PUTATIVE EARLY LIFE EPIGENETIC BIOMARKERS OF HEPATOCELLULAR CARCINOMA IN MICE PERINATALLY EXPOSED TO BISPHENOL A

by

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DEDICATION

I dedicate this thesis to my mother, Simi, who was diagnosed with pancreatic cancer in December 2011. Mom, it has been my honor to be one of the many family members holding your hand during the past few years. I greatly respect the way that you have chosen to fight, with complete autonomy and ownership of your care, with a quiet strength and deep courage. Large parts of this thesis were completed at your kitchen table – that makes this one both yours and mine and I'm glad that you got to see it through.

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ABSTRACT

Bisphenol A (BPA) is a high production-volume chemical with hormone-like properties that has been implicated as a potential carcinogen. Early life exposure has been linked to increased risk for precancerous lesions in mammary and prostate glands and the uterus, but no prior study has shown a significant association between cancer development and exposure to BPA alone. The overall goal of this dissertation was to test the central hypothesis that early life BPA exposure dysregulates the DNA methylome and thereby modifies risk for adult liver tumors. Chapter 2 describes a monotonic increase in hepatic tumors with increasing dose of perinatal BPA. Chapter 2 further characterizes the observed liver tumor phenotype in a murine model and notes a lack of sexual dimorphism in incidence, as well as a lack of regenerative response to injury, suggesting a solely proliferative response to BPA. Chapter 3 provides proof of principle for a novel method for identification of epigenetic biomarkers of exposure and outcome across the life-course and across species. One of three candidate genes that we tested with this method, *Stat3*, displayed dose-dependent DNA methylation changes in 10-month mice with liver tumors as compared to those without liver tumors, as well as dose-dependent methylation changes in 3-week sibling mice from the same exposure study, implicating *Stat3* as a potential epigenetic biomarker of both early life BPA exposure and adult disease in mice. Chapter 4 demonstrates

that epigenome-wide discovery experiments in animal models are effective tools for identification and understanding of paralagous epimutations in cell signaling pathways salient to human disease. Pathway enrichment analysis revealed mouse and human genes linked to BPA exposure related to intracellular Jak/STAT and MAPK signaling pathways likely linked to sexual dimorphism of HCC. Taken together, these findings are indicators of the relevance of the hepatic tumor phenotype seen in BPA-exposed mice to human health. This work combines a state-of-the-art epigenomic discovery approach with high resolution, quantitative epigenetic techniques to identify dose-dependent alterations in the fetal and adult epigenomes that correlate with HCC status. As such, this research represents a critical link between early life environment and a specific phenotypic outcome in later life, necessary to the determination of human health risk assessment and human disease prevention, diagnosis, and treatment.

CHAPTER 1

Introduction

1.1 Overview of Dissertation

Epidemiological studies and animal experiments have now critically established that environmental exposures during early embryonic development have the ability to induce aberrant epigenomic programs that influence adverse health outcomes in adulthood (Barker, 2004; Sayer et al., 1997). Bisphenol A (BPA) is a high production volume monomer used in manufacture of polycarbonate plastic and epoxy resins. Prior animal studies have associated pre- or perinatal BPA exposure with a plethora of later life health outcomes, including liver damage (Moon et al., 2012), insulin resistance (Wei et al., 2014), decreased sperm production (Liu et al., 2013, Ma et al., 2013), and prostate (Prins, et al. 2008) and breast cancer development. Epidemiological data have linked BPA exposure with increased risks of metabolic and hepatic dysfunction (Rubin, 2011). The present study focuses on the role of early life BPA exposure in the development of hepatocellular carcinoma (HCC). The central hypothesis is that perinatal BPA exposure leaves stable imprints on the fetal epigenome that influence the development of hepatocellular carcinoma in adulthood in both mice and humans. First, I characterize the incidence and dose-relationship of hepatic tumors in mice perinatally exposed to 50 ng, 50 μg, or 50 mg BPA per kg maternal diet (**Figure 1.1**).

Second, I characterize putative early life epigenetic biomarkers across the life-course and across species. Third, I profile epigenome-wide changes in genes and pathways associated with perinatal BPA exposure in 10-month mice. This work combines a state-of-the-art epigenomic discovery approach with high resolution, quantitative epigenetic techniques to identify dose-dependent alterations in the fetal and adult epigenomes that correlate with HCC status. As such, this research represents a critical link between early life environment and a specific phenotypic outcome in later life, necessary for the determination of human health risk assessment and human disease prevention, diagnosis, and treatment.

1.2. Background

1.2.1. Epigenetics and Developmental Origins of Health and Disease

It is increasingly recognized that exposure to chemical, nutritional, and behavioral factors alters gene expression and affects health and disease by modifying the epigenome, formally defined as the heritable changes in gene expression not governed by underlying genotype (Barker, 2004; Dolinoy and Jirtle, 2008). These changes are moderated by epigenetic marks, including DNA methylation, histone modifications, and chromatin remodeling proteins; of these, DNA methylation is currently accepted as the most stable and well studied (How Kit et al., 2012). Epigenetic marks control the timing and magnitude of gene expression. Various cell types exhibit discrete epigenomic profiles, or genome-wide collections of epigenetic marks, that enable their unique cellular identities (Cheedipudi et al., 2014). Epigenetic marks, unlike the DNA sequence itself, are both dynamic, in that they undergo programmed temporal and spatial change, and plastic, in that they respond to fluctuations in the environment (Baccarelli and Bollati, 2009; Dolinoy et al., 2007). The period of greatest change, and therefore greatest vulnerability, is embryonic development, during which DNA methylation profiles are systematically erased and re-established (Oswald et al., 2000; Reik

et al., 2001; Surani et al., 1986). Environmental perturbations during embryogenesis may prevent faithful re-establishment of epigenetic marks, thereby inducing an aberrant program that may alter susceptibility to later adult disease (Barker 2004; Sayer et al., 1997). As such, differential exposures in early life may enable a single genotype to give rise to a range of adult phenotypes (Dolinoy et al., 2007; Waterland and Jirtle, 2003). These data underscore the plasticity of the epigenome and the need for identifying regions of dysregulation following toxicant exposure for potential mitigation via public health intervention.

1.2.2. Hepatocellular Carcinoma (HCC) and Epigenetics

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third most common cause of cancer-related deaths worldwide, with a prognosis of 10% survival five years after diagnosis (Giannitrapani et al., 2006). Risk factors for HCC include: cirrhosis, steatosis, obesity, diet, hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, *Helicobacter pylori* infection, alcohol abuse, tobacco smoking, aflatoxin B1 (AFB1) exposure, and oral contraceptive use (Blonski et al., 2010; Giannitrapani et al., 2006; Xuan et al., 2008). Genetic risk factors for HCC have been well documented (Shimizu et al., 1998; Tsuei et al., 2011). As the liver tumors described in Chapter 2 arose in an isogenic mouse population exposed perinatally to BPA, the mechanism of carcinogenesis is likely not solely genotoxic. Epigenetic mechanisms may explain the ability of non-mutagens to promote cancer phenotypes.

Aberrant promoter methylation is a major inactivation mechanism of tumor suppressor genes involved in progression of human cancers. Promoter hypermethylation and concomitant transcriptional downregulation has been reported in DNA damage response genes in human HCC tissues and cell lines (Li et al., 2012). Hypomethylation of repetitive element *LINE-1* is prevalent in human HCC (Ramzy et al., 2011); Shitani et al. identified

four gene promoters that, together with the methylation profiles of *LINE-1* elements, strongly discriminate between cancerous and non-cancerous tissue in primary clinical HCC samples (Shitani et al., 2012). Recent work suggests that molecular gene expression profiles of hepatocellular carcinomas in B6C3F1 mice are similar to those of humans (Hoenerhoff et al. 2011), supporting the utility of *in vivo* animal models to inform human HCC prevention and treatment.

1.2.3. Bisphenol A (BPA) and Epigenetics

With over 6 billion pounds produced annually, bisphenol A (BPA), a monomer used in manufacture of plastic and epoxy resins, is one of the highest volume chemicals produced globally (Vandenberg et al., 2012). Early life BPA exposure in animal models is associated with liver damage, insulin resistance, decreased sperm production, and neoplastic development in rodent prostate and mammary glands (Acevedo et al., 2013; Liu et al., 2013; Ma et al., 2013; Moon et al., 2012; Prins et al., 2008; Wei et al., 2014). Human epidemiological data have linked BPA with metabolic and hepatic dysfunction (Rubin, 2011). BPA is a non-steroidal, synthetic environmental estrogen, or xenoestrogen (Bromer et al., 2010) that may promote tumorigenic activity via epigenotoxic mechanisms (Bernal and Jirtle, 2010). Altered developmental programming following BPA exposure has been shown to dysregulate four classes of epigenetic marks: DNA methylation, histone tail modifications, non-coding RNA, and chromatin remodeling proteins (Avissar-Whiting et al., 2010; Baccarelli and Bollati, 2009; Dolinoy et al., 2007; Greathouse et al., 2012). BPA exposure has been specifically linked to dysregulation of potential mediators of hepatic tumorigenesis, in both *in vivo* rodent and *in vitro* human studies. Holtzman rats exposed neonatally to 2.4 μg BPA/day injections exhibited significant hypermethylation of both nuclear estrogen receptors ERs (Doshi et al., 2011), which have been implicated in xenoestrogen-induced breast cancer

development (Pupo et al., 2012). Human developmental transcription factor *HOXA10* has been shown to play a role in the progression of gastric, breast, and brain cancers (Chen et al., 2012; Di Vinci et al., 2012; Sentani et al., 2012); protein and mRNA levels of *Hoxa10* increased in uterine tracts of CD-1 mice exposed prenatally to intraperitoneal injections of 5 mg/kg BPA (Bromer et al., 2010). Concomitant promoter hypomethylation at *Hoxa10/HOXA10* in mice and human breast carcinoma MCF-7 cells facilitated increased binding of ER-α to promoter estrogen response elements (ERE), leading to increased ERE-driven expression (Bromer et al., 2010). Protein and mRNA levels of histone methyltransferase enhancer of Zeste homolog 2 (*EZH2*), an epigenetic regulator of tumorigenesis, were also increased 2- to 3-fold in adult mammary tissue of CD-1 mice injected with 5 mg/kg BPA during gestation and in BPA-treated (10^{-8} M and 10^{-6} M) human MCF-7 cells (Doherty al., 2010). BPA treatment of three human transformed placental cell lines (3A, TCL-1, HTR-8) induced changes in 21 miRNAs, most notably overexpression of *miR-146a*, which is involved in cellular proliferation (Avissar-Whiting et al., 2010). Literature-derived examples of BPA induction of dysregulated epigenetic marks associated with tumor development support the hypothesis of BPA-mediated carcinogenicity.

