Influence of diet and maternal dioxin on endocrine disruption: puberty, metabolic syndrome, and breast cancer

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ABSTRACT

Influence of diet and maternal dioxin on endocrine disruption: puberty, metabolic
syndrome, and breast cancer
(Under the direction of Drs. David W. Threadgill and Linda S. Birnbaum)

Total lifetime estrogen (E2) exposure increases mammary cancer risk. TCDD indirectly
affects E2 signaling and early life TCDD exposures are also implicated in breast cancer.
E2 produced by adipose tissue is an underlying mechanism by which obesity contributes
to early puberty, and if obesity and weight gain extend into adulthood, increased post-
menopausal breast cancer risk. These observations are further complicated by greater
TCDD stores in the adipose of obese- compared to lean- individuals. How several
environmental exposures, maternal exposure to TCDD, lifelong exposure to high fat diet
(HFD), and pubertal exposure to DMBA, modify the mammary gland was examined. It
was expected that HFD and maternal exposure to TCDD would increase mammary
cancer risk, through both structural remodeling of the pubertal gland and through
molecular changes. Interactions of these exposures and phenotypes were examined using
several mouse strains and mouse models of breast cancer, but objectives were primarily
pursued using the DMBA mouse model of breast carcinogenesis. Growth trajectories,
adiposity, blood glucose levels, and mammary gland development effects of HFD and
maternal TCDD were examined as potential causal players in DMBA carcinogenesis. The
growth trajectory, adiposity, and fasting blood glucose were increased in DMBA on HFD
relative to low-fat diet (LFD). Maternal TCDD exposure increased blood glucose levels
and depressed mammary gland growth at PND in DBA/2J mice fed HFD. Maternal
TCDD exposure also increased blood glucose levels in DMBA transiently. Mammary
lesion incidence was increased by HFD and maternal TCDD exposure. TCDD exposure
increased the number of terminal end buds in DMBA on LFD, but decreased them at
PND 35 in DMBA on HFD. Among DMBA on HFD, maternal TCDD increased
epithelial *Cyp1b1* and decreased *Comt* mRNA in PND 50 mammary glands. Maternal

TCDD exposure also increased *Cyp1b1* in mammary tumors of offspring. Thus increased steady state levels of E2 and its metabolites were likely associated with increased mammary tumor incidence in DMBA. The interactions of HFD and maternal TCDD exposure on type II diabetes risk, pubertal mammary morphology, mammary tumor incidence and estrogen pathway regulation should be further explored.

DEDICATION

To Antonia Jolivette, and to my children.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AR	Androgen receptor
Arnt	Aryl hydrocarbon receptor nuclear translocator
BMI	Body mass index
BW	Body weight
Ccdm1	Cyclin D1 mRNA
C/EBPβ	CCAAT/enhancer binding protein beta
Cmyc	C-myc mRNA
COMT	Catechol-O-Methyltransferase
CYP	Cytochrome P450 enzyme family
D2	DBA/2J
DEXA	Duel energy x-ray absorptiometry
DHT	dihydrotestosterone
DMBA	7,12-dimethyl-benz[a]anthracene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DPC	Days post coitus
DRE	Dioxin responsive element
E2	17-β estradiol or estrogen
EC	Effective concentration
Egf	Epidermal growth factor

ERα	Estrogen receptor α subtype protein
ERβ	Estrogen receptor β subtype protein
ERE	Estrogen response element
Ereg	Epiregulin mRNA
Esr1	Estrogen receptor α subtype mRNA
Esr2	Estrogen receptor β subtype mRNA
FVB	FVB/NJ
H&E	Hematoxylin and eosin
HFD	High fat diet
HER2	Tg(MMTV-Neu)202Mul/J
HRT	Hormone replacement therapy
Insig	Insulin-induced gene 1 mRNA
LFD	Low fat diet
LOAEL	Lowest observed adverse effect level
p	p-value
PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
PHAH	Polyhalogenated aromatic hydrocarbons
PND	Post natal day
PyMT	TgN(MMTV-PyMT)634Mul/J
ROS	Reactive oxygen species
SAH	S-adenosyl-L-homocysteine
SE	Standard error

TAG   Triacylglyceride or triglyceride

TCDD   2,3,7,8-tetrachlorodibenzo-p-dioxin

TEB   Terminal end buds

CHAPTER 1

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a potent polyhalogenated

aromatic hydrocarbon (PHAH), with one of the lowest observed adverse effect levels

(LOAELs) identified for developmental effects in animals. TCDD and other dioxins are

persistent byproducts of industrial processes including incineration, pulp bleaching, and

Agent Orange-production. These sources cause contamination of water and food

resources. Because of the lipophilic nature of dioxins, the greatest source of human

exposure is consumption of animal-based fatty foods, where dioxins bioconcentrate. In

humans, dietary dioxin intake is correlated to net dioxin absorption (Harrad *et al.* 2003).

