HUMAN BISPHENOL A BIOMONITORING AND BIOTRANSFORMATION PROGRAMMING IN THE DEVELOPING FETUS

by

Muna S. Nahar

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Doctoral Committee:

Assistant Professor Dana C. Dolinoy, Chair
Professor Craig Harris
Professor Paul Hollenberg
Assistant Professor Sung Kyun Park

DEDICATION

I dedicate this thesis to my family and my two undergraduate research advisors: Dr. S.G. Holmes and the late Dr. L. Appel. They nurtured my curiosity for science and believed in me long before I was confident in my abilities.

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LIST OF ABBREVIATIONS

AP1	Activator Protein1
B2M	β2-microglobulin
BSC	Bisulfite converted
bp	Base pair
BPA	Bisphenol A
CDC	U.S. Centers for Disease Control and Prevention
CES2	Carboxylesterase 2
COMT	Catechol o-methyltransferase
CpG	Cytosine guanine dinucleotides
CT	Threshold cycle
DOHaD	Developmental origins of health and disease
EAC	Endocrine active compound
ETS	E-Twenty Six
FPKM	Fragments per kilobase of exon per million fragments mapped
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GUSB	β-glucuronidase
HPLC coupled ESI-MS/MS	High-performance liquid chromatography coupled with API 2000 electrospray triple-quadrupole mass spectrometer
IQR	Interquartile Range
LINE1	Long interspersed transposable element-1
LSHE	Laboratory for the Study of Human Embryology
LOQ	Limit of Quantification
LUMA	Luminometric Methylation Assay
NGS	Next generation sequencing
PAPS	3'-phosphoadenosine-5'phosphosulfate
PBPK	Physiologically based pharmacokinetic
RIN	RNA integrity numbers
RT-qPCR	Real time quantitative polymerase chain reaction
STS	Steroid sulfatase
SULT1A1	Sulfotransferase isoform 1A1
SULT2A1	Sulfotransferase isoform 2A1
TFBS	Transcription factor binding site
TSS	Transcription start site
UBC	Ubiquitin C
UGT2B15	UDP-glucuronosyltransferase isoform 2B15
XME	Xenobiotic metabolizing enzyme

ABSTRACT

The ubiquitous monomer, bisphenol A (BPA), is an endocrine active compound used in the production of polycarbonate plastics and epoxy resin. Environmental biomonitoring and epidemiology studies report continuous exposure in humans that are associated with different adverse health outcomes. Although regulatory agencies are concerned with BPA's potential to harm pregnant women, fetuses, and young children, BPA's toxicity in humans remains highly debated. Animal and *in vitro* studies support BPA exposure-disease relationships, but metabolic, genetic and physiological differences ultimately limit translatability to humans. Thus, the objective of this dissertation was to identify BPA concentrations and early biological outcomes directly in humans, focusing on the developing fetus. Using healthy 1^{st}-2^{nd} trimester human clinical specimens obtained from a fetal biobank, we hypothesized that BPA concentration and biotransformation will differ across age and tissue compartments, altering biotransformation programming in the developing fetus.

The overall goal of this work was to characterize BPA toxicokinetic and toxicodynamic profiles that influence tissue-specific BPA biotransformation via xenobiotic metabolizing enzymes (XME) and elicit subsequent cellular changes. First, we reported BPA concentrations in N=50 fetal liver specimens (total BPA range: below limit of quantification - 96.8 ng/g), where the concentration of free BPA was three times that of BPA conjugates. Both concentrations and metabolic profiles varied across age with significant reduction in BPA-specific XME gene expression of *UGT2B15*, *SULT1A1*, and *STS* in fetal versus adult livers. Next, we examined matched fetal liver, kidney, and

placenta in N=12 subjects and observed significant tissue-dependent differences in BPA concentrations, XME expression profiles, and global DNA methylation. Fetal livers exhibited higher BPA concentrations compared to matched tissues; however, XME expression profiles suggest an increased likelihood of BPA-glucuronide deconjugation and BPA-sulfate conjugation across the fetal compartments. With organ-specific differences in the epigenome, only placental global methylation measurements were associated with BPA. Finally, we investigated BPA's role in pathway specific biological outcomes and regulation in fetal liver. In particular, we identified 14 different XME candidate genes that were down regulated with higher BPA concentrations. After identifying common transcription factor binding sites across all candidate gene promoters, we reported increased methylation at these functionally relevant sites with higher BPA concentrations at several candidate genes.

In summary, results suggest that the 1^{st}-2^{nd} trimester human fetus is exposed to a considerable amount of BPA *in utero*, especially of the active free BPA form. XME expression profiles reveal an altered capacity for BPA biotransformation in the fetus compared to adults, with distinct metabolic profiles across different tissues. Interestingly, higher BPA concentrations in fetal liver were associated with reduced expression of novel XME candidate genes mediated by epigenetic mechanisms. These findings indicate that environmentally relevant concentrations of BPA, even across a short window of development, result in detectable changes in the host's toxicological defense system.

CHAPTER 1
Introduction

1.1 Children's Environmental Health Relevance

Since the beginning of the 20th century, newborn and infant mortality has rapidly declined as a result of improvements in nutrition, sanitation practices, and infectious disease control (K. S. Lee, 2007). Despite these healthcare reforms, pregnancy-related complications and infant morbidities still exist in the population today and give rise to excessive medical costs. Prematurity and low birth weights are commonly associated with neonatal morbidities, contributing to long-term adverse health consequences as well as death in children under 5 (Goldenberg & Culhane, 2007). According to the World Health Organization, approximately 13 million babies were born prematurely across the globe in 2005 and this number is rising (Beck et al., 2010). Although the focus in the last century has been on maternal and childhood survival, the current public health issues require the need for reduction in newborn morbidities that can influence quality and longevity of life. Like many diseases, the determinants of abnormal fetal development and birth-related complications are quite complex, influenced by a variety of environmental, social, and genetic factors (Gunning-Schepers & Hagen, 1987). Social reform and innovative medical practices will continue to benefit maternal and child health; however, to apply the best preventative measures, we must first explore gene-

environment interactions throughout pregnancy to understand early life disease etiologies.

Developmental origins of health and disease

Following a seminal study by Barker and colleagues from the University of Southampton in the 1980s, several associations between low birth weight and adult chronic diseases such as coronary heart disease, hypertension, and type 2 diabetes have been reported (Barker, 2007). These epidemiological studies along with recent animal studies support the developmental origins of health and disease (DOHaD) hypothesis. The DOHaD hypothesis states that early life exposures to various environmental stressors can perturb normal biological programming and these maladaptations can lead to disease later in life (Barker, 1990). Malnutrition and infectious agents can influence development when exposures occur throughout sensitive windows of development, especially in the prenatal, postnatal, and adolescent stages of life. Perturbations at different life stages can influence unique physiological processes with effects ranging from metabolic disorders to cancer development (Wright & Christiani, 2010). Disease outcomes are dependent on the type of insults and timing of exposure in relation to these sensitive windows of development.

With the growing concern over chemicals that are ubiquitous in our environment, DOHaD as well as general environmental health research has shifted from the study of nutrition and microbial infection to environmental contaminants. In fact, the focus on understanding the link between chemical exposures in early life and disease susceptibility has led to the development of the large-scale National Children's Study (Pak & Souders,

2012). My overall dissertation project parallels many of the epidemiological studies in children's environmental health, but focuses on the characterization of chemical exposure within the developing human fetus and identification of early genetic and epigenetic changes associated with exposure. Findings from this dissertation help bridge many of the knowledge gaps that remain using animal and epidemiological studies, while contributing to both human environmental exposure and health risk assessment.

1.2 The Vulnerable Human Fetus

For the proper development of an organism, a sequence of major biological processes must take place within a complex milieu. The timing of these events and complexity of the biological structures vary across species given the morphological and genomic differences (Xue, Yi, Huang, Shi, & Li, 2011); however, general developmental and regulatory mechanisms may be well conserved. The human fetus typically gestates for 266 days, unlike its rodent counterparts where gestation lasts from 19-23 days. Gestation is divided into several stages including fertilization, pre-implantation, post-implantation, and fetal periods. Seven days after human fertilization, the newly formed blastocyst implants into the uterus, marking the beginning of embryogenesis (Downs, 2008). Once the three germ layers have formed between day 7 to 20, organogenesis takes place until week 8 (Jirasek, 2000). These dramatic changes occur throughout the first trimester, followed by rapid growth and functional maturation throughout the fetal period until birth at approximately 40 weeks of gestation.

Vulnerable window of development

Throughout prenatal development, the most dynamic physiological changes occur in the embryonic stage; thus, this sensitive period is most susceptible to many teratogenic effects. Embryonic development is normally described using the Carnegie stages (C1-23) of development, based on the 23 distinct external appearances of the embryo within the first 2 months of conception (O'Rahilly & Muller, 2010). By stages C10-23, genes related to "stemness" are down-regulated and those related to differentiation and organ development are up-regulated (Xue, et al., 2011). After neurulation, the heart is the first organ to develop, followed by liver, adrenal gland, and then kidney (Hill, 2007). Toxic environments that coincide with diverse developmental processes throughout organogenesis are more likely to cause adverse effects such as interference with proper growth trajectories, malformations, or miscarriages (Rutledge, 1997). The exact uterine environment necessary for sustaining a healthy human conceptus and the maturing fetus is not well defined. Thus, characterizing both the normal uterine environment and its changes will be necessary to understand organ-specific developmental alterations that contribute to postnatal morbidity and mortality.

Role of hormones in pregnancy

Hormones play an important role in the initiation and establishment of fertilization, implantation, and other pregnancy-related events, and their regulation has been studied extensively in humans. Continuous crosstalk between the maternal and fetal endocrine system is necessary for modulating correct metabolic and developmental alterations in pregnancy (Feldt-Rasmussen & Mathiesen, 2011). In the early stages,

elevated progesterone and estrogen help prepare the endometrium for blastocyst implantation (Halasz & Szekeres-Bartho, 2013). Utilizing stimulatory feedback mechanisms, the developing placenta starts to excrete chorionic gonadotropin to prolong progesterone activity. The placenta becomes the major site for active steroidogenesis, providing precursor molecules to the fetal adrenal gland for the production of important hormones like glucocorticoids (Sherwood, 2008). By week 8, the placenta is a functionally mature endocrine organ with significant placental secretions that can sustain pregnancy despite ovariectomy (Evain-Brion & Malassine, 2003). By the end of pregnancy, progesterone concentrations are reduced, accompanied by increased levels of estrogen, prostaglandin, oxytocin, and other hormones that help ripen the cervix in preparation for parturition (Terzidou, 2007). Without proper hormonal checks and balances, the uterine environment becomes hostile to the fetus, leading to pregnancy terminations or morbidity.

Endocrine disruption

Given the important role of hormones throughout pregnancy, foreign chemicals or xenobiotics that mimic or antagonize endogenous steroids can easily modify developmental programming and subsequently affect organ function. Endocrine active compounds (EACs) are a class of chemicals originally designed for industrial and agricultural purposes. EACs can alter normal endocrine function by disrupting hormone production and metabolism or by changing hormone distribution throughout the body at very low doses (Schug, Janesick, Blumberg, & Heindel, 2011). The compounds use a broad spectrum of mechanisms ranging from nuclear receptor signaling to epigenetic

modification to influence the body in a tissue and time-specific manner. Thus, typical dose-response curves and toxicological assessments that are routinely used for other environmental contaminants have not been useful for EAC research (McLachlan, 2001). While EAC-related studies have rapidly grown since the 1990s with emphasis on vulnerable windows of development, further research is necessary to characterize low dose exposures and biological effects in the developing human fetus. The goal of my dissertation project is to fill this knowledge gap in regards to the most controversial EAC, bisphenol A.

1.3 Bisphenol A and Health

Bisphenol A: uses and fate

Bisphenol A (BPA), a synthetic estrogen used in the manufacturing of polycarbonate plastics and epoxy resin, is one of the highest production volume chemicals worldwide with annual production of more than 8 billion pounds (L. N. Vandenberg et al., 2010). First synthesized in 1891, its estrogenic properties were identified in the 1930s with commercial use of epoxy resin beginning in the 1950s. By the 1970s after the discovery of its use for polycarbonate plastic, BPA production in the USA reached half a billion pounds (S. A. Vogel, 2009). BPA continues to play a fundamental role in the plastics industry with its various desirable properties including transparency, high impact strength, malleability, and superior adhesive properties (Ben-Jonathan & Steinmetz, 1998). Consumer products that contain BPA include food and beverage containers, thermal paper, electronics, medical supplies, building materials,

adhesives, and safety and leisure equipments like helmets. BPA exposure from these consumer products is continuous and ubiquitous, raising concern for health effects especially in vulnerable populations.

BPA has the ability to leach from these consumer products, creating many opportunities for environmental and human exposure. Introduction to high temperatures, acidic or basic solutions, and physical damage can lead to the hydrolysis of plastic polymers into the estrogenic monomer. Breakdown of BPA has been reported in several products including baby bottles, water bottles, polyvinyl chloride (PVC) tubing, and plastic containers (Carwile et al., 2009; Lopez-Cervantes & Paseiro-Losada, 2003; Mountfort, Kelly, Jickells, & Castle, 1997; Nerin, Fernandez, Domeno, & Salafranca, 2003; Sajiki & Yonekubo, 2004). BPA can enter our ecosystems when consumer products are disposed into wastewater systems and landfills. There, in the environment, BPA containing polymers remain as debris or undergo biodegradation and photodegradation (Crain et al., 2007). Environmental contamination can also occur through large-scale manufacturing wastes during processing, handling, and transportation of the compound; for example, in 1993 approximately 109 tons of BPA was released into air, water, and wastewater treatment facilities (Staples, Dorn, Klecka, O'Block, & Harris, 1998).

Humans encounter BPA through various routes, but ingestion through dietary sources like food packaging and canned beverages are the primary means of BPA exposure as determined by dietary intervention studies. In these studies, volunteers who were asked to consume a set amount of canned or packaged meals showed increased urinary BPA concentrations (Carwile, Ye, Zhou, Calafat, & Michels, 2011), while those

asked to eat fresh unprocessed foods had decreased urinary concentrations compared to baseline (Rudel et al., 2011). Investigation into the quantification of free BPA in foodstuff has revealed significant variation between brands, across food type and composition, and from lot-to-lot (Cao et al., 2011; Noonan, Ackerman, & Begley, 2011). Other important routes of exposure include inhalation from aerosolized BPA and dermal uptake from contact with thermal paper or medical supplies (Biedermann, Tschudin, & Grob, 2010; Calafat et al., 2009; Fleisch, Sheffield, Chinn, Edelstein, & Landrigan, 2010). Since the identification of every unique source of exposure can be difficult, environmental and biological specimens like urine have been analyzed to estimate total body burden in the population. These studies estimate that human daily intake range from 0.1-1 µg/kg body weight per day (L. N. Vandenberg, et al., 2010).

Bisphenol A and health effects

Once in the body, the parent compound (free BPA) is metabolized into inactive BPA conjugates in the liver during first pass metabolism (Figure 1.1). In humans, the compound has a short half-life of 6 to 43 hours and once metabolized, conjugates are excreted mainly through urine (Dybing & Soderlund, 1999a; Volkel, Colnot, Csanady, Filser, & Dekant, 2002). Rodent species, on the other hand, excrete BPA conjugates through bile and feces; the half-life of BPA may be longer as a result of intestinal deconjugation and enterohepatic recirculation (Chapin et al., 2008). Even with rapid metabolism and excretion of the estrogenic free BPA species, dozens of studies have identified adverse health effects associated with exposure in both human and animal models.

In the late 1970s, one of the first national BPA studies was conducted by the National Cancer Institute and later continued by the National Toxicology Program. The 2 year study, based on high dose exposures to adult rodent models, found "no convincing evidence" for carcinogenicity and provided the basis for the BPA safety standard or reference dose that is still used today at 50 µg/kg of body weight per day (Carcinogenesis Bioassay of Bisphenol A (CAS No. 80-05-7) in F344 Rats and B6C3F1 Mice (Feed Study), 1982). While originally dismissed because of its weak estrogenicity, BPA started to become scrutinized for its hormone-like effects as research into EACs gained momentum in the 1990s. Challenging the traditional toxicity and regulatory testing paradigm, the first BPA toxicity studies reported BPA dependent changes to developmental and reproductive endpoints such as prostate weight and mammary proliferation using low dose exposures or pregnant rodent models (Colerangle & Roy, 1997; Nagel et al., 1997). Since then, rodent studies have associated pre- or perinatal BPA exposure with altered body weight, impaired brain development, altered reproductive function, changes in immune function, metabolism, and increased cancer susceptibility along with other chronic diseases (Chapin, et al., 2008; Maffini, Rubin, Sonnenschein, & Soto, 2006; Laura N. Vandenberg et al., 2013). Low dose concentrations used to expose animals typically fall below the reference dose, but often do not reflect environmentally relevant concentrations encountered in a real world setting.

Over the past few years, environmental biomonitoring studies in conjunction with epidemiological research have established links between relevant human BPA exposure and diseases. In 2008, the first comprehensive environmental epidemiology study for

BPA reported significant positive associations between urinary BPA concentrations and risk for cardiovascular disease, type 2 diabetes, and liver enzyme abnormalities (Lang et al., 2008). Since then, a number of studies have correlated increased BPA exposure with adverse health outcomes across human populations that include increased sex hormone concentrations, decreased semen quality, altered childhood behavior, and recurrent miscarriages along with other metabolic and reproductive dysfunction (Braun et al., 2011; Galloway et al., 2010; Meeker et al., 2010; Sugiura-Ogasawara, Ozaki, Sonta, Makino, & Suzumori, 2005; Laura N. Vandenberg, et al., 2013). While these findings do not imply causation and do not take into account co-exposures, results indicate that concentrations relevant to human exposures may be associated with both short-term and long-term disease outcomes.

Bisphenol A mechanisms of action and toxicity

BPA is a compound classically known for its weak estrogenic activity. As an EAC, BPA can perturb endocrine function by binding weakly to several steroid receptors including estrogen receptors (ER α and β), as well as thyroid and androgen hormone receptors (Gould et al., 1998; Kuiper et al., 1998; Moriyama et al., 2002; Welshons et al., 2003). In addition, BPA has a strong binding affinity for the trans-membrane ER, G protein-coupled receptor 30 (GPR30), and the orphan nuclear receptor, estrogen related receptor gamma (ERRγ) (Takayanagi et al., 2006; Thomas & Dong, 2006). BPA can also activate transcription factors, such as peroxisome x receptor (PXR) and the aryl hydrocarbon receptor (AhR), which are often involved in cross talk with steroid receptors (Kruger, Long, & Bonefeld-Jorgensen, 2008; Sui et al., 2012). Concentrations required

for receptor-mediated responses are tissue-dependent, given that receptor expression and occupancy differ from one cell type to another (Welshons, et al., 2003).

As an EAC, BPA can also act through non-genomic signaling pathways. Unlike hormone receptor-mediated mechanisms, non-genomic systems can produce rapid, amplified responses after activation at low doses down to the pM-fM concentrations (Welshons, Nagel, & vom Saal, 2006). In mammalian models, BPA can increase intracellular calcium influx as well as help modify different kinases and nuclear factors such as cAMP, MAPK, ERK, JNK, and NFkB (S. Lee et al., 2008; Song, Lee, & Choi, 2002; Walsh, Dockery, & Doolan, 2005; Wozniak, Bulayeva, & Watson, 2005). The exact signal transduction mechanisms utilized by BPA are not well understood but are likely tissue-dependent.

BPA's mechanisms of action also include genomic toxicity and oxidative damage. In rats exposed to various concentrations of BPA, increased frequency of micronuclei, chromosomal aberrations, and DNA damage was observed in blood (Tiwari et al., 2012). Furthermore, DNA adducts as well as mitotic and meiotic aneuploidy were reported after exposure across several cell lines and animal models (De Flora et al., 2011; George et al., 2008; Izzotti, Kanitz, D'Agostini, Camoirano, & De Flora, 2009; Johnson & Parry, 2008). In addition to genomic instability, several studies report that BPA induce reactive oxygen species and oxidative stress by altering mitochondrial function (Kabuto, Amakawa, & Shishibori, 2004; Lin et al., 2013; Moon et al., 2012; Ooe, Taira, Iguchi-Ariga, & Ariga, 2005).

With crosstalk between cell membrane, cytosolic, nuclear, and genetic factors within the cell, EACs like BPA may act through several mechanisms simultaneously (Silva, Kabil, & Kortenkamp, 2010). Many of the receptors and factors play an important role in gene regulation important for normal differentiation and maturation processes, with implications for embryonic and fetal development. Thus, stimulation or inhibition of signaling pathways or damage to regulatory proteins in early development can produce a wide range of adverse cellular responses. The type of mechanism used and subsequent responses are dependent on BPA concentration, protein expression, cofactor availability, tissue location, and timing.

Bisphenol A and epigenetic regulation

There is evidence to suggest that epigenetic mechanisms aid in endocrine disruption. Alterations to the epigenome through changes in DNA methylation, histone modification, or non-coding RNAs help regulate gene activity without varying the DNA sequence. Environmentally induced changes in expression and phenotype can occur through one or more stable modifications that persist throughout the lifetime (Wolffe & Matzke, 1999). These heritable markers are especially important for developmental gene regulation and can modify disease susceptibility; thus, epigenetic modifications are likely mechanisms that support the DOHaD hypothesis. For example, EACs like diethylstilbestrol (DES) cause increases in breast cancer risk in adulthood as well as reproductive tract abnormalities several generations after initial exposure. These transgenerational effects from environmental estrogens are likely explained by epigenetic modifications to the germ line (Crews & McLachlan, 2006).

In several animal studies, developmental exposure to BPA caused hypermethylation of the estrogen receptor promoter regions in rat testis, hypomethylation of *Hoxa10* in CD-1 mice reproductive tract, hypomethylation of the *Pde4d4* gene in rat prostrate, and altered methylation of the A^{vy} gene in viable yellow agouti mice tails (O. Anderson et al., 2012; Bromer, Zhou, Taylor, Doherty, & Taylor, 2010; Doshi, Mehta, Dighe, Balasinor, & Vanage, 2011; S. Ho, W. Tang, J. Belmonte de Frausto, & G. Prins, 2006). These DNA methylation changes were associated with decreased fertility, metabolic disorder, and prostate cancer. BPA also demonstrated increases in histone H3 trimethylation and Enhancer of Zeste Homolog (EZH3) histone methyltransferase expression in mice mammary tissue (Doherty, Bromer, Zhou, Aldad, & Taylor, 2010). The newest studies in epigenetic research show that BPA is associated with alterations in several miRNAs including miR-146a in human placenta, mouse sertoli cell lines, and the ewe ovary (Avissar-Whiting et al., 2010; Cho et al., 2010; Nagel & Bromfield, 2013). Still, more studies are required that explore the interaction between multiple epigenetic pathways, especially in humans.

1.4 Study Overview

This dissertation research builds upon the theme of environmental exposures at vulnerable windows of development. Given its widespread presence and controversy, it is necessary to understand whether BPA poses a risk to environmental and community health. Without proper measurements of the true exposures and subsequent effects of BPA as well as other chemicals within our population, haphazard changes in policies and

social norms can be economically costly. On the other hand, confirmation of BPA related adverse health effects is essential to drive immediate regulatory changes or interventions. Even with the hundreds of BPA studies reported every year, this dissertation project contributes to the general body of literature, focusing on the most vulnerable of populations: the developing human fetus.

BPA toxicology studies using animal models show strong evidence for an exposure-disease relationship, especially at low dose exposures with health effects related to developmental and reproductive dysfunction. Extrapolating these findings to humans is difficult given physiological and genetic differences from one species to another. Even after accounting for environmental exposures, how chemicals change within the body (toxicokinetics) and how the body responds to these chemicals (toxicodynamics) vary between species, across tissues, and with time (Dybing & Soderlund, 1999b). Human physiologically based pharmacokinetic (PBPK) models may be utilized, which mathematically simulate how chemicals are absorbed, distributed, metabolized, and excreted by relying on *a priori* knowledge of the human anatomical and biochemical makeup. However, the robustness and reliability of these predictions are a major concern, and current PBPK models for a variety of chemicals have limited applications for the pediatric and obstetric community (Barrett, Della Casa Alberighi, Laer, & Meibohm, 2012). Environmental epidemiology studies are helpful for determining exposure-disease associations under real world conditions; however, expensive and time-consuming longitudinal datasets are required to assess causality. Thus, underlying mechanisms are often identified using *in vitro* or animal model systems. To properly understand exposure-disease relationships, it is ideal to characterize both the

toxicokinetic and toxicodynamic potential of a compound within humans. Accessibility of human target tissue for these analyses, while challenging, can be very informative for environmental health risk assessment. Acknowledging the limitations with traditional animal toxicological and epidemiological studies, this dissertation project bridges the knowledge gap by employing human clinical specimens to characterize different aspects of BPA toxicokinetics and toxicodynamics.

A key factor in toxicological assessment is chemical biotransformation or xenobiotic metabolism. In the body, xenobiotic metabolism enzymes (XME) help activate or detoxify xenobiotics by altering the physiochemical properties of the compound within any given site. The chemical reactions are catalyzed by two groups of enzymes called phase I and phase II XMEs. Phase I XMEs catalyze hydrolysis, reduction, and oxidation reactions, while phase II XMEs are involved in glucuronidation, sulfation, acetylation, methylation, and other conjugation reactions (Casarett, Klaassen, & Watkins, 2003). These chemical transformations increase hydrophilicity in order to assist in the elimination of foreign agents from the body. The enzymes are sensitive to the environment with expression or activity varying by cofactor and nutrient concentrations, genetic polymorphisms, age, tissue-type, and environmental exposures.