Cancer is classically characterized by global epigenomic changes, including hypomethylation, aberrant histone modifications, and altered non-coding RNA profiles (Herceg and Paliwal, 2011; Sandoval and Esteller, 2012). BPA has also been shown to induce global alterations to the epigenome. BPA-induced up-regulation of global epigenomic mediators is evident in both rodents and humans. Holtzman rats injected gestationally with 2.4 μg BPA/day exhibited increased expression of repressive DNA methyltransferases *Dnmt3a/b* (Doshi et al., 2011). The same methyltransferases, as well as methyl binding proteins *Mbd2/4* showed early overexpression in BPA (10 μg/kg)-exposed Sprague-Dawley

rat prostate glands (Tang et al., 2012). Interestingly, BPA (10^{-8} M and 10^{-6} M) increased expression of histones H2A, H2B, H3, and H4 in *ER-α+/ER-β+* human MCF-7 cells, but not in cell lines negative for one or both ERs (Doherty et al., 2010; Zhu et al., 2009). Alterations in DNA methyltransferases, histone proteins involved in nucleosome formation and regulation, and methyl binding proteins which bind and further repress expression at methylated gene promoters, have the potential to induce epigenome-wide changes such as those characteristic of epigenomic profiles found in cancer (Herath et al., 2006).

1.2.4. HCC and Endocrine Active Chemicals

HCC incidence is increasing in the U.S., particularly in young patients. Risk factor data suggest incidence is not only increasing, but also is likely under-represented in the literature (Shaw and Shah, 2011). HCC is classically sexually dimorphic. Male: female ratios are estimated to be between 2:1 and 4:1 (Giannitrapani et al., 2006). This skewed sex ratio indicates a primary role for sex-specific steroid hormones in carcinogenesis. However, data on the relative roles of endogenous estrogens, xenoestrogens, and androgens in HCC development are conflicting. Nuclear estrogen receptors, *ER-α* and *ER-β*, and androgen receptor (*AR*) are expressed in the liver. Some studies note that *AR* is overexpressed in HCC, and androgens promote hepatocarcinogenesis (De Maria et al., 2002). Further, elevated serum testosterone and an increased testosterone: estrogen ratio have been noted to increase HCC risk (Shimizu et al., 1998). In contrast, males with HCC typically present with a relative hyper-estrogenic state (De Maria et al., 2002; Farinati et al., 1995). However, liver-specific *ER* expression is decreased in HCC, as compared to healthy samples, suggesting that estrogen sensitivity in the liver is decreased, as well. Endogenous estrogen estradiol has been implicated as both a potent endogenous antioxidant that protects against tumorigenic events and a carcinogen responsible for sexual dimorphism in HCC. Estradiol inhibits cellular

proliferation and lipid peroxidation, attenuates inflammatory target hepatic stellate cell (HSC) activation, and induces apoptosis (Huang et al., 2006; Omoya et al., 2001; Shimizu et al., 1998). Estrogen-mediated inhibition of IL-6 production in mice eliminated gender differences in HCC risk (Naugler et al., 2007), providing evidence that estrogen simultaneously drives sexual dimorphism in HCC and protects against tumor progression. Conversely, estradiol induces hepatocyte (Granata et al., 2002) and uterine cancer cell proliferation (Miyake et al., 2009) and produces free oxygen radicals (Robins et al., 2011).

Synthetic estrogens, or xenoestrogens, have also been associated with HCC pathogenesis. Metabolites of DDT, an organochlorine pesticide and endocrine disruptor, displayed dose-dependent HCC risk in 346 HCC cases in China (Zhao et al., 2011). Most notably, strength and duration of oral contraceptive (OC) use, commonly as combinations of xenoestrogen 17α-ethinylestradiol and synthetic progestin, have been associated with benign (liver hemangioma [LH], hepatic adenoma [HA], and focal nodular hyperplasia [FNH]) and, more controversially, malignant (HCC) hepatic tumors. Rare before widespread OC use, LH, HA, and FNH occur predominantly in young women, emphasizing their hormonal etiologies (Giannitrapani et al., 2006; Rosenberg, 1991). Data from five case-controls studies in low risk populations (2 England, 2 U.S., 1 Italy) indicated an association between 5+ years OC use and HCC, but two studies in developing countries with high-risk (HBV-endemic) populations showed no association (Rosenberg, 1991). A 2007 meta-analysis of <5 years OC use and HCC yielded an adjusted pooled OR estimate of 1.45 (95% CI: 0.93-2.27, p=0.11). Although excluded from analysis, six of the 12 studies showed 2- to 20-fold increase in HCC risk with longer durations of OC use (Maheshwari et al., 2007). The International Agency for Research on Cancer considers the existing evidence sufficient to support the carcinogenicity

of OC use in populations with low HBV prevalence and chronic liver disease (Farges and Dokmak, 2010).

Interestingly, the risks of benign and malignant disease are not unrelated. The literature cites 17 cases of HA with focal malignant transformation to HCC in women taking OCs, with duration of use ranging from one month to >20 years (Blonski et al., 2010; Ferrell, 1993; Foster and Berman, 1994; Gordon et al., 1986; Gyorffy et al., 1989.; Korula et al., 1991; Micchelli et al., 2008; Perret et al., 1996; Tesluk and Lawrie, 1981). Several studies agree that approximately 5% of patients with resected HA presented pathological evidence of HCC (Farges and Dokmak, 2010). Therefore, even an anecdotal increase in transformation rates with OC use is notable. This summary body of evidence linking estrogen and estrogen-like chemicals with increased incidence of HCC illustrates the need to explore a possible connection between HCC and the endocrine disruptor BPA.

1.3. Study Design Overview

Recent evidence indicates that components of the early life environment, such as nutrition, maternal care, and toxic exposures, affect adult phenotypic outcomes by altering epigenetic regulatory marks (Barker, 2004; Sayer et al., 1997). The epigenome is particularly vulnerable to environmental perturbations during embryogenesis because the elaborate DNA methylation patterning and chromatin structure required for normal growth is established early in development. Ubiquitous endocrine disruptor bisphenol A (BPA) is an example of a chemical that has been implicated in epigenetic developmental programming and subsequent increased susceptibility to multiple health endpoints. Preliminary data now indicate that perinatal exposure to BPA may epigenetically mediate the risk of hepatocellular carcinoma (HCC), a widespread disease with poor prognosis (Shaw and Shah, 2011). Thus, there is a critical need for identification of predictive biomarkers for epigenotoxic developmental programs induced by

high impact chemicals like BPA in diseases with global relevance and poor therapeutic options and prognostic outlooks, such as hepatocellular carcinoma (HCC).

Using rodent and human samples (**Figure 1.2**), this dissertation investigates epigenetic mechanisms associated with early life BPA exposure and the development of HCC later in life. This novel approach differs from classic cancer studies in its focus on perinatal exposure and early origins of disease, as opposed to *a priori* interrogation of a chosen phenotype. Here, I test the central hypothesis that perinatal BPA exposure leaves stable imprints on the fetal epigenome that lead to development of hepatocellular carcinoma in adulthood. First, I characterize the incidence and dose-relationship of various hepatic lesions, including hepatocellular carcinomas and hepatic adenomas, in 10-month mice perinatally exposed to 50 ng, 50 μg, or 50 mg BPA per kg maternal diet (**Figures 1.1** and **1.2**). Second, I characterize putative early life epigenetic biomarkers across the life-course and across species via bisulfite sequencing within agnostically-identified regions within functionally-relevant murine and human candidate genes in BPA-exposed 10-month-old mice, sibling 3-week mice from the same exposure study, and human fetal livers (**Figures 1.1** and **1.2**). Third, I perform and validate promoter DNA methylation tiling microarray experiments to profile epigenome-wide changes in genes and pathways associated with perinatal BPA exposure in 10-month mice that are both relevant to the lack of sexual dimorphism in our observed hepatic tumor phenotype and linked to human BPA exposure (**Figures 1.1** and **1.2**). This work combines a state-of-the-art epigenomic discovery approach with high resolution, quantitative epigenetic techniques to identify dose-dependent alterations in the fetal and adult epigenomes that correlate with HCC status.