Thus dioxins are commonly found in the adipose tissue and blood of all humans,

including newborns (Brody *et al.* 2007).

About 50 to 90% of dietary TCDD is absorbed (Huff *et al.* 1991). Consequently,

TCDD levels in food are important to monitor. Compared to background levels, elevated

TCDD levels have been detected in catfish and chicken eggs across the USA, and in

butter, lamb fat and cottonseed oil from rural Kazakhstan (Hayward *et al.* 1999).

Uzbekistani sheep fat, chicken fat and egg samples had TCDD-dominated dioxin levels

that exceed European Commission standards (Muntean *et al.* 2003). Further, serum

dioxin levels are directly correlated with consumption of beef and eggs containing

various levels of dioxins (Goldman *et al.* 2000). Exposure to dioxins in an omnivorous

diet exceedes exposure from a vegan diet (Harrad *et al.* 2003), and dioxin levels in

nursing babies exceed US and European safety standards (Brody *et al.* 2007). TCDD is a

known human carcinogen that targets multiple sites (Birnbaum and Fenton 2003).

Various lines of evidence implicate a modulatory role of TCDD in breast cancer. TCDD

binds the aryl hydrocarbon receptor (AhR), and the complex translocates to the nucleus,

where it binds dioxin responsive elements (DRE) (Nebert *et al.* 2004). DREs activate

transcription of genes involved in estrogen (E2) metabolism and also xenobiotic

transformation, *e.g. Cyp1a1, Cyp1a2, Cyp1b1* (Nebert *et al.* 2004; Rushmore and Kong

2002) (Fig. 1-1).

The CYP1 family of proteins, induced by TCDD, have differing expression

patterns in various organ systems. CYP1B1 is expressed constitutively and is moderately

inducible in steroidogenic and steroid-responsive tissues like breast (Badawi *et al.* 2000;

Buters *et al.* 1999). CYP1A2 is primarily expressed constitutively in the liver (Uchida *et*

al. 2002). CYP1A1 is not expressed constitutively, but is inducible in most tissues,

including breast (Badawi *et al.* 2000; Buters *et al.* 1999). Variability of CYP1A induction

within these tissues comes from variable concentrations of endogenous and exogenous

inducers, physiological factors interfering with various steps of the induction process, and

genetic variation in CYP1A and AhR (Ma and Lu 2003). CYP1A1 and CYP1B1

induction in humans is highly variable, as are their link to cancer incidence; high

inducibility of CYP1A1 and CYP1B1 is linked to increased breast cancer incidence in

some populations, but not others (Justenhoven *et al.* 2007; Masson *et al.* 2005; Wen *et al.*

2007). This discrepancy may result from overly simplified or under-powered studies that

fail to detect gene by environment interactions on cancer risk. For example, *CYP1A1* polymorphisms are associated with a modification of the association between breast cancer risk and polychlorinated biphenyl (PCB) exposure, though earlier studies found conflicting relationships between breast and serum PCB levels and breast cancer (Laden *et al.* 2002; Li *et al.* 2005b).

Unlike many xenobiotics that activate the CYP1 family, TCDD is poorly metabolized. TCDD is very persistent, with a half-life of 11.0 to 24.4 days in mice (Gasiewicz *et al.* 1983; Leung *et al.* 1990), and over seven years in humans (Pitot III and Dragan 2001). TCDD clearance is also gender-dependent. Maternofetal transfer is a mode of TCDD excretion in females; transfer of TCDD is largely through the milk, though some is also transferred via the placenta (Koch *et al.* 1995; Sinjari *et al.* 1996). Through lactational exposure, TCDD concentrations adipose and liver of the pup increases (Ikeda *et al.* 2005). The tissue distribution of TCDD in neonates is also linked to dose-dependent CYP1A2 induction, where at 1 μg TCDD/ dam kg, CYP1A2 influenced fetal concentrations but at lower doses, CYP1A2 induction did not impact distribution (Emond *et al.* 2004).