Research objectives

The overall goal for this project is to address different components of BPA toxicokinetic and toxicodynamic principles, specifically related to metabolism, which can be important for human development. The three main chapters included in this dissertation focus on BPA exposure in tissue and the xenobiotic metabolism pathway

(Figure 1.2). In the first half of the dissertation, we identify differences in tissue-specific BPA concentrations as well as expression of BPA-specific XMEs in fetal versus adult livers (Aim 1, Chapter 2) or across various fetal tissues (Aim 2, Chapter 3). In the latter half, we explore BPA exposure-dependent changes in the expression of the general biotransformation system along with their epigenetic and genetic regulation (Aim 3, Chapter 4). While the dissertation project takes only a cross-sectional snapshot of a chemical across the developing human fetus in relation to xenobiotic metabolism, we investigate our scientific questions by using unique human clinical specimens and new high throughput technologies. These findings are relevant to human exposure and health risk assessment, contributing to the latest research in children's environmental health and DOHaD.

Study samples

In general, human studies are limited to surrogate tissues like saliva and blood to inform xenobiotic concentration or physiological changes related to a target tissue. To address our three specific aims in this dissertation, we utilized human fetal specimens. Pre-existing human fetal tissue samples, ranging from gestational day 70 to 120, were obtained from the NIH-funded University of Washington Laboratory for the Study of Human Embryology (LSHE) fetal tissue bank (2R24 HD000836-47). These healthy tissue specimens were originally collected from volunteers undergoing elective terminations during the 1st or 2nd trimester of pregnancy. After surgery and proper consent for donation, organs were flash-frozen and subsequently stored in polycarbonate-free polypropylene tubing at -80 °C, prior to shipment to the University of Michigan

(UM) on dry ice. Other than gestational age, and occasionally sex and race, no identifiable information regarding the clinical samples that could be traced to subjects was provided. Thus, samples met the criteria for IRB exemption for human subject research (UM IRB exemption: HUM00024929). For Aim 1 and 3, we analyzed BPA concentrations and XME genes across N=50 fetal liver specimens. Of the 50 samples, N=12 fetal liver specimens were matched to fetal kidney and placental samples (N=12 each) from the same subject for Aim 2 analysis.

Figure 1.1 BPA Biotransformation and Elimination

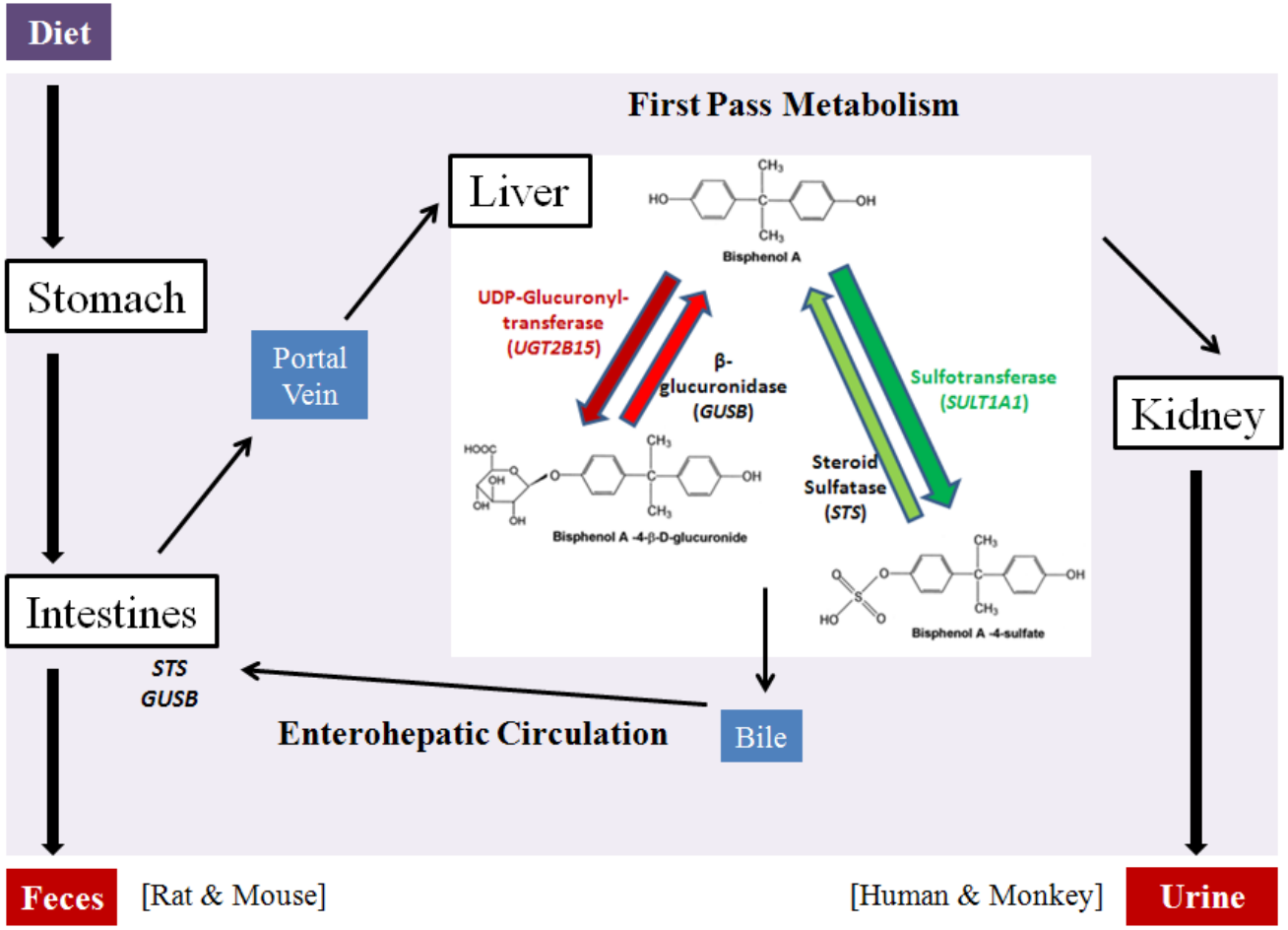
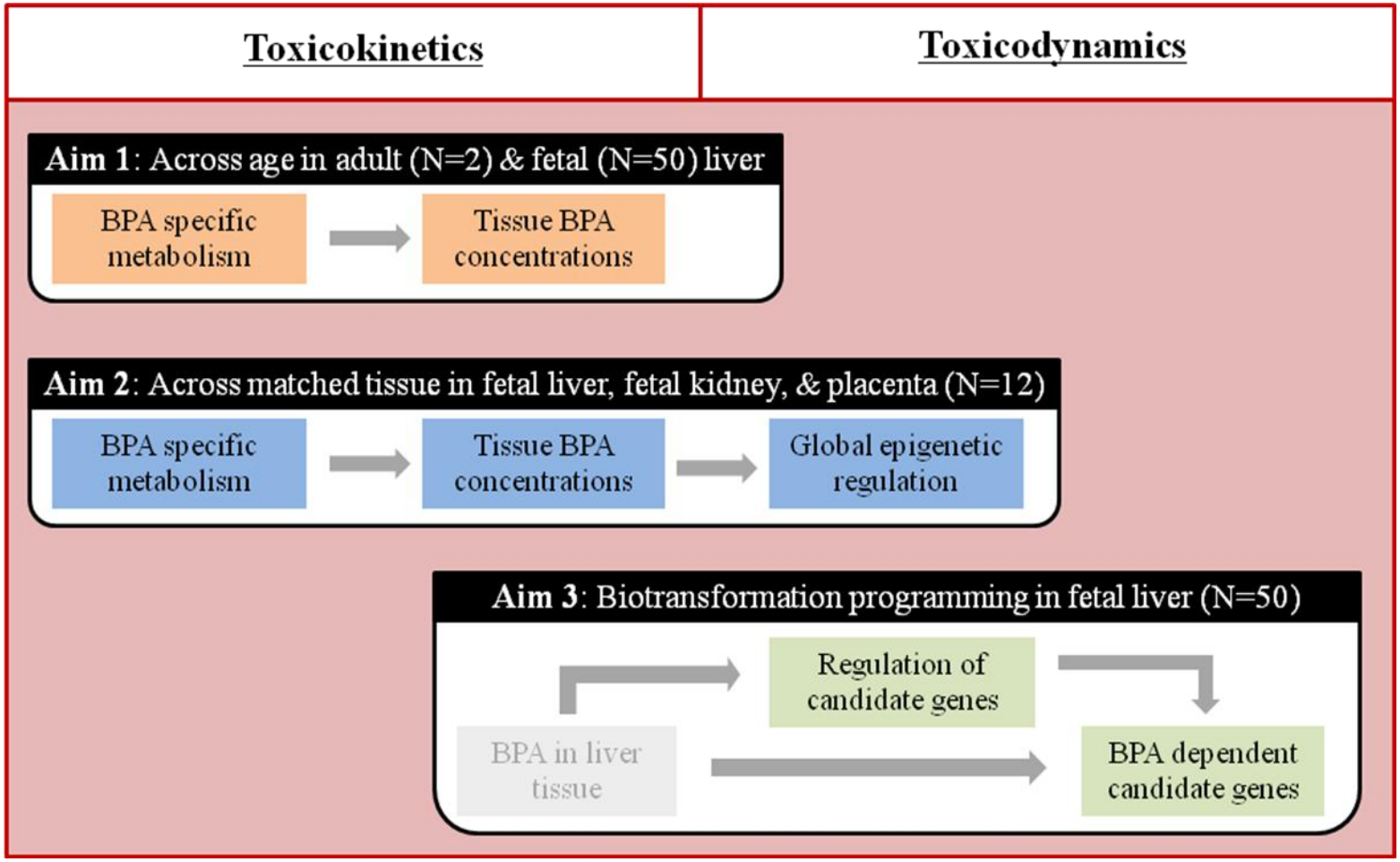


Figure 1.2 Conceptual Overview of Dissertation Aims



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CHAPTER 2

In Utero Bisphenol A Concentration and Biotransformation Gene Expression Across Human Fetal and Adult Liver Specimens

Text reused and modified from Nahar, M.S., Liao, C., Kannan, K., Dolinoy, D.C., 2013. Fetal Liver Bisphenol A Concentrations and Biotransformation Gene Expression Reveal Variable Exposure and Altered Capacity for Metabolism in Humans. J Biochem Mol Toxicol. 27(2):116-123. Permission for reuse approved by Rightslink and Copyright Clearance Center, Inc.

2.1 Abstract

Widespread exposure to the endocrine active compound, bisphenol A (BPA), is well documented in humans. A growing body of literature suggests adverse health outcomes associated with varying BPA concentrations. Here, we measured tissue concentrations of free BPA and conjugated BPA, and evaluated gene expression of xenobiotic metabolizing enzymes (XMEs) specific for BPA biotransformation in fifty 1st and 2nd trimester human fetal liver samples. Both free BPA and conjugated BPA concentrations varied widely, with free BPA exhibiting three times higher concentrations than conjugated BPA concentrations. As compared to gender-matched adult liver controls, UDP-glucuronosyltransferase (*UGT2B15*), sulfotransferase (*SULT1A1*), and steroid sulfatase (*STS*) genes exhibited reduced expression while β-glucuronidase (*GUSB*) mRNA expression remained unchanged in the fetal tissues. This study provides

evidence that there is considerable exposure to BPA during human pregnancy and that the capacity for BPA metabolism is altered in the human fetal liver.

2.2 Introduction

Human exposure to BPA is widespread, and several studies have reported detectable concentrations of total urinary BPA in a majority of individuals in populations around the world, including the United States, China, and Korea (Calafat, Ye, Wong, Reidy, & Needham, 2008; Z. Zhang et al., 2011). Since the first BPA biomonitoring study conducted at the U.S. Centers for Disease Control and Prevention (CDC) in 2001, dozens of spot or pooled urine specimens have been examined across different cohorts for BPA, accounting for exposure from a variety of sources (Brock et al., 2001). Detectable urinary BPA concentrations have been observed across various age groups; the body burden generally decreases with age, indicating higher BPA concentrations in children compared to adults (Calafat, et al., 2008). Although most human biomonitoring studies report total BPA concentrations in urine, the different individual BPA species are typically unspecified and concentrations do not reflect the absorbed or internal dose.

In the BPA scientific community, there is a consensus for proper sampling and improved technical analysis. Recent studies have addressed BPA internal dose by measuring circulating concentrations in serum, plasma, and cord blood (He et al., 2009; Kaddar et al., 2009; Volkel, Bittner, & Dekant, 2005; M. Yang, Ryu, Jeon, Kang, & Yoo, 2009). Furthermore, techniques that account for contamination and detect BPA at lower concentrations are becoming popular, although remain limited by high costs. Studies that

specifically report the internal dose of BPA in tissue are scarce. Access to tissue is often difficult and optimizing analyses for this matrix prove to be tricky. Still, quantifying tissue BPA concentrations, especially in humans, is necessary for better risk assessment and determination of BPA associated adverse health outcomes.

While the debate on human BPA exposure-disease relationships continues, the National Toxicology Program (NTP) declared in 2008 that there is concern for BPA toxicity in sensitive populations such as pregnant women, fetuses, and children. So far, BPA has been reported in pregnancy-related biological fluids, including breast milk, amniotic fluid, and follicular fluid (Edlow, Chen, Smith, Lu, & McElrath, 2012; Engel, Levy, Liu, Kaplan, & Wolff, 2006; Ikezuki, Tsutsumi, Takai, Kamei, & Taketani, 2002; Sun et al., 2004; Yamada et al., 2002; Ye, Kuklenyik, Needham, & Calafat, 2006). In adults, human pharmacokinetic data suggest rapid metabolism of estrogenic free BPA into the non-estrogenic BPA-glucuronide and BPA-sulfate conjugates, via UDP-glucuronosyltransferase (*UGT2B15*) and sulfotransferase (*SULT1A1*) enzymes, followed by urinary elimination (Hanioka, Naito, & Narimatsu, 2008; Nishiyama et al., 2002). In human and rodent fetuses and neonates, however, there is generally reduced or altered capacity for chemical detoxification (Hines & McCarver, 2002; McCarver & Hines, 2002); thus, metabolism may not ensure negligible risk in these vulnerable populations. Furthermore, β-glucuronidase (*GUSB*) and steroid sulfatase (*STS*), enzymes that catalyze the breakdown of inactive BPA conjugates to free BPA, have been detected in mammalian placenta (Kriz, Bicikova, & Hampl, 2008; Sperker, Backman, & Kroemer, 1997). Studies report detectable concentrations of estrogenic free BPA in the human placenta and amniotic fluid, indicating the potential for dysregulation of important

biological processes necessary for fetal development and disease progression later in life (Cao et al., 2012; Ikezuki, et al., 2002; Jimenez-Diaz et al., 2010).

For this dissertation aim, 1st and 2nd trimester human liver specimens were analyzed using a high performance liquid chromatography based technique for endogenous BPA concentrations to assess *in utero* exposure. In order to assess the ability of the fetus to detoxify BPA, tissue-specific BPA concentrations were compared to expression of metabolizing enzyme isoforms specific for BPA biotransformation. In this chapter, the primary objective was to compare BPA concentrations and mRNA expression between fetal and adult liver specimens in order to test the hypothesis that the developing fetus is at a greater risk for BPA exposure and subsequently, BPA toxicity.

2.3 Materials and Methods

Tissue samples

From the fetal tissue biobank, N=50 fetal liver specimens weighing more than 700 mg were chosen. With the exception of one sample, all flash frozen fetal livers were stored at -80°C for less than 2 years with more than 75% of samples stored for less than 1 year. Furthermore, multiple tissue freeze-thaw events were minimized to prevent BPA and mRNA degradation. Previously, BPA in urine samples stored at -20°C over a year exhibited minimum degradation (Calafat et al., 2005), and thus is not expected to be a considerable limitation in our study. No significant correlation was observed between time in -80°C storage and free BPA concentrations (r <0.03). While tissue type and gestational age was available for all specimens, several samples had missing sex

information (N=10). Sex was determined from these particular tissues by assaying for the Y chromosome-specific *SRY* and the X chromosome-specific *ATL1* genes using a nested PCR analysis as described previously, with slight modifications (Tungwiwat, Fucharoen, Ratanasiri, Sanchaisuriya, & Fucharoen, 2003).

As controls, healthy frozen adult liver samples were procured (Asterand, Detroit, MI). These normal adult liver specimens, belonging to one male and one female 50-year-old Caucasian subjects, were recovered from routine autopsies, stored in polycarbonate-free polypropylene tubing, and immediately flash frozen for storage. The liver specimens did not exhibit any pathological characteristics.

BPA analysis and quality control

Frozen liver samples were processed for quantification of free BPA and conjugated BPA species. An average of 630 mg (between 400-970 mg) of excised frozen tissue was pulverized into fine powder using a stainless steel mortar and ceramic pestle above liquid nitrogen and stored in a 2 mL polypropylene eppendorf tube. The homogenized tissue was shipped to the Wadsworth Center (New York State Department of Health, Albany, NY) overnight on dry ice, where samples were processed. Homogenized liver samples were spiked with 5 ng of the internal standard, $^{13}C_{12}$-BPA, and further homogenized in 5 mL of acetonitrile. The method for the analysis of free BPA and conjugated BPA has been previously described (O. S. Anderson et al., 2012). Briefly, tissue samples were extracted several times with acetonitrile, and reconstituted with 10% dichloromethane in hexane. Sample extract was then loaded onto a Strata NH_2 cartridge (200mg/3cc, Phenomenex, Torrance, CA) pre-conditioned with 5 mL of 80%

methanol in acetone and 5 mL of hexane. The cartridge was washed with 5 mL of hexane and eluted with 5 mL of 80% methanol in acetone. The eluate was concentrated to 0.5 mL under a gentle stream of nitrogen. The resulting extract represents free BPA, while the residual pellet was further processed for conjugated BPA quantification. The pellet, after addition of 5 ng of the internal standard and 1 mL of water, was digested in 1 mL of 1 M ammonium acetate buffer containing 2 µL/mL β-glucuronidase (with 0.6% sulfatase activity; from *Helix pomatia*, 145700 unit/mL, Sigma, St Louis, MO) at 37ºC for 12 hrs. This conjugated BPA fraction was extracted thrice with ethyl acetate. The sample extract was concentrated to near dryness, reconstituted with 10% dichloromethane in hexane, and purified by passing through a Strata NH_2 cartridge as described above. The final eluate was concentrated to 0.5 mL.

BPA and $^{13}C_{12}$-BPA detection from both extracts were quantified using a high-performance liquid chromatography (HPLC) coupled with API 2000 electrospray triple-quadrupole mass spectrometer (ESI-MS/MS; Applied Biosystems, Foster City, CA), and modified from a protocol published previously (Padmanabhan et al., 2008). Ten µL of each extract was injected onto an analytical column, set at 25°C and a flow rate of 300 µL/min with the mobile phase comprised of varying methanol and water mixtures (25-99% methanol gradient). Instrument parameters were as follows: MS/MS was set to electrospray negative mode, cone voltage set at -30 V, collision energy set at -25 V, capillary voltage set at -4.5 KV, and desolvation temperature set at 400°C. For detection, transitions were monitored at 227>212 for BPA and 239>224 for $^{13}C_{12}$-BPA using multiple reaction monitoring (MRM).

Several quality assurance and quality control measurements were taken to assure the validity of the analytical technique. Selected sample matrices were spiked with BPA standards and then passed through the entire analytical procedure. Results indicated an average recovery of 104% (90-120%) for spiked BPA and 85% (65-120%) for spiked $^{13}C_{12}$-BPA. An external calibration curve was prepared by injecting standards at varying concentrations (10 µL of 0.05-100 ng/mL), resulting in a calibration coefficient >0.99. In addition, a procedural blank (water) was analyzed between every 10 samples that were analyzed. To prevent contamination, only polypropylene or BPA-free supplies were used during sample processing. Furthermore, BPA-free water was stored at -80°C and then pulverized over the frozen mortar and pestle apparatus, similar to the process described above, to assess contamination introduced during homogenization. Free BPA and conjugated BPA concentrations were both below the limit of quantification (LOQ) of 0.1 ng/g in controls and blanks. For data analysis, liver BPA concentrations below the LOQ were assigned a value of 0.071 ng/g, which was estimated by dividing the LOQ by the square root of 2.

RNA extraction and cDNA synthesis

Total RNA, in addition to genomic DNA and total protein, was purified from frozen liver tissue using the AllPrep DNA/RNA/Protein kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Before extraction, 10-20 mg of tissue was added to a 2 mL round bottom micro-centrifuge tube containing 600 µL of Buffer RLT with 1% β-mercaptoethanol and a 5 mm stainless steel bead, and subsequently homogenized twice for 2 min at 20 Hz in the TissueLyser II (Qiagen). RNA quality and

quantity was assessed using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

To generate complementary DNA for each sample, approximately 1 µg of total RNA template was used with the iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA) following the manufacturer's protocol. The thermocycler settings for cDNA synthesis required incubation at 25°C for 5 min, 42°C for 60 min, and 90°C for 5 min.

Primer design and real-time quantitative PCR

Primer pairs for real time quantitative polymerase chain reaction (RT-qPCR) were designed for four genes related to BPA metabolism using GenScript Real Time PCR (TaqMan) Primer Design Tool (GenScript, Piscataway, NJ). The final *UGT2B15* primer sequence was obtained from the Harvard Primer Bank. The primers for human liver reference genes, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Ubiquitin C (*UBC*), and β2-microglobulin (*B2M*), were similarly designed using GenScript. All RT-qPCR primers (Table 2.1) were verified using Primer BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and chosen avoiding sequence alignment over polymorphisms or multiple binding sites. Most primers span introns or bridge exon-exon junctions, resulting in unique amplicons 50-200 base pairs long. All primer sets were tested for PCR efficiency using 10-fold serial dilutions of a standard template. Primers with the best standard curve slope and R^2 values (>0.97) were chosen as the final RT-qPCR assay.

Template amplification was set up in a 25 µL reaction for RT-qPCR using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Each reaction, containing 2 µL of 10-

fold diluted cDNA template and 100 nM primer concentration, was carried out in triplicate on a C100 Thermal Cycler with a CFX96 Real-Time head (Bio-Rad). Each 96-well plate contained No Template Controls (NTC) for individual primer sets as well as Plate Control (PC) samples to adjust for inter-plate variability. RT-qPCR cycle parameters were set to 95°C for 3 min, and 40 cycles of 95°C for 10 sec and 55°C for 30 sec, followed by 95°C for 10 sec and 65°C for 5 sec.

The average threshold cycle (CT) from triplicate runs was calculated for individual genes using the CFX Manager Version 1.6 software (Bio-Rad). An inter-run calibration using the plate controls automatically adjusted data points between plates. For normalized expression, the software takes the CT for each gene and then normalizes the values to the average CT of the endogenous reference genes: *GAPDH*, *UBC*, and *B2M*. Results are reported as normalized fold expression change ($2^{-\Delta\Delta CT}$) with standard error of the mean. The normalized fold change value reported herein represents fetal expression relative to expression in a gender matched adult control, where fold change equals one.

Statistical analysis

Distribution of BPA in liver tissue was examined and did not appear to be normal. Free BPA, conjugated BPA, and total BPA (ng/g wet weight) concentrations were presented as percentiles and geometric means. Bivariate nonparametric tests (Wilcoxon rank sum and Spearman correlation) were used to analyze free BPA and conjugated BPA concentrations with covariates such as sex and gestational age. Similar bivariate analyses were conducted separately with the primary predictors: mRNA expression of *UGT2B15*, *GUSB*, *SULT1A1*, and *STS*. Simple and multiple linear regression models, in which BPA

concentration outcomes were log transformed, were tested to assess the relationship between BPA tissue concentrations and gene expression. Each of the four genes were individually analyzed with free BPA, and separately with free to conjugated BPA ratio, in a simple linear regression model. Regression models were not analyzed for conjugated BPA species given that the exact BPA-glucuronide and BPA-sulfate conjugate composition was unknown. In the multiple linear regression models, gene expression and free BPA or free: conjugated BPA ratio were analyzed after adjusting for gestational age, as a confounder, and sex, as an effect modifier. Regression coefficients (β) were analyzed and calculated as percent change [100(e^β -1)] with p-values <0.05 representing statistical significance. All statistical analyses were conducted using the stats and epicalc packages in R (version 2.14.2; The R Foundation for Statistical Computing 2012).

2.4 Results

BPA concentrations in liver

In the adult liver controls, the concentrations of conjugated BPA were both below the limit of quantification (<LOQ), while the concentrations of free BPA were <LOQ and 0.38 ng/g (Table 2.2). Greater than 70% of the 50 fetal liver samples, however, contained detectable concentrations of free BPA and conjugated BPA, ranging from <LOQ to 50.5 ng/g (geometric mean: 2.26 ng/g) and <LOQ to 49.5 ng/g (geometric mean: 0.65 ng/g), respectively (Table 2.2 & Table 2.3). The majority (78%) of samples exhibited a free BPA to conjugated BPA ratio greater than 1, with a mean of 6.91 and median of 3.75, across all gestational age and sex (Table 2.2). No significant differences in free BPA (p-

value: 0.675) or conjugated BPA (p-value: 0.976) concentrations were observed between male and female specimens. After assessing BPA concentrations over developmental time span (Figure 2.1), gestational age was found to be significantly correlated with free BPA (r=0.316; p-value: 0.025) but only marginally significant with conjugated BPA (r=0.267; p-value: 0.059) across all samples. In a meta-analysis of free BPA across adult tissues, pregnancy related biological fluids, and pregnancy related tissues from published studies (Figure 2.2), concentrations in our adult and fetal liver specimens were comparable to similar cohorts that use various analytical techniques.

BPA-specific XME gene expression in liver

In general, mRNA expression of *UGT2B15* and *SULT1A1* was significantly reduced (p-values: 0.039 and 0.044, respectively), while *STS* expression was marginally reduced (p-value: 0.054) in fetal liver compared to adult liver samples. Furthermore, normalized mRNA expression was significantly higher in male compared to female fetal livers, especially for *UGT2B15, SULT1A1,* and *STS* expression (p-values <0.005; Figure 2.3). *GUSB* displayed no significant difference in expression between fetal and adult liver samples (p-value: 0.197). There were no significant correlations between expression of the four BPA biotransformation genes and gestational age (p-values >0.200).