1.4. Significance

BPA is a high production volume chemical that has been implicated in cardiovascular, immune, reproductive, and cancer endpoints (Rubin, 2011). However, BPA's toxicity remains

controversial, as several studies display inconsistent or contradictory results and concerns about laboratory contamination remains an issue (Vom Saal, Nagel, Coe, Angle, & Taylor, 2012). In addition, the dose-response curve has not been fully determined, making the incorporation of various physiologically-relevant doses of BPA a crucial aspect of experimental design (Vandenberg et al., 2010). BPA has been shown to be present in 93% of 394 human urine samples collected during a recent Centers for Disease Control and Prevention (CDC) study (Calafat et al., 2005) supporting the chemical's environmental ubiquity and the relevance of BPA exposure studies to U.S. population health. BPA has been classified as an endocrine disruptor due to its ability to bind nuclear estrogen receptors ER-α and ER-β; however, BPA has been reported to bind nuclear ERs with ~10-fold lower affinity than endogenous estradiol. Further, past studies showing deleterious effects of BPA exposure have been criticized for exposing model organisms to levels higher than environmentally-relevant human doses (Sekizawa, 2008; Vandenberg et al., 2008). Newer evidence indicates that BPA is as potent an inductor of estrogenic effects in mice and humans as estradiol, perhaps via nongenomic intracellular signaling mediated by membrane-bound estrogen receptor GPER (Vandenberg et al., 2010). Perinatal exposure to environmentally-relevant doses of BPA has also been shown to induce aberrant developmental and epigenetic programs in mice (Kundakovic and Champagne, 2011).

Data presented in this dissertation indicate that BPA may epigenetically mediate the risk of hepatocellular carcinoma (HCC) (Shaw and Shah, 2011). This dissertation represents a first step toward identifying potentially modifiable epigenetic risk factors associated with multiple, environmentally-relevant BPA exposures and hepatocellular carcinoma, a disease with high global burden and few effective treatments. Characterization of epigenetic biomarkers indicative of early BPA epigenotoxicity and predictive of HCC development will allow at-risk individuals to be identified long before they develop disease, opening new avenues for potential disease

prevention strategies, such as dietary supplementation or pharmaceutical intervention (Dolinoy et al., 2007; Kalra et al., 2008). Further, therapeutic modification of these epialleles in individuals with existing HCC may facilitate reversal of disease progression, due to the plasticity of the epigenome (Waterland and Jirtle, 2003).

1.5. Innovation

Despite the importance of identifying epigenetic biomarkers of exposures and diseases, biomarker studies reported in the literature are limited in design. Past studies have characterized biomarkers in the context of either exposure or disease (Kundakovic and Champagne, 2011; Pogribny and Rusyn, 2012; Stein, 2012). Few studies have followed individuals positive for exposure biomarker(s) over time to evaluate the power of these markers to predict later disease in the same individuals. This project is therefore innovative because it represents a proof of concept of predictive epigenetic biomarkers that are both a consequence of early environmental exposure and present in the adult phenotype.

Further, many past studies have either focused exclusively on rodent models; few studies have incorporated *in vitro* experiments in primary human cell lines (Baccarelli and Bollati, 2009; Pogribny and Rusyn, 2012), which are often derived from diseased human tissue and cannot inform human responses to early life chemical exposures prior to disease development. Therefore, this research is innovative in its translational implications to human health by coupling the use of an experimentally-relevant mouse model with analyses of human fetal tissue samples with known tissue BPA levels. Technologically, epigenome-wide arrays are a relatively new addition to the epigenomic experimental toolbox. In this dissertation, I use an innovative 'tiered focus approach': epigenome-wide DNA methylation promoter tiling arrays that function as broad discovery tools for epigenetic biomarkers of perinatal BPA exposures and adult hepatic tumors, followed by focused validation of altered

methylation candidate loci. Prior studies in our research group have successfully utilized similar epigenome-wide discovery scans (Bakulski et al., 2012; Kim et al., 2014; Sartor et al., 2011) supporting the approach's feasibility and utility in identifying candidates. As a consequence of the innovative design described above, this project is expected to yield one or more candidate epigenetic biomarkers of both perinatal BPA exposure and HCC with preliminary translation to human disease.

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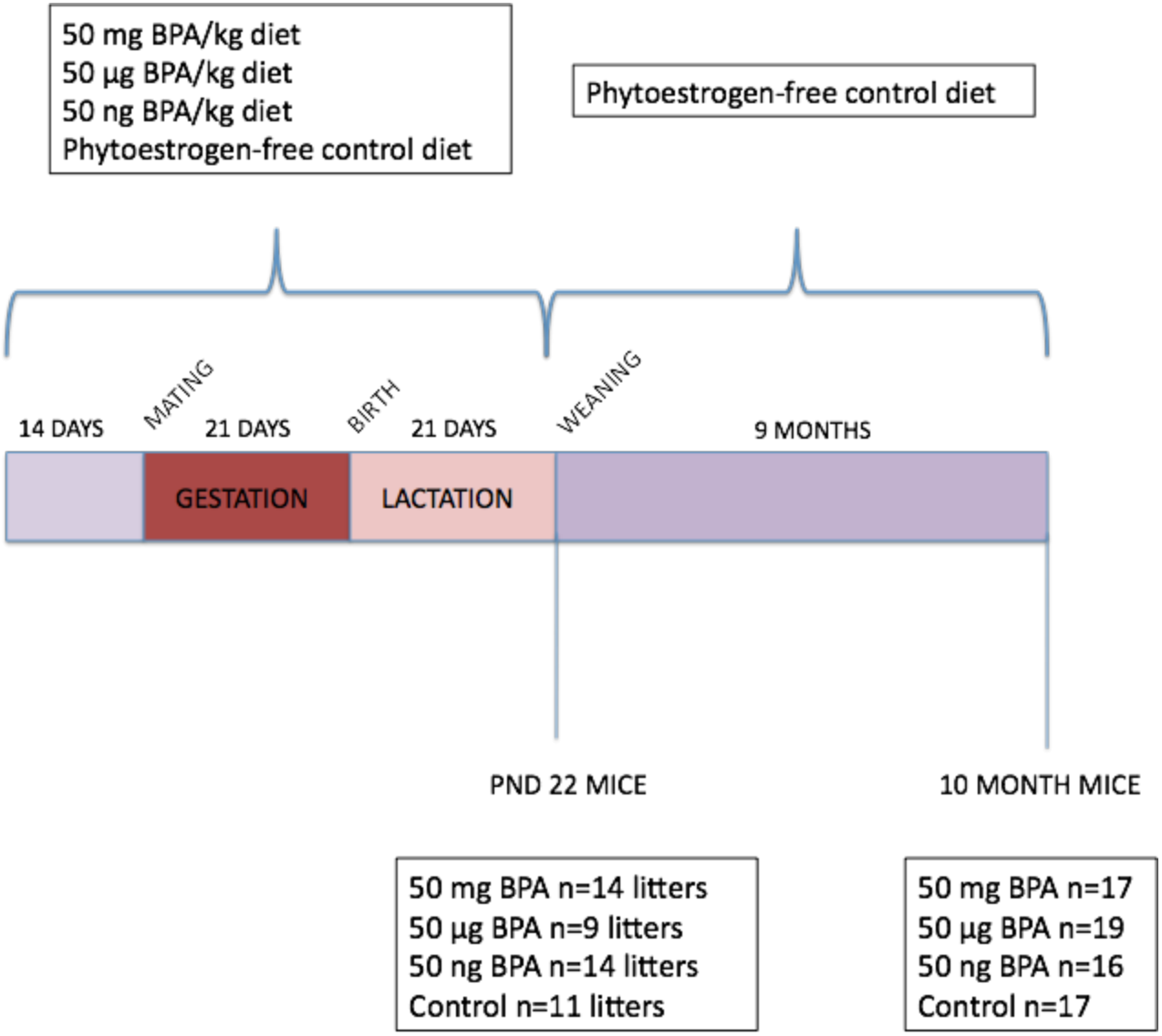