The lactational TCDD exposure of offspring may effected by the ability of TCDD-exposed mothers to lactate. Gestational exposure to TCDD has been shown to alter mammary gland development in dams (Vorderstrasse *et al.* 2004). Organochlorines are associated with shortened breast feeding duration among mothers in Mexico and in North Carolina as well as decreased milk volume and lower fat content in nursing Dutch women (Boersma and Lanting 2000; Gladen and Rogan 1995; Rogan *et al.* 1987). Mammary glands of pregnant C57BL/6J mice exposed to TCDD during gestation weigh

less, have decreased branching, decreased formation of lobular alveolar structures and decreased expression of whey acidic protein, resulting in pup death within 24 hours of birth (Vorderstrasse *et al*. 2004). A study of gestational TCDD exposure in rats found decreased pup size, suggesting TCDD again disrupted dam lactation (Fenton *et al*. 2000). In rats, TCDD was shown to impact pup survival by interfering with milk production (Vorderstrasse *et al*. 2004). While such disruptions would likely lower the total dose of TCDD to the pups, breast-feeding is known to lower breast cancer risk (Shantakumar *et al*. 2007). Thus it is likely that lactation disruption due to TCDD exposure contributes to a net increase in rodent breast cancer risk.

AhR and estrogen receptor (ER) orchestrate multiple gene transcription events to alter E2 homeostasis (Fig. 1-1). During times of cellular proliferation and carcinogenesis, E2 binding to ER activates the MAPK signal transduction pathway, causing increased *Ccnd1* and *Myc* transcription and transition into S phase (Currier *et al*. 2005) Fig. 1-1). When mitogenic activity should cease, AhR and ER downregulate transcription of members of this E2-ER pathway to reduce E2 availability (Currier *et al*. 2005). Further, AhR and ER interact to increase *Cyp1a1* and *Cyp1b1* transcription to cause E2 metabolism. Yet some E2 metabolites cause DNA damage (Cavalieri and Rogan 2004; Yu *et al*. 2001) Fig. 1-1). These anti- and pro-carcinogenic TCDD mechanisms have been explored extensively and are reviewed in greater depth below.

Anti-breast cancer activity of TCDD has been investigated mostly using *in vitro* (Wang *et al*. 1998; Wormke *et al*. 2003) and adult rodent models (Gierthy *et al*. 1993; Holcomb and Safe 1994). Because of mitogenic E2 activity, it is thought that E2 metabolism by CYP1 is anti-estrogenic and decreases breast cancer risk (Holcomb and

Safe 1994). Transcriptional activation of *Cyp1* also suppresses transcription of E2 and ER. When AhR and TCDD bind the DRE in the *Cyp1* promoter, ER is prevented from binding an estrogen response element (ERE) that is required for E2 responsiveness (Safe *et al*. 2000). The indirect effect of CYP1B1 on estrogenicity is demonstrated by its loss in a *Cyp1b1* deficient model (Takemoto *et al*. 2004). TCDD suppresses E2-induced cell proliferation in ERα positive human breast cancer cells and tumor growth rates in mice with xenographed tumors (Gierthy *et al*. 1993; Gierthy *et al*. 1987)Fig. 1-1). This decreased cell proliferation is caused by inhibition of cell cycle genes and proteins that E2 induce for movement into S phase (Safe *et al*. 2000) Fig. 1-1). TCDD also inhibits *in vitro* ERα-mediated upregulation of *Esr2* (Kietz *et al*. 2004) and nuclear ER (Zacharewski *et al*. 1991).