BPA concentration and XME gene expression associations in liver

The association between free BPA concentrations and expression of BPA-specific XME genes in fetal liver was assessed using linear regression. No significant associations were observed between free BPA concentrations and expression levels in simple

regression modeling (*UGT2B15* p-value: 0.441, *SULT1A1* p-value: 0.914, *GUSB* p-value: 0.477, and *STS* p-value: 0.753). The correlation between free BPA and many of the gene expression profiles, however, differed between male and female liver specimens, suggesting that sex may act as a potential effect modifier. Therefore, in the final model, an interaction term between sex and expression was added along with adjustment for gestational age. There were no significant relationships between free BPA tissue concentration and individual biotransformation gene expression values adjusting for gestational age, sex, and their interaction (p-values >0.500). Actual maternal BPA exposures are likely to differ from one study subject to another; thus, free to conjugated BPA ratio may be a better metric for understanding metabolism capacity. We conducted a similar analysis between free: conjugated BPA and the gene expression profiles. For both simple linear and adjusted regression models, no significant associations were reported between free: conjugated ratio and mRNA expression (*UGT2B15* p-value >0.767, *SULT1A1* p-value >0.347, *GUSB* p-value >0.828, and *STS* p-value > 0.357).

2.5 Discussion

Studies reporting internal dose of BPA in human tissue at vulnerable developmental stages are presently lacking. When we measured endogenous BPA concentrations in human fetal liver, more than 70% of samples exhibited detectable concentrations of free and conjugated species, ranging from <LOQ to 50 ng/g (or parts per billion). Comparable concentrations of free BPA in pregnancy related tissue have been previously reported in 49 term placentas collected from Spanish women (<LOQ-

22.2 ng/g), 248 amniotic fluids from 2nd trimester Japanese women (<LOQ-5.62 ng/mL), and 28 fetal livers from a Canadian population (<LOQ-37.7 ng/g) (Cao, et al., 2012; Jimenez-Diaz, et al., 2010; Yamada, et al., 2002). Often, these studies only quantify free BPA or total BPA, and the LOQs are higher than 0.1 ng/g. Still, the exposure distribution profiles of our samples were equivalent to other cohorts that used different analytical techniques as seen in Figure 2.2.

A higher free BPA to conjugated BPA ratio in this sample population is especially notable, given that free BPA normally represents 10-30% of total BPA in adult urinary excretions (Koch, Kolossa-Gehring, Schroter-Kermani, Angerer, & Bruning, 2012; Liao & Kannan, 2012). The discrepancy in our clinical samples may be attributed to varying metabolic potential across biomonitoring samples and time points. For example, given the nature of BPA metabolism and excretion in adults, limited concentration of free BPA in circulating blood and a high percentage of conjugated species in urine specimen are expected. Contrary to this initial model, free BPA has been detected in blood measurements across various studies and these inconsistencies have brought to light the issue of BPA contamination during sample processing and analysis (L. N. Vandenberg et al., 2012a). In this study, several experimental precautions were taken, including use of polycarbonate-free equipment and negative controls throughout processing. Any exogenous BPA introduced during tissue procurement likely affected all samples; however, the wide range of free BPA and <LOQ concentrations observed implies otherwise. Toxicokinetic differences in the fetal versus adult organism may be a probable explanation for the observance of high free to conjugated ratio in our clinical samples. In a study examining BPA in 2nd and 3rd trimester amniotic fluid from American women

found that free BPA accounted for more than 80% of total BPA concentrations (Edlow, et al., 2012). Furthermore, a study measuring BPA in midgestational umbilical cord serum determined that on average BPA-glucuronide make up 19% and free BPA make up 36% of total BPA in the sample population (Gerona et al., 2013). While significant differences in structure and function between animal species make it hard to extrapolate to humans, one study in rats reported a decrease in % free BPA in tissue with increasing age after iv administration, consistent with age related increases in Phase II conjugation (Doerge, Twaddle, Vanlandingham, Brown, & Fisher, 2011).

The biotransformation system undergoes significant changes during hepatic maturation, resulting in differential response to xenobiotic chemicals. Hepatic mRNA and protein expression of 13 *UGT* genes, including *UGT2B15,* were not detectable in the fetus and were reduced in children compared to adults (Strassburg et al., 2002). In our larger collection of samples, we also found significantly reduced mRNA expression of *UGT2B15* in fetal liver. While fetal *GUSB* did not show differential expression, *STS* exhibited significantly reduced expression compared to adult livers, as supported by other studies (Collier, Tingle, Paxton, Mitchell, & Keelan, 2002; Lucier, Sonawane, & McDaniel, 1977; Miki et al., 2002). Hepatic *SULT* genes, especially *SULT1A1*, often exhibit protein expression levels that are equivalent to adult levels (Duanmu et al., 2006; Stanley, Hume, & Coughtrie, 2005). Although we observed reduced mRNA expression of *SULT1A1* in fetal liver, there was wide variability in our samples. Future studies can be improved by increasing the number of adult liver controls to account for inter-subject variability resulting from not only differential exposure but also genetic variation contributing to altered xenobiotic metabolism. Furthermore, integration of gene

ontogeny with genetic variants involved in differential BPA metabolism, such as the *UGT2B15* D85Y substitution (Hanioka, Oka, Nagaoka, Ikushiro, & Narimatsu, 2011), among others, will be necessary for complete understanding of altered BPA metabolism in human fetal samples.

When the relationship between biotransformation gene expression and free BPA concentration was examined, no significant correlations were observed. Important epidemiological information for individual clinical specimen was unavailable for this study. Access to certain maternal data may have shed light on true maternal BPA exposures and other co-exposures throughout pregnancy. Therefore, to account for differences attributed to initial maternal exposures, we also examined correlations using the free to conjugated BPA ratio but did not find any significant associations. In addition to small sample size, several reasons may help explain the lack of association, even after adjustment for gestational age and gender. First, the mRNA levels quantified in these 1st and 2nd trimester liver samples may not be a good proxy for measuring functional activity of biotransformation enzymes at this particular window of development. Thus, experiments that assess protein activity of BPA biotransformation enzymes in fresh human tissue will be valuable. For proper metabolism, enzyme reactions are also limited by tissue cofactor concentrations and nutrient availability (Bidlack, Brown, & Mohan, 1986); low levels of cofactors such as 3'-phophoadenosine 5'-phosphosulfate (PAPS) and UDP-glucuronic acid may limit BPA conjugate production even with optimal protein expression. Second, there is also the possibility that, despite experimental precautions described above, free BPA was introduced exogenously during sample procurement, and this contamination may have negated any true associations between expression and

exposure. Therefore, the optimal regression model should evaluate biotransformation gene expression with their respective BPA species (i.e. *SULT1A1* with BPA-sulfate only, *UGT2B15* with BPA-glucuronide only). Future analyses will require separate characterization of both BPA-glucuronide and BPA-sulfate in tissue. Finally, the high free BPA to conjugated BPA ratio observed may also be a result of deconjugation in the placenta or differential biotransformation from physiological changes occurring in the mother during pregnancy (Abduljalil, Furness, Johnson, Rostami-Hodjegan, & Soltani, 2012; Lucier, et al., 1977). To assess BPA fate throughout human pregnancy, investigation of BPA metabolism and transport in the placenta, maternal liver, and across other fetal compartments is required. Thus, in a subsequent chapter, we address BPA concentration and metabolism across matched tissues from individual subjects.

As is true in many human studies, bioethical issues and the paucity of appropriate samples limit our ability to comprehend the role of BPA biotransformation during this sensitive window of development. This study is one of the first of its kind to investigate both BPA conjugating and deconjugating enzyme gene expression in relation to endogenous BPA exposure in the developing human fetus. Characterizing biotransformation gene ontogeny with BPA exposure from early gestation to birth will enhance our understanding of BPA fate and subsequent health effects.

Table 2.1 RT-qPCR Primers for BPA XME Analysis

Gene Type	Gene	Full Name	GenBank Number	Strand	Primers (5' to 3')	Tm (°C)	Amplicon Size
Conjugation	*UGT2B15**	UDP-glucurono-syltransferase	NM_001076	Forward	CCAACCAATGAAGCCCCTG	60.7	94
				Reverse	GTTGTGAGCTGCGACTCGAA	62.7	
Conjugation	*SULT1A1*	Sulfotransferase	NM_001055	Forward	TCAAGGTGGTCTATGTTGCC	58.6	149
				Reverse	CCAGGATCCGTAGGACACTT	59.0	
Deconjugation	*STS*	Steroid Sulfatase	NM_000351	Forward	AGCACTGATAGGGAAATGGC	59.2	82
				Reverse	GAAGCCGTGATGTAAAGGGT	59.1	
Deconjugation	*GUSB*	B-Glucuronidase	NM_000181	Forward	ATCGCCATCAACAACACACT	59.0	82
				Reverse	TGGGATACTTGGAGGTGTCA	58.9	
Reference	*GAPDH*	Glyceraldehyde 3-phosphate dehydrogenase	NM_002046	Forward	CATCAATGGAAATCCCATCA	59.1	90
				Reverse	GACTCCACGACGTACTCAGC	58.5	
Reference	*UBC*	Ubiquitin C	NM_021009	Forward	GATCGCTGTGATCGTCACTT	58.8	112
				Reverse	TCTTTGCCTTGACATTCTCG	59.0	
Reference	*B2M*	β2-microglobulin	NM_004048	Forward	TCGCTCCGTGGCCTTAGCTG	58.9	161
				Reverse	CAATGTCGGATGGATGAAACCCAG	56.1	

*Primer sequence from Harvard Primer Bank. All other primers were designed by GenScript.

Table 2.2 BPA Concentrations in Liver Specimen

Sample Type	ID	Age	Sex	Free BPA (ng/g)	Conjugated BPA (ng/g)	Free/Conjugated Ratio
Adult Liver	1	50 Y	M	0.38	<LOQ	5.30
Adult Liver	2	50 Y	F	<LOQ	<LOQ	1.00
Fetal Liver	1	GD 101	M	47.23	49.54	0.95
Fetal Liver	2	GD 110	F	31.24	7.01	4.46
Fetal Liver	3	GD 91	F	25.54	9.90	2.58
Fetal Liver	4	GD 98	F	31.25	7.78	4.01
Fetal Liver	5	GD 103	F	16.91	4.22	4.00
Fetal Liver	6	GD 105	M	11.77	4.33	2.72
Fetal Liver	7	GD 107	M	3.63	0.61	5.94
Fetal Liver	8	GD 110	F	3.93	0.59	6.70
Fetal Liver	9	GD 115	M	40.22	15.85	2.54
Fetal Liver	10	GD 101	F	12.05	13.03	0.92
Fetal Liver	11	GD 98	F	3.49	2.29	1.52
Fetal Liver	12	GD 86	F	1.68	1.18	1.42
Fetal Liver	13	GD 89	F	12.47	1.05	11.93
Fetal Liver	14	GD 93	F	16.47	3.99	4.13
Fetal Liver	15	GD 94	M	3.68	0.47	7.79
Fetal Liver	16	GD 96	M	1.81	0.29	6.24
Fetal Liver	17	GD 108	M	12.98	4.67	2.78
Fetal Liver	18	GD 113	M	3.45	0.40	8.52
Fetal Liver	19	GD 114	F	2.12	0.81	2.60
Fetal Liver	20	GD 120	M	2.71	0.68	3.99
Fetal Liver	21	GD 96	M	1.71	0.45	3.81
Fetal Liver	22	GD 108	F	2.00	0.50	3.98
Fetal Liver	23	GD 105	M	50.50	3.23	15.63
Fetal Liver	24	GD 115	M	4.69	0.87	5.41
Fetal Liver	25	GD 101	F	3.76	1.02	3.69
Fetal Liver	26	GD 96	M	0.54	0.66	0.82
Fetal Liver	27	GD 101	F	1.55	0.50	3.11
Fetal Liver	28	GD 105	M	0.66	0.65	1.01
Fetal Liver	29	GD 91	M	4.77	22.70	0.21
Fetal Liver	30	GD 98	F	0.78	<LOQ	11.09
Fetal Liver	31	GD 101	M	1.82	1.28	1.42
Fetal Liver	32	GD 96	F	1.18	<LOQ	16.69
Fetal Liver	33	GD 94	M	5.90	0.48	12.34
Fetal Liver	34	GD 94	M	1.80	0.65	2.76
Fetal Liver	35	GD 96	F	3.27	0.22	14.60
Fetal Liver	36	GD 96	F	0.83	<LOQ	11.71
Fetal Liver	37	GD 89	M	<LOQ	<LOQ	1.00
Fetal Liver	38	GD 98	F	0.32	0.42	0.75
Fetal Liver	39	GD 105	M	<LOQ	<LOQ	1.00
Fetal Liver	40	GD 113	M	<LOQ	<LOQ	1.00
Fetal Liver	41	GD 80	F	0.16	<LOQ	2.23
Fetal Liver	42	GD 98	M	4.52	<LOQ	63.93
Fetal Liver	43	GD 104	M	6.63	1.28	5.18
Fetal Liver	44	GD 74	M	0.43	<LOQ	6.11
Fetal Liver	45	GD 94	F	1.12	<LOQ	15.84
Fetal Liver	46	GD 82	F	<LOQ	<LOQ	1.00
Fetal Liver	47	GD 85	M	<LOQ	<LOQ	1.00

Fetal Liver	48	GD 95	M	<LOQ	<LOQ	1.00
Fetal Liver	49	GD 113	F	3.50	<LOQ	49.50
Fetal Liver	51	GD 103	M	1.19	0.70	1.70

Y= years; GD= gestational day; M= males, F=females

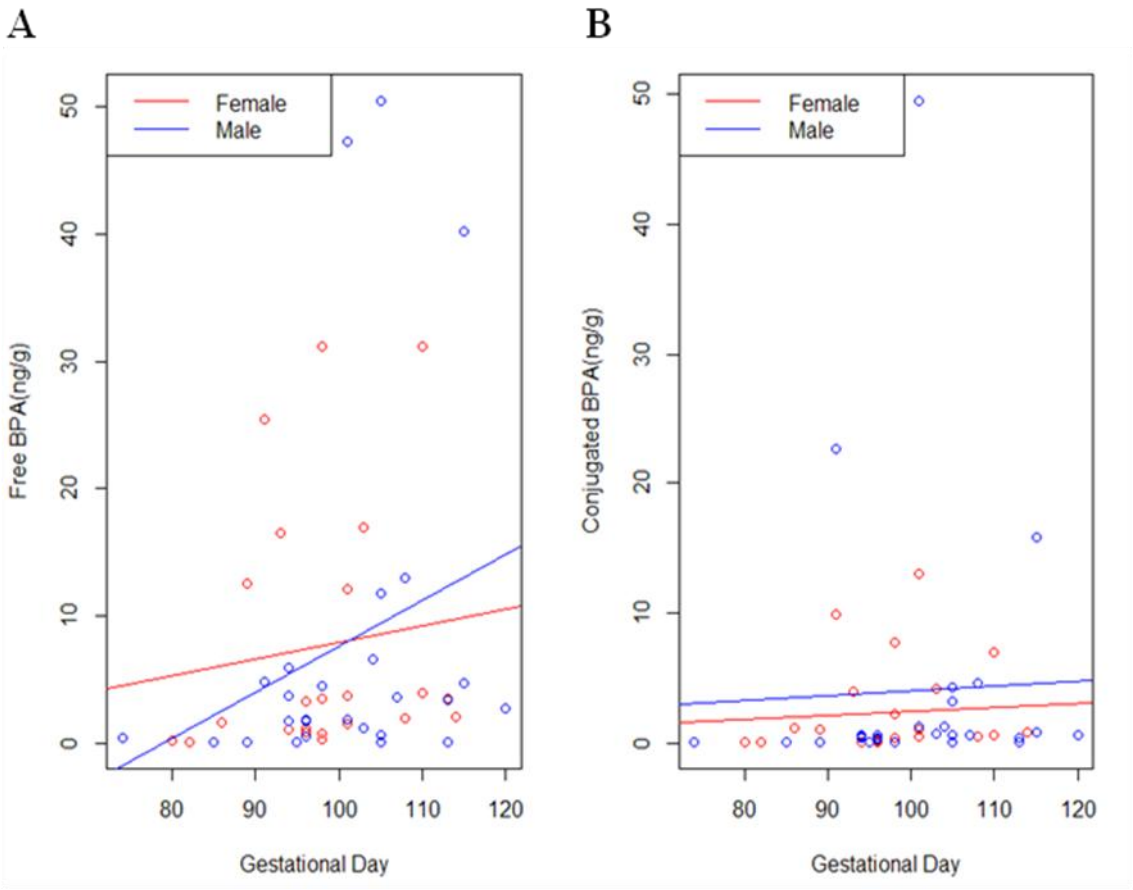
Table 2.3 Distribution of Free, Conjugated, and Total BPA (ng/g) in Human Fetal Liver Tissues (Gestational Age: day 74-120, N=50)

BPA Species	*Above LOQ[a] (%)*	*Percentile*						
		Min	*10th*	*25th*	*50th*	*75th*	*90th*	*Max*
Free	88	<LOQ	<LOQ	0.90	2.99	6.45	26.1	50.5
Conjugated	72	<LOQ	<LOQ	<LOQ	0.63	2.04	8.00	49.5
Total		<LOQ	<LOQ	1.18	3.44	12.1	35.7	96.8

[a]LOQ = 0.1 ng/g

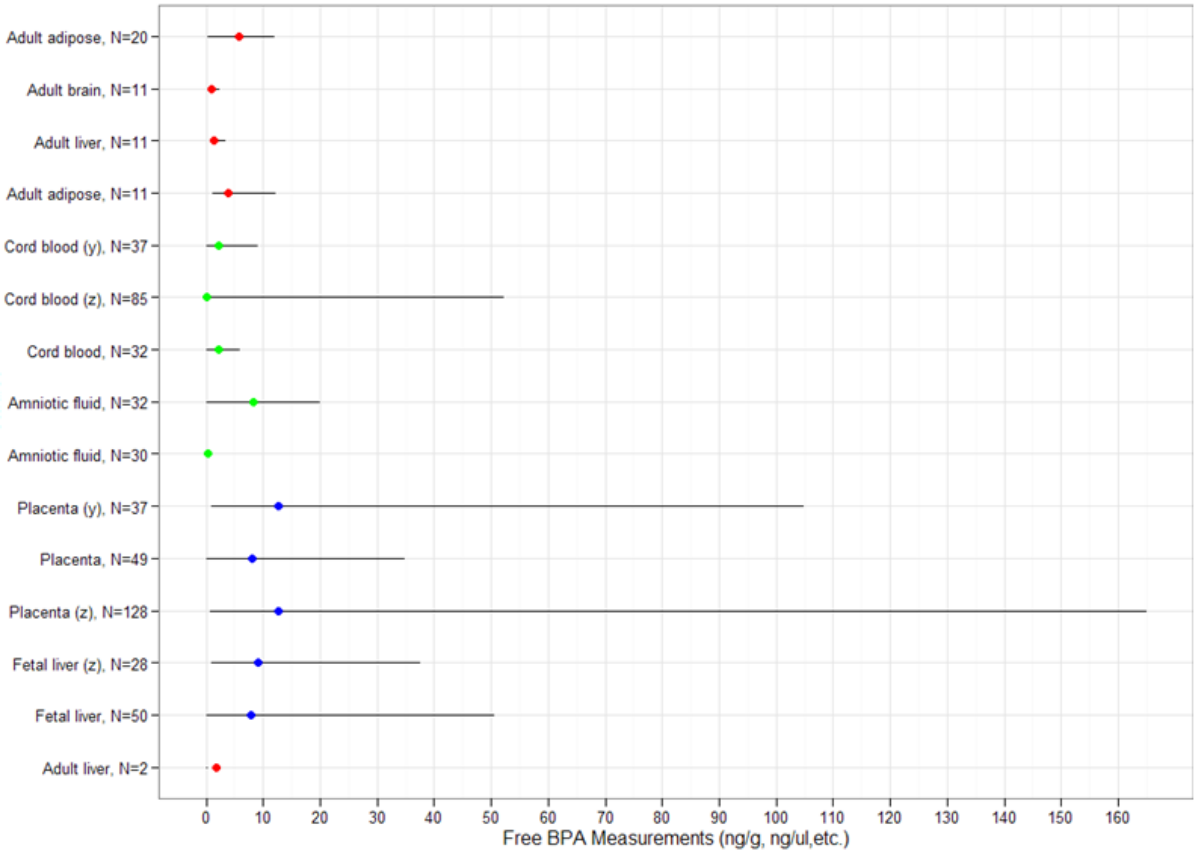
<LOQ = 0.1/sqr(2)= 0.071 ng/g

Figure 2.1 Scatter Plot Displaying the Association Between Gestational Age and BPA Concentration



(A) A significant positive correlation between free BPA concentration and gestational age is observed (overall *r:* 0.316, p-value: 0.025). When the exposure-age correlation was stratified by sex, no significant differences were observed (female *r:* 0.333, p-value: 0.120; male *r:* 0.345, p-value: 0.078). **(B)** A marginally significant trend was found between conjugated BPA and gestational age (overall *r:* 0.269 and p-value: 0.059; female *r:* 0.183 and p-value: 0.404; male *r:* 0.062 and p-value: 0.062).

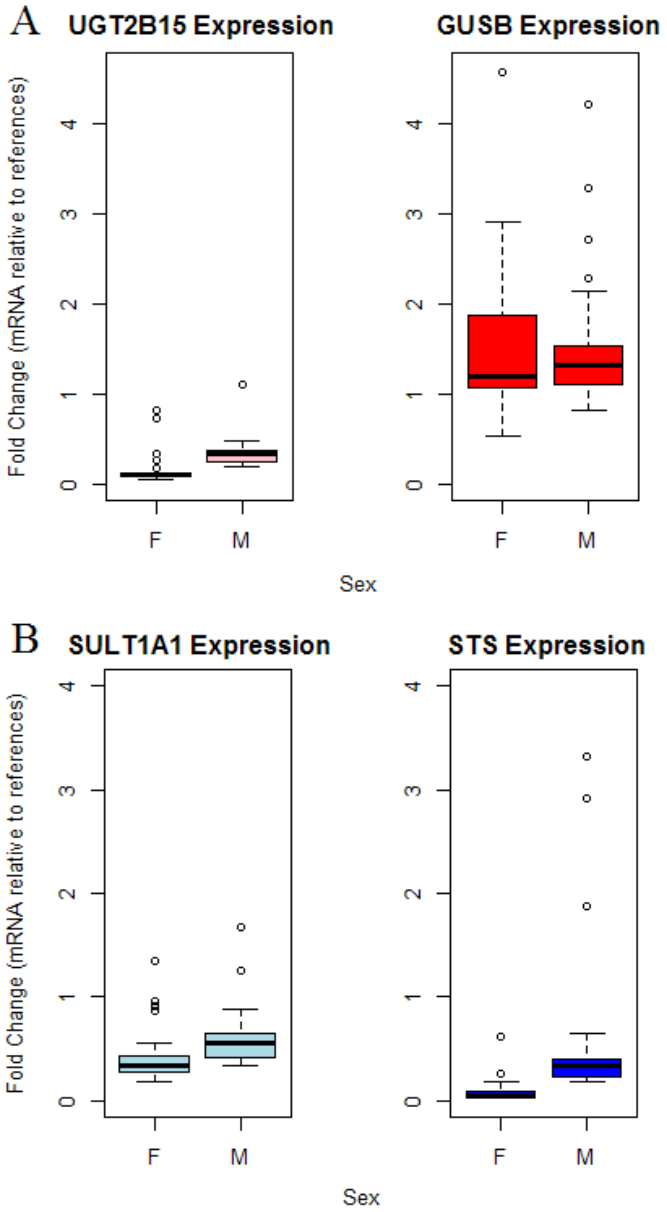
Figure 2.2 Meta-Analysis of Free BPA Across Different Human Specimen



Authors	Country	Age
MF Fernandez 2007	Spain	24-81 yr
T Geens 2012	Belgium	9-96 yr
T Geens 2012	Belgium	9-96 yr
T Geens 2012	Belgium	9-96 yr
G Schonfelder 2002	Germany	32-24 wk
R Gerona 2013	USA	13-24 wk
Y Ikezuki 2002	Japan	Term
Y Ikezuki 2002	Japan	15-18 wk
AG Edlow 2012	USA	2nd tri
G Schonfelder 2002	Germany	32-41 wk
I Jemenez-Diaz 2010	Spain	Term
XL Cao 2012	Canada	10-20 wk
XL Cao 2012	Canada	10-20 wk
MS Nahar 2012	USA	10-17 wk
MS Nahar 2012	USA	50 yr

The forest plot displays the distribution of free BPA reported across all published studies addressing BPA internal dose in humans. Each line indicates the minimum and maximum with the dot indicative of the average BPA concentration. Several studies did not report the mean, so the median (y) or geometric mean (z) was reported instead. Red dots represent studies reporting free BPA quantified in adult tissue, green dots represent studies that quantified free BPA in pregnancy related biological fluids like cord blood and amniotic fluid, and blue dots represent those in pregnancy related tissue specimen. As indicated by the plot, our study is the first to quantify free BPA in adult and pregnancy related tissue in the USA, with considerable sample size for fetal liver. The range of free BPA in our samples is comparable to other studies.

Figure 2.3 Boxplots Representing Relative Expression of BPA-specific XMEs in Fetal Liver



Boxplots represent sex stratified biotransformation enzyme expression in fetal livers compared to adult livers, normalized to 1 fold change for each gene. **(A)** Fetal liver expression of *UGT2B15* is significantly reduced compared to adults (overall p-value: 0.039). Here, normalized male expression is significantly higher than female expression (p-value <0.001). *GUSB*, however, does not exhibit significant difference in expression between fetal and adult samples (overall p-value: 0.197). Also, there is no significant difference in normalized expression between male and female (p-value: 0.463). **(B)** Fetal liver expression of *SULT1A1* is also significantly reduced compared to adults (overall p-value: 0.044). The difference in *STS* expression between fetal compared to adult samples is only marginally significant (overall p-value: 0.054). Normalized male expression is significantly higher than female expression in both *SULT1A1* and *STS* (p-value: 0.001 and p-value <0.001), respectively).