Figure 1.1 Experimental Exposure Scheme

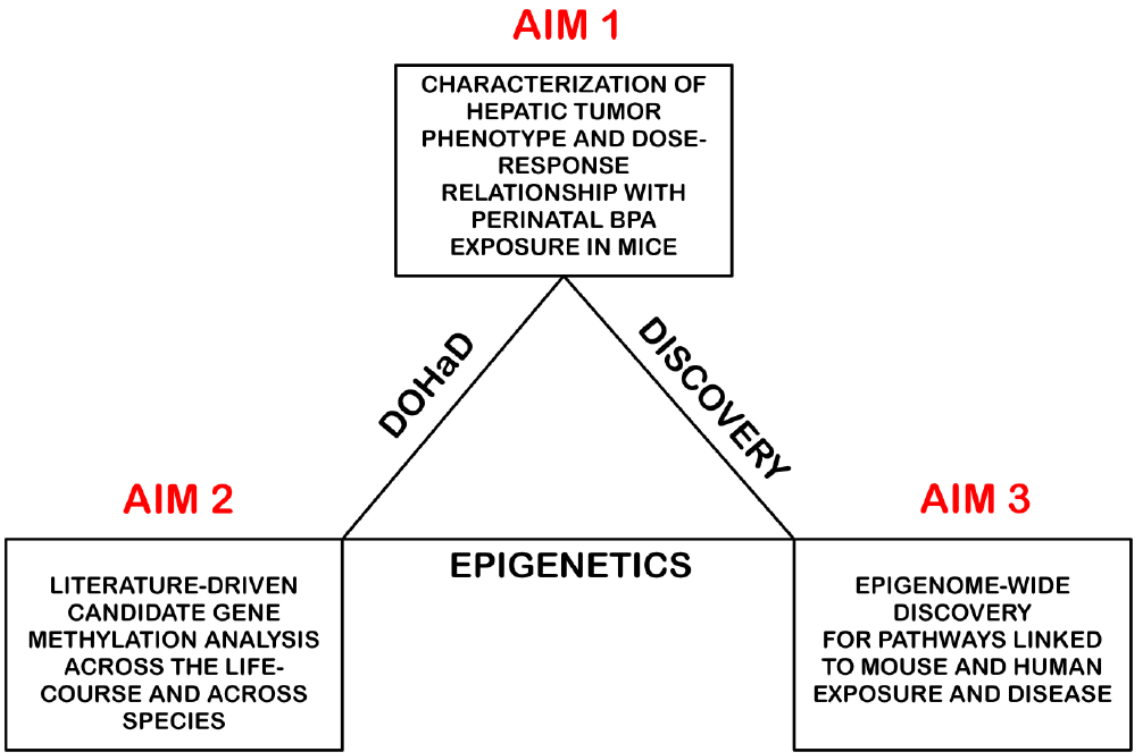

Figure 1.2 Conceptual Overview of Dissertation Aims

CHAPTER 2
Dose-Dependent Incidence of Hepatic Tumors in Adult Mice following Perinatal Exposure to Bisphenol A

2.1 Abstract

Bisphenol A (BPA) is a high production-volume chemical with hormone-like properties that has been implicated as a potential carcinogen. Early life exposure has been linked to increased risk for precancerous lesions in mammary and prostate glands and the uterus, but no prior study has shown a significant association between BPA exposure and cancer development. We explored the effects of exposure to BPA during gestation and lactation on adult incidence of hepatic tumors in mice. Isogenic mice were perinatally exposed to BPA through maternal diets containing one of four environmentally relevant doses (0, 50 ng, 50 μg, or 50 mg of BPA per kg diet) and approximately one male and one female per litter were followed until 10 months of age. Animals were tested for known risk factors for hepatocellular carcinoma, including bacterial and viral infections. We report dose-dependent incidence of hepatic tumors in exposed 10-month mice. 23% of offspring presented with hepatic tumors or preneoplastic lesions. A statistically significant dose-response relationship was observed, with an odds ratio for neoplastic and preneoplastic lesions of 7.23 (95% CI: 3.23, 16.17) for mice exposed to 50 mg BPA per kg diet compared with unexposed controls. Observed early disease onset, absence of bacterial or viral infection, and lack of characteristic sexual dimorphism in tumor incidence support a non-

classical etiology. To our knowledge, this is the first report of a statistically significant association between BPA exposure and frank tumors in any organ. Our results link early life exposure to BPA with the development of hepatic tumors in rodents, with potential implications for human health and disease.

2.2 Introduction

Bisphenol A (BPA) is an environmentally ubiquitous, high production-volume chemical that has been linked to cardiovascular, immune, neuroendocrine, and reproductive endpoints (Diamanti-Kandarakis et al. 2009). Biomonitoring studies routinely detect levels of BPA in urine in greater than 90% of adults in the United States, indicating that exposure to BPA is widespread (Calafat et al. 2008). BPA has been classified as an endocrine disruptor, and has been implicated in alterations in tissue enzyme and hormone receptor levels, interaction with hormone response systems, and cellular changes suggestive of carcinogenic potential (vom Saal et al. 2007).

The last large-scale evaluation of BPA's potential carcinogenicity in multiple target organs was a National Toxicology Program (NTP) 2-year chronic feed study conducted in 1982, which employed doses ranging from 1,000-10,000 ppm BPA. Results provided inconclusive evidence for BPA's carcinogenicity in the context of adult exposure. Non-significant incidences of liver tumors were reported in both sexes of rats and mice (National Toxicology Program (NTP) 1982). Subsequent early life BPA exposure studies that examined mammary (Vandenberg et al. 2007) and prostate (Prins et al. 2008) glands and the uterus (Bergeron et al. 1999) reported precancerous lesions following perinatal BPA exposure, but none have shown direct tumor development. Thus far, research on BPA and cancer has focused on reproductive estrogen-target organs, despite the ability of non-reproductive organs, such as the liver, to express estrogen receptors and respond to steroid hormone signaling (Cui et al. 2013) . Here we evaluate the

effects of perinatal exposure to BPA at three environmentally relevant levels and show dose-dependent incidence of hepatic tumors in adult mice at 10 months of age. To our knowledge, this is the first statistically significant report of frank tumors, in addition to precancerous lesions, in any organ following perinatal or adult BPA exposure. Classically, both male humans and rodents are two to four times as likely to develop hepatocellular carcinoma (HCC) as compared to females (Hoenerhoff et al. 2011). Liver tumors are uncommon in rodents prior to 12 months of age and often present at or later than 20 months (Maronpot 2009). The combination of observed early disease onset and lack of characteristic sexual dimorphism in tumor incidence support a non-classical etiology.

2.3 Materials and Methods

2.3.1 Animals and Diets

Mice were obtained from a colony that has been maintained with sibling mating and forced heterozygosity for the viable yellow *Agouti* (A^{vy}) allele, resulting in a genetically invariant background (Waterland and Jirtle 2003). The A^{vy} mutation initially arose spontaneously in C3H/HeJ mice; animals carrying the mutation were backcrossed with C57BL/6J mice, followed by >220 generations of sibling mating. Based on these crosses, animals are calculated to be genetically 6.25%-25% C3H/HeJ and 75%-93.75% C57BL/6J (Waterland and Jirtle 2003). The reported rate of spontaneous or induced hepatocellular carcinoma in C57BL/6J mice is variably reported as 2%-10%. The C57BL/6J strain as been classified in numerous publications as "relatively resistant" to hepatocellular carcinoma (Maronpot 2009). The incidence rate observed in our control animals is consistent with the reported rate in C57BL/6J mice.

Virgin wild-type *a/a* dams were randomly assigned to phytoestrogen-free AIN-93G diets (diet 95092, with 7% corn oil substituted for 7% soybean oil) supplemented with one of four

doses of BPA (0, 50 ng, 50 µg, or 50 mg BPA per kg diet). All diet ingredients were supplied by Harlan Teklad, except BPA, which was supplied by the National Toxicology Program (NTP, Durham, NC). Diet composition is available online at www.harlan.com.

Wildtype (*a/a*) , 6-week-old, virgin dams were exposed to their assigned BPA diets for two weeks prior to mating and housed in polycarbonate-free cages with *ad libitum* access to diet and BPA-free water. At eight weeks of age, virgin dams were mated once to A^{vy}/a sires and were impregnated within 0.5 to 5 days following co-housing with males. Sires were briefly exposed to diets containing BPA during the mating period (0.5 to 5 days). Pups were housed with their respective dams and fed their respective BPA diets until weaning at postnatal day 22. Pups were then housed with a same-sex A^{vy}/a sibling on standard phytoestrogen-free control diet until 10 months of age (Anderson et al. 2012, Anderson et al. 2013).

This mating scheme produces ~50% wildtype (*a/a*) offspring and ~50% heterozygous (A^{vy}/a) offspring. For this study, a subset of wildtype animals, approximately 1 male and 1 female per litter, was followed until 10 months of age: control diet (n=19 offspring; n=10 males and n=9 females), 50 ng BPA/kg diet (n=20 offspring; n=10 males and n=10 females), 50 µg BPA/kg diet (n=21 offspring, n=10 males and n=11 females), or 50 mg BPA/kg diet (n=18 offspring, n=9 males and n=9 females). This subset of offspring mice was assessed for metabolic and activity outcomes (Anderson et al. 2013).

Associated estimates of daily BPA exposure levels, based on a dam weighing 25 g consuming 5 g chow daily, are 0, 10 ng BPA/kg body weight/day, 10 µg BPA/kg body weight/day, and 10 mg BPA/kg body weight/day, respectively. These diets were chosen to capture both mean and maximum human environmental exposures to BPA, reported recently to range from 0.1-5 µg /kg body weight/day (Vandenberg et al., 2013). BPA exposure within

human relevant ranges was confirmed with direct measurements in liver tissue of a subset of exposed and control animals (Anderson et al. 2012). For example, total liver BPA measurements in animals fed the highest dose of 50 mg BPA /kg chow ranged from 9.5-870 μg BPA /kg liver, which captures the maximum human environmental exposure indicated by human fetal liver measurements ranging from below the limit of detection to 96.8 μg BPA /kg liver (Anderson et al. 2012). Livers from mice fed diets containing 50 μg BPA /kg chow and 50 ng BPA /kg chow exhibited <LOQ-11.3 μg BPA /kg liver (mean 2 μg /kg; median 0.6 μg /kg) and <LOQ-13 μg BPA /kg liver (mean 2.8 μg /kg; median 0.3 μg/kg), respectively (Anderson et al. 2012). To prevent possible BPA contamination, animals were singly housed in polypropylene cages, no polycarbonate plastics were used in animal management, and animal drinking water was tested once prior to the beginning of the exposure study by an independent, accredited public health and safety organization (NSF International, Ann Arbor, Michigan, *www.nsf.org*). These mice were housed in an AAALAC –approved facility with a 12-hr light cycle, ~50% relative humidity, 72±2 °C. Animals used in this study were maintained in accordance with the Institute of Laboratory Animal Resources guidelines (ILAR 1996) and were treated humanely and with regard for alleviation of suffering. The study protocol was approved by the University of Michigan Committee on Use and Care of Animals.