Some estrogen metabolites may increase breast cancer risk through oxidative stress leading to DNA damage (Cavalieri and Rogan 2004; Cavalieri *et al*. 1997) Fig. 1). AhR agonists, including TCDD, recruit ERα to CYP1A1 and CYP1B1 (Beischlag and Perdew 2005; Matthews *et al*. 2005). This appears to occur through the direct interaction of AhR/Arnt and ERα (Beischlag and Perdew 2005), where ER binding to the ERE in the *Cyp1* promoter regulates *Cyp1* transcription (Tsuchiya *et al*. 2004). CYP1A1 and CYP1B1 metabolize E2 to 2OH-E2 and 4OH-E2, respectively (Hayes *et al*. 1996). However, the major E2 metabolic pathway is through formation of reactive 4OH-E2 by CYP1B1 (Lee and Zhu 2006). These E2-catechol metabolites are inactivated by catechol-O-methyltransferase (COMT) or are involved in redox chemistry. Semi-quinones and quinones pump out reactive oxygen species (ROS; (Cavalieri and Rogan 2004). These ROS result in DNA stable- and depurination- adduct formation and DNA mutations

(Cavalieri and Rogan 2004; Chakravarti *et al*. 2001; DiGiovanni *et al*. 1986; Mitrunen and Hirvonen 2003).

Human data suggests that TCDD can increase mammary cancer susceptibility. Dioxin exposure is associated with breast cancer in Russian woman (Revich *et al.* 2001). However most humans are exposed to a mixture of chemicals and the Russian study was no exception (Brody *et al.* 2007). In Seveso, Italy, where 45,000 persons have elevated TCDD in serum due to an industrial accident, a positive dose-response relationship demonstrates that increasing serum dioxin levels correlate to increased breast cancer incidence years later (Warner *et al.* 2002). This finding is remarkable given that demonstrating a positive association between hormone replacement therapy (HRT) and breast cancer required more than 150,000 woman (Kortenkamp 2006). Perhaps the infrequent association of xeno-estrogens with breast cancer risk human studies occurs in part because they typically fail to measure the estrogenic exposure during the appropriate temporal window.

There is substantial evidence in animal models that adult disease risk can be modified by environmental factors early in development, and evidence of endocrine action on the fetal epigenome modifying adult endocrine cancers is growing (Dolinoy *et al.* 2007a; Junien and Nathanielsz 2007). Virtually all identified breast cancer risk factors increase exposure to E2 in one form or another, including early menarche, late pregnancy, short duration of breast feeding, HRT, post-menopausal obesity and total xeno-estrogen body burden (Clemons and Goss 2001; Ibarluzea Jm *et al.* 2004). Natural elevation in E2 during gestation is associated with increased breast cancer in offspring (Weiss *et al.* 1997). Similarly, tamoxifen exposure during late gestation increases

susceptibility to DMBA-induced mammary tumors through E2 agonism, yet acts as a E2 antagonist in the adult breast when used as a breast cancer chemotherapeutic (Hilakivi-Clarke *et al.* 2000). This developmental-stage dependent estrogenicity increases the plausibility of TCDD acting both as a pro- and anti-estrogen. A pro-estrogenic role of early life TCDD exposures in modifying breast cancer susceptibility is supported by the Seveso study, where breast cancer incidence is associated with early life, but not late life TCDD exposures (Warner *et al.* 2002). Such observations point to the existence of critical periods in development that increase susceptibility of the mammary gland to cancer, potentially by sensitizing the gland to hormonally active chemicals.

Unequivocal evidence from two environmental exposures supports the importance of early life exposures in carcinogenesis. X-ray exposures to the fetus increased leukemia and other types of childhood cancer, and x-ray exposure to infants increased their adult breast cancer risk (Birnbaum and Fenton 2003). The E2 agonist diethylstilbestrol (DES) caused rare vaginal adenocarcinomas in women who were exposed to DES as fetuses (Birnbaum and Fenton 2003). Californian women with high levels of serum DDT, another xeno-estrogen, prior to age 14 had a five-fold increased risk of breast cancer, but after that age, there was no relationship between serum DDT levels and breast cancer risk (Cohn *et al.* 2007). Several of these examples suggest that while parental exposures may not be sufficient to induce cancer in offspring, a majority of parental exposures increase the susceptibility to carcinogen exposure in adult offspring (Birnbaum and Fenton 2003). In other words, the children of exposed parents have a higher cancer incidence than those children from unexposed parents.

The role of early life TCDD exposure in adult mammary carcinogenesis has been investigated in several rodent studies. Gestational TCDD exposure doubled the frequency of DMBA-induced mammary adenocarcinomas in rats (Brown *et al.* 1998). Exposure to TCDD on PND 18 also increased the incidence of MNU-induced mammary tumors in rats (Desaulniers *et al.* 2001). However the window of susceptibility of breast cancer due to perinatal TCDD is narrow; rats were exposed to a mixture containing TCDD on PND 21 had no dose dependent effects on mammary tumor morphology, latency or prevalence (Desaulniers *et al.* 2004).