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CHAPTER 3

In Utero Bisphenol A Concentration, Biotransformation, and Global DNA Methylation Across Matched Placenta, Kidney, and Liver in the Human Fetus

3.1 Abstract

While urine has been an easily accessible and feasible matrix for human biomonitoring, accurate environmental exposure and epidemiology assessments require internal dose measurements using blood and tissue to properly determine disease etiology. This is especially important for the endocrine active compound, bisphenol A (BPA), where studies investigating internal dose at sensitive periods of human development are currently lacking. For this project, we quantified BPA concentrations and BPA-specific metabolizing enzyme gene expression across three matched tissues in individual1st-2nd trimester human subjects (N=12). The metabolically active placenta, fetal liver, and fetal kidney specimens were also analyzed for exposure-dependent alterations in global DNA methylation in order to characterize BPA's early regulatory effects. Compared to fetal liver, BPA concentrations were lower in matched placenta and kidney specimens. BPA-specific metabolism gene expression levels differed across each tissue type; however, conjugation and deconjugation patterns were similar across the fetus. In general, the expression of *GUSB* and *SULT1A1* was higher than the respective *UGT2B15* and *STS* genes, indicating that BPA-glucuronide deconjugation and BPA sulfation reactions are likely to predominate in the early fetus. Tissue-specific DNA

methylation differences in global repetitive content, as measured by LINE1 (p-value <0.001) and global CCGG content, as measured by LUMA (p-value <0.001) were observed. Average LINE1 and CCGG global methylation were 58.27 and 59.15% in placenta, 79.49 and 66.42% in fetal liver, and 77.92 and 76.99% in fetal kidney. Total BPA concentrations were significantly associated with global methylation only for placenta using the LINE1 assay (p-value: 0.002), suggesting organ-specific biological effects after fetal exposure. This study takes advantage of human clinical specimens to assess BPA biomonitoring, toxicokinetics, and toxicodynamics within matched tissues across individuals. Findings are novel and provide evidence for BPA body burden and adverse effects in the developing human fetus.

3.2 Introduction

Characterizing internal dose, defined as the absorbed concentration, is essential for accurately detecting associations between exposures and adverse health outcomes. Therefore, biomonitoring focus has recently shifted from urinary measurements to quantification of xenobiotics in circulating blood and tissue, especially for human bisphenol A (BPA) studies. Urine measurements may be sufficient for understanding general BPA exposure given its short half-life in adults (Volkel, et al., 2002), but tissue measurements provide evidence for contact at target sites with implications for organ-specific biological effects. Continuous exposure to even minute amounts of BPA may cause the rate of absorption to exceed the rate of elimination, resulting in accumulation. Bioaccumulation and lipophilic properties have been previously suggested for BPA. One

human *in vitro* study noted a high fat: blood partition coefficient for BPA (Csanady et al., 2002), and in a later study BPA was detected in adipose tissue collected from Spanish women (Fernandez et al., 2007). Thus far, there are only a handful of studies reporting BPA concentration in tissue because human tissue acquisition is difficult and analytical techniques still require optimization in these complex matrices.

Information regarding human BPA internal dose is currently limited to studies quantifying the compound in biological fluids like blood, saliva, and breast milk or estimated from physiologically based pharmacokinetic (PBPK) models (L. N. Vandenberg, et al., 2012a). There is often conflict between biomonitoring data and these PBPK models, leading to considerable debate in the BPA community (L. N. Vandenberg, Hunt, Myers, & Vom Saal, 2013). For example, under the assumption that BPA is completely metabolized to its conjugates and rapidly excreted via urine, BPA is expected to be undetected in blood, yet several studies have reported detectable BPA concentrations in circulation (Kaddar, et al., 2009; Y. J. Lee et al., 2008; Padmanabhan, et al., 2008). Discrepancies exist because our understanding of human BPA toxicokinetics is inconclusive, especially across different age groups.

Comprehensive BPA toxicokinetic studies have been established using animal models, and are sometimes inappropriately extrapolated to humans. In a study comparing BPA clearance rates after 400 µg/kg/day oral dose of deuterated-BPA in rhesus macaques and female CD1 mice, researchers noted similar serum BPA profiles over time with a peak concentration 1 hr after initial exposure (Taylor et al., 2011). The estimated kinetic parameters were similar across mice, monkeys, and humans, with findings suggestive of considerable exposure through non-oral routes and incomplete first-pass metabolism.

More recently, studies have addressed high BPA exposure and risk concerns in pregnant animal populations. After subcutaneous dosing of pregnant CD1 mice to 25 µg/kg of radioactive BPA, the percent of total administered dose recovered after 24 hours was 45% in digestive tract, 21% in feces, 5% in urine, 4% in fetal litter, 2.5% in liver, and 1.8% in bile (Zalko et al., 2003). Also, in a rat uterine perfusion model when 2 µM of BPA-glucuronide was administered for 20 minutes, the conjugate was shown to pass through the placenta using Mrp1 and Oat4a1 transporters and become deconjugated in the fetal liver and amniotic fluid (Nishikawa et al., 2010). The few studies that have attempted to determine BPA toxicokinetics in humans show major flaws in dose frequency, analytical techniques, sample size, and selection of sample population (Teeguarden et al., 2011; Volkel, et al., 2005; Volkel, et al., 2002). Traditional methods of assessing BPA toxicokinetics are especially difficult in human pregnant adults and the developing fetus, but if tissues are available, biomonitoring with sensitive analytical techniques may be a first step to addressing the BPA knowledge gap.

The exposure-disease relationships in humans identified through epidemiological studies currently lack important mechanistic data that inform us about direct alterations in structure and function. These subtle exposure-dependent changes can gradually manifest as diseases much later in life, most likely occurring through epigenetic mechanisms (Gluckman, Hanson, & Low, 2011). DNA methylation at cytosine-guanine dinucleotides (CpG) is one commonly studied epigenetic marker that undergoes extensive reprogramming during pre-implantation and gametogenesis in early fetal development followed by tissue-dependent epigenetic differentiation (Faulk & Dolinoy, 2011). Several animal studies have already identified methylation as an important intermediate in BPA

related adverse health outcomes (O. S. Anderson, et al., 2012; Bromer, et al., 2010; S. M. Ho, W. Y. Tang, J. Belmonte de Frausto, & G. S. Prins, 2006). Research addressing human BPA-methylation associations is necessary especially throughout early development. Tissue utilized for biomonitoring can also be an excellent source for recognizing early dose-dependent changes that occur in expression and regulation.

Using matched placenta, fetal liver, and fetal kidney specimens from 12 different 1^{st}-2^{nd} trimester human fetuses, the aim of this study is to assess tissue-specific BPA concentrations, biotransformation gene expression, and exposure-dependent effects on global methylation. Identifying tissue-specific differences in BPA concentration and xenobiotic metabolizing enzyme expression will inform BPA toxicokinetics for improving human PBPK models specific for the developing fetus. The investigation of tissue-specific global methylation differences is particularly important for understanding BPA toxicodynamics and identifying early exposure-dependent regulatory changes that may lead to long-term adverse health effects.

3.3 Methods and Materials

Sample selection

For this project, we took a subset of the fetal liver specimens from the extended sample clinical cohort (N=50) and acquired matching tissue from the NIH-funded University of Washington Laboratory for the Study of Human Embryology (LSHE) fetal biobank (2R24 HD000836-47). Tissue BPA concentrations were previously measured in several 1^{st}-2^{nd} trimester fetal liver samples at the Wadsworth Center (New York State

Department of Health), indicating broad exposure ranging from below limit of quantification (LOQ) up to 96.8 ng/g of total tissue BPA (Nahar, Liao, Kannan, & Dolinoy, 2013). In particular, we identified fetal kidney (N=12) and placental (N=12) tissues matching BPA-characterized fetal liver samples that represent low exposure (total BPA: 1.2-2.9 ng/g; N=6) and high exposure (total BPA: 25.1-53.7 ng/g; N=6), for which sufficient sample was available for BPA quantification and DNA/RNA extraction.

Tissue BPA analysis and quality control

All flash-frozen fetal specimen were stored in polycarbonate-free tubing at -80°C and homogenized before processing. Like liver specimen, both free BPA and BPA conjugates were quantified in matched kidney and placental samples using high-performance liquid chromatography coupled with API 2000 electrospray triple-quadrupole mass spectrometer (HPLC ESI-MS/MS). Free BPA and conjugated BPA were homogenized from tissue weighing 550-710 mg. As mentioned in the last chapter, several quality assurance and quality control measurements were implemented for BPA analysis, including a procedural blank to check for laboratory contamination. By the start of this project, the LOQ was improved to 0.05 ng/g; thus, fetal kidney and placental tissue concentrations below the LOQ were assigned a value of 0.035 ng/g, estimated from dividing LOQ by the square root of 2.

RNA and DNA extraction

Similar to the fetal livers, total RNA and DNA was isolated from matched frozen fetal kidney and placental tissue using the AllPrep DNA/RNA/Protein kit following

manufacturer's instructions, (Qiagen, Valencia, CA). Approximately 10-20 mg of tissue was homogenized in 600 µL of Buffer RLT solution (containing 1% β-mercaptoethanol) for 2 min at 20 Hz (2x) in the TissueLyser II (Qiagen). DNA quantity and purity was measured using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE), while the RNA concentration and purity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). High quality mRNA was extracted from most specimens, with RNA integrity numbers (RIN) averaging 9.5 for kidney, 7.1 for placenta, and 7.6 for liver. RIN values for individual subjects and their respective tissues are reported in Table 3.1.

cDNA synthesis and real-time quantitative PCR

To produce complementary DNA, 1 µg of total RNA template was processed using the iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA) as per manufacturer's instructions. The thermocycler settings for cDNA synthesis required incubation at 25°C for 5 min, 42°C for 60 min, and 90°C for 5 min. Four BPA-related metabolism genes (*UGT2B15*, *GUSB*, *SULT1A1*, and *STS*) along with three reference genes (*GAPDH*, *UBC*, and *B2M*) were previously optimized using fetal liver tissue and listed in Table 2.1. Using the same protocol from the last chapter, we amplified templates for each primer set for the new kidney and placenta samples using the same cycle parameters, running each unique reaction out in triplicate.

The CFX Manager Version 1.6 software (Bio-Rad) was used to calculate the average threshold cycle (CT) across the triplicate runs. Unlike the previous chapter, results are reported as normalized expression with standard deviation. The normalized

expression of a particular target gene for each sample is calculated by normalizing the relative quantity ($RQ=Efficiency_{primers}^{[CT(minimum)-CT(target\ sample)]}$) of the gene of interest to that of the reference genes.

$$\text{Normalized Expression} = RQ_{\text{gene of interest}} / [(RQ_{GAPDH} * RQ_{UBC} * RQ_{B2M})^{\wedge}(1/3)]$$

Bisulfite conversion, and LINE1 and LUMA global methylation analysis

For the production of bisulfite converted (BSC) DNA, 1 µg of genomic DNA was treated with sodium bisulfite solution using the Qiagen Epitect kit automated on the Qiacube (Qiagen, Valencia, CA) following manufacturer's protocol. The converted DNA was diluted in 20 µL of EB Buffer, yielding a final concentration of approximately 50 ng/µL. Epitect methylated and unmethylated human BSC standards (Qiagen) were purchased and used as positive controls.

Two independent DNA methylation assays were employed using the Pyrosequencing platform (Pyrosequencing, Westborough, MA) for global methylation analysis: the long interspersed transposable element-1 (LINE1) assay and the Luminometric Methylation Assay (LUMA). For amplification of LINE1, 2 µL of BSC DNA was added to a 30 µL PCR reaction containing 15 µL of HotStarTaq master mix (Qiagen), 375 nM forward primer, and 187.5 nM of biotin-labeled reverse primer. LINE1 product amplification was set up using the following PCR cycling parameters: 95°C for 14.5 minutes followed by 45 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, then by 5 min at 72°C. After amplicon verification via gel electrophoresis, 10 µL of PCR product was added to LINE1 sequencing primers and analyzed using a pre-determined sequence to analyze run. Each amplicon was run in duplicate in the

PyroMark™Q96 MD Pyrosequencing System. The PyroMark software computes % methylation at 4 CpG sites across the assay for each sample and control. Primer information is detailed in Table 3.2 and previously described (Virani et al., 2012).

Unlike LINE1, LUMA detects methylation at 5'-CCGG-3' sequences both within and outside of repetitive elements across the genome. This restriction digest based assay requires unconverted genomic DNA and MspI, HpaII, and EcoRI restriction enzymes, as described previously (Bjornsson et al., 2008; Karimi et al., 2006). For each sample digest, two parallel reactions were set up containing 300 ng of genomic DNA, an internal standard enzyme (5 units of EcoRI), and 10x Buffer Tango™ with BSA (Fermentes). We incubated these mixtures at 37°C for 4 hours after adding 5 units of a methylation insensitive (MspI) enzyme to one reaction, and 5 units of methylation sensitive enzyme (HpaII) to the other. Digested products were assessed in duplicates using the PyroMark MD SNP software using the nucleotide dispensation sequence order: GTGTCACATGTGTG. Percent global methylation results were calculated by looking at the ratio of digested versus undigested CCGG sites using the following equation:

$$1-[(\text{HpaII(G)}/\text{EcoRI(T)})/(\text{MspI(G)}/\text{EcoRI(T)})] \times 100$$

In the equation, G and T represent pyrosequencing signal peak intensities from the 3rd G and T nucleotides of the dispensation order.

Statistical analysis

Univariate analyses were conducted across all exposure, gene expression, and global methylation variables across the different tissue types. Mean differences across matched tissue were evaluated using ANOVA with Tukey post-hoc analysis. To test the

association between BPA and mRNA expression, we identified the Pearson's correlation coefficient and p-value between BPA species and individual assays for each tissue type.

Pearson's correlation analysis was used to test the association between total BPA concentration and average LUMA methylation for each tissue type. The LINE1 assay evaluates % methylation across 4 adjacent CpG sites per reaction. To account for correlations between sites for each reaction, a linear mixed effects model was used. While total BPA concentration was held as the fixed effects and percent methylation was used as the dependent variable, CpG site number was used as the random effect. For each model tested using kidney, liver, and placental samples, the inter-subject variability was higher than the variability across CpG sites. All statistical analyses for this project were conducted using the stats, epicalc, and lme4 packages in R (version 2.14.2; The R Foundation for Statistical Computing 2012).

3.4 Results

BPA concentrations across tissue

Subjects with above average total BPA in liver and subjects with below average total BPA in liver that had matching placenta and kidney specimens were first identified. The average was defined previously using the normal distribution of the full N=50 fetal liver cohort (Nahar, et al., 2013). We classified N=6 subjects (A-F) into the high exposure group (total liver BPA: 25.1-53.7 ng/g) and N=6 subjects (G-L) into the low exposure group (total liver BPA: 1.2-2.9 ng/g), representing a 10 fold difference in concentrations between the groups. Given these differences in liver concentrations across

the groups, we expected similar patterns in BPA concentrations in placenta and fetal kidney specimens.

In general, free and conjugated BPA concentrations for placenta and fetal kidney significantly differed from matched fetal liver with p-values at 0.005 and 0.004, respectively. In Table 3.3, 100% of liver, 100% of kidney, and 83% of placental samples reported detectable concentrations of free BPA. While all 12 fetal liver samples exhibited detectable concentrations of conjugated BPA, the conjugated species was detected in only 58% of kidney and 42% of placental samples. Free BPA was higher than conjugated BPA concentrations across most tissues except for the placenta in subject H. Figure 3.1 displays BPA for individual subjects (A- L) and their tissue types, with free BPA species in panel A and conjugated BPA species in panel B. No obvious patterns were observed for either free or conjugated BPA concentrations. Unlike fetal liver specimen, BPA concentrations did not significantly differ by low and high exposure group for matched placentas (free p-value: 0.532, conjugated p-value: 0.336) or for matched kidneys (free p-value: 0.377, conjugated p-value: 0.735). The discrepancy in BPA concentrations from one tissue to another in any given subject is likely due to physiological differences across organs related to xenobiotic transport, metabolism, and storage. Interestingly, a few specimens, especially in the placenta, displayed free BPA or BPA conjugates at concentrations below the LOQ.

BPA-specific XME gene expression across tissue

The placenta, liver, and kidney are organs that have some metabolic capacity in the 1^{st}-2^{nd} trimester developing fetus. Each tissue was analyzed for mRNA expression for

xenobiotic metabolizing enzymes important for the conjugation (*UGT2B15, SULT1A1*) or deconjugation (*GUSB, STS*) of BPA, as denoted by Figure 1.1. Figure 3.2 illustrates the normalized expression pattern of these four genes together for each tissue type. In general, the three organs have distinct metabolic profiles with varying mRNA levels.

For the glucuronidation conjugation and deconjugation pathway, kidney and placenta *GUSB* expression were significantly higher than *UGT2B15* expression (p-values <0.001). Although the two genes did not significantly differ from one another in liver (p-value: 0.727), the mRNA levels were relatively higher in liver compared to other organs. For the sulfation conjugation and deconjugation pathway, *SULT1A1* expression was significantly higher than *STS* expression in kidney and liver specimen (p-values <0.001). The placenta mRNA profile is quite distinct given that *STS* expression is significantly higher than the remaining BPA-metabolism genes. In the developing fetus, these expression patterns may denote higher activity for BPA-glucuronide deconjugation across the three tissues, and higher activity for BPA sulfation in all tissues except for the placenta.

BPA concentration and XME gene expression associations across tissue

The sulfotransferase and UDP-glucuronosyltransferase enzymes contribute to higher concentrations of BPA-glucuronide and BPA-sulfate conjugates, while the steroid sulfatase and β-glucuronidase deconjugation enzymes contribute to higher concentrations of the parent compound. Since we did not separately measure the two BPA conjugates, we only assessed the correlation between free BPA and the four metabolism genes for each tissue, expecting a positive correlation with deconjugating genes and negative

correlation with conjugating genes. As noted in Table 3.4, most associations were negative and weak across fetal liver, fetal kidney, and placenta. Furthermore, no significant BPA-expression correlations were identified across each tissue (p-values >0.200).

Global methylation across tissue

LINE1 retrotransposons are found in approximately 17% of the genome (Ostertag & Kazazian, 2001), and CCGG sequences assayed by LUMA make up 8% of all CpG sites in the epigenome (X. Xu et al., 2012). Here, we measured both LINE1 and CCGG global DNA methylation across three matched tissues. Average LINE1 methylation across N=12 subjects was 77.9% in kidney, 79.5% in liver, and 58.3% in placenta, while the average LUMA methylation was 77.0% in kidney, 66.4% in liver, and 59.2% in placenta. Although the LINE1 and LUMA assays measure different parts of the genome, methylation values were comparable especially across the kidney and placenta samples. The standard deviations for each tissue, however, were greater for LUMA compared to LINE1 methylation measurements. As expected, there were significant differences in methylation from one tissue to another within an individual subject. For example, both LINE1 and LUMA assay exhibited significant hypomethylation in placenta compared to matched liver and kidneys (p-values <0.001, Figure 3.3).

BPA concentration and global methylation associations across tisue

While free BPA is the only species that can actively bind to hormone receptors, it is uncertain whether both free and conjugated forms influence epigenetic processes.

Therefore, we reported the relationship between global methylation and total BPA concentrations instead of the separate BPA species. As listed in Table 3.5, the Pearson's correlation between average % methylation using LUMA and total BPA was not significant with p-values at 0.934, 0.113, and 0.453 for kidney, liver, and placenta, respectively. Similarly, we did not identify any significant associations between average LUMA methylation and free BPA or BPA conjugates (data not shown).

Each LINE1 reaction reads methylation values at 4 adjacent CpG sites. To consider site-specific differences, a mixed effects model was used to test the relationship between total BPA and LINE1 methylation at all four sites, separately for each tissue. The β1 estimates in Table 3.5 for the BPA-methylation relationship were insignificant for kidney and liver, but significant for placenta. Accounting for CpG site-specific variation, with every 1 ng/g increase in total BPA, there was a 0.23% increase in global methylation in placental samples (p-value <0.002). A significant association was also observed with LINE1 methylation and free BPA but not with conjugated BPA in placenta (data not shown). Thus, the tissue BPA concentration and methylation association is likely tissue-dependent.

3.5 Discussion

The internal dose for nutrients, pharmaceuticals, and toxicants may differ across various tissues within an individual, depending on the fat content, capacity for uptake and elimination, or metabolic potential of an organ. Which biological sample is selected for exposure assessment depends on tissue availability, cost and ease of extraction,

optimization of environmental analysis in matrix, and expected adverse effects. Research concerning the internal dose of BPA across various human tissues is currently limited. Geens et al. were the first to measure BPA concentrations across several postpartum organs in adults, detecting 1-10 ng/g of free BPA across liver, brain, and adipose tissues in N=11 human subjects (Geens, Neels, & Covaci, 2012). In this study, we quantified both free and conjugated BPA across three organs from individuals representing a sensitive population, with N=6 from a low exposure and N=6 from a high exposure group. With the exception of one subject (H), we demonstrated that BPA during 1^{st}-2^{nd} trimester pregnancy tends to accumulate in fetal liver (median free: 7.09 ng/g) more so than in placenta (median free: 1.36 ng/g) and fetal kidney (median free: 0.536 ng/g), regardless of initial exposure. In the first pregnancy-related tissue biomonitoring studies, researchers detected an average of 11.2 ng/g of free BPA in term placenta from 37 German women, and reported 5.7-22.2 ng/g of free BPA in 10 out of 49 term placenta from Spanish women (Jimenez-Diaz, et al., 2010; Schonfelder et al., 2002). Recently, a Health Canada study reported free BPA in early and mid-gestational fetuses at concentrations of 0.6-64.0 ng/g in placenta and 1.3-27.0 ng/g in fetal liver (J. Zhang, Cooke, Curran, Goodyer, & Cao, 2011). Unlike our study, samples were not matched and free BPA was detected at higher rates and concentrations in placenta compared to fetal liver, as summarized in Figure 2.2.

Most of the findings from these tissue biomonitoring studies suggest considerable BPA exposure directly to the human fetus during development. The exact range of tissue concentration and detection frequency from one population to another may differ, but is likely explained by the various sensitivities and analytical techniques employed. The

HPLC coupled ESI-MS/MS method utilized is an important strength of this project, given its high sensitivity (LOQ= 0.05 ng/g) and use as the "gold standard" for BPA analysis (L. N. Vandenberg, et al., 2010). Even with stringent quality controls throughout tissue processing and analysis in the lab, BPA contamination from tissue acquisition and storage is a major concern for all human BPA biomonitoring studies. While the details of the extraction procedure for each fetus were not available to us, findings from this study suggest that BPA contamination during tissue acquisition is negligible in this sample set. Assuming similar instrument use within a given procedure and consistent background contamination at the collection site, any BPA contamination occurring throughout surgical processing would be expected to have distributed equally to the various matched tissues within a single donor, which were then immediately flash frozen. In this study, several subjects with measurable levels of free BPA in liver showed undetectable concentrations in matching placenta and low concentrations in kidney, suggesting that free BPA was attributed to *in utero* instead of *ex vivo* contamination. Furthermore, tissue storage in polypropylene tubing at -80°C with minimal freeze-thaw events should negate contamination during storage. Still, even if some contamination occurred during tissue extraction, the presence of biologically activated BPA conjugates in our fetal tissue demonstrates considerable exposure *in utero*. The disadvantage with observing major differences in tissue concentrations within an individual is the inability to use one tissue as a proxy for another. While the placenta may be an accessible tissue, according to these findings, BPA concentrations in this tissue do not accurately represent and may underestimate the internal dose in fetal liver and other target sites.

Identifying toxicokinetic characteristics, such as prevailing detoxifying reactions, may explain tissue differences in BPA concentrations observed within subjects. Therefore, we examined mRNA expression specific for BPA metabolism across placenta, fetal liver, and fetal kidney. In this study, we noted distinct tissue-specific expression profiles, with the highest *SULT1A1*, *GUSB*, and *UGT2B15* mRNA levels in fetal liver compared to other tissues. The higher *GUSB* expression compared to *UGT2B15* expression previously identified in liver was also observed across placenta and fetal kidney (Nahar, et al., 2013). As expected, *SULT1A1* expression was higher than *STS* expression in fetal tissues (O'Shaughnessy, Monteiro, Bhattacharya, Fraser, & Fowler, 2013), but with opposite trends in placenta. While sulfation reactions are generally higher in the developing fetus for xenobiotic defense, high STS activity is necessary in the placenta for the regulation of estrone and DHEA (Stanley, et al., 2005). If mRNA levels accurately reflect enzyme activity, findings suggest that BPA-glucuronide and BPA-sulfate conjugates in maternal circulation are more likely to be cleaved back into the estrogenic parent form in the placenta. Once in the fetal liver, both BPA-glucuronide conjugation and deconjugation may occur, but any excess free BPA can become conjugated to BPA-sulfate. In the fetal kidneys, similar reactions can occur, although at a lower rate. Future biomonitoring studies will require separate measurements of BPA-glucuronide and BPA-sulfate, as new evidence reveals that BPA-sulfate may be the predominant conjugate in the human fetus (Gerona, et al., 2013).

To evaluate toxicokinetics in this project, we only assessed biotransformation through gene expression of XMEs that are important for BPA across various tissues. Simply correlating BPA concentrations with mRNA expression did not reveal significant

associations for any particular organ. As mentioned in chapter 2, the null results suggest that other factors may influence BPA toxicokinetics such as 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and UDP-glucuronic acid cofactor concentrations, protein modification of enzymes, and xenobiotic transporters for absorption and elimination across tissue. Variability in the transporters specific for free BPA or its conjugates, although currently unknown, are likely to explain the different concentrations observed in our matched tissues. For example, a particular set of transporters may be required for BPA to actively cross the placenta and then be eliminated from the fetal kidneys into amniotic fluid, bypassing bioaccumulation in these organs.