2.3.2 Histopathologic Evaluation

Upon dissection at 10 months of age, liver tissue was flash frozen in liquid nitrogen and later formalin fixed and paraffin embedded; for each mouse, 2-3 slides containing liver sections, both with and without grossly visible masses, were evaluated for histopathology. Liver lesions were classified by light microscopy by an exposure-blinded, board-certified veterinary pathologist (ILB), according to recently revised, standardized guidelines established by the

International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) project (Thoolen et al. 2010). This project represents consensus criteria for histopathological lesions in rodents as established by the North American, European, British, and Japanese Societies of Toxicologic Pathology. Hyperplastic nodules were not classified as "regenerative" or "non-regenerative," as specified in INHAND, because hepatopathic lesions, such as inflammation and oval cell hyperplasia, were present, but no significant markers of liver injury, such as necrosis or fibrosis were observed. Representative photomicrographs were taken using an Olympus DP72 12.5 megapixel digital camera mounted on an Olympus BX45 light microscope with manufacturer's software (DP2-BSW, Olympus). Photo processing and composite plate construction were performed in Adobe Photoshop CS4.

2.3.3 Bacterial and Viral Screens

A total of 8 animals from our colony (n=4 10-month-old mice and n=4 post-natal day 22 (PND22) mice) were tested by PCR for infection with *H. hepaticus* or *H. mastomyrinus* using previously published primers and positive controls obtained from Judith S. Opp in the laboratory of Vincent Young at University of Michigan, under published PCR conditions (Eaton et al. 2011). Four of the eight animals tested were 10-month animals included in the present study (one from each exposure group: two with hepatic tumors, one each from the medium and high dose groups; two without hepatic tumors, one each from the control and low dose groups.) The remaining four animals were siblings of the animals in the present study euthanized at PND22 (Anderson et al. 2012); one animal from each exposure group was tested. Testing was performed after the completion of the present study. We confirmed our animal selection and screening protocol with a board-certified veterinary pathologist (ILB).

Serology testing for murine hepatitis virus (MHV) was performed via enzyme-linked immunosorbent assay (ELISA) every 6-8 weeks on sentinel animals not included in the present study (3 animals per 50-70 experimental cages) housed in cages with small amounts of soiled bedding from randomly sampled experimental cages, changed once weekly (Wunderlich et al. 2011). Sentinel animals were co-housed in our animal facilities during our exposure study.

2.3.4 SNP Genotyping

DNA was isolated from spleens of three male animals 200 days old [one A^{vy}/a, one wild-type *a/a*, which were provided by Dr. Jirtle (Duke University) and one C57BL/6J purchased from The Jackson Laboratory (stock #000664) as a 21-day old weanling]. These mice were maintained in an AAALAC –approved facility with a 12-hr light cycle, ~50% relative humidity, 72±2 °C, and fed Purina LabDiet 5001 in shoebox-style polycarbonate cages (27 cm x 15 cm x 13 cm) with corn cob bedding (Bed-O' Cobs ¼®, Maumee, OH). The DNA was purified using a standard protocol of phenol/chloroform extraction followed by ethanol precipitation and dissolved in water. The mice were genotyped by Geneseek (Neogen) on the Mega Mouse Universal Genotyping Array (MegaMUGA) for 74,800 microsatellite markers, spaced at ~33 KB intervals throughout the mouse genome. Data were processed in PLINK (v1.07, *pngu.mgh.harvard.edu/~purcell/plink/*) and SAS (v9.3, SAS Institute, Cary, NC). Genotypes are available from the Mouse Phenome Database, MPD:484 at http://phenome.jax.org.

2.3.5 Data Analysis

We histologically identified 16 total tumors, benign and malignant, as well as four hyperplastic nodules, two of which co-occurred with tumors. Neoplastic and pre-neoplastic lesions were grouped in four different binary variables (present/absent): (1) malignant hepatocellular carcinomas (HCC) only (n=13); (2) benign hepatic adenomas (HA) only (n=3);

(3) all tumors combined (HCC and HA; n=16); and (4) combined tumors and hyperplastic nodules (n=18). Additional hepatic lesions analyzed as binary variables included: steatosis, inflammation, Kupffer cell hyperplasia, oval cell hyperplasia, multinucleated hepatocytes, hepatocyte hypertrophy, and lipofuscin deposition. Total hepatic lesions (including tumors and all additional lesions listed) were evaluated as summary scores (1 point for presence of each lesion, summed across lesions). As steatosis and inflammation may represent non-specific background lesions whose inclusion may mask a true association, each score was tested in three ways: (1) inclusion of all hepatic lesions; (2) inclusion of all hepatic lesions, except steatosis; and (3) inclusion of all hepatic lesions, except steatosis and inflammation. Associations between dose groups and hepatic lesions (9 variables) and summary scores (3 variables) were tested in the models below.

To facilitate comparison of results in our data with those of the 1982 NTP carcinogenicity bioassay on BPA, a nearly identical statistical strategy was employed. A total of 15 associations were tested between BPA exposure level and hepatic lesion(s); all associations were tested with both exact tests and logistic regression models, to account for bias inherent in each method, for a total of 30 models. Fisher's exact tests and Cochran-Armitage exact tests of trend were used to detect associations between dose groups and hepatopathic lesions listed above, and trends in those lesions by dose, respectively. Fisher's exact tests and Cochran-Armitage exact tests of trend were were run using the PROC FREQ statement with the EXACT option in SAS v9.3. Exact tests allow for conservative estimation of association significance given small cell counts, as compared to potential overestimation of significance by Chi-squared tests of association; therefore, we stratified data by sex in exact test analyses only, to prevent exacerbation of this bias. Logistic regression models, adjusted for clustering of mice within litters using generalized

estimating equations (GEE), were used to test the same associations and trends. Poisson regression models, adjusted for clustering by litter, were run on summary score variables. Clustering prevents overestimation of association significance due to errant assumption of animal independence. Neither exact tests nor logistic regression models allow for simultaneous adjustment for small cell counts and litter; bias inherent in both methods tends to overestimate significance. Statistical significance was defined as p-value < 0.05 for all analyses. All statistical analyses were completed using SAS (v9.3, SAS Institute, Cary, NC).

2.4 Results

2.4.1 Histopathologic evaluation

We exposed mice during gestation and lactation through maternal diets containing one of four environmentally relevant doses of BPA (0, 50 ng, 50 μg, or 50 mg of BPA per kg diet) and followed approximately one male and one female offspring per litter until 10 months of age (n=19, n=20, n=21, and n=18, respectively). Upon dissection, 23.08% (n=18/78) of offspring presented with neoplastic lesions (hepatocellular carcinomas or hepatic adenomas) or pre-neoplastic lesion (hyperplastic nodules), with an odds ratio of 7.23 (95% CI: 3.23, 16.17; p=0.014) for the 50 mg group compared with controls, and a significant dose-response on both Cochran-Armitage exact (p=0.014) and logistic regression (p= 0.022) tests of trend (**Figures 2.1A-C, 2.2, and 2.3; Tables 2.1-2.3**). As murine hepatic adenomas and carcinomas are related pathologies (Hoenerhoff et al. 2011), and preneoplastic lesions, including hyperplastic nodules, are often included in risk calculations following short-term carcinogenicity studies (Allen et al. 2007), we grouped benign adenomas, malignant carcinomas, and hyperplastic nodules for analysis. Results remained significant when preneoplastic lesions were excluded from analysis (**Figure 2.3; Tables 2.1-2.3**). Upon stratification by offspring sex, we report a significant linear

dose-response in a combination of neoplastic and preneoplastic lesions in female animals (**Figures 2.2A and 2.3D; Table 2.2**). The presence of a statistically significant dose-response in females but not in males does not necessarily indicate that the dose-responses were significantly different between males and females.

Almost half of animals presented with oval cell, or hepatobiliary stem cell, hyperplasia (43.95%, n=34/78), with significant odds ratios for the two highest dose groups (50 µg OR=5.40; 95% CI: 3.26, 8.93, *p*=0.001; 50 mg OR=2.67; 95% CI: 1.75, 4.06, *p*=0.020) (**Figure 2.1D; Tables 2.1-2.3**). Approximately one-third of animals presented with hepatocyte hypertrophy (32.05%, n=25/78), with a significant OR for the highest dose group (50 mg OR=5.66; 95% CI: 2.57, 12.50, *p*=0.028) (**Figure 2.1E; Tables 2.1-2.3**). Incidences of oval cell hyperplasia and hepatocyte hypertrophy were significantly associated with increasing dose (**Figure 2.4A and 2.4C; Tables 2.2 and 2.3**). Animals with neoplastic lesions were significantly more likely to co-present with oval cell hyperplasia, hepatocyte hypertrophy, and Kupffer cell hyperplasia, suggesting a proliferative response to perinatal BPA exposure (**Tables 2.4 and 2.5**). We observed multinucleated hepatocytes in 8 animals (10.26%, n=8/78), primarily in males in low-dose groups, although the association with BPA exposure was not statistically significant (**Figures 2.1F, 2.2B, and 2.4D; Table 2.1**). Inflammation (50.00%, n=39/78) and steatosis (50.00%, n= 39/78) may represent non-specific markers of liver damage with age, rather than markers of chemical toxicity, as these lesions were distributed fairly uniformly across doses and controls, without any apparent pattern (**Figure 2.1E, Table 2.1**). Notably, no evidence of liver injury, such as fibrosis or necrosis, was present, suggesting that the proliferative lesions noted were not a regenerative response to injury. When inflammation and steatosis were excluded from analysis, the total number of hepatic lesions increased with dose, indicating that hepatic lesions

that were significantly associated with perinatal BPA exposure co-presented in the same animals (**Tables 2.6 and 2.7**). Exposed dams did not present with any overt signs of obesity or other adverse health outcomes.