Evidence suggests that gestational TCDD exposures may increase breast cancer risk through remodeling of the developing mammary gland (Birnbaum and Fenton 2003). In the same model, phytoestrogen genistein, tamoxifen and TCDD gestational exposures increased mammary cancer risk in offspring, Brown *et al.* found an increase in undifferentiated terminal end buds (TEB), and decreased lobules in Sprague Dawley rats exposed to TCDD during gestation (Brown *et al.* 1998; Hilakivi-Clarke *et al.* 2000; Hilakivi-Clarke *et al.* 1999). In the control mice, TEB had differentiated mostly into lobules, a sign of sexual maturity of the gland. It has been known for 30 years that TEB undergo rapid proliferation, and the presence of these structures is a crucial element of susceptibility to carcinogenic initiation (Russo and Russo 1978). Together, these results indicate that by delaying proliferation and differentiation of pubertal mammary glands, TCDD extends the period that mammary glands are most susceptible to tumor-forming insults (Birnbaum and Fenton 2003; Brown *et al.* 1998).

Similar effects have been seen in other rat strains; Long Evans rats from exposed grandmothers had disrupted mammary gland development (Fenton *et al.* 2000). When

Long Evans rats were exposed to TCDD on GD 15, they had fewer than half the number of TEB, as well as decreased epithelial elongation, reduced alveolar buds, and lateral and primary branches that persisted to PND 68, long after these mammary glands normally mature (Fenton *et al.* 2002). Mammary alterations were detected as early as PND 4, and the morphogenic changes seen in the maturing gland were found when TCDD exposure occurred at GD 15, but not at GD 21 or postnatally (Fenton *et al.* 2002). These observations further support a key role of gestational, rather than lactational, TCDD exposure on fetal reprogramming of the mammary gland that may result in mammary carcinogenesis in later life.

Human data on developmental effects of parental exposure to TCDD are available (Fenton 2006). Delayed breast development in Flemish adolescents was associated with premenarcheal TCDD exposure (Den Hond *et al.* 2002). Menstrual cycle length was increased with premenarcheal TCDD exposure in Italians of the Seveso Women's Health Study (SWHS), which monitors health outcomes in a cohort exposed to an industrial explosion resulting in the highest known population exposure to TCDD (Warner *et al.* 2004). Early life TCDD exposure is also associated with similar endocrine defects in rodent offspring. Maternal TCDD caused a 30% permanent reduction in body weight as well as altered and likely delayed estrous cyclicity in rodents, even in doses lower than those producing maternal effects (Fenton *et al.* 2002; Flaws *et al.* 1997; Gray and Ostby 1995; Wolf *et al.* 1999).

Overweight and obesity have also emerged as public health issues in children and adults worldwide (Adair 2004; Popkin and Doak 1998; Popkin and Udry 1998). Rising obesity prevalence is a concern for many reasons. The risk of life threatening diseases,

such as diabetes and cancer, is also increased in obese persons. An American Cancer Society (ACS) study found that overweight women are 60% more likely to die from breast cancer than normal weight women (Calle *et al.* 2003). ACS suggests that rising breast cancer incidence rates may reflect increased prevalence of obesity (Jemal *et al.* 2005; Society 2005). While diets high in fat and calories are associated with obesity, it is obesity and weight gain, and not any dietary factor, that consistently correlates with breast cancer risk (Michels *et al.* 2007). Here the links between obesity, environmental endocrine disruptors, and breast development and cancer are examined.

The timing of exposure to obesity, like that of E2, plays a modulatory role on breast cancer risk. Adipose tissue produces E2 by aromatizing androgens, and excess adipose increases peripheral E2 levels (Simpson 2003). Though adipose tissue produces E2, it is thought that adipose production of E2 is not a major source of E2 until after menopause when obesity increases susceptibility to breast cancer (De Assis and Hilakivi-Clarke 2006; Hankinson *et al.* 2004). Elevated steady state E2 levels in breast tissue may expose the breast to elevated E2-quinone metabolites, which can cause DNA damage. Further, obese woman with ER positive tumors have a poorer prognosis than ER negative tumor bearing obese women, although ER positive tumors are generally associated with a more positive prognosis (Maehle and Tretli 1996; Sorlie *et al.* 2001). This genetically susceptible group of obese women may be at a disproportional risk of breast cancer mortality because of the interaction of increased ER and its ligand, E2 (Lorincz and Sukumar 2006).