Despite bioaccumulation, individual organs may exhibit differential sensitivity to BPA, eliciting distinct biological responses. Environmental exposures during sensitive windows of development can alter the baseline regulatory markers or developmental trajectories that lead to noticeable biological effects at a later time point (Faulk & Dolinoy, 2011). Thus, epigenetic regulatory markers such as DNA methylation may be the best biomarker for assessing short-term biological effects from exposure, especially if longitudinal data is unavailable. Global DNA hypomethylation is commonly found in several adverse health outcomes such as cancer, indicating genomic instability (Torano, Petrus, Fernandez, & Fraga, 2012). Utilizing two crude global methylation assays in this study, we identified significant differences in average methylation across tissues, where placental samples showed the lowest global methylation levels. Several studies have already reported low CpG methylation in placenta versus other tissues using a variety of global methylation techniques (Ehrlich et al., 1982; Fuke et al., 2004). For example, Price et al. observed LINE1 methylation at approximately 60% in placenta but around 80%

methylation in fetal somatic tissue (Price et al., 2012). DNA hypomethylation, especially within repetitive elements, is assumed to play an evolutionary role in invasive and proliferative function of the placenta.

Assays like LUMA and LINE1 are currently used as a surrogate for global DNA methylation measurements, each representing different genomic regions with varying strengths and weaknesses. In a recent methods paper, results from LINE1 showed the least replicate variability akin to the HPLC gold standard, while LUMA values were most variable (Lisanti et al., 2013). Large variability, coupled with a relatively small sample size in our LUMA data, may have masked any BPA concentration-dependent effects on global methylation. Using the less variable LINE1 assay, we identified a small but significant positive association between BPA concentration and methylation only in the placenta. Still, several limitations of this study necessitate a cautious interpretation of results and further investigation into this association. Global methylation assays are crude, efficient methods to look at exposure-dependent changes, but BPA may influence methylation in a locus or gene-specific manner. Furthermore, epigenome-wide methylation patterns are not only tissue-specific but also cell-specific (Michels et al., 2013). Thus, once cell-specific methylation techniques are optimized and cost-effective, BPA's impact on cell-specific methylation changes should be assessed. Exposure-epigenetic associations are also likely to differ by the type of epigenetic marker examined. In recent years, other DNA base modifications like 5-hydroxymethylcytosine (5hmC) have been discovered with evidence for its role in fetal liver development (Ivanov et al., 2013). Nevertheless, this is the first study to report environmentally relevant BPA concentrations and altered capacity for metabolism across several human

tissues. Although subtle BPA dependent changes were identified, findings support the need for early investigation of biological effects and assessment across various tissues.

Table 3.1 RNA Integrity Number Across Individual Tissues and Subjects

RNA Integrity Number (RIN)			
Subject	**Placenta**	**Liver**	**Kidney**
A	8.0	8.5	9.4
B	3.0	9.0	9.6
C	3.6	8.4	9.5
D	8.8	7.8	9.6
E	7.5	7.4	9.7
F	8.8	8.6	9.6
G	7.7	5.8	9.6
H	7.2	4.5	9.6
I	7.1	8.7	9.6
J	8.7	6.4	9.7
K	7.0	8.2	9.1
L	8.1	7.8	9.7

Table 3.2 LINE1 PCR and Pyrosequencing Information

	Sequence
Forward Primer	5'-TTGAGTTAGGTGTGGGATATAGTT-3'
Reverse Biotinylated Primer	5'-CAAAAAATCAAAAAATTCCCTTTCC-3'
Sequencing Primer	5'-AGGTGTGGATATAGT-3'
Sequence to Analyze	TTT/CGTGGTGT/CGTT/CGTTTTTTAAGTT/CGGTTTGAAAAG

Table 3.3 BPA Concentrations Across All Tissue Types

Tissue	**Free BPA**			**Conjugated BPA**		
	% Above LOQ	**Median (ng/g)**	**Range (ng/g)**	**% Above LOQ**	**Median (ng/g)**	**Range (ng/g)**
Kidney (N=12)	100	0.536	0.079-11.10	58	0.092	<LOQ-0.751
Liver (N=12)	100	7.085	0.540-50.50	100	2.255	0.450-15.85
Placenta (N=12)	83	1.355	<LOQ-25.40	42	0.071	<LOQ-9.810
ANOVA*			0.0053			0.0038

*Values represent p-values. Significant differences are denoted by values <0.05

Table 3.4 Pearson's Correlation Between Free BPA and Gene Expression (N=12 Per Tissue)

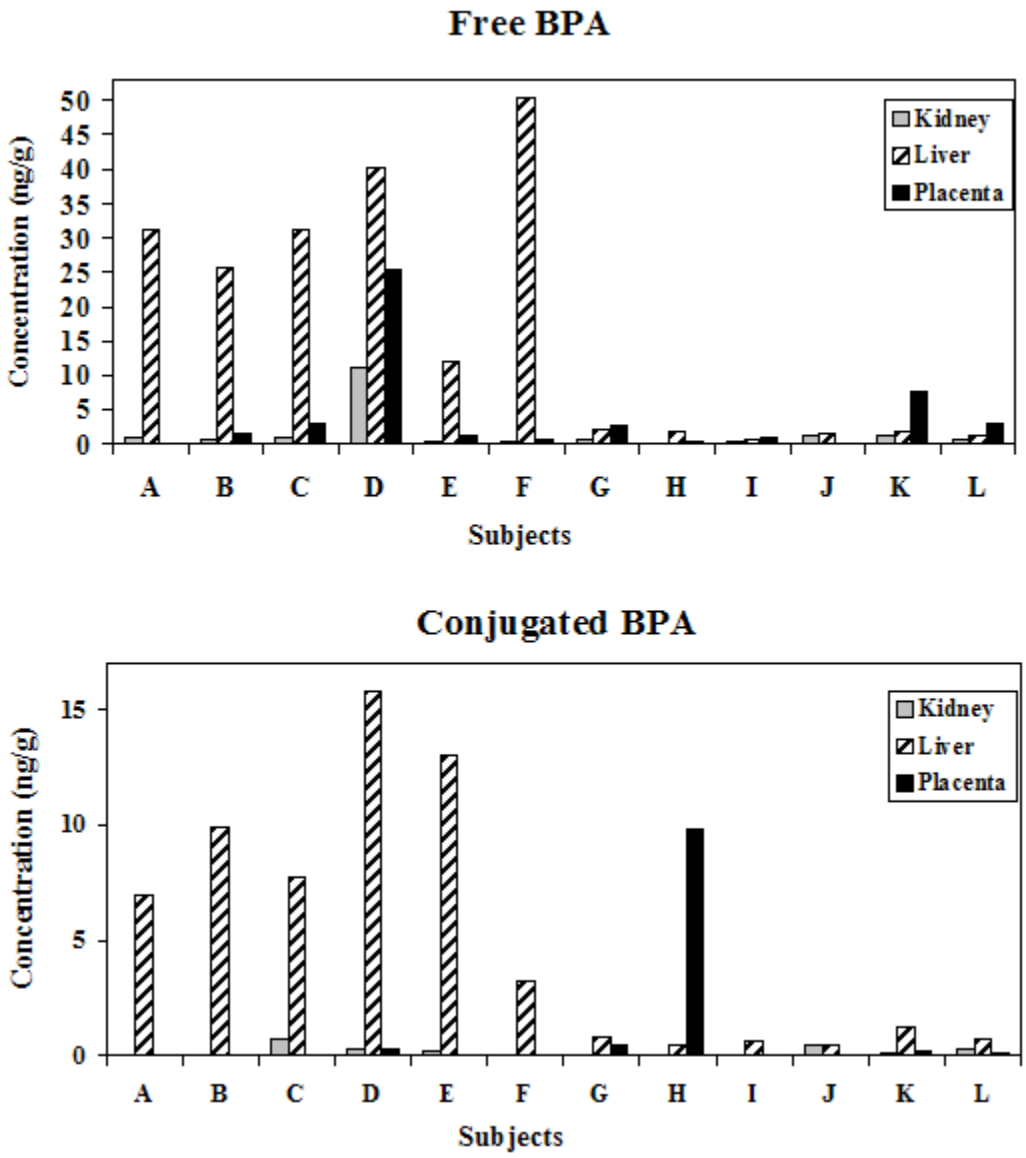
	Fetal Kidney	Fetal Liver	Placenta
GUSB	-0.260, p: 0.414	-0.196, p: 0.541	-0.190, p: 0.555
UGT2B15	-0.285, p: 0.370	-0.204, p: 0.524	0.147, p: 0.649
STS	-0.184, p: 0.567	-0.084, p: 0.795	-0.124, p: 0.701
SULT1A1	-0.249, p: 0.436	0.167, p: 0.631	-0.170, p: 0.598

Table 3.5 Associations Between Total BPA Concentration and Global Methylation

	LINE1†		LUMA	
	β1 Estimate	p-value	Pearson's correlation	p-value
Kidney	0.066	0.424	0.027	0.934
Liver	0.021	0.425	0.482	0.113
Placenta	0.225	0.002*	-0.240	0.453

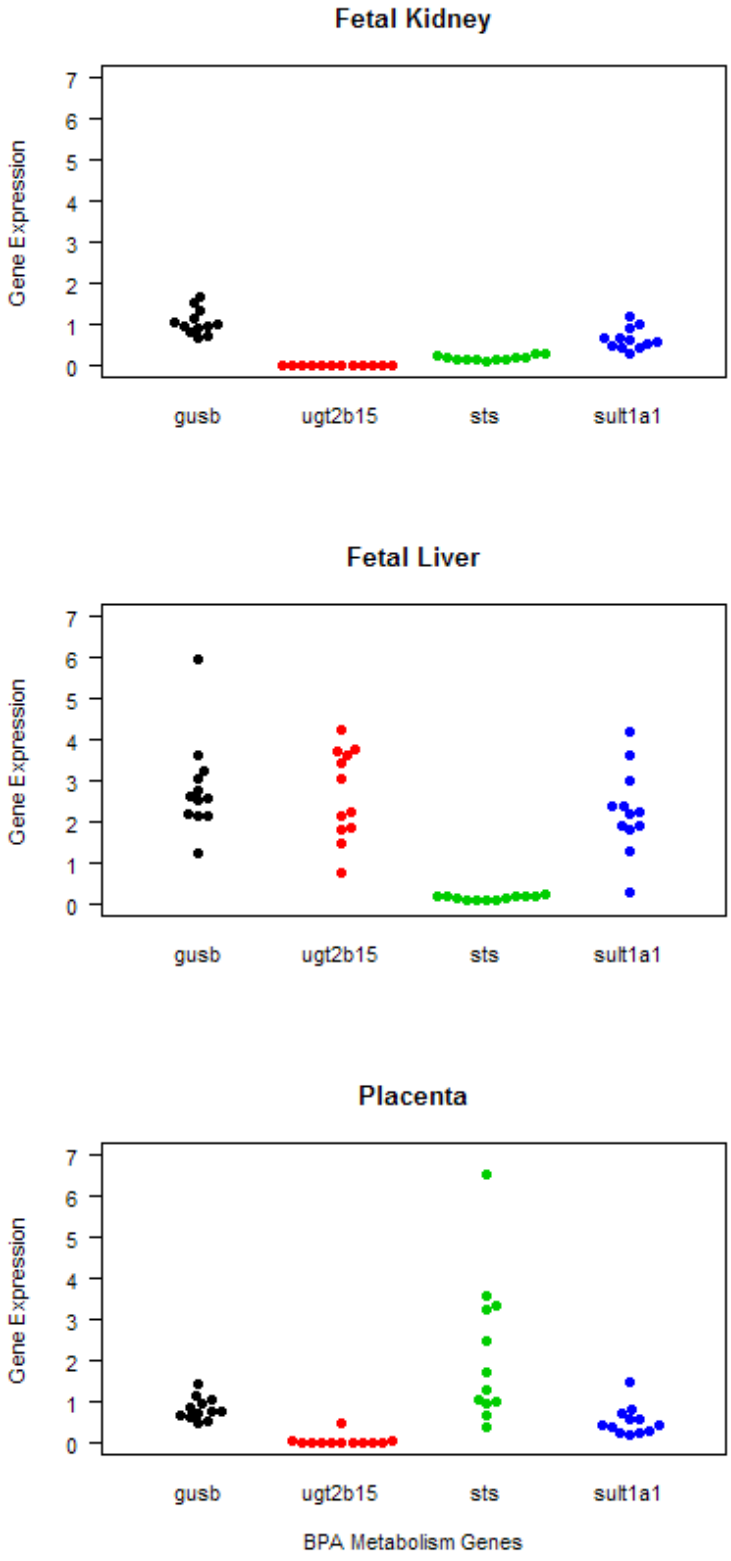
† Mixed effects model controlling for random effect by CpG site
*p-value<0.05 show significant association

Figure 3.1 Free and Conjugated BPA Concentrations Across Tissue from N=12 Subjects



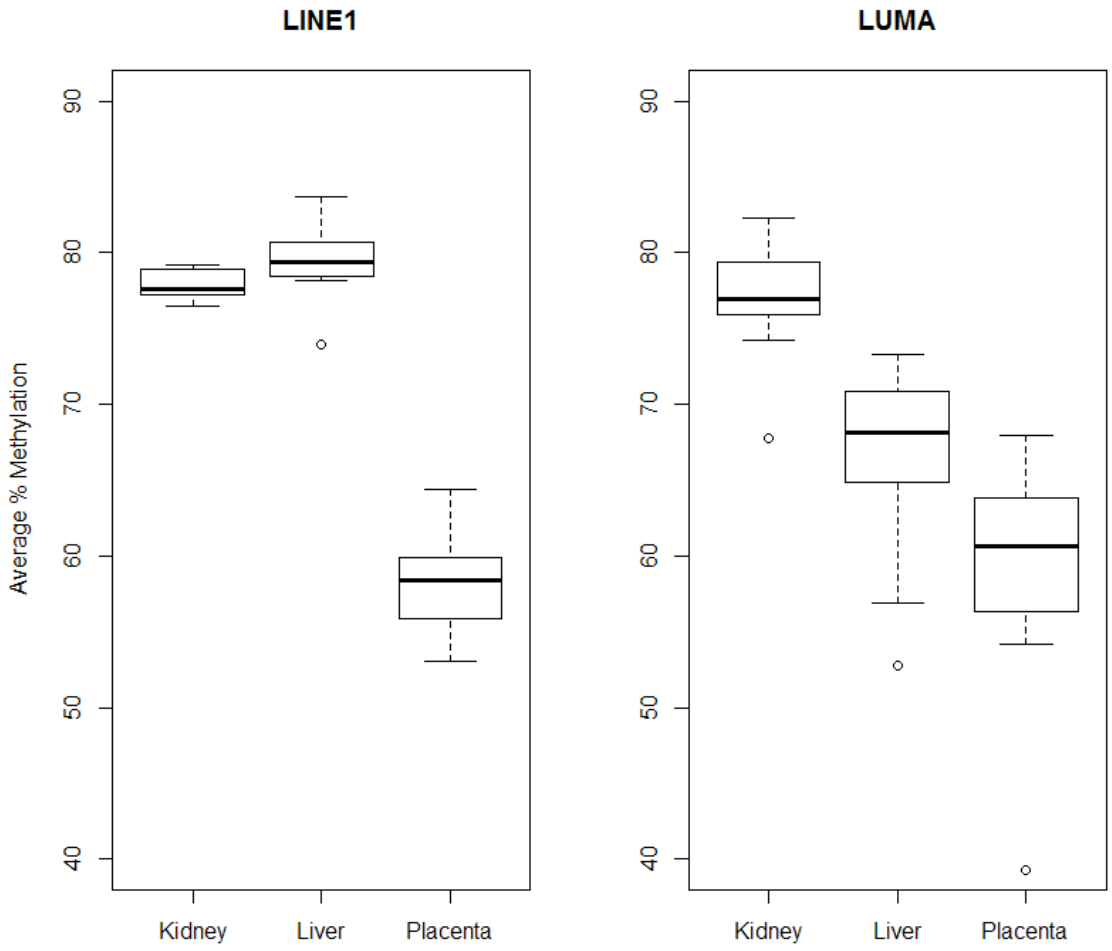
BPA concentrations differ across tissue and individuals. Subjects A-F (N=6) represent the high BPA exposure group while subjects G-L (N=6) represent the low BPA exposure group. Within each subject, three tissues were analyzed: placenta, fetal liver, and fetal kidney. Two separate graphs for all subjects and tissues display **(A)** free BPA concentrations and **(B)** conjugated BPA concentrations.

Figure 3.2 BPA-specific XME Gene Expression Across Tissue



BPA-related XME gene expression profiles differ by tissue. The dot plot displays the normalized gene expression values for all 12 subjects for four genes: *GUSB*, *UGT2B15*, *STS*, and *SULT1A1*. Each panel shows the expression profiles by tissue type: (A) fetal kidney (B) fetal liver and (C) placenta. When an ANOVA test was conducted across the three tissues for *GUSB*, *UGT2B15*, *STS*, and *SULT1A1* separately, there were significant tissue-dependent differences in expression (p-values <0.001).

Figure 3.3 LINE1 and LUMA Global Methylation Across Tissue



Global methylation levels differ across tissue. Boxplots represent the average global % methylation values for placenta, fetal liver, and fetal kidney using the assay for **(A)** LINE1 on the left panel and **(B)** LUMA on the right panel. Average LINE1 methylation was 77.92% in kidney, 79.49% in liver, and 58.27% in placenta, while average LUMA methylation was 76.99% in kidney, 66.42% in liver, and 59.15% in placenta. Percent global methylation measured by LINE1 and LUMA were significantly different across tissues with significant hypomethylation in placenta compared to either fetal liver or fetal kidney (p-values <0.001).

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CHAPTER 4

Bisphenol A Associated Alterations in Candidate Biotransformation Gene Expression and Regulation in Human Fetal Liver

Text modified from Nahar, M. S., Kim, J. H., Sartor, M. A., & Dolinoy, D. C., 2014. Bisphenol A-associated alterations in the expression and epigenetic regulation of genes encoding xenobiotic metabolizing enzymes in human fetal liver. *Environ Mol Mutagen, 9*(10), 21823. Permission for reuse approved by Rightslink and Copyright Clearance Center, Inc.

4.1 Abstract

Alterations in xenobiotic metabolizing enzyme (XME) expression across the life course, along with genetic, nutritional, and environmental regulation, can influence how organisms respond to toxic insults. In this study, we investigated the hypothesis that *in utero* exposure to the endocrine active compound, bisphenol A (BPA), influences expression and epigenetic regulation of phase I and II XME genes during development. Using healthy 1^{st}-2^{nd} trimester human fetal liver specimens quantified for BPA concentrations, we examined XME gene expression using qPCR Array (N=8) and RNA-sequencing (N=12) platforms. Of the greater than 160 XME genes assayed, two phase I and twelve phase II genes exhibited significantly reduced expression with higher BPA concentrations, including isoforms from the carboxylesterase, catechol o-methyltransferase, glutathione s-transferase, sulfotransferase, and UDP-

glucuronosyltransferase families. When the promoters of these candidate genes were evaluated *in silico*, putative binding sites for the E-Twenty Six (ETS) and Activator Protein1 (AP1) related transcription factor families were identified and unique to 97% of all candidate transcripts. Interestingly, many ETS binding sites contain cytosine-guanine dinucleotides (CpGs) within their consensus sequences. Using Methylplex-next generation sequencing (M-NGS) data, we identified regions of variable methylation that overlap with ETS binding sites to design site-specific DNA methylation assays. Quantitative analysis of CpG methylation of three candidate genes was conducted across N=50 samples and analyzed with tissue BPA concentrations. Higher total BPA concentrations were associated with increased site-specific methylation at *COMT* (p-value <0.005) and increased average methylation at *SULT2A1* (p-value <0.020) promoters. While toxicological studies have traditionally focused on high dose effects and hormonal receptor mediated regulation, our findings suggest the importance of low dose effects and non-classical mechanisms of endocrine disruption during development.

4.2 Introduction

A compound's absorption, distribution, metabolism, and excretion via biotransformation pathways dictate its therapeutic or toxic potential. Specifically, changes in phase I and II xenobiotic metabolizing enzyme (XME) gene expression and activity can alter drug efficacy and toxicity, with the greatest discrepancies observed in children compared to adults (Alcorn & McNamara, 2003). Preliminary studies indicate that relative XME expression and activity among different classes and isoforms change

drastically throughout the life course (Hines, 2008). For example, the phase II UDP-glucuronosyltransferase isoforms, *UGT1A1* and *UGT1A6*, are both expressed at low levels in the human fetus; while *UGT1A1* attains adult levels within months after birth, the *UGT1A6* isoform reaches adult levels at 10 years of age (McCarver & Hines, 2002). Therefore, the extent of biotransformation of a xenobiotic chemical is dependent on age and ontogeny, or the maturation of specific XMEs (Allegaert, van den Anker, Naulaers, & de Hoon, 2007). Much of our understanding of human XME ontogeny arises from adverse pharmaceutical exposures or diseases throughout birth and infancy, or extrapolations from rodent models (Saghir, Khan, & McCoy, 2012). Comprehensive studies examining metabolism in early human fetal development are limited; for example, recent studies characterize only a handful of XMEs, such as steroidogenic enzymes in 2nd trimester fetal liver (O'Shaughnessy, et al., 2013) or cytochrome p450s and glutathione s-transferases in fetal liver and adrenals (Wang et al., 2008). Evaluating ontogeny, especially throughout human gestation, is thus of great importance in assessing toxicity; however, obtaining suitable human specimens may pose ethical and technical challenges.

In addition to drug metabolism, XMEs play an important role in steroid homeostasis, neuroendocrine function, and growth. Using feedback signaling, both endogenous and exogenous compounds help regulate XME ontogeny and subsequently, metabolic function. More recently, studies demonstrating the importance of hormonal regulation on the establishment of hepatic metabolism throughout pregnancy and development have emerged (Jeong, 2010; Kennedy, 2008). Xenobiotics that can mimic endogenous hormones, such as bisphenol a (BPA), can potentially modify baseline hormonal regulation of xenobiotic metabolism and response to environmental stressors

during critical windows of development. While endogenous and exogenous agents commonly act through nuclear or steroid receptor mediated pathways for modulating XME expression, currently there is considerable interest in exploring the role of epigenetic markers along with signal transduction in XME regulation (Klaassen, Lu, & Cui, 2011; Zhong & Leeder, 2013).

To date, research addressing BPA's influence on XME expression and activity has been limited to animal and *in vitro* models. For example, rat hepatic microsome studies show that BPA inhibits cytochrome p450 enzymes, including activity for CYP1A2, CYP2C11, and CYP2E1 (Hanioka, Jinno, Tanaka-Kagawa, Nishimura, & Ando, 2000; Pfeiffer & Metzler, 2004), yeast and human liver microsome studies associate BPA with both competitive and noncompetitive inhibition of UGT1A6 (Hanioka et al., 2008), and human endometrial Ishikawa cell line studies show increased *ALDH3A1* expression following BPA exposure (Naciff et al., 2010). Interspecies differences in metabolism and/or relatively high BPA exposure levels, however, prevent the translatability of these findings to assess human risk.

The influence of xenoestrogens on the maturation and regulation of phase I and II xenobiotic metabolizing enzymes have yet to be studied in humans. In this final dissertation chapter, we utilize high-throughput approaches for evaluating XME expression and regulation associated with tissue BPA concentrations. Here, we identify novel candidate XME genes that are associated with physiologically relevant concentrations of total BPA, assess relative abundance of these XME mRNAs in the developing human fetal liver, explore common regulatory factors for XME candidates,

and investigate DNA methylation as a potential mechanism influencing biotransformation response to BPA exposure.

4.3 Methods and Materials

Tissue sample selection

While free BPA is the only species to activate receptor-mediated pathways, the role that conjugated BPA plays on non-receptor mediated mechanisms is currently unknown. Thus, we used total BPA concentrations to identify exposure-dependent differences in gene expression and methylation. As described in chapter 2, total BPA concentrations measured in fetal liver tissue ranged from below the limit of quantification at 0.071 ng/g (LOQ/√2, where LOQ=0.1 ng/g) up to 96.8 ng/g (Nahar, et al., 2013). For candidate gene identification and quantification, samples were binned into low or high exposure categories based on total tissue BPA concentrations. The category descriptions are detailed for each technique below.

RNA and DNA preparation

As described in the previous two chapters, total RNA and DNA was isolated from all frozen liver tissue with quality and quantity assessed using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only high quality samples with RNA integrity number (RIN) >7 were used for RT^2 Profiler PCR Arrays (N=8) and high-throughput

RNA-sequencing (N=12). For PCR gene expression analysis, RNA templates were converted to complementary DNA as previously described in chapter 2.

Human drug metabolism RT^2 Profiler^{TM} PCR Array

Differential mRNA expression of various genes from the XME pathway were assessed by using two different cataloged human arrays from SuperArray Biosciences (Frederick, MD): the Phase I Enzyme PCR Array (PAH-068E4) and the Phase II Enzyme PCR Array (PAH-069E4). Each SYBR Green-based 96-well PCR Array platform interrogates multiple genes and isoforms relevant to a specific biological pathway along with five reference genes and several controls per sample. Based on the distribution of total BPA concentrations measured in 50 fetal liver samples (Nahar, et al., 2013), the median BPA concentration (3.44 ng/g) was used as a cutoff for group categorization. The final samples were chosen based on the highest RIN scores and a >10 fold difference in average total BPA between low and high exposure groups. Four liver RNA samples exhibiting low total BPA concentrations (1.2-2.9 ng/g) and four liver RNA samples exhibiting high total BPA concentrations (35.4-56.1 ng/g) were submitted to the UM Sequencing Core facility and run on both PCR Array platforms using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems).

PCR Array gene expression analysis

The threshold cycle (CT) was obtained for each target gene, and an average reference CT was calculated for the five endogenous reference genes: β2-microglobulin (*B2M*), hypoxanthine phosphoribosyltransferase I (*HPRT1*), ribosomal protein L13a

(*RPL13A*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and β-actin (*ACTβ*). Positive PCR and reverse transcription controls were either undetected or had an extremely high CT value. Results are reported as ΔCT, which represents the difference between the CT of the target gene versus the average CT of the reference genes. The average ΔCT of the low exposure samples were subtracted from the average ΔCT of the high exposure samples to obtain the ΔΔCT value, and fold change was calculated as $2^{-\Delta\Delta CT}$. A linear model designed for microarray analysis was utilized to increase the power to detect fold change differences in target arrays by pooling information using the limma package of Bioconductor in R statistical software version 2.13.2 (Smyth, 2004). Furthermore, p-values were adjusted for multiple comparisons using false discovery rate (Benjamini & Hochberg, 1995). Candidate genes were chosen based on greater than 2 fold or less than 0.5 fold change and p-value <0.05.

Next generation RNA sequencing

To characterize the relative mRNA abundance of XMEs in human fetal liver, regardless of sex or exposure, we utilized information from a second set of samples (N=12, including 3 samples also used in the PCR Arrays) submitted for high throughput next generation mRNA sequencing (RNA-seq) using the Illumina HiSeq 2000 platform (Illumina, San Diego, CA). General workflow for next generation sequencing library construction consists of enrichment of poly-A RNA, fragmentation, RNA clean up, cDNA synthesis using hexamer primers, end repair, and adaptor ligation, as previously described (Sengupta et al., 2011). The raw sequencing image data was analyzed with the Illumina analysis pipeline, using single reads up to 150 base pairs (bp) in length. Over

438 million reads across 12 samples were obtained, with an average read number of 36.5 million per sample. The FastQC tool was used to perform quality control checks on raw data. In order to utilize reads with the best quality calls (FastQC per base sequence quality score above 28), sequences were trimmed to 70 bp in length, and reads were aligned to the hg19 human reference genome using TopHat. Parameters with the best alignment and increased search time were used in order to improve sensitivity. After removing duplicate reads resulting from PCR duplicates with SamTools, transcript abundance was measured by CuffDiff v2.0.1 software as fragments per kilobase of exon per million fragments mapped (FPKM). Total fragment reads were calculated and normalized across 12 samples. Approximately 152 genes of the phase I and phase II genes on the PCR Arrays were identified in the RNA-seq dataset along with their normalized FPKM reads. The bottom and top quartile reads in FPKM are reported, indicative of low and high relative expression of phase I and phase II XME genes during development.

Transcription factor binding site identification

An *in silico* bioinformatics approach was used to identify common promoter regulatory elements within BPA-associated candidate genes identified from the PCR Arrays. Gene2Promoter (Genomatix, Munich, Germany) software utilizes position weight matrices to predict transcription factor binding sites (TFBS) 500 bp upstream and 100 bp downstream of transcription start sites (TSS) of candidate genes (Cartharius et al., 2005). Using MatInspector, fasta-formatted input sequences of the PCR Array candidate gene promoters were compared, and common transcription factors were clustered

together into TFBS families or "matrix families" based on similarities in binding domain or function. Regulatory families were reported when TFBS were identified in >97% of all relevant transcripts of the candidate XME genes.

Methylplex next generation sequencing profiles

In a parallel study, we used the epigenome-wide Methylplex next generation sequencing (M-NGS) platform to identify novel exposure-dependent regions of altered methylation within a subset of the fetal liver specimens (N=18). For each sample, the Methylplex library synthesis and GC-enrichment was carried out using a proprietary kit (Rubicon Genomics, Ann Arbor, MI). Subsequent library sequencing took place at the UM DNA Sequencing Core using the Illumina HiSeq platform, which produces 100 cycles of single-end reads for each sample. Detailed protocols for M-NGS library construction, sequencing, and alignment have been described previously (Kim et al., 2014). Using the Illumina analysis pipeline, we obtained approximately 104 million reads per sample (ranging from 78 to 125 million reads) with 83.9% of these reads uniquely mapped to the human hg19 reference genome. For this dissertation, we mined the methylome dataset to extract the methylation reads 5000 bp upstream and downstream surrounding the TSS for our candidate genes to aid in our Sequenom methylation assay design. In particular, we report the compiled methylation profiles for three candidate genes: *CES2, COMT,* and *SULT2A1*.

Bisulfite conversion and Sequenom methylation analysis

To quantitatively assess methylation at biologically relevant promoter regions, we designed Sequenom assays for three of our candidate genes using Methylplex and Genomatix data across N=50 fetal liver bisulfite converted (BSC) DNA. Chapter 3 describes the instructions for bisulfite conversion using the Qiagen Epitect kit. Epitect methylated and unmethylated human BSC samples (Qiagen) were used as positive controls. T7 tagged primers for the moderately expressed *CES2*, *COMT*, and *SULT2A1* candidate genes were designed for promoter methylation analysis using the EpiDesigner Web tool (http://www.epidesigner.com/, Table 4.1). Candidate gene promoter regions containing consensus sequences for ETS related transcription factors were amplified using HotStarTaq master mix in a 30 µl PCR reaction. A standard HotStarTaq thermal cycling setting was used for the three assays with the following changes: *CES2* required annealing at 56°C for 30 sec at 35 cycles, *COMT* required annealing at 60°C for 30 sec at 35 cycles, and *SULT2A1* required annealing at 56°C for 30 sec at 40 cycles.

After *in vitro* transcription and uracil-cleavage of amplicons as previously described (Coolen, Statham, Gardiner-Garden, & Clark, 2007), promoter methylation was quantified using the mass-spectrometry based Sequenom MassArray EpiTYPER platform (Sequenom, San Diego, CA) in the UM DNA Sequencing Core. Associations between amplicon average DNA methylation and total BPA tissue concentrations were assessed using a linear mixed effects model. For this model, percent methylation was the dependent variable and CpG unit number was the random effect, while total BPA concentration, sex, and gestational age were set as fixed effects. The analysis incorporated multiple site-specific CpG units (4 for *CES2* and *SULT2A1*; 17 for *COMT*)

and accounted for correlations between adjacent dinucleotides for each assay. The association between CpG site-specific methylation of ETS consensus sequences and total BPA was assessed using multiple linear regression after adjusting for sex and gestational age. For each statistical analysis, regression diagnostics were used to identify samples that deviate from standard linear regression assumptions (e.g. constant variance and normal distribution of residuals). Residuals were determined to be within cook's distance cutoff (<1); thus no data points were excluded from the final analysis. Complete methylation data was analyzed for the following number of samples: N=50 for *CES2* and *COMT*, and N=40 for *SULT2A1*. Due to technical limitations, 10 samples failed the *SULT2A1* Sequenom assay, and *SULT2A1* CpG site #1, which falls within the ETS consensus sequence, was not resolvable on any sample via the Sequenom platform.

4.4 Results

Candidate XME gene identification by tissue BPA

Using the RT^2 Profiler^{TM} PCR Array, we analyzed 167 genes involved in xenobiotic metabolism (93 phase I and 74 phase II enzyme isoforms) in human fetal liver specimens. Based on gene selection criteria (p-value <0.05 and fold change >2 or <0.5), two phase I genes and twelve phase II genes exhibited altered expression based on low (1.2-2.9 ng/g; N=4) versus high (35.4-56.1 ng/g; N=4) tissue total BPA concentrations (Table 4.2). All differentially expressed candidate genes exhibited significantly decreased expression in liver samples exhibiting high compared to low tissue BPA concentrations. The phase I genes include the *CES2* and *CES5A* carboxylesterase isoforms, while the

phase II genes include catechol o-methyltransferase (*COMT*), glutathione s-transferase (*GSTA5*), sulfotransferase (*SULT2A1, 1B1, 6B1, 1C3*), and UDP-glucuronosyltransferase (*UGT1A3, 1A4, 1A6, 1A8, 1A9, 1A10*).

Relative expression levels of XME genes

RNA deep sequencing was employed to qualitatively characterize the relative expression of 152 of the 167 phase I and phase II XME genes contained on the PCR Arrays. While real time quantitative polymerase chain reaction (RT-qPCR) based techniques are the gold standard for quantitative expression, RNA-seq platforms are important for simultaneous characterization of hundreds or thousands of genes; thus, this platform was utilized for exposure independent comparative expression rather than identification of differential expression. RNA-seq reads were reported as fragments per kilobase of exon per million fragments mapped (FPKM) using CuffDiff v2.0.1. Table 4.3 depicts the bottom and top quartile FPKM reads separately for phase I and phase II XME gene isoforms, with PCR Array candidate genes in bold.

For phase I XME genes, the epoxide hydrolase (*EPHX*), hydroxysteroid dehydrogenase (*HSD*), and monoamine oxygenase (*MAO*) genes were expressed at high levels while carboxylesterase and cyclooxygenase (*PTGS*) genes were primarily expressed at low levels. In several instances, an isoform of a gene family exhibited low expression while a second isoform of the same family exhibited high expression. For example, while the alcohol dehydrogenase and aldehyde dehydrogenase isoforms, *ADH1A* and *ALDH1A1*, were both expressed at high levels, the *ADH7* and *ALDH3B2* isoforms were both expressed at low levels. Similarly, the predominantly expressed

cytochrome p450 and flavin-dependent monoxygenase isoforms were *CYP3A7* and *FMO5*, while the *CYP11B2* and *FMO2* isoforms were expressed at low levels.

Among the phase II genes, several methyltransferases (*GAMT*, *HNMT*) and the thiosulfate sulfotransferase (*TST*) gene were expressed at high levels, while many N-acetyltransferase genes like arylalkalamine and arylamine N-acetyltransferases (*AANAT*, *NAT2*) were expressed at low levels. The more common conjugating XME families such as glutathione s-transferase, sulfotransferases, and UDP-glucuronosyltransferase displayed isoforms that were variably expressed.

Interestingly, several BPA-associated candidate XME genes identified through the PCR Arrays exhibited relatively low expression in the 1^{st}-2^{nd} trimester fetal liver. They include *CES5A*, *GSTA5*, *SULT6B1*, *SULT1B1*, *SULT1C3*, and the *UGT1A* related isoforms. While none of the differentially expressed genes were expressed at high levels in early development, *CES2*, *COMT*, and *SULT2A1* showed moderate expression and were subsequently assayed for epigenetic analysis.

Identification of common transcription factors in candidate genes

Genomatix was used to assess common transcription factor binding sites 500 bp upstream and 100 bp downstream of the TSS belonging to nine unique BPA-associated loci (all *UGT1A* functional genes are regulated by a common promoter). Two transcription factor families were common in at least 97% of the promoters of all relevant transcripts. Both Activator protein1 (AP1) and E-twenty-six (ETS) related transcription factor binding sites were identified at least once in the promoter of each locus as seen on the graphical presentation in Figure 4.1 (depicted in purple and green, respectively). The

AP1 family consists of MAF and AP1 related factors such as BACH1/2, MAFA/B/F/G/K, NRL, NFE2L1/2/3 (NRF1/2/3), and NRL. The ETS family consists of a variety of transcription factors containing the Ets DNA binding domain including ELF1/2/4, ETS1/2, ETV1/4/5, SPI1/B/C, among others. The consensus sequence for each family and subfamily along with key transcription factors are listed in Table 4.4. Interestingly, a majority of transcription factor binding sites belonging to the ETS family contain CpG dinucleotides in the consensus sequence, especially in the ELF, ELK, and ETV subfamilies.

Candidate gene methylation profiling

M-NGS methylation reads were plotted every 100 bp for each sample in order to produce DNA methylation profiles of candidate genes and their promoters. The methylation profiles for *CES2*, *COMT*, and *SULT2A1* displayed in Figure 4.2, Figure 4.3, and Figure 4.4 span 5000 bp upstream and downstream of the TSS (profiles for the remaining 11 genes are not shown). The profiles show distinct differences in methylation read numbers and location of variable methylation from one gene to another. Regions of variable methylation that overlap with ETS consensus sequences, located approximately 1600-1900 bp upstream of the TSS, were highlighted for both *CES2* (Figure 4.2) and *SULT2A1* (Figure 4.4). For *COMT* (Figure 4.3), the highlighted region also coincides with ETS transcription factor binding but does not show large variability in methylation reads. This region was chosen because the 300 bp section across the TSS has been noted for its CpG island and was successfully assayed showing significant associations between methylation and disease outcomes (Abdolmaleky et al., 2006; Q. Xu, Ma, Payne, & Li,

2010). All highlighted regions on the Methylplex figures were important for designing site-specific DNA methylation assays that may be biologically relevant.

ETS-specific methylation analysis at candidate gene promoters

We studied CpG methylation in and surrounding ETS transcription factor binding sites at the promoters of three candidate genes: *CES2*, *COMT*, and *SULT2A1*. These genes were chosen based on relatively moderate expression levels across fetal specimens irrespective of age, sex, and exposure according to RNA-seq reads. Figure 4.5 displays the genomic location of the methylation assays with number of CpG sites, CpG units, and ETS transcription factor binding sites. Each assay analyzes multiple CpG units: 4 for *CES2* and *SULT2A1*, and 17 for *COMT*. Methylation across all sites ranged between 89-96% for *CES2*, 3-8% for *COMT*, and 59-81% for *SULT2A1*. Within each assay, we also focused on specific CpG sites located on the ETS consensus sequence: CpG site #3 for *CES2* and CpG sites #30 & #31 for *COMT*. CpG site #1 for *SULT2A1* falls within the ETS transcription factor consensus sequences, but was unresolvable with Sequenom.

The average methylation across all detectable sites in the *COMT* assay was not significantly associated with tissue BPA after adjusting for age and sex (p-value: 0.108); however, one out of the two CpG sites spanning the putative ETS consensus sequence showed a significant positive trend (p-value: 0.002). An interquartile range (IQR; 10.93 ng/g) increase in total tissue BPA was associated with 0.416% increase in *COMT* methylation at CpG site #31 (Figure 4.5; Table 4.5). Similarly, an IQR (8.28 ng/g) increase in total tissue BPA was associated with a significant 0.821% increase across all six *SULT2A1* CpG sites (Figure 4.5; Table 4.5; p-value: 0.042). The *CES2* assay,

however, displayed decreased site-specific methylation at CpG site #3 and average methylation across all sites with increasing BPA concentrations, although neither association was significant (p-value: 0.409 and 0.953, respectively).

4.5 Discussion

The impact of nutritional and environmental exposures on the human xenobiotic metabolism system during early development is not well understood. As previously described, we quantified total BPA concentrations in fifty 1^{st}-2^{nd} trimester fetal liver specimens, ranging from below the LOQ to 96.8 ng/g total BPA in tissue (Nahar, et al., 2013). In the current study, we compared gene expression of over 160 XME genes within a subset of fetal liver specimens, dichotomized into low (1.2-2.9 ng/g; N=4) and high (35.4-56.1 ng/g; N=4) total tissue BPA. In general, 14 candidate genes were identified that exhibit significantly reduced expression in fetal liver with higher tissue BPA concentrations. With the exception of the phase I carboxylesterase isoforms, *CES2* and *CES5A*, the majority of BPA-related XME candidate genes belong to the phase II gene family.

Traditionally, the induction of phase I enzymes following drug and xenobiotic exposure is commonly observed, but here we observe BPA-associated repression of predominantly phase II XME expression. Only a handful of studies have previously reported xenobiotic-dependent repression of primarily phase II metabolizing genes. In a 2008 study examining endocrine disruptors' ability to modify estrogen bioavailability, 80 µM of BPA or 70 µM of genistein exposure to ER-negative HepG2 cells was associated

with decreased expression of several phase II metabolism enzymes, including *UGT2B7, UGT2B15,* and *SULT1E1* genes (Hanet et al., 2008). Of our newly identified BPA-associated XME genes, only the UDP-glucuronosyltransferases have been previously associated with BPA as identified from the Comparative Toxicogenomic Database (http://ctdbase.org/). When MCF7 cells were exposed to 10 nM of BPA instead of the control DMSO vehicle, there was a reduction in *UGT1A3* mRNA expression (Buterin, Koch, & Naegeli, 2006). In a second study, BPA was reported to competitively inhibit UGT1A6 and subsequently alter metabolism of serotonin in liver microsomes (Hanioka, Takeda, et al., 2008).

Characterizing XME activity and regulation, especially throughout fetal development, is challenging given the difficulty in acquiring relevant human specimens from sensitive populations. With our unique samples, we were able to compare relative expression for hundreds of genes simultaneously using mRNA-next generation sequencing technology. After compiling all XME related genes, the top and bottom 25% of normalized RNA-seq reads for phase I and phase II metabolizing enzymes were reported for several 1st and 2nd trimester fetal liver specimen in humans, listed in Table 4.2. Detectable protein levels of CYP2C9, CYP2C19, CYP3A4, CYP3A5, and CYP3A7 were previously reported in 1st-2nd trimester human liver (Hines, 2007). Specific isoforms for other biotransformation proteins, such as alcohol dehydrogenase (ADH1A), epoxide hydrolase (EPHX1, EPHX2), glutathione s-transferase (GSTA1), and sulfotransferase (SULT1A1) have also been reported in the early fetal liver (Duanmu, et al., 2006; Omiecinski, Aicher, & Swenson, 1994; Smith, Hopkinson, & Harris, 1971; Strange et al., 1989). Activity of phase I and II enzymes vary between gene families and isoforms with

relative abundance drastically altering over time until adult levels are reached (Hines & McCarver, 2002; McCarver & Hines, 2002). Interestingly, a majority of the BPA related candidate genes that were identified exhibited relatively low RNA-seq reads as compared to other phase I and phase II metabolism genes. Thus, the negative association between BPA and specific underdeveloped XME genes may be indicative of repressive effects or delayed maturation of the candidate XME genes to adult levels, potentially altering the response to toxicants later in life.

BPA is commonly known to interact with nuclear receptors such as estrogen receptor (ERα and β), estrogen related receptor gamma (ERRy), pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR), androgen receptor, and thyroid receptor (Molina-Molina et al., 2013; Wetherill et al., 2007). More recently, studies suggest that endocrine active compounds (EACs) induce rapid responses via non-genomic signal transduction pathways, and disease susceptibility may depend on the coordination between genomic and non-genomic pathways (Watson, Jeng, & Guptarak, 2011). In this study, the co-repression of candidate genes warranted a closer look at common regulators in order to provide insight into novel mechanism of action for BPA that are relevant to the XME pathway. In our bioinformatic investigation of regulatory sites, we identified binding sites for the highly conserved ETS and AP1 related transcription factors. Extracellular signals affecting the MAPK and JNK signaling pathways often converge both upstream and downstream at ETS proteins, which consist of over 25 family members that can heterodimerize with other transcription factor families, such as the Fos and Jun domains of AP-1 related proteins (Bassuk & Leiden, 1995; Kyriakis & Avruch, 2001; Yordy & Muise-Helmericks, 2000). Combinatorial control of specific ETS and

AP1 factors, along with other regulators, is likely to influence transcriptional XME specificity during early fetal development, a period in which estriol (E3) predominates and activates non-genomic processes (Watson, et al., 2011). While evidence for estrogen receptor independent mechanisms for BPA is increasing, only a few studies thus far have investigated BPA's influence on ETS and AP1 factor targets. BPA has been reported to stimulate the PU.1 transcription factor, a relative of the SP1A, SP1B, and SP1C regulators, in promyelocytic cells for neutrophilic differentiation (Watanabe et al., 2003). In human embryonic kidney cells, BPA at μM concentrations activated Nrf1 and Nrf2 transactivation activity and induced antioxidant response element (ARE) target genes (Chepelev et al., 2013). Subsequent studies are necessary to investigate specific ETS and AP1 transcription factors and their genomic localization using methods such as chromatin immunoprecipitation coupled with sequencing (CHIP-seq).

Cis-acting regulation at ETS and AP1 related transcription factor binding sites and promoter occupancy, in addition to post-transcriptional modifications, may alter environmental response via signal transduction. In particular, DNA methylation at CpG sites along the consensus sequence motifs, especially for ETS, can inhibit transcription factor binding and transcriptional activity (Hollenhorst, McIntosh, & Graves, 2011). In our *in silico* analysis, ETS proteins were prevalent in the candidate gene promoters and many of the ETS consensus sequences contained at least one CpG site. When Hogart et al. characterized epigenome-wide patterns across several different primary murine cells, ETS consensus sequences were overrepresented within methylated regions that were abundant especially in hematopoietic stem cells (Hogart et al., 2012). By overlapping ETS binding sites with variable regions of methylation from M-NGS data in our study,

we used a comprehensive approach to designing epigenetic biomarkers at biologically relevant targets in humans. In particular, we wanted to examine whether decreased expression along with increased tissue BPA concentrations were associated with increased promoter CpG methylation at ETS binding sequences. Both site-specific methylation at the *COMT* promoter and average CpG methylation at the *SULT2A1* promoter significantly increased with higher BPA concentrations, although the increase in DNA methylation was relatively modest. The less than 1% increase in promoter methylation at the ETS sites of differentially expressed genes suggests that additional regulatory elements, including other epigenetic mechanisms, influence expression. For example, the environmentally sensitive MAPK signaling proteins can associate with chromatin modifying complexes and directly regulate histone modifications (Suganuma & Workman, 2012). Future work will require further integration of signal transduction pathways with various epigenetic mechanisms, including histone modifications and RNA interference, in response to environmental stress at critical windows of development.

Traditionally toxicological studies employed enzymatic activity assays to understand xenobiotic metabolism *in vitro* or in animal models; however, analyzing protein activity in the developing fetal liver is far more difficult when access is limited to small amounts of sensitive biobanked specimens. With the improvement of real-time PCR and utilization of next generation sequencing, hundreds of genes can be reliably profiled simultaneously using small mRNA sample volume and compared to protein activity. In general, mRNA expression is a good proxy for protein level, but gene expression does not always correlate to protein abundance. This discrepancy can be attributed to differences in the rate of protein or mRNA synthesis and turn over, or post-

transcriptional regulation (C. Vogel & Marcotte, 2012). Hence, examination of target protein activity and post-transcriptional modification of regulatory elements in suitable samples will be necessary in future studies for complete characterization of fetal xenobiotic biotransformation. Still, in the case that healthy specimens are available, longitudinal analysis of the development of the human XME pathway is challenging given the bioethical concerns for fetal tissue, especially in 3rd trimester. Despite these limitations, gene expression profiling, even at a narrow window of development, can be helpful for exploring early genomic regulation that occur with a changing environment.

While the task of investigating BPA's influence on XMEs may be clearer in animal models in which investigators exercise control over experimental design and model choice, here we utilize human fetal liver samples from an NIH-funded biobank. These samples are derived from individuals who may have been exposed to a wide array of compounds and maternal factors. Thus, a variety of unmeasured but related factors, such as co-exposure to other EACs, may confound our results.

In the presence of endogenous hormones and growth factors, phase I and phase II XME gene expression and enzyme function matures until adult baseline levels are attained, either after birth, during childhood, or in adolescence. Therefore, exposure to environmental compounds, especially during early life when the capacity for xenobiotic metabolism is suboptimal, may increase susceptibility to disease in the developing fetus and children (Allegaert et al., 2008). When XME gene expression was compared in fetal liver exposed *in utero* to physiologically low and high BPA concentrations, subtle decreases in specific phase I and II XME genes were observed. Although BPA may not immediately dysregulate essential proteins or directly cause disease, the EAC may stunt

XME maturation via signaling pathways and epigenetic mechanisms. Considering the daily and cumulative exposure to a myriad of compounds in humans, the inability to adequately respond to these insults suggests potential consequences for health later in life.

Table 4.1 Sequenom Epityper Primer Assays for DNA Methylation

Gene Name	GenBank Number	Strand	Primers (5'- 3")	Tm (°C)	Amplicon Size	Amplicon Location*
Carboxylesterase 2 (*CES2*)	NM_003869	Forward[a]	aggaagagagTTTTGGTTTTGTATATTTGGTGAGA	58.93	310	-1900bp to -1590bp
		Reverse[b]	cagtaatacgactcactatagggagaaggctATTCAAACCCTAATTATCTCCCTCC	61.01		
Catechol o-methyltransferase (*COMT*)†	NM_000754	Forward[a]	aggaagagagTTTTAGTTTTTTTATTTGGGAAGGG	60.00	343	-245bp to +98bp
		Reverse[b]	cagtaatacgactcactatagggagaaggctAACAACCCTAACTACCCCAAAAAC	60.70		
Sulfotransferase 2A1 (*SULT2A1*)	NM_003167	Forward[a]	aggaagagagAGGTTGTTTTGTATAGATGTGGGTTAT	59.48	294	-1953bp to -1659bp
		Reverse[b]	cagtaatacgactcactatagggagaaggctTCCCAACTACTCAAAAAACTAAAACA	59.56		

*Surrounding transcription start site (TSS) where (-) represents upstream and (+) represents downstream of TSS
[a]All forward methylation primers contain a 10mer tag on the 5' end, represented in lower case letters
[b]All reverse methylation primers include a T7 promoter tag on the 5' end, represented in lower case letters
†Methylation designed for the MB-COMT (membrane bound) promoter

Table 4.2 Differentially Expressed Phase I and II XME Genes by BPA Exposure

Xenobiotic Metabolism Enzyme	Symbol	Description	Fold Change*	Adjusted p-value
Phase I	*CES2*	Carboxylesterase	0.186	0.0495
Phase I	*CES5A*		0.124	0.0495
Phase II	*COMT*	Catechol o-methyltransferase	0.245	0.0251
Phase II	*GSTA5*	Glutathione s-transferase	0.140	0.0495
Phase II	*SULT2A1*	Sulfotransferase	0.248	0.0251
Phase II	*SULT1B1*		0.159	0.0261
Phase II	*SULT6B1*		0.054	0.0261
Phase II	*SULT1C3*		0.185	0.0261
Phase II	*UGT1A3*	UDP-glucuronosyltransferase	0.122	0.0343
Phase II	*UGT1A4*		0.121	0.0284
Phase II	*UGT1A6*		0.249	0.0373
Phase II	*UGT1A8*		0.048	0.0251
Phase II	*UGT1A9*		0.094	0.0449
Phase II	*UGT1A10*		0.187	0.0261

*Fold Change <0.5 is down regulation; Fold Change >2 is up regulation in high BPA exposure group compared to low BPA exposure group

Table 4.3 Bottom and Top Quartile Expression (FPKM Reads from RNA-seq) of Phase I and Phase II XME Genes in N=12 Human Fetal Liver

Biotransformation Pathway	Gene Family Names	Low Expression (bottom 25%)	High Expression (top 25%)
Phase I	Alcohol dehydrogenase	*ADH7*	*ADH1A, ADH6, ADH5*
	Aldehyde dehydrogenase	*ALDH3B2, ALD1A3*	*ALDH1A1, ALDH2, ALDH4A1*
	Hydroxysteroid (17β) dehydrogenase		*HSD17B10*
	Carboxylesterase	***CES5A,*** *CES3*	
	Cytochrome P450	*CYP4F8, CYP26C1, CYP11B2*	*CYP3A7, CYP27A1, CYP19A1*
	Epoxide hydrolase		*EPHX1, EPHX2*
	Oxygenase	*FMO2, PTGS2*	*FMO5, MAOB*
Phase II	Acyl-CoA synthetase	*ACSM1*	*ACSL4, ACSL1*
	Glutathione s-transferase	***GSTA5,*** *PTGES*	*GSTA1, GSTP1, GSTO1, MGST3, MGST1, MGST2*
	Methyltransferase	*NNMT*	*GAMT, HNMT*
	N-acetyltransferase	*AANAT, NAT2*	
	Sulfotransferase	***SULT6B1, SULT1B1, SULT1C3***	*SULT1A1*
	Thiosulfate sulfurtransferase		*TST*
	UDP-glucuronosyltransferase	*UGT2A1, UGT2B17,* ***UGT1A1-10***	*UGT2B10, UGT2B4*

Phase I XME genes with FPKM reads <0.1 and FPKM reads >9 represent the bottom and top quartile of XME expression. For phase II XME, the genes at the bottom and top quartile of XME expression exhibit FPKM reads <0.2 and FPKM reads >12, respectively. Genes in bold are BPA associated candidate XMEs identified from PCR Arrays. While these genes show low expression, the remaining candidate genes (*CES2, COMT,* and *SULT2A1*) show relatively moderate expression or FPKM reads.

Table 4.4 Consensus Sequences of Common Transcription Factor Binding Sites (TFBS) at Candidate XME Gene Promoters

TFBS Family	Transcription Factor	Genomatix IUPAC Sequence
AP1 (AP1 and MAF related factors)	BACH1	nsa**TGAGtca**tgny
	BACH2	nr**TG**A**Gtca**nn
	MAFA	**TGCWg**mnynngcn
	MAFB	naawn**tgCTGAc**nwarn
	MAFK	nw**a**aaw**TGCTgac**tn
	NFE2	h**gCTGAgtca**y
	NFE2L1 (NRF1)	vn**cGCGC**ab**gcgc**vnv
	NFE2L2 (NRF2)	nmc**CGGAagtg**ac
	NRL	nnc**TGCTga**sn
ETS (Human and murine ETS1 factors)	EHF	nna**cCGGAagt**nn
	ELF1	nnc**cGGAAg**ygnn
	ELF2	nnrnca**GGAAg**nr
	ELF3	naancc**GGAA**rtwnn
	ELF4	nawm**ccGGAAgt**nn
	ELF5	**a**nsm**GGAAgt**wn
	ELK1	nrcc**GGAA**rynn
	ELK3	nna**cCGGAa**gynn
	ELK4	nna**cCGGAa**rynn
	ETS1	vsm**GGAAg**ygn
	ETS2	da**cAGGAa**ryvnk**t**
	ETV1	nnc**aGGAAg**nn
	ETV3	nnac**CGGAa**rtnn
	ETV4	nna**cCGGA**wrtnn
	ETV5	nna**cCGGA**w**gt**tn
	ETV6	anc**cGGAAgt**ann
	GABPA	nna**cCGGAagt**nn
	GABPB1	nmc**CGGAagtg**ac
	SPDEF	nnat**cCGGA**tgynn
	SPI1	ngn**GGAA**stn
	SPIB	na**a**wgm**GGAA**gtn
	SPIC	naaagm**GGAAgt**wn

The table displays individual transcription factors and their consensus sequence motifs for each TFBS family. The sequence motifs follow the standards set by the International Union of Pure and Applied Chemistry (IUPAC), with highly conserved nucleotides in bold. Upper case letters (A, C, G, T) show the most frequent nucleotides occurring at greater than 50% frequency. The degenerate code (R, Y, K, M, S, W) represents two nucleotides that occur at >75% frequency, but individually contribute to <50% frequency. All other frequency distributions are represented by the letter "n". In particular, several motifs belonging to the ETS family contain cytosine-guanine dinucleotides within their highly conserved sequence.

Table 4.5 Mixed Effects and Linear Regression Models Comparing Average or Individual CpG (within ETS binding sites) Methylation with BPA Concentration

Methylation Assay		N	Change in % Methylation for an IQR Change in Tissue Total BPA (ng/g)	p-value
CES2				
	Average (4 total CpG; 4 CpG units)[a]	50	-0.008	0.953
	CpG #3[b]	50	-0.114	0.409
COMT				
	Average (30 total CpG; 17 CpG units)[a]	50	0.080	0.108
	CpG #30[b]	50	-0.010	0.851
	CpG #31[b]	50	0.416	0.002
SULT2A1				
	Average (6 total CpG; 4 CpG units)[a]	40	0.821	0.042

Inter-quartile Range (IQR) = 10.93 ng/g for *CES2* and *COMT*, and 8.28 ng/g for *SULT2A1*;
[a] Mixed-effects model to account for multiple adjacent CpG sites, adjusted for gestation age and sex;
[b] Linear regression at biologically relevant CpG sites, adjusted for gestational age and sex

Figure 4.1 Diagram Displaying Common Transcription Factor Binding Sites (TFBS) on Candidate XME Gene Promoters

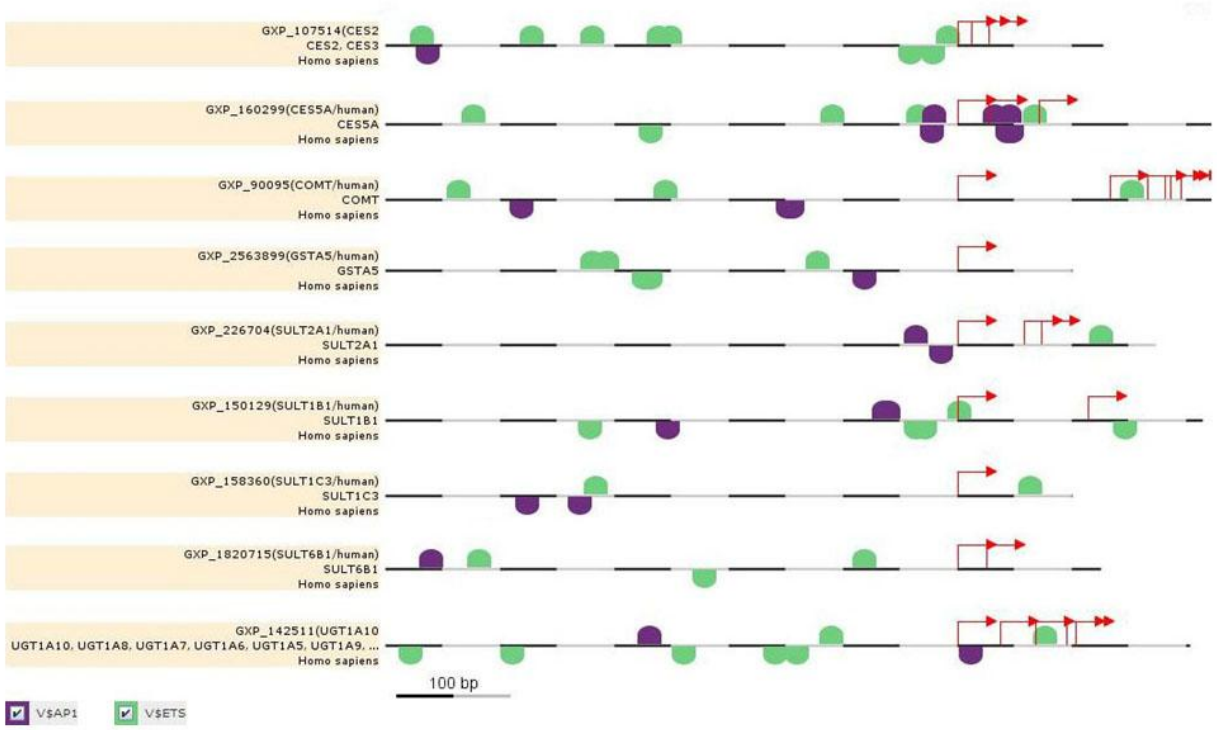


Diagram displaying conserved consensus sequence binding sites for AP1 (purple) and ETS (green) related factors. The two groups represent transcription factor families at the promoters of BPA-associated candidate genes *CES2*, *CES5A*, *COMT*, *GSTA5*, *SULT2A1*, *SULT1B1*, *SULT1C3*, *SULT6B1*, and *UGT1A1-10* retrieved from MatInspector. The view includes 500 bp upstream and 100 bp downstream of the TSS. Although transcription factor binding sites were identified for multiple transcripts per gene, only one promoter transcript is displayed above

Figure 4.2 Methylation Profile of *CES2*

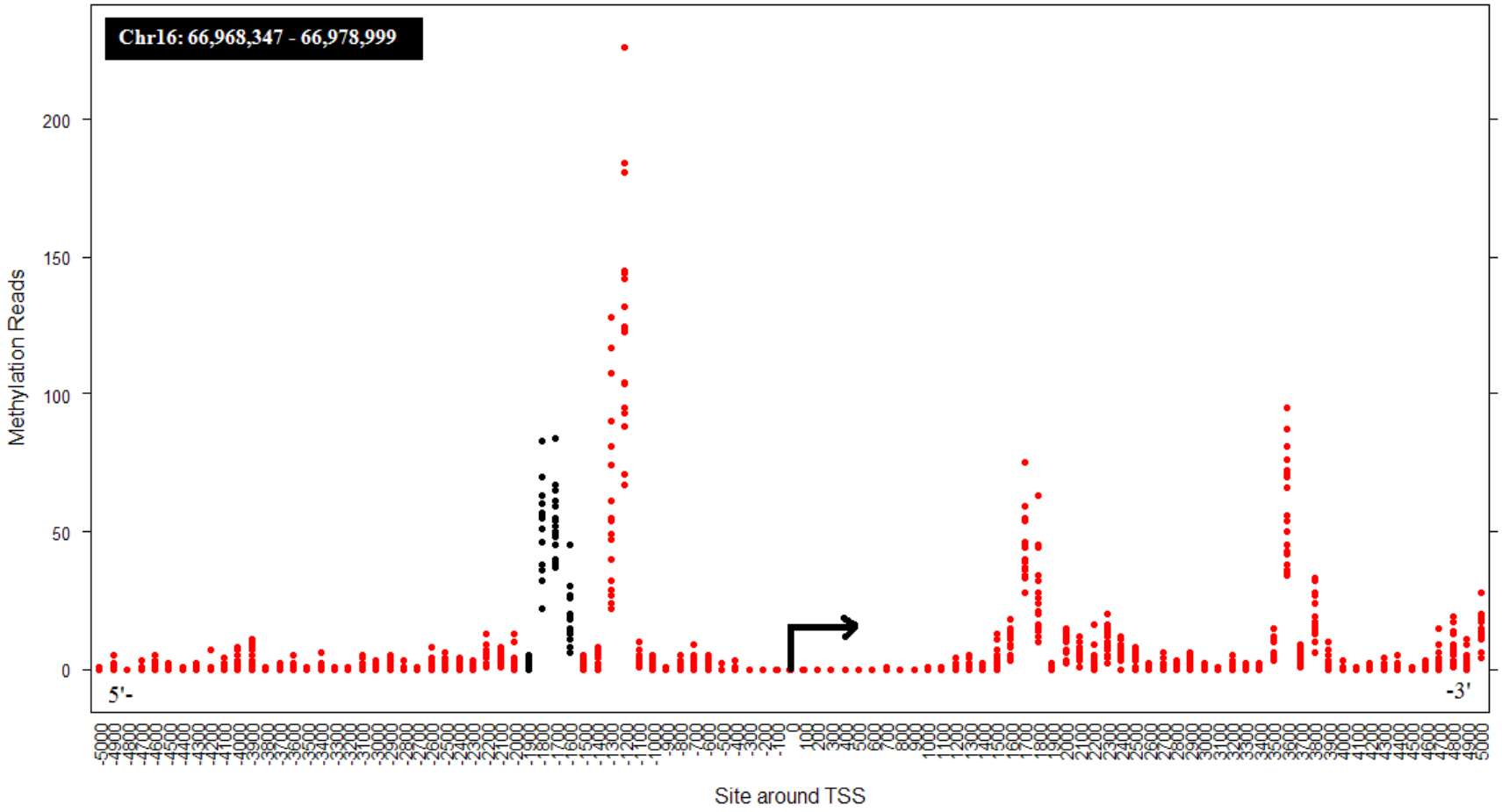


Figure 4.3 Methylation Profile of *COMT*

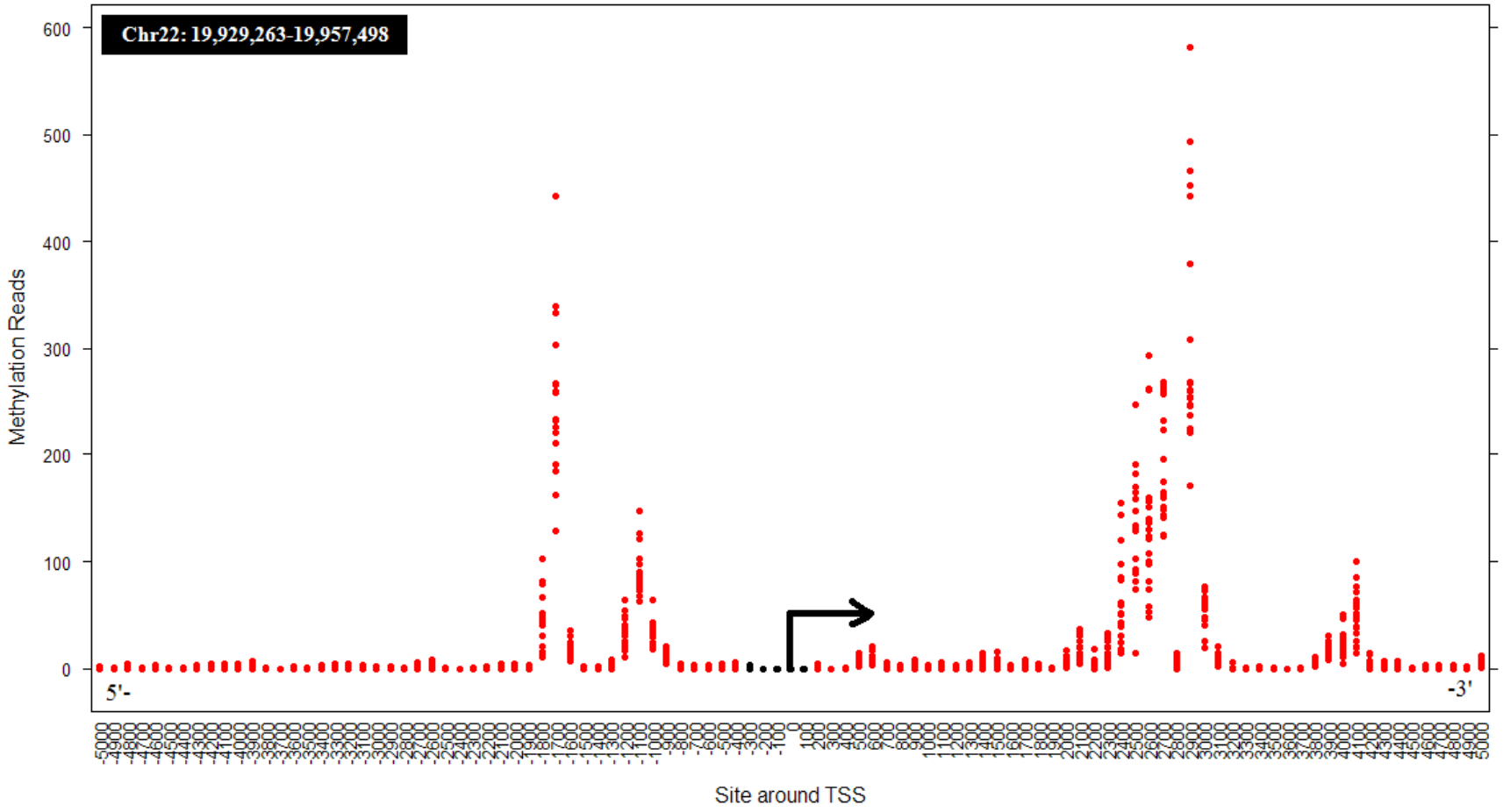


Figure 4.4 Methylation Profile of *SULT2A1*

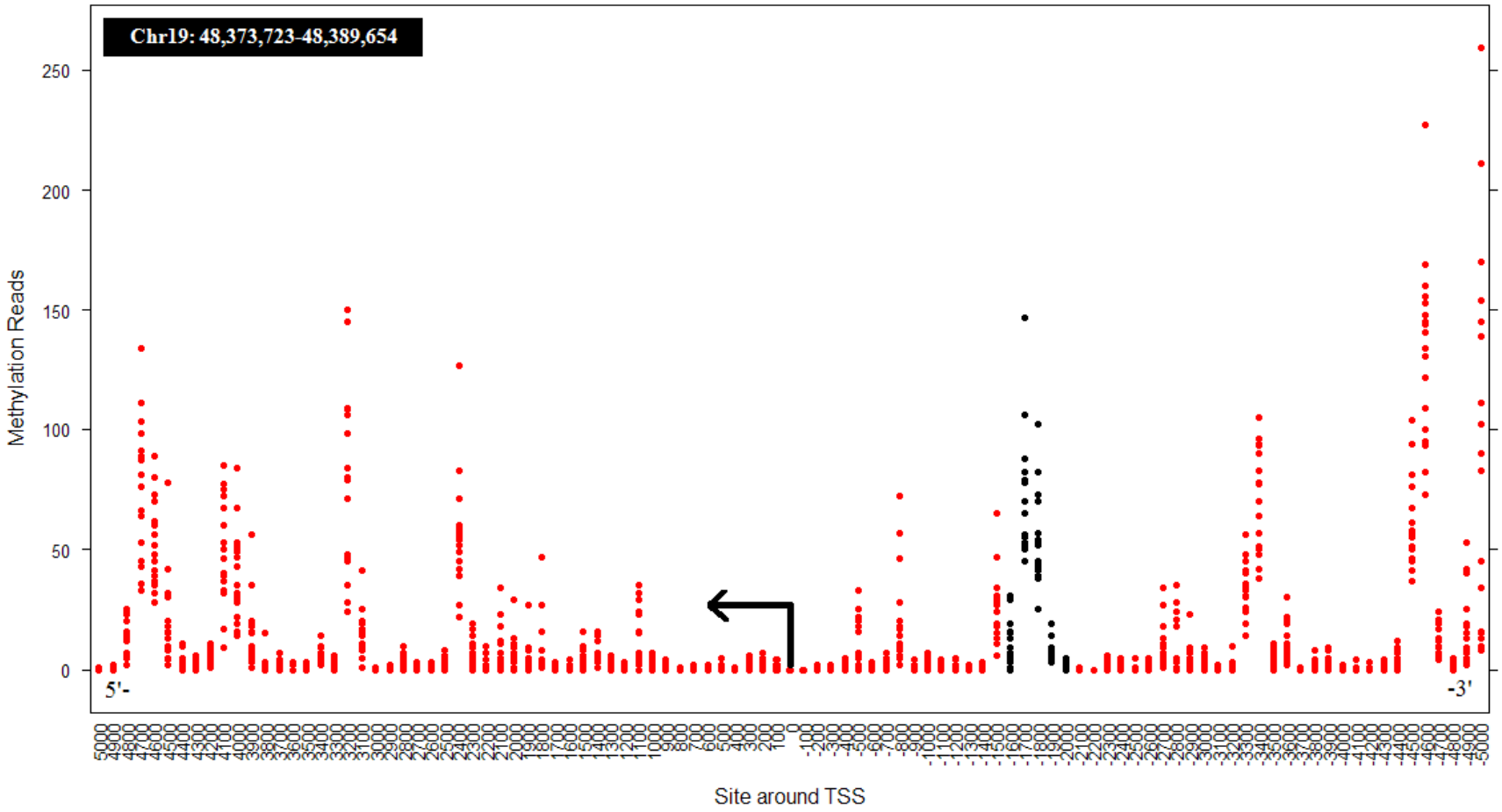
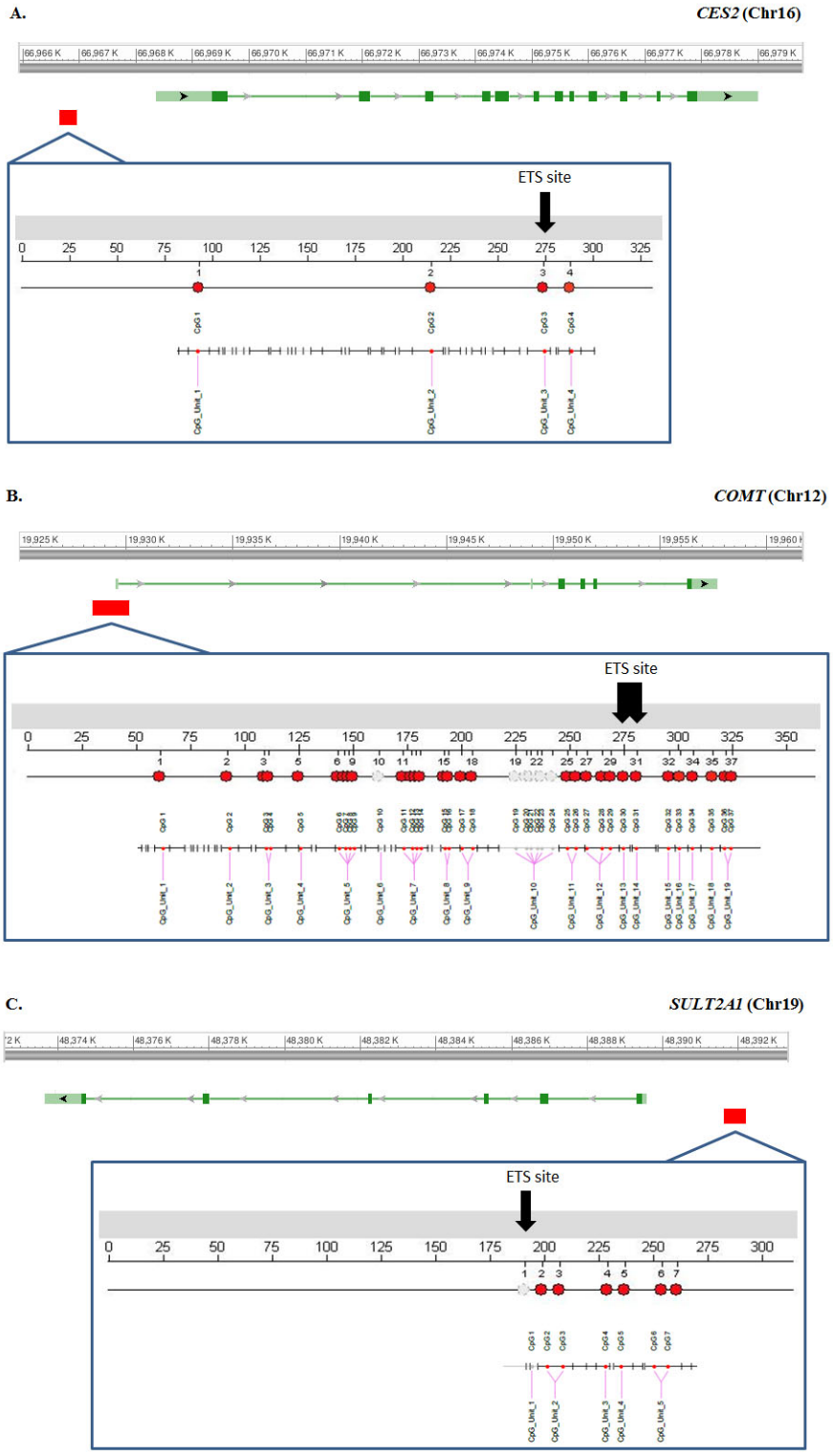


Figure 4.2-4.4. Dot plots representing methylation profiles 5000 bp upstream and downstream of the TSS. Unadjusted methylation reads were graphed at every 100 bp fragment with individual dots representing N=18 fetal liver specimens. Regions highlighted in black were chosen for subsequent Sequenom methylation analysis. (**A**) The *CES2* gene has three distinct regions with variable methylation including one peak at -1300 to -1800 bp upstream, and two peaks at 1700 to 1800 bp and 3600 to 3800bp downstream of TSS. An ETS site coincides within the variable region upstream, highlighted in black. (**B)** The *COMT* gene also has three distinct regions of variable methylation: two peaks at -1200 to -1000bp and -1700 to -1800bp, and another at 2400 to 2900bp from TSS. While methylation variability was low, the region from -300 to 100bp around TSS was selected and highlighted in black because it overlapped with ETS binding site and was previously assayed successfully for epigenetic biomarker (Q. Xu, et al., 2010). **(C)** The *SULT2A1* gene on the antisense strand exhibits variable methylation activity upstream and downstream of the TSS. The variable region at -1600 to -1900 bp is highlighted because it coincides with an ETS binding factor site.

Figure 4.5 Graphs Displaying Sequenom EpiTYPER Assays with Transcription Factor Binding Sites



(**A)** The *CES2* assay examines 4 individual CpG sites located approximately 1600-1900 bp upstream of the TSS. The *CES2* CpG site #3 is found within an ETS transcription factor-binding site. (**B)** The *COMT* assay covers 30 CpGs sites, although due to technical limitations (e.g. small fragment sizes are not detectable) Sequenom EpiTYPER outputs only 17 CpG units, spanning approximately 250 bp upstream and 100 bp downstream of the TSS. An ETS transcription factor binding site coincides with CpG sites #30 and #31 on *COMT*. (**C)** Although CpG methylation on the ETS consensus sequence of *SULT2A1*could not be directly assessed (CpG site #1), the assay investigates 6 individual CpG sites (4 CpG units) surrounding the target site, spanning approximately 1650-1950 bp upstream of the TSS.

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CHAPTER 5
Discussion

5.1 Review of Dissertation Findings

The widespread apprehension over BPA and its health effects in our society today is primarily fueled by toxicology studies using animal and *in vitro* model systems, but the translatability of the findings to relevant human health risk continues to be a topic of extensive debate. The overall goal of this dissertation was to address BPA exposure-outcome associations directly in humans, especially pertaining to the vulnerable fetus. Here, we utilized human clinical tissues obtained from elective pregnancy terminations to quantify *in utero* BPA exposure, understand fetal capacity for metabolism, and identify subtle exposure-dependent changes to gene expression and regulation important for biotransformation.

In chapter 2, we identified a wide distribution of BPA concentrations in the ng/g or parts-per-billion range within fetal liver specimens that changed with gestational age, indicating substantial yet variable fetal exposure during pregnancy. Metabolic potential for eliminating BPA in fetal liver differed from adult livers. Overall, we observed reduced hepatic expression of the BPA-specific metabolizing enzymes: *UGT2B15*, *SULT1A1*, and *STS*. When we explored differences in BPA toxicokinetics and toxicodynamics across fetal liver, placenta, and fetal kidney in chapter 3, we found

significant tissue-dependent differences in BPA concentrations, BPA-related metabolism gene expression, as well as global DNA methylation. Given the greater likelihood of BPA in fetal liver compared to matched tissue, we next examined exposure-dependent changes in gene expression and regulation of the biotransformation pathway only in the liver. In chapter 4, we identified over a dozen candidate XME genes that were down regulated with higher tissue BPA concentrations, all of which had common AP1 and ETS related transcription factor binding sites in the promoters. In a subset of these candidate genes, we identified increased methylation at biologically relevant promoter sites with increased BPA concentrations.

In general, findings from the entire dissertation can be compiled into two distinct sections: toxicokinetics (Figure 5.1) and toxicodynamics (Figure 5.2). Instead of employing classical toxicokinetic methods such as enzyme kinetics, we characterized tissue BPA concentrations and quantified gene expression of XMEs most relevant for BPA metabolism in the first section. Our results are of interest because they indicate that the 1^{st}-2^{nd} trimester human fetus is exposed to detectable concentrations of BPA *in utero*. Compared to adult liver tissue, BPA metabolizing enzymes are expressed at suboptimal levels in the fetal liver, which may explain the high free to conjugated ratio. Furthermore, greater expression of *SULT1A1* in the sulfation reactions and *GUSB* in the glucuronidation reactions suggests that BPA-sulfate rather than BPA-glucuronide is the major conjugate in the fetus. In the latter half of the dissertation, we explore cellular responses associated with physiologically relevant concentrations of BPA, focusing on cis-acting regulatory markers and the genome. While BPA concentrations and global methylation vary across tissue, the placental epigenome was most sensitive to exposure.

Focusing on pathway specific effects, BPA associated changes were noted for XME gene expression and methylation in fetal liver. These findings indicate that environmentally relevant concentrations of BPA, even across a short gestational window in the fetus, can cause detectable alterations in biotransformation programming.

5.2 Significance

Over the past several years, regulatory agencies have determined that BPA may be potentially harmful for human development, especially for pregnant women, the developing fetus, and young children. While evidence for environmental exposure to humans is well established, studies that address how much BPA is reaching target sites and subsequently altering structure and function in these vulnerable populations are lacking. This dissertation project is noteworthy in that it is one of the first studies to reveal significant internal dose or exposure to tissue in developing humans and identify subtle changes in expression and regulation at target sites within the human fetus. In particular, BPA's impact on the biotransformation pathway is important because it may contribute to inter-individual variations in succeeding drug and environmental toxicant response. Pharmacological studies have already considered nonlinear growth and maturation processes, genetic differences, and sex as important factors for shaping drug efficacy and toxicity, which is also relevant to environmental exposures (McCarver, 2004). Results from this dissertation add to the complexity, demonstrating that early BPA exposure can alter metabolism and response to other nutrients, hormones, and xenobiotics that can influence proper development.

Furthermore, BPA's relationship with XME signal transduction and epigenetic mechanisms has implications for xenobiotic response adaptations and susceptibility to diseases linked to toxicity, thus supporting the DOHaD hypothesis. Traditionally, pharmacogenomic studies have provided much of the evidence for the association between altered biotransformation capacity and adverse health outcomes, but the focus is shifting to prevalent environmental exposures. For example, studies now show that *CYP1A1*, *GSTM1*, and *GSTT1* polymorphisms can alter polycyclic aromatic hydrocarbon metabolism and contribute to inflammatory processes that predispose individuals to atherosclerosis (Marinkovic, Pasalic, & Potocki, 2013). In the fetus, when xenobiotic metabolizing enzymes and transporters are not fully developed, genetic differences are less likely to explain variability in xenobiotic biotransformation. Thus, interest in epigenetic regulation of absorption, distribution, metabolism, and excretion is emerging (Klaassen, et al., 2011; Zhong & Leeder, 2013). The dissertation findings show preliminary evidence for environmental exposures and their influence on biotransformation programming in early development. Furthermore, this research fills an important knowledge gap in early life epigenetics given that investigation of global, epigenome-wide, and gene-specific methylation patterns in the 1^{st} and 2^{nd} trimester human fetus is limited. In the future, studies that combine epigenetics with developmental toxicology or pharmacology will be important for the improvement of environmental risk assessment and interventions to decrease disease susceptibility.

5.3 Study Strengths and Limitations

The use of state-of-the-art techniques for BPA quantification and epigenetic analysis is a major strength of this dissertation. Advancements in analytical chemistry over the past decade has allowed for better biomonitoring of environmental compounds with increased sensitivity and precision. While a variety of methods and matrices are utilized for BPA detection, the CDC has optimized on-line solid-phase extraction coupled to HPLC-isotope dilution tandem mass spectrometry for biomonitoring. With the limit of detection decreasing from 0.4 to 0.1 ng/mL over the years, this analytical method is recommended as a gold standard for urinary BPA measurements (L. N. Vandenberg et al., 2012b; Zhou, Kramer, Calafat, & Ye, 2014). The HPLC coupled ESI-MS/MS method used for this project, however, is one of the most sensitive techniques optimized for tissue BPA biomonitoring with LOQs at 0.1 and 0.05 ng/g. Furthermore, we used the universal gene expression procedure, RT-qPCR, and novel next generation sequencing based methods to identify candidate genes and altered methylation patterns within our clinical samples.

Using proper extraction methodology, careful experimental design, and validation, fetal samples were analyzed for biomarkers of exposure to inform environmental health. Considering the sensitive nature of fetal biospecimens, our study included a higher sample size for chemical analysis in fetal liver (N=50) and across matched tissue (N=12), whereas analogous studies investigated BPA biomonitoring in only N=28 Canadian fetal livers and N=11 matched adult postmortem tissues (Cao, et al., 2012; Geens, et al., 2012). Furthermore, the dissertation aims attempt to address many questions that arise in the debate over BPA's risk to humans with one particular

uncertainty being the role of deconjugating enzymes during BPA metabolism (Ginsberg & Rice, 2009). Here, we analyze gene expression of both conjugating and deconjugating enzymes to explain BPA speciation, a discreet but pertinent distinction for understanding metabolism and risk.

Another unique aspect to this dissertation research is the use of human fetal clinical specimen for BPA biomonitoring and toxicodynamics. Once human tissues are available with proper patient consent, clinical samples can be ideal matrices for identifying organ specific concentrations and physiological modifications relevant for humans. In fact, they eliminate the need for costly animal models that are often inappropriate for extrapolation to human. In the BPA biomonitoring field, however, some scientists are skeptical about the use of biobanked specimens. Even with the application of proper quality controls to prevent contamination during chemical analysis, there is room for uncertainty given limited patient data and information about tissue extraction processes. Reports suggest that free BPA contamination can occur from breakdown of surgical equipment and plastic lab ware, deconjugation of tissue BPA during storage, and *ex vivo* contributions from reagents like water (Dekant & Volkel, 2008; L. N. Vandenberg, et al., 2012b). Interestingly, the study design and results from chapter 3 suggest otherwise, demonstrating that BPA contamination is negligible or absent within our clinical cohort. Among individual subjects, matched tissues that have the same environmental and genetic characteristics are extracted at similar times and under similar conditions before storage. Thus, BPA contamination during surgical extraction should be equally distributed across matching tissues. Yet, 3 out of 12 subjects exhibited undetectable concentrations of free BPA in one matched tissue, indicating that the

measurements in the remaining tissues are reflective of endogenous levels rather than contamination. Any potential contamination from tissue processing may lead to only a small overestimation of free BPA measurements, but detection of conjugated BPA alone is quite worrisome as it suggests substantial *in utero* BPA exposure in the human fetus.

While we were fortunate to have access to valuable fetal specimen, there were several drawbacks to our study design using this clinical cohort. Due to the bioethical nature of the tissues, addressing our primary research goals over a wider gestational window including 3rd trimester could not be feasible. Although rare, 3rd trimester fetal tissues exclusively come from patients with morbidities and pregnancy complications, which could potentially confound any BPA exposure-outcome associations. Additionally, unlike other biomonitoring studies that only report free BPA or total BPA concentrations, separate analysis of free BPA and BPA conjugate concentrations consumed most of the tissue reserves. Therefore, subsequent biomolecular testing was limited to genetic analyses that require only a small sample volume. A major limitation of this project was the failure to account for cell specificity in expression and methylation. The cellular makeup of fetal organs can quickly change over a short period of time, especially by the end of 1st trimester, as each cell population maintains a distinct expression and epigenetic trajectory (Gellen, 1976; Isagawa et al., 2011). For example, the fetal liver is composed of a heterogeneous mixture of hepatocytes and hematopoietic cells, with cell composition changing given the decrease in hematopoietic potential over time (Brunner et al., 2009). In this dissertation, we explored epigenetic and genetic analysis across homogenized tissues by the end of human organogenesis without considering cell composition or tissue location mainly because of cost and sample volume. Furthermore, many of the flash

frozen tissues characterized in this study are highly perfused with blood during prenatal development, so there is some uncertainty as to whether measurements are true tissue concentrations or include blood concentrations. Separation of blood and heterogeneous cells can be problematic, introducing exogenous BPA and altering structure and function. Nevertheless, technological advancements in cell sorting processes and cell-specific molecular analysis are ongoing, but are not yet cost effective for everyday use on precious specimen and unrealistic for samples with small volume.

5.4 Future Directions

The results and shortcomings from this dissertation bring to light new questions that must be tackled for future BPA research. Separating free BPA and BPA conjugates is just the first step, but biomonitoring projects must consider measuring different BPA conjugate species. Traditionally scientists have viewed BPA-glucuronide as the primary conjugate in adults, but new evidence suggests that BPA-sulfate conjugates may predominate in the fetus. In this study, we noted higher fetal liver *SULT1A1* compared to *UGT2B15* and *STS* expression, and a new study reported BPA-sulfate concentrations to exceed BPA-glucuronide and free BPA concentrations in midgestational umbilical cord serum (Gerona, et al., 2013). Ideally, we would measure both conjugates to corroborate our gene expression results across multiple fetal organs. Our collaborators at Wadsworth and other analytical chemistry labs are currently optimizing cost-effective techniques that simultaneously measure multiple BPA metabolites as well as BPA-like compounds. For example, the CDC has begun to analyze BPA and 11 other similar phenols together in

urine (Zhou, et al., 2014), but similar chemical analyses will take longer to optimize in solid tissue matrices given the varying fat content. In the natural environment, people are exposed to several types of environmental contaminant. It is likely that these fetal specimen also exhibit detectable concentrations of phthalates and other phenols, with biological programming affected by co-exposure interactions. To investigate toxicant interactions and co-exposure toxicities in future environmental health research, improvements in sample processing and analytical techniques will be necessary.

As mentioned previously, exposure-outcome associations require examination in a cell-specific and time-specific manner. Accounting for cell specificity using these sensitive clinical specimens will be difficult with the current technology, but other biological models are available. Studies using human cell lines derived from fetal tissue may be beneficial for determining BPA toxicity. Thus far, BPA is found to induce preterm associated corticotrophin-releasing hormones in placental JEG3 choriocarcinoma cells, and promote proliferation of human mammary epithelial cells from embryonic stem cells (Huang, Tan, Wang, & Leung, 2012; L. Yang et al., 2013). Unfortunately, primary human fetal cell lines are scarce, and methods for cell separation or isolation from fresh tissue can be tedious and low yielding. While these human cell lines may provide greater flexibility for BPA toxicity assays, they fail to emphasize *in utero* exposures and cell-cell interactions. Understanding BPA toxicity in the context of human organ ontogeny or development is far more complicated given bioethical challenges as previously noted. Comparing BPA concentrations with physiological changes over time in humans is limited to cross-sectional rather than longitudinal study designs. In a recent review evaluating metabolism and transport ontogeny across species, changes in

biotransformation were reported through mRNA and protein expression with developmental data in the human fetus grossly underrepresented (Moscovitz & Aleksunes, 2013). Thus, forthcoming research in toxicology will require new study samples and methodologies to address developmental biotransformation or ontogeny in humans.

For subsequent research projects, our clinical specimens can still be valuable for addressing BPA accumulation, biotransformation regulation, and toxicological endpoints. In this dissertation, we measured BPA species across placenta, fetal liver, and fetal kidney because they have important functions in xenobiotic metabolism. Other fetal organs are also available in the lab and may be interesting to check for BPA accumulation. Since studies suggest that BPA can influence neuroendocrine and reproductive structure and function (Cantonwine, Hauser, & Meeker, 2013; Frye et al., 2012), measuring BPA and metabolic potential in additional tissues like the fetal brain and fetal gonads may be informative. Comparison of various organs can help determine whether expendable tissues (i.e. blood, placenta) can act as a reliable substitute for target tissues that are hard to access (i.e. liver, brain, etc.). Furthermore, multi-organ comparison of BPA concentration within a handful of fetal subjects can support BPA physiologically based pharmacokinetic (PBPK) models for estimating tissue deposition at other gestational ages. In this dissertation, we examined the role of gestational age only for BPA concentrations in fetal livers and adjusted for age in many of our statistical models. We could characterize time-dependent exposure, expression, and epigenetic changes for each tissue type by increasing sample size and selecting samples across a wider gestational window.

The divergence in BPA concentrations across matched fetal tissues brings to question tissue-specific differences in factors other than phase I and II enzymes, including transporters and cofactor concentrations. In general, two large classes of transporters, the solute carrier (Slc) and ATP-binding cassette (Abc), are known for substance influx and efflux across different organs (Konig, Muller, & Fromm, 2013). Smaller uncharged molecules are more likely to favor passive diffusion, while bulky molecules like BPA-glucuronide require active transport. Thus far, studies report conflicting evidence for the P-glycoprotein (MDR) efflux transporter and its ability to bind free BPA in various cell lines (Jin & Audus, 2005; Yoshikawa et al., 2002). A recent study demonstrated that free BPA stimulated the MDR1, MRP2, and BCRP human efflux transporters and BPA-glucuronide stimulated MRP3 using ATPase activity assays (Mazur et al., 2012). Similar to phase I and II XMEs, expression of transporters likely differ throughout prenatal development; although, this is not well characterized in the developing human fetus. Our clinical fetal specimens can be a viable tissue to characterize transporters that specifically bind to BPA species, comparing expression across different gestational weeks and different tissue types. Furthermore, if enough tissue is available, we can measure cofactors such as UDP-glucuronic acid and 3'-phosphoadenosine-5'phosphosulfate (PAPS) across different tissue compartments. Combining research in xenobiotic metabolism, transporter, and cofactor concentrations analysis will provide an all-encompassing depiction of BPA internal dose in the developing fetus.

5.5 Health Behavior and Policy

Toxicology studies using animal or *in vitro* models have traditionally been used to understand human health, but human clinical specimen are an incomparable asset that can be most informative for driving science policy. Currently, issues surrounding inappropriate translation across species and BPA contamination have lead to skepticism and doubt in BPA research. Addressing these concerns, findings from this study and other human studies directly support the 2008 National Toxicology Program (NTP) conclusions in that there is "some concern for developmental toxicity for fetuses, infants, and children" (Chapin, et al., 2008). Many of the BPA toxicities are subtle, indirect, or manifest at a later period. Whereas conventional policies are determined by factors that demonstrate obvious dangers to general human health, science-based regulations should now create specific guidelines that consider different populations, different chemical characteristics, timing, and low dose exposures. In doing so, many environmental compounds like BPA would become important contenders for stringent regulation.

Although copious research surrounding BPA exposure and health has surfaced over the last two decades, opposition from influential chemical and plastic companies has brought BPA to the forefront of international debate. BPA's desirable properties have led to the creation of a multi-billion dollar industry, supplying convenient products to a global consumer market. Policies regulating this high-stake product is challenging given the economic cost and difficulty in finding safe chemical replacements. In the USA, regulatory agencies have been slow to determine safety levels, with the Environmental Protection Agency still inaccurately using the 1993 lowest observed adverse effect level at 50 mg/kg per bodyweight. Regulatory agencies across different countries and within

the USA display conflicting views on BPA; for example, in 2008 Health Canada declared BPA as a health hazard, while Food and Drug Administration announced that research for BPA risk was inconclusive (Erler & Novak, 2010; Resnik & Elliott, 2014). In general, stringent regulatory action against BPA is difficult especially since the social and economic costs to industry and health care agencies are unknown.

In recent years, the growing public alarm over BPA risk have prompted several companies like Wal-Mart and Toys R Us to voluntarily stop the sale of different BPA containing products. Consumer response has driven manufacturers to investigate BPA substitutes and to ensure better labeling (Barraza, 2013). While some safe alternatives include glass, steal, and polypropylene, BPA synthetic alternatives like bisphenol-S and bisphenol-F are proving to be just as harmful as BPA (Chen, Ike, & Fujita, 2002; S. Lee, Liu, Takeda, & Choi, 2013). In general, health care providers and environmental advocacy groups have promoted behavioral changes to reduce exposures. Reducing canned food consumption, avoiding polycarbonate plastic storage containers to heat food, and using reusable BPA-free water bottles are some common recommendations for consumers. Until further legislation, the public must be kept informed about new potential risks, with vulnerable populations taking extra precautions with BPA containing products.

5.6 Conclusion

In this dissertation, identification of BPA species at different developmental periods and fetal tissues along with concentration-dependent changes in XME gene

expression and regulation signify considerable *in utero* BPA exposure and potential risk during pregnancy. The findings have implications across multiple disciplines, informative especially for developmental toxicology and pharmacology. In particular, this project sheds light on programming in early life to support the developmental origins of health and disease hypothesis. Additionally, application of sensitive and high throughput techniques to human clinical specimens help address natural exposures and biological changes that are most relevant to the human population. Thus, results are important for public health and policy not only important for BPA regulation but also useful for other environmental contaminants, nutrients, and pharmaceuticals.

Figure 5.1 BPA Toxicokinetic Result Overview

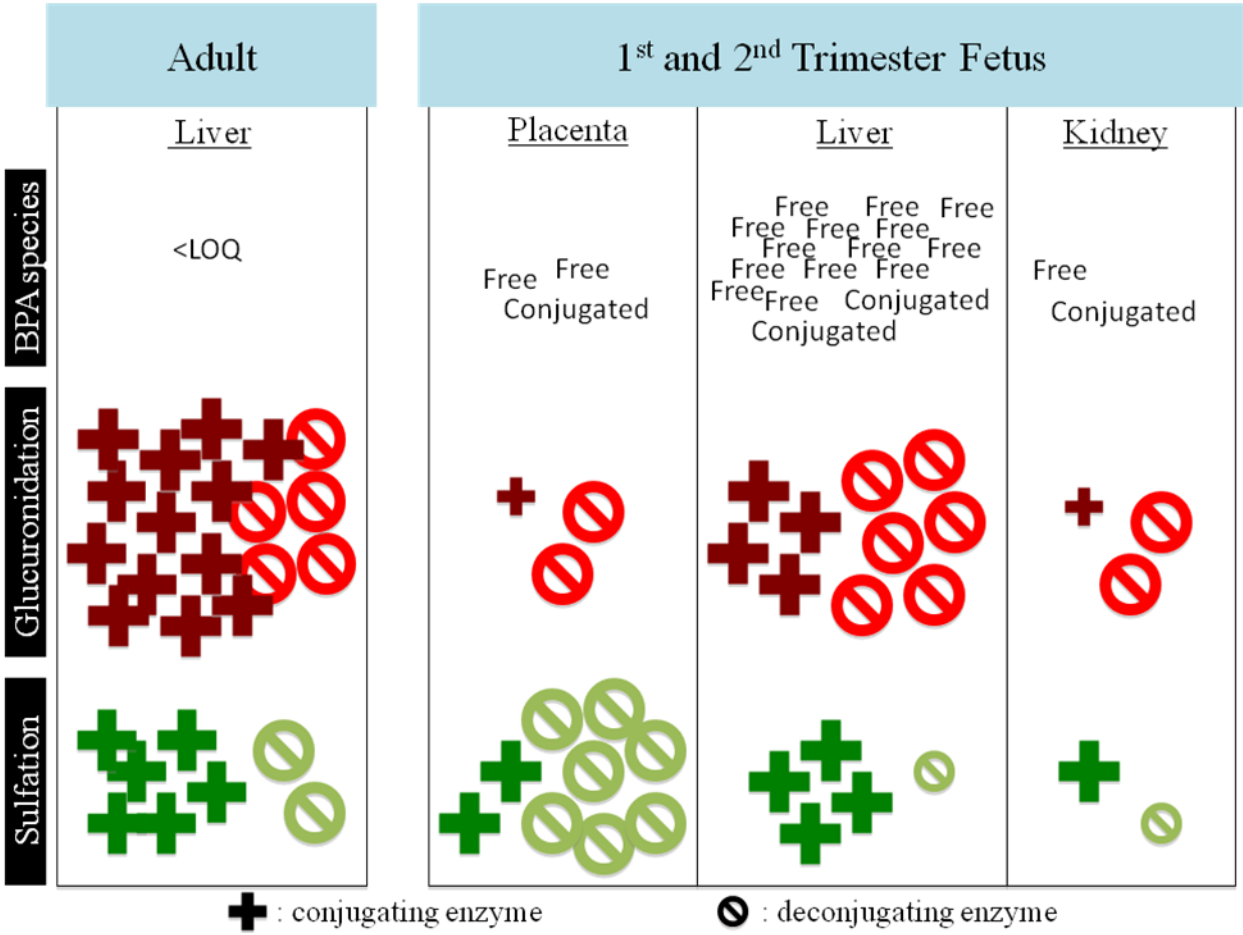
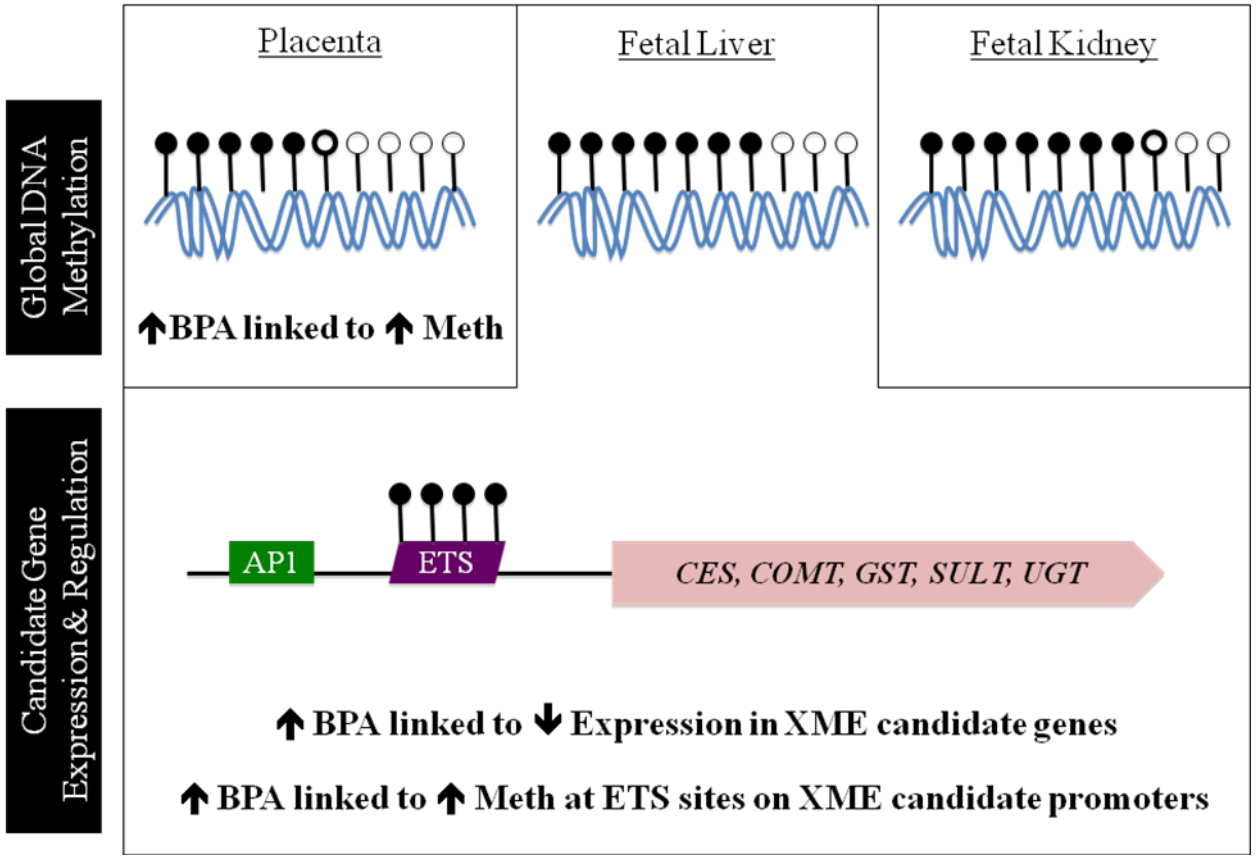


Figure 5.2 BPA Toxicodynamic Result Overview



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