2.4.2 Bacterial and viral screens

In order to rule out known bacterial and viral disease risk factors, we performed a representative PCR screen for potential bacterial infection with *Helicobacter hepaticus* or *Helicobacter mastomyrinus* and assessed murine hepatitis viral load via serology measurements. All animals evaluated tested negative on all bacterial and viral screens.

2.4.3 SNP genotyping

The mouse strain used in these experiments was previously calculated to contain 6.25%-25% of the C3H/HeJ genome and 75%-93.75% of the C57BL/6J genome (Waterland and Jirtle, 2003). C3H/HeJ mice are prone to spontaneous hepatocellular neoplasms and C57BL/6J are relatively resistant (Maronpot 2009). Up to 85% of the greater susceptibility of the C3H mouse to hepatocellular carcinomas can be attributed to the *Hcs7* (*Hepatocarcinogenicity sensitivity 7*) locus, located on the distal arm of chromosome 1 (Bilger et al. 2004; Drinkwater 1994). In order to empirically confirm the overlap between our strain's genome and the C57BL/6J genome, we genotyped 74,830 SNPs in two male mice derived from our colony and one male C57BL/6J mouse purchased from The Jackson Laboratory. Our strain's genome differed from the C57BL/6J genome at 5,247 SNPs in total, and at only six of 5,416 SNPs on chromosome 1, indicating that our mice are genetically 93% C57BL/6J overall and >99% C57BL/6J on chromosome 1 (**Table 2.8**). Thus, our strain is genetically C57BL/6J at the *Hcs7* locus and, therefore, likely relatively resistant to spontaneous hepatocellular carcinoma.

2.5 Discussion

Here, we report findings of dose-dependent incidence of hepatic tumors following perinatal exposure to BPA in an isogenic mouse model. Although mammary carcinomas have been reported in rodents following perinatal BPA exposure (Acevedo et al. 2013), the link was not statistically significant. To our knowledge, this is the first study to demonstrate a statistically significant relationship between BPA exposure and frank tumors of any reproductive or non-reproductive estrogen-target organ. These tumors may be classified as early onset disease, as liver tumors are uncommon in all laboratory mouse strains prior to 12 months of age and often present at or later than 20 months (Maronpot 2009). We did not note any apparent sexual dimorphism in disease incidence, except in control animals. Classically, both male humans and rodents are two to four times as likely to develop hepatocellular carcinoma as compared to females (Hoenerhoff et al. 2011). The combination of observed early disease onset and lack of characteristic sexual dimorphism in tumor incidence support a non-classical etiology. These findings appear to be a function of dose and/or exposure timing, as the adult rats and mice in the National Toxicology Program's 1982 carcinogenicity bioassay on BPA were exposed to doses estimated to be 20 times to 200 times higher than the doses employed in this study, but no significant increase in hepatic tumors was reported (NTP 1982).

Interestingly, we replicated the NTP study observation of dose-dependent multinucleated hepatocytes (NTP 1982); the association between this lesion and BPA exposure was not statistically significant in either study. These abnormal cells may be found in aged mice but appear at younger ages following xenoestrogen exposure and may be associated with increased hepatocyte proliferation (Hayashi et al. 2008; Scampini et al. 1993). These data represent increased ploidy in mice without visible liver masses. BPA has previously been shown to induce meiotic aneuploidy in female mice (Hunt et al. 2003). Aneuploidy is the most common

characteristic of solid tumors in humans (Kops et al. 2005). The presence of several proliferative lesions in exposed mice, including multinucleated hepatocytes and oval and Kupffer cell hyperplasia, in the absence of cellular necrosis or fibrosis, indicates an isolated proliferative response, and not a regenerative response following liver injury (Thoolen et al. 2010). Prior studies have noted a connection between BPA exposure and oxidative stress (Babu et al. 2013; Moon et al. 2012); perhaps an exposure-mediated increase in reactive oxygen species (ROS) led to a concomitant increase in cellular proliferation in exposed mice via ROS signaling (Goodson et al. 2011; Hassan et al. 2012).

Animals tested negative on all bacterial and viral screens for infectious agents known to be promoters of hepatocellular carcinoma in rodents. As previously reported, gestational BPA exposure in these animals did not significantly influence litter size, survival, genotypic ratio, or sex ratio in comparison to control offspring (Anderson et al. 2012). Obesity and diabetes are well-documented risk factors for hepatocellular carcinoma in both rodents and humans. However, at 9 months of age, the exposed offspring examined in this study, regardless of tumor presence, exhibited body weights and serum glucose and insulin measurements at or below levels found in control animals (Anderson et al. 2013).

Since rodents have a high capacity for hepatocellular proliferation in response to liver damage, non-genotoxic factors may or may not be relevant to human exposures, although recent work suggests that molecular gene expression profiles of hepatocellular carcinomas in B6C3F1 mice are similar to those of humans (Hoenerhoff et al. 2011). Hepatocellular carcinoma is the sixth most common malignancy and the third most common cause of cancer-related deaths globally. Mortality rates in the United States are increasing more rapidly than for any other

leading cancer, and age-adjusted incidence rates have doubled in the past thirty years, with an increase in early onset disease in both sexes (Shaw and Shah 2011).

Although the majority (80%) of hepatocellular carcinomas in humans can be attributed to hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, further study of BPA's role as a potential risk factor is warranted. Historically, use of first-generation oral contraceptives containing high doses of estradiol has been associated with increased rates of hepatic neoplasms, particularly hepatic adenomas (Giannitrapani et al. 2006). Recent studies have indicated that endogenous sex hormone levels can increase rates of carcinogenic conversion in HBV+ individuals (Wu et al. 2010). Ramirez et al., demonstrated that female rats given daily subcutaneous injections of 50 or 500 µg BPA (equivalent to 2.5-6.25 mg/kg BW and 25-62.5 mg/kg BW, respectively) from PND1-10 experienced a loss of growth hormone-dependent sexual dimorphism in the liver's ability to metabolize toxicants (Ramirez et al. 2012). Moon et al., showed that intraperitoneal doses of 0.05-1.2 mg/kg BW/day administered to mice for five days induced hepatic mitochondrial dysfunction (Moon et al. 2012). An epidemiological study of 1455 adults, aged 18-74, demonstrated a statistical association between increased urinary BPA and clinically abnormal concentrations of liver enzymes gamma-glutamyltransferase and alkaline phosphatase (Lang et al. 2008). Further, Betancourt et al., found that exposing lactating female rats to 250 µg/kg BW/day (estimated exposure to offspring 2.5-25 ng BPA/kg BW/day) led to an increase in offspring susceptibility to subsequent chemical carcinogenesis (Betancourt et al. 2012).

Our study design has several notable strengths. We exposed mice to three doses that span several orders of magnitude, and the lower two doses are classified as 'low dose' by two well-accepted definitions: a dose not exceeding the threshold of the EPA's reference dose of 50 µg/kg

BW/day; and a dose within the range of observed human environmental exposure levels (Vandenberg et al. 2013). Our model was an inbred rodent strain that is well accepted and relatively resistant to hepatic tumor development. We exposed animals through the diet, currently accepted as a dominant route of exposure to BPA in humans (vom Saal et al. 2007). Animals were exposed during the perinatal period, capturing outcomes that may depend on exposure during critical developmental time points. Finally, we statistically clustered our data by litter, a method not used in many earlier BPA studies, which represents a significant criticism of and barrier to interpretation of prior studies.

A limitation of this study is the absence of direct maternal and fetal internal BPA dose measurements. However, comprehensive maternal and fetal measurements have been previously described. Zalko *et al.* determined that fetal free BPA levels peaked at approximately 4 ng/g 30 minutes following subcutaneous injection of pregnant CD-1 mice with 25 μg BPA/kg BW, indicating that fetuses were exposed to approximately 6.25% of the administered dose (Zalko et al. 2003). Sieli et al. demonstrated that bioavailability of BPA is higher in adult female C57BL/6J mice following dietary exposure (100 mg BPA-d_6/kg diet, similar to this study's maximum dose of 50 mg BPA/kg diet), as compared to oral bolus administration, despite less efficient absorption of BPA when ingested (Sieli et al. 2011). In addition, mice exposed via diet exhibited higher maximum serum BPA concentrations and greater temporal delay in reaching maximum serum BPA concentrations as compared to those receiving oral bolus, indicating sustained circulating concentrations of BPA following dietary exposure (Sieli et al. 2011).

Our animal model and exposure scheme were initially chosen to evaluate the effects of perinatal BPA exposure on the mouse epigenome (Anderson et al. 2012) and adult obesity risk (Anderson et al. 2013), rather than liver tumorigenesis. However, SNP genotyping performed in

this study confirms that our model is genetically similar to C57BL/6J mice at *Hcs7*, the locus reported to be associated with this strain's resistance to hepatocellular carcinoma; thus the mice evaluated in this study represent a conservative model for liver cancer development. The limitations of this model are similar to that of any animal model, in that no direct conclusions can be drawn from this study on human health risk, particularly as human populations are genetically diverse and our model is isogenic. The use of an isogenic model, however, also represents a study strength, in that we were able to detect statistically significant outcomes without potentially confounding effects of individual differences in genetic susceptibility.

2.6 Conclusion

The significance of this study may be summarized as follows: (1) to our knowledge, these data represent the first report of frank tumors in any organ following perinatal or adult BPA exposure; (2) these findings underscore the critical importance of exposure timing when evaluating adverse outcomes, particularly in light of non-significant liver tumor data in peripubertally exposed rodents in the noted 1982 NTP study; and (3) these results implicate perinatal exposure to an environmentally ubiquitous chemical in the development of hepatic tumors, with potential implications for human health and disease.

2.7 References

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Table 2.1 Frequency of hepatic lesions in mice exposed perinatally to BPA. Frequencies of hepatic lesions in mice exposed perinatally to a control diet or to one of three doses of BPA (50 ng/kg diet, 50 µg/kg diet, or 50 mg/kg diet), by dose and sex. All values are shown as *percent (proportion)*

Hepatic lesion	Dose (kg/diet)	Total animals	Males	Females
Hepatic adenoma	Total	3.85 (3/78)	5.00 (2/40)	2.63 (1/38)
	Control	0 (0/19)	0 (0/10)	0 (0/9)
	50 ng BPA	0 (0/20)	0 (0/10)	0 (0/10)
	50 µg BPA	0 (0/21)	0 (0/11)	0 (0/10)
	50 mg BPA	16.67 (3/18)	22.22 (2/9)	11.11 (1/9)
Hepatocellular carcinoma	Total	16.67 (13/78)	17.50 (7/40)	15.79 (6/38)
	Control	10.53 (2/19)	20.00 (2/10)	0 (0/9)
	50 ng BPA	15.00 (3/20)	14.29 (1/10)	22.22 (2/10)
	50 µg BPA	14.29 (3/21)	18.18 (2/11)	12.50 (1/10)
	50 mg BPA	27.78 (5/18)	22.22 (2/9)	33.33 (3/9)
Neoplastic lesions[a]	Total	20.51 (16/78)	22.50 (9/40)	18.42 (7/38)
	Control	10.53 (2/19)	20.00 (2/10)	0 (0/9)
	50 ng BPA	15.00 (3/20)	10.00 (1/10)	22.22 (2/10)
	50 µg BPA	14.29 (3/21)	18.18 (2/11)	10.00 (1/10)
	50 mg BPA	44.44 (8/18)	44.44 (4/9)	44.44 (4/9)
Neoplastic and	Total	23.08 (18/78)	25.00 (10/40)	21.05 (8/38)
preneoplastic lesions[b]	Control	10.53 (2/19)	20.00 (2/10)	0 (0/9)
	50 ng BPA	15.00 (3/20)	10.00 (1/10)	20.00 (2/10)
	50 µg BPA	23.81 (5/21)	27.27 (3/11)	20.00 (2/10)
	50 mg BPA	44.44(8/18)	44.44 (4/9)	44.44 (4/9)
Oval cell hyperplasia	Total	43.95 (34/78)	45.00 (18/40)	42.11 (16/38)
	Control	26.32 (5/19)	40.00 (4/10)	11.11 (1/9)
	50 ng BPA	30.00 (6/20)	20.00 (2/10)	40.00 (4/10)
	50 µg BPA	66.67 (14/21)	72.73 (8/11)	60.00 (6/10)
	50 mg BPA	50.00 (9/18)	44.44 (4/9)	55.56 (5/9)
Kupffer cell hyperplasia	Total	12.82 (10/78)	7.50 (3/40)	18.42 (7/38)
	Control	15.79 (3/19)	20.00 (2/10)	11.11 (1/9)
	50 ng BPA	15.00 (3/20)	0 (0/10)	30.00 (3/10)
	50 µg BPA	9.52 (2/21)	9.09 (1/11)	10.00 (1/10)
	50 mg BPA	11.11 (2/18)	0 (0/9)	22.22 (2/9)
Multinucleated hepatocytes	Total	10.26 (8/78)	17.50 (7/40)	2.63 (1/38)
	Control	0 (0/19)	0 (0/10)	0 (0/9)
	50 ng BPA	20.00 (4/20)	30.00 (3/10)	10.00 (1/10)

	50 μg BPA	14.29 (3/21)	27.27 (3/11)	0 (0/10)
	50 mg BPA	5.56 (1/18)	11.11 (1/9)	0 (0/9)
Steatosis	Total	50.00 (39/78)	47.50 (19/40)	52.63 (20/38)
	Control	52.63 (10/19)	50.00 (5/10)	55.56 (5/9)
	50 ng BPA	45.00 (9/20)	40.00 (4/10)	50.00 (5/10)
	50 μg BPA	57.14 (12/21)	54.55 (6/11)	60.00 (6/10)
	50 mg BPA	44.44 (8/18)	44.44 (4/9)	44.44 (4/9)
Inflammation	Total	50.00 (39/78)	42.50 (17/40)	57.89 (22/38)
	Control	57.89 (11/19)	50.00 (5/10)	66.67 (6/9)
	50 ng BPA	45.00 (9/20)	30.00 (3/10)	60.00 (6/10)
	50 μg BPA	42.86 (9/21)	36.36 (4/11)	50.00 (5/10)
	50 mg BPA	55.56 (10/18)	55.56 (5/9)	55.56 (5/9)
Hepatocyte hypertrophy	Total	32.05 (25/78)	27.50 (11/40)	36.84 (14/38)
	Control	15.79 (3/19)	20.00 (2/10)	11.11 (1/9)
	50 ng BPA	30.00 (6/20)	20.00 (2/10)	40.00 (4/10)
	50 μg BPA	33.33 (7/21)	27.27 (3/11)	40.00 (4/10)
	50 mg BPA	50.00 (9/18)	44.44 (4/9)	55.56 (5/9)
Lipofuscin deposition	Total	16.67 (13/78)	7.50 (3/40)	26.32 (10/38)
	Control	5.26 (1/19)	0 (0/10)	11.11 (1/9)
	50 ng BPA	15.00 (3/20)	0 (0/10)	30.00 (3/10)
	50 μg BPA	23.81 (5/21)	18.18 (2/11)	30.00 (3/10)
	50 mg BPA	22.22 (4/18)	11.11 (1/9)	33.33 (3/9)

aNeoplastic lesions are defined as a combination of benign adenomas and malignant carcinomas.
bNeoplastic and pre-neoplastic lesions include adenomas, carcinomas, and pre-neoplastic nodules.

Table 2.2 Exact tests of hepatic lesions by dose in mice exposed perinatally to BPA. Fisher's exact tests and Cochran-Armitage exact tests of trend for hepatic lesions in mice exposed perinatally to control diet or to one of three doses of BPA (50 ng/kg diet, 50 µg/kg diet, or 50 mg/kg diet).

	Total		**Males**		**Females**	
Hepatic lesion	**Fisher's exact test (*p*-value)**	**Cochran-Armitage exact test of trend (*p*-value)**	**Fisher's exact test (*p*-value)**	**Cochran-Armitage exact test of trend (*p*-value)**	**Fisher's exact test (*p*-value)**	**Cochran-Armitage exact test of trend (*p*-value)**
Hepatic adenoma	*0.011*	*0.011*	*0.046*	*0.046*	0.474	0.474
Hepatocellular carcinoma	0.570	0.218	0.946	0.855	0.273	0.162
Neoplastic lesions	0.061	*0.021*	0.384	0.236	0.089	0.056
Neoplastic and preneoplastic lesions[a]	0.089	*0.014*	0.390	0.190	0.166	*0.046*
Oval cell hyperplasia	*0.036*	*0.037*	0.115	0.392	0.133	0.052
Kupffer cell hyperplasia	0.939	0.649	0.489	0.288	0.734	1.000
Multinucleated hepatocytes	0.174	0.741	0.256	0.582	1.000	1.000
Steatosis	0.840	0.918	0.972	1.000	0.970	0.884
Inflammation	0.758	0.918	0.695	0.886	0.968	0.659
Hepatocyte hypertrophy	0.178	*0.036*	0.642	0.267	0.277	0.094
Lipofuscin deposition	0.384	0.131	0.486	0.193	0.740	0.410

Table 2.3 Logistic regressions of hepatic lesions on dose in mice exposed perinatally to BPA. Risk ratios for hepatic lesions in mice exposed perinatally to one of three doses of BPA (control, 50 ng/kg diet, 50 µg/kg diet, or 50 mg/kg diet) were generated using logistic regression models, adjusted for clustering of mice within litters using Generalized Estimating Equations (GEE).

			95% confidence interval			
Hepatic lesion	**Dose** ***(per kg diet)***	**Risk ratio**	**Lower limit**	**Upper limit**	**Parameter** ***p*-value**	***p* for trend**
Hepatocellular carcinoma	Control	Reference				
	50 ng BPA	1.36	0.53	3.53	0.744	
	50 µg BPA	1.37	0.70	2.67	0.643	
	50 mg BPA	3.01	1.45	6.27	0.131	
						0.185
Neoplastic lesions	Control	Reference				
	50 ng BPA	1.49	0.57	3.86	0.676	
	50 µg BPA	1.41	0.70	2.84	0.620	
	50 mg BPA	6.75	3.03	15.00	*0.017*	
						0.040
Neoplastic and preneoplastic lesions†	Control	Reference				
	50 ng BPA	1.59	0.61	4.12	0.627	
	50 µg BPA	2.69	1.27	5.72	0.189	
	50 mg BPA	7.23	3.23	16.17	*0.014*	
						0.022
Oval cell hyperplasia	Control	Reference				
	50 ng BPA	1.15	0.55	2.41	0.850	
	50 µg BPA	5.40	3.26	8.93	*0.001*	
	50 mg BPA	2.67	1.75	4.06	*0.020*	
						0.007
Kupffer cell hyperplasia	Control	Reference				
	50 ng BPA	1.27	0.45	3.60	0.816	
	50 µg BPA	0.67	0.25	1.78	0.679	
	50 mg BPA	0.68	0.28	1.66	0.666	
						0.541
Steatosis	Control	Reference				
	50 ng BPA	1.07	0.47	2.49	0.929	
	50 µg BPA	1.20	0.55	2.63	0.815	
	50 mg BPA	1.11	0.51	2.43	0.890	
						0.857
Inflammation	Control	Reference				
	50 ng BPA	0.59	0.28	1.26	0.489	
	50 µg BPA	0.54	0.32	0.93	0.254	
	50 mg BPA	0.90	0.50	1.63	0.862	
						0.849
Hepatocyte hypertrophy	Control	Reference				
	50 ng BPA	2.40	0.93	6.22	0.358	
	50 µg BPA	2.78	1.33	5.82	0.165	
	50 mg BPA	5.66	2.57	12.50	*0.028*	
						0.031
Lipofuscin deposition	Control	Reference				
	50 ng BPA	3.06	0.98	9.60	0.328	
	50 µg BPA	5.47	2.15	13.93	0.069	
	50 mg BPA	4.99	1.81	13.81	0.114	
						0.087

**p*-values <0.05 are shown in italics. †Neoplastic and pre-neoplastic lesions include adenomas, carcinomas, and pre-neoplastic nodules.

‡Risk ratios for hepatic adenoma and multinucleated hepatocytes were not estimated, due to low lesion counts and subsequent lack of model convergence.

Table 2.4 Exact tests of associations between tumor status and hepatic lesions linked to cellular proliferation, in mice exposed perinatally to BPA. Fisher's exact tests for associations between tumor status and hepatic lesions linked to cellular proliferation, in mice exposed perinatally to control diet or to one of three doses of BPA (50 ng/kg diet, 50 µg/kg diet, or 50 mg/kg diet), stratified by sex. Lesion percents and proportions are shown as the fraction of animals presenting with the lesion of interest among animals with neoplasms and among animals without neoplasms.

		Total		Males		Females	
Hepatic lesion	**Tumor status**	**Percent (Proportion)**	**Fisher's exact test (*p*-value)**	**Percent (Proportion)**	**Fisher's exact test (*p*-value)**	**Percent (Proportion)**	**Fisher's exact test (*p*-value)**
Oval cell hyperplasia	Neoplastic lesion	93.75 (15/16)	*5E-6*	88.89 (8/9)	*0.006*	100.00 (7/7)	*9E-4*
	No neoplastic lesion	30.65 (19/62)					
Kupffer cell hyperplasia	Neoplastic lesion	37.50 (6/16)	*0.004*	22.22 (2/9)	0.122	57.14 (4/7)	*0.013*
	No neoplastic lesion	6.45 (4/62)					
Hepatocyte hypertrophy	Neoplastic lesion	93.75 (15/16)	*1E-8*	88.89 (8/9)	*2E-5*	100.00 (7/7)	*3E-4*
	No neoplastic lesion	16.13 (10/62)					

**p*-values <0.05 are shown in italics.
†Neoplastic lesions include hepatocellular carcinomas and hepatic adenomas.
‡Multinucleated hepatocytes may be associated with hepatocyte proliferation but did not co-present with a hepatic tumor in any liver sample.

Table 2.5 Logistic regressions of hepatic lesions linked to cellular proliferation on tumor status in mice exposed perinatally to BPA. Risk ratios for hepatic lesions predicted by tumor status in mice exposed perinatally to one of three doses of BPA (control, 50 ng/kg diet, 50 µg/kg diet, or 50 mg/kg diet) were generated using logistic regression models, adjusted for clustering of mice within litters using Generalized Estimating Equations (GEE).

			95% confidence interval		
Hepatic lesion		**Risk ratio**	**Lower limit**	**Upper limit**	**Parameter *p*-value**
Oval cell hyperplasia	No neoplastic lesion	Reference			
	Neoplastic lesion	33.95	12.76	90.30	*3E-4*
Kupffer cell hyperplasia	No neoplastic lesion	Reference			
	Neoplastic lesion	8.75	3.02	25.31	*<1E-4*
Hepatocyte hypertrophy	No neoplastic lesion	Reference			
	Neoplastic lesion	78.16	27.02	226.08	*<1E-4*

**p*-values <0.05 are shown in italics.
†Neoplastic lesions include hepatocellular carcinomas and hepatic adenomas.
‡Multinucleated hepatocytes may be associated with hepatocyte proliferation but did not co-present with a hepatic tumor in any liver sample.

Table 2.6 Total hepatic lesion scores in mice exposed perinatally to BPA. Frequencies of co-occurring hepatic lesions in mice exposed perinatally to control diet or to one of three doses of BPA (50 ng/kg diet, 50 μg/kg diet, or 50 mg/kg diet), by dose.

		Total number of hepatic lesions								
Total hepatic lesions score model*	**Dose** *(per kg diet)*	**0 lesions** *Percent (Proportion)*	**1 lesion** *Percent (Proportion)*	**2 lesions** *Percent (Proportion)*	**3 lesions** *Percent (Proportion)*	**4 lesions** *Percent (Proportion)*	**5 lesions** *Percent (Proportion)*	**6 lesions** *Percent (Proportion)*	**7 lesions** *Percent (Proportion)*	**8 lesions** *Percent (Proportion)*
Summary score (all lesions)	Control	36.84 (7/19)	10.53 (2/19)	21.05 (4/19)	10.53 (2/19)	5.26 (1/19)	15.79 (3/19)	0 (0/19)	0 (0/19)	0 (0/19)
	50 ng BPA	25.00 (5/20)	35.00 (7/20)	15.00 (3/20)	0 (0/20)	0 (0/20)	10.00 (2/20)	5.00 (1/20)	5.00 (1/20)	5.00 (1/20)
	50 μg BPA	0 (0/21)	42.86 (9/21)	14.29 (3/21)	4.76 (1/21)	9.52 (2/21)	0 (0/21)	19.05 (4/21)	4.76 (1/21)	0 (0/21)
	50 mg BPA	16.67 (3/18)	27.78 (5/18)	11.11 (2/18)	5.56 (1/18)	5.56 (1/18)	0 (0/18)	33.33 (6/18)	0 (0/18)	0 (0/18)
	Total	20.51 (16/78)	29.49 (23/78)	15.38 (12/78)	5.13 (4/78)	5.13 (4/78)	6.41 (5/78)	14.10 (11/78)	2.56 (2/78)	1.28 (1/78)
Summary score (less steatosis)	Control	42.11 (8/19)	26.32 (5/19)	10.53 (2/19)	5.26 (1/19)	10.53 (2/19)	5.26 (1/19)	0 (0/19)	0 (0/19)	-
	50 ng BPA	40.00 (8/20)	25.00 (5/20)	10.00 (2/20)	0 (0/20)	10.00 (2/20)	5.00 (1/20)	5.00 (1/20)	5.00 (1/20)	-
	50 μg BPA	19.05 (4/21)	38.10 (8/21)	4.76 (1/21)	14.29 (3/21)	0 (0/21)	19.05 (4/21)	4.76 (1/21)	0 (0/21)	-
	50 mg BPA	27.78 (5/18)	16.67 (3/18)	16.67 (3/18)	0 (0/18)	5.56 (1/18)	27.78 (5/18)	5.56 (1/18)	0 (0/18)	-
	Total	32.05 (25/78)	26.92 (21/78)	10.26 (8/78)	5.13 (4/78)	6.41 (5/78)	14.10 (11/78)	3.85 (3/78)	1.28 (1/78)	-
Summary score (less steatosis and inflammation)	Control	68.42 (13/19)	10.53 (2/19)	5.26 (1/19)	10.53 (2/19)	5.26 (1/19)	0 (0/19)	0 (0/19)	-	-
	50 ng BPA	55.00 (11/20)	15.00 (3/20)	5.00 (1/20)	10.00 (2/20)	5.00 (1/20)	5.00 (1/20)	5.00 (1/20)	-	-
	50 μg BPA	23.81 (5/21)	33.33 (7/21)	19.05 (4/21)	0 (0/21)	19.05 (4/21)	4.76 (1/21)	0 (0/21)	-	-
	50 mg BPA	38.89 (7/18)	11.11 (2/18)	11.11 (2/18)	5.56 (1/18)	27.78 (5/18)	5.56 (1/18)	0 (0/18)	-	-
	Total	46.15 (36/78)	17.95 (14/78)	10.26 (8/78)	6.41 (5/78)	14.10 (11/78)	3.85 (3/78)	1.28 (1/78)	-	-

*Total summary scores included the following ten lesions: hepatic adenoma; hepatocellular carcinoma; hyperplastic nodule; oval cell hyperplasia; Kupffer cell hyperplasia; multinucleated hepatocytes; steatosis; inflammation; hepatocyte hypertrophy; lipofuscin deposition. No animal presented with greater than eight (8) lesions. Two additional scores were computed, excluding steatosis or both steatosis and inflammation, to avoid masking true effects with highly prevalent background lesions.