Unlike postmenopausal obesity, pre-menopausal-, adolescent-, and childhood-obesity is consistently associated with decreased breast cancer risk (Hankinson *et al.*

2004). Being overweight as a young woman may perturb the pituitary-hypothalamic axis, resulting in suppressed ovarian production of E2 and decreased ovulation frequency (De Assis and Hilakivi-Clarke 2006; Hankinson *et al.* 2004). This decrease in systemic E2 exposure is thought to lower risk of breast cancer among obese premenopausal women (Potischman *et al.* 1996). However, ovariectomized mice have shorter latency and higher incidence when obese than when lean (Hakkak *et al.* 2007). Further, prepubertal body mass index (BMI) correlates to BMI in young adults, and BMI in young adults correlates to BMI in adults (Weiderpass *et al.* 2004). Since adult weight gain also increases post-menopausal breast cancer risk, weight gain may play a key role in the transition from protection to danger of BMI on breast cancer risk (De Assis and Hilakivi-Clarke 2006). Thus it may be that extragonadal E2 production in obese young women that eventually contributes to increased breast cancer risk if weight gain continues into later life.

Increased breast cancer susceptibility associated with parental exposure to TCDD may be altered by obesity due to the altered pharmacokinetics and bioavailability of TCDD. Though TCDD is lipophilic, its distribution among rodent and human tissues is *Cyp1a2*- and dose-dependent (Emond *et al.* 2006). Higher *Cyp1a2* induction results in a greater portion of TCDD in liver relative to fat (Emond *et al.* 2004; Evans and Andersen 2000). Given the unique pharmacokinetics of TCDD in obese women, it is likely that they will have different physiological responses to TCDD.

In addition to the effect of obesity on TCDD distribution, obesity also slows TCDD elimination. The elimination of TCDD in obese women is slowed significantly with their increasing adiposity (Michalek and Tripathi 1999). Treatment of C57BL/6J mice with a high fat diet results in an elimination half-life of TCDD that is 2.4 and 1.4

times higher in liver and adipose, respectively, compared to those on a normal diet (DeVito *et al.* 2003). The dose of TCDD might further be reduced by early discontinuation of breastfeeding, which is a risk in woman with higher pre-pregnant BMI (Hilson *et al.* 2004).

Obesity may also impact the elimination of TCDD through milk. Shifts in the milk fat content associated with a high fat diet may alter the concentration and total levels of TCDD lactationally excreted. In a high fat context, one would expect higher fat milk, but this appears not to be the case (Aoki *et al.* 1999). Milk fat levels are not higher in obese women. TCDD does not get mobilized readily during milk production and is sequestered from mobilized adipose, potentially resulting in less TCDD delivered in obese women (Chao *et al.* 2005). This conclusion is supported by work in several mammal models. There is less dose-dependent TCDD induction of CYP1A2 in obesity-associated diabetic C57BL/6J mice than those on a normal diet without diabetes or obesity (Godin *et al.* 2003). Oral acute 30-day LD50s of TCDD are negatively correlated to total body fat content (Geyer *et al.* 1993). The authors suggest that storage of lipophilics, such as TCDD in adipose tissue acts as a detoxification mechanism by removing TCDD from tissues targeted for toxic action, *e.g.* liver and mammary organs. This implies that overweight and obese populations are less susceptible to TCDD toxicity than their normal weight counterparts.

In summary, mammary cancer is the second most common cancer among women, and its etiology consistently converges on E2 exposures from a variety of sources. The timing of exposure to endogenous- and exogenous-E2, obesity and TCDD also plays a key role in susceptibility to breast cancer. The interaction of environmental

susceptibilities is further complicated by genetics. Thus breast carcinogenesis results from a change in probability of mammary cancer occurrence through time, as the environment changes and the genetics respond to the changing environment.

This dissertation aims to examine environmental exposures, and how they modify the molecular and physical structure of the mammary gland. Specifically these exposures are maternal exposure to TCDD, lifelong exposure to high fat diet (HFD) and pubertal exposure to DMBA. The effects of these exposures were examined in the phenotypes of pubertal mammary gland development, mammary cancer incidence and pathology. Molecular changes potentially underlying these phenotypes were evaluated with particular emphasis on E2. The physiological effects, *e.g.* growth trajectories, adiposity and blood glucose levels, of HFD and TCDD were examined as potential causal intermediates for the mammary gland phenotypes. Finally, various interactions of these exposures and phenotypes were examined in several mouse strains and mouse models of breast cancer. The central hypothesis is that HFD and gestational exposure to TCDD interact to increase mammary cancer risk, through both structural remodeling of the pubertal gland and through action on E2. This hypothesis was primarily tested using the DMBA mouse model of breast carcinogenesis.

DMBA is a polyaromatic hydrocarbon (PAH) that is frequently used to induce tumors in skin and mammary glands. PAHs are typically found in cigarettes and air pollution and these exposures have been linked to breast cancer (Brody *et al.* 2007). DMBA weakly binds AhR, causing hepatic and mammary transcription of *Cyp1a1, Cyp1a2,* and *Cyp1b1* (Rowlands *et al.* 2001). Unlike TCDD, DMBA is a procarcinogen,

which through 7-methylhydroxylation, is activated by the P450s (Parkinson 2001). Both dietary constituents and TCDD have been shown to modify DMBA carcinogenesis.

In chapter 2, the ability of dietary fat and maternal TCDD to modify pubertal physiology and DMBA activation, via *Cyp1* transcription, is explored in a mouse strain relatively resistant to AhR-mediated toxicity. Here we also examine changes in pubertal mammary gland remodeling and blood glucose levels that occur due to maternal TCDD exposure in mice, on either a HFD or a low fat diet (LFD).

In chapter 3, the interaction of dietary fat and maternal TCDD exposure on metabolic syndrome in several mouse models of breast cancer is examined. Here, the body growth trajectory is modeled to explore the age at pubertal growth spurt, the rate of growth spurt across different models, and how these measures vary with HFD. Longitudinal change in blood glucose and percent body fat was also examined across diets and cancer models. Lastly, changes in blood triglycerides are examined to complete the evaluation into what extent TCDD and HFD induce metabolic syndrome in these cancer models.

In chapter 4, the interaction of dietary fat and maternal TCDD on mammary cancer is examined. The mechanisms of this interaction are explored. Pubertal mammary gland morphology is examined during cancer initiation and progression. Gene expression changes during cancer progression are also explored.

In conclusion, the interactive relationships between HFD and maternal TCDD exposure across metabolic and mammary phenotypes are highlighted. Exploration of the remaining questions ensues, with particular emphasis on future research in order to begin generalizing our observations to human hazard models for the overweight population.

Figure 1-1. Mechanisms of breast carcinogenesis: initiation and promotion (Cavalieri and Rogan 2004; DiGiovanni *et al.* 1986; Mitrunen and Hirvonen 2003). The effects of high-fat diet and maternal TCDD seen in this dissertation are also shown. COMT denotes catechol-O-methyltransferase, CYP Cytochrome P450 Family, E2 17b-estradiol, ER Estrogen Receptor, OH-E2 hydroxyestradiol, Q semiquinone, SQ seminquinone.

E2

E2 metabolism　　**TCDD & HD**　　**E2 activation**
　　　　　　　　　　　　　　　　　　　E2-ERα bind

CYP1A1　　　　CYP1B1

　　　　　　　　　　　　　　　　　　DNA promoter binding　MAPK signal
2-OH-E2　　4-OH-E2　　　　by E2-ERα complex　amplification
COMT　　　　　　COMT

2-Me-E2　　　　　4-Me-E2　　　　Cyclin D1　　　　C-myc
　　　　　　　　　　　　　　　　　transcription　　　transcription

E2-2,3-SQ　　E2-3,4-SQ
Oxidative　　Redox　　Oxidative　　　　Transition to S phase
stress　　　cycling　　stress
E2-2,3-Q　　E2-3,4-Q

　　　　　　　　　　　　　　　　　Increased cell proliferation

Stable　　　Depurinating
DNA adducts　DNA adducts　　　　　**Mitotic cancer promotion**

Genotoxic cancer initiation

Figure 1-1.

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CHAPTER 2

DIETARY FAT ALTERS PUBERTAL BODY COMPOSITION AