Welshons W V, Nagel SC, vom Saal FS. Large Effects from Small Exposures. III. Endocrine Mechanisms Mediating Effects of Bisphenol A at Levels of Human Exposure. Endocrinology. 2006;147: S56-69.

14. Bhandari R, Xiao J, Shankar A. Urinary Bisphenol A and Obesity in US Children. Am J Epidemiol. 2013; 1–8.

15. Carwile JL, Michels KB. Urinary Bisphenol A and Obesity: NHANES 2003-2006. Environ Res. Elsevier; 2011;111: 825–30.

16. Wang T, Li M, Chen B, Xu M, Xu Y, Huang Y, et al. Urinary Bisphenol A (BPA) Concentration Associates with Obesity and Insulin Resistance. J Clin Endocrinol Metab. 2012;97: E223–E227.

17. Alonso-Magdalena P, Quesada I, Nadal A. Prenatal Exposure to BPA and Offspring Outcomes: The Diabesogenic Behavior of BPA. Dose Response. 2015; 1–8.

18. Peretz J, Vrooman L, Ricke WA, Hunt PA, Ehrlich S, Hauser R, et al. Bisphenol A and Reproductive Health: Update of Experimental and Human Evidence, 2007-2013. Env Heal Perpsect. 2014;122: 775–786.

19. Ben-Jonathan N, Hugo ER, Brandebourg TD. Effects of Bisphenol A on Adipokine Release from Human Adipose Tissue: Implications for the Metabolic Syndrome. Mol Cell Endocrinol. 2009;304: 49–54.

20. Gassman NR. Induction of Oxidative Stress by Bisphenol A and Its Pleiotropic Effects. Environ Mol Mutagen. 2017;58: 60–71.

21. Anderson OS, Peterson KE, Sanchez BN, Zhang Z, Mancuso P, Dolinoy DC. Perinatal Bisphenol A Exposure Promotes Hyperactivity, Lean Body Composition, and Hormonal Responses across the Murine Life Course. FASEB J. 2013;27: 1784–1792.

22. van Esterik J, Dollé M, Lamoree M, van Leeuwen S, Hamers T, Legler J, et al. Programming of Metabolic Effects in C57BL/6JxFVB Mice by Exposure to Bisphenol A during Gestation and Lactation. Toxicology. Elsevier Ireland Ltd; 2014;321: 40–52.

23. Mirmira P, Evans-molina C. Bisphenol A, Obesity, and Type 2 Diabetes Mellitus: Genuine Concern or Unnecessary Preoccupation? Transl Res. Mosby, Inc; 2014;164: 13–21.

24. Srivastava S, Gupta P, Chandolia A, Alam I. Bisphenol A: A Threat to Human Health? J Environ Health. 2015;77: 20–27.

25. National Toxicology Program. NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A.

26. Cao X-L, Dufresne G, Belisle S, Clement G, Falicki M, Beraldin F, et al. Levels of Bisphenol A in Canned Liquid Infant Formula Products in Canada and Dietary Intake Estimates. J Agric Food Chem. 2008;56: 7919–7924.

27. Directorate B of CSF. Health Canada's Updated Assessment of Bisphenol A (BPA) Exposure from Food Sources. 2012.

28. Mui YQ. Wal-Mart to Pull Bottles Made with Chemical BPA. Washington Post. 2008: 1.

29. Austen I. Bottle Maker to Stop Using Plastic Linked to Health Concerns. New York Times. 2008: 1–3.

30. National Toxicology Program. Carcinogenesis Bioassay of Bisphenol A (CAS No. 80-05-7) in F344 Rats and B6C3F1 Mice (Feed Study). 1982.

31. Lin F, Ph D, Hfs- C, Lin FS, Keefe DM. 2014 Updated Safety Assessment of Bisphenol A (BPA) for Use in Food Contact Applications. Dep Heal Hum Serv. 2014; 8–12.

32. Padmanabhan V, Siefert K, Ransom S, Johnson T, Pinkerton J, Anderson L, et al. Maternal Bisphenol-A Levels at Delivery: A Looming Problem? J Perinatol. 2008;28: 258–263.

33. Nahar MS, Liao C, Kannan K, Harris C, Dolinoy DC. In Utero Bisphenol A Concentration, Metabolism, and Global DNA Methylation across Matched Placenta, Kidney, and Liver in the Human Fetus. Chemosphere. 2015;124: 54–60.

34. Balakrishnan B, Henare K, Thorstensen EB, Ponnampalam AP, Mitchell MD. Transfer of Bisphenol A across the Human Placenta. Am J Obstet Gynecol. Elsevier Inc.; 2010;202: 393.e1-393.e7.

35. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons W V. Human Exposure to Bisphenol A (BPA). Reprod Toxicol. 2007;24: 139–177.

36. Nahar M, Kim J, Sartor M, Dolinoy D. Bisphenol A-Associated Alterations in the Expression and Epigenetic Regulation of Genes Encoding Xenobiotic Metaboloizing Enzymes in Human Fetal Liver. Environ Mol Mutagen. 2014;55: 184–195.

37. Dolinoy DC, Huang D, Jirtle RL. Maternal Nutrient Supplementation Counteracts Bisphenol A-Induced DNA Hypomethylation in Early Development. Proc Natl Acad Sci. 2007;104: 13056–13061.

38. Wang M, Zhang X, Feng K, He C, Li P, Hu Y, et al. Dietary α-Linolenic Acid-Rich Flaxseed Oil Prevents Against Alcoholic Hepatic Steatosis via Ameliorating Lipid Homeostasis at Adipose Tissue-Liver Axis in Mice. Sci Rep. 2016;6: e26826.

39. Kucera O, Cervinkova Z. Experimental Models of Non-Alcoholic Fatty Liver Disease in Rats. World J Gastroenterol. 2014;20: 8364–8376.

40. Ding S, Zuo X, Fan Y, Li H, Zhao N, Yang H, et al. Environmentally Relevant Dose of Bisphenol A Does Not Affect Lipid Metabolism and Has No Synergetic or Antagonistic Effects on Genistein's Beneficial Roles on Lipid Metabolism. PLoS One. 2016;11: 1–15.

41. Wei J, Lin Y, Li Y, Ying C, Chen J, Song L, et al. Perinatal Exposure to Bisphenol A at Reference Dose Predisposes Offspring to Metabolic Syndrome in Adult Rats on a High-Fat Diet. Endocrinology. 2011;152: 3049–3061.

42. Wei J, Sun X, Chen Y, Li Y, Song L, Zhou Z, et al. Perinatal Exposure to Bisphenol A

Exacerbates Nonalcoholic Steatohepatitis-Like Phenotype in Male Rat Offspring Fed on a High-Fat Diet. J Endocrinol. 2014;222: 313–325.

43. Strakovsky RS, Wang H, Engeseth NJ, Flaws JA, Helferich WG, Pan Y, et al. Developmental Bisphenol A (BPA) Exposure Leads to Sex-Specific Modification of Hepatic Gene Expression and Epigenome at Birth that May Exacerbate High-Fat Diet-Induced Hepatic Steatosis. Toxicol Appl Pharmacol. 2015;284: 101–112.

44. Cheng Y, Ma Z, Kim B, Wu W, Cayting P, Boyle AP, et al. Principles of Regulatory Information Conservation Between Mouse and Human. Nature. 2014;515: 371–375.

45. Yue F, Consortium ME. A Comparative Encyclopedia of DNA Elements in the Mouse Genome. Nature. 2014;515: 355–364.

46. Ferramosca AF, Savy VS, Zara VZ. Olive Oil Increases the Hepatic Triacylglycerol Content in Mice by a Distinct Influence on the Synthesis and Oxidation of Fatty Acids. Biosci Biotechnol Biochem. 2008;72: 62–69.

47. Ferramosca A. Modulation of Hepatic Steatosis by Dietary Fatty Acids. World J Gastroenterol. 2014;20: 1746.

48. Bowers EC, McCullough SD. Linking the Epigenome with Exposure Effects and Susceptibility: The Epigenetic Seed and Soil Model. Toxicol Sci. 2017;155: 302–314.

49. McCullough SD, Bowers EC, On DM, Morgan DS, Dailey LA, Hines RN, et al. Baseline Chromatin Modification Levels May Predict Interindividual Variability in Ozone-Induced Gene Expression. Toxicol Sci. 2016;150: 216–224.

50. Chappell G, Kobets T, O'Brien B, Tretyakova N, Sangaraju D, Kosyk O, et al. Epigenetic Events Determine Tissue-Specific Toxicity of Inhalational Exposure to the Genotoxic Chemical 1,3-butadiene in Male C57BL/6J Mice. Toxicol Sci. 2014;142: 375–384.

51. Compare D, Coccoli P, Rocco A, Nardone OM, De Maria S, Cartenì M, et al. Gut-Liver Axis: The Impact of Gut Microbiota on Non Alcoholic Fatty Liver Disease. Nutr Metab Cardiovasc Dis. 2012;22: 471–476.

52. Kirpich IA, Marsano LS, McClain CJ. Gut-Liver Axis, Nutrition, and Non-Alcoholic Fatty Liver Disease. Clin Biochem. The Canadian Society of Clinical Chemists; 2015;48: 923–930.

53. Duseja A, Chawla YK. Obesity and NAFLD. The Role of Bacteria and Microbiota. Clin Liver Dis. Elsevier Inc; 2014;18: 59–71.

54. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic Endotoxemia Initiates Obesity and Insulin Resistance. Diabetes. 2007;56: 1761–1772.

55. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, et al. Selective Increases of Bifidobacteria in Gut Microflora Improve High-Fat-Diet-Induced Diabetes in

Mice through a Mechanism Associated with Endotoxaemia. Diabetologia. 2007;50: 2374–2383.

56. Cani PD, Bibiloni R, Knauf C, Neyrinck AM, Delzenne NM. Changes in Gut Microbiota Control Metabolic Diet–Induced Obesity and Diabetes in Mice. Diabetes. 2008;57: 1470–81.

57. Cani PD, Delzenne NM. Interplay between Obesity and Associated Metabolic Disorders: New Insights into the Gut Microbiota. Curr Opin Pharmacol. 2009;9: 737–743.

58. Boutagy NE, McMillan RP, Frisard MI, Hulver MW. Metabolic Endotoxemia with Obesity: Is it Real and is it Relevant? Biochimie. 2016;124: 11–20.

59. Bäckhed F, Ding H, Wang T, Hooper L V, Koh GY, Nagy A, et al. The Gut Microbiota as an Environmental Factor that Regulates Fat Storage. Proc Natl Acad Sci. 2004;101: 15718–23.

60. Jumpertz R, Le DS, Turnbaugh PJ, Trinidad C, Bogardus C, Gordon JI, et al. Energy-Balance Studies Reveal Associations between Gut Microbes, Caloric Load, and Nutrient Absorption in Humans. Am J Clin Nutr. 2011;94: 58–65.

61. Becker N, Kunath J, Loh G, Blaut M. Human Intestinal Microbiota: Characterization of a Simplified and Stable Gnotobiotic Rat Model. Gut Microbes. 2011;2: 25–33.

62. Wolin M. Fermentation in the Rumen and Human Large Intestine. Science (80). 1981;213: 1463–1468.

63. Høverstad T, Midtvedt T. Short-Chain Fatty Acids in Germfree Mice and Rats. J Nutr. 1986;116: 1772–1776.

64. Dumas M-E, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, et al. Metabolic Profiling Reveals a Contribution of Gut Microbiota to Fatty Liver Phenotype in Insulin-Resistant Mice. Proc Natl Acad Sci. 2006;103: 12511–12516.

65. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison, Bruce S, Dugar B, et al. Gut Flora Metabolism of Phophatidylcholine Promotes Cardivovascular Disease. Nature. 2011;472: 57–63.

66. Muccioli GG, Naslain D, Backhed F, Reigstad CS, Lambert DM, Delzenne NM, et al. The Endocannabinoid System Links Gut Microbiota to Adipogenesis. Mol Syst Biol. 2010;6: 1–15.

67. Cani PD. Crosstalk between the Gut Microbiota and the Endocannabinoid System: Impact on the Gut Barrier Function and the Adipose Tissue. Clin Microbiol Infect. 2012;18: 50–53.

68. Ma X, Hua J, Li Z. Probiotics Improve High Fat Diet-Induced Hepatic Steatosis and Insulin Resistance by Increasing Hepatic NKT Cells. J Hepatol. 2008;49: 821–830.

69. Li Z, Yang S, Lin H, Huang J, Watkins PA, Moser AB, et al. Probiotics and Antibodies to TNF Inhibit Inflammatory Activity and Improve Nonalcoholic Fatty Liver Disease. Hepatology. 2003;37: 343–350.

70. Lee HY, Park JH, Seok SH, Baek MW, Kim DJ, Lee KE, et al. Human Originated Bacteria, *Lactobacillus rhamnosus* PL60, Produce Conjugated Linoleic Acid and Show Anti-Obesity Effects in Diet-Induced Obese Mice. Biochim Biophys Acta. 2006;1761: 736–744.

71. Nardone G, Compare D, Liguori E, Di Mauro V, Rocco A, Barone M, et al. Protective Effects of *Lactobacillus paracasei* F19 in a Rat Model of Oxidative and Metabolic Hepatic Injury. Am J Physiol Gastrointest Liver Physiol. 2010;299: G669-676.

72. Xu R, Wan Y, Fang Q, Lu W, Cai W. Supplementation with Probiotics Modifies Gut Flora and Attenuates Liver Fat Accumulation in Rat Nonalcoholic Fatty Liver Disease Model. J Clin Biochem Nutr. 2012;50: 72–77.

73. John G, Wang L, Nanavati J, Twose C, Singh R, Mullin G. Dietary Alteration of the Gut Microbiome and Its Impact on Weight and Fat Mass: A Systematic Review and Meta-Analysis. Genes (Basel). 2018;9: 167.

74. Ma Y-Y, Li L, Yu C-H, Shen Z, Chen L-H, Li Y-M. Effects of Probiotics on Nonalcoholic Fatty Liver Disease: A Meta-Analysis. World J Gastroenterol. 2013;19: 6911–6918.

75. Lai KP, Chung YT, Li R, Wan HT, Wong CKC. Bisphenol A Alters Gut Microbiome: Comparative Metagenomics Analysis. Environ Pollut. 2016;218: 923–930.

76. Malaisé Y, Menard S, Cartier C, Gaultier E, Lasserre F, Lencina C, et al. Gut Dysbiosis and Impairment of Immune System Homeostasis in Perinatally-Exposed Mice to Bisphenol A Precede Obese Phenotype Development. Sci Rep. 2017;7: 1–12.

77. Younossi ZM, Henry L. Economic and Quality-of-Life Implications of Non-Alcoholic Fatty Liver Disease. Pharmacoeconomics. 2015;33: 1245–1253.

78. Dan AA, Kallman JB, Wheller A, Younoszai Z, Collantes R, Bondini S, et al. Health-Related Quality of Life in Patients with Non-Alcoholic Fatty Liver Disease. Aliment Pharmacol Ther. 2007;26: 815–820.

79. Afendy A, Kallman JB, Stepanova M, Younoszai Z, Aquino RD, Bianchi G, et al. Predictors of Health-Related Quality of Life in Patients with Chronic Liver Disease. Aliment Pharmacol Ther. 2009;30: 469–476.

80. David K, Kowdley K V., Unalp A, Kanwal F, Brunt EM, Schwimmer JB, et al. Quality of Life in Adults with Nonalcoholic Fatty Liver Disease: Baseline Data from the Nonalcoholic Steatohepatitis Clinical Research Network. Hepatology. 2009;49: 1904–1912.

81. Ekstedt M, Hagström H, Nasr P, Fredrikson M, Stål P, Kechagias S, et al. Fibrosis Stage is the Strongest Predictor for Disease-Specific Mortality in NAFLD After Up to 33 Years of Follow-Up. Hepatology. 2015;61: 1547–1554.

82. Mary Martini E, Garrett N, Lindquist T, Isham GJ. The Boomers are Coming: A Total Cost of Care Model of the Impact of Population Aging on Health Care Costs in the United States by Major Practice Category. Health Serv Res. 2007;42: 201–218.

83. Angulo P. Nonalcoholic Fatty Liver Disease and Liver Transplantation. Liver Transplant. 2006;12: 523–534.

84. Seto W, Yuen M. Nonalcoholic Fatty Liver Disease in Asia: Emerging Perspectives. J Gastroenterol. 2017;52: 164–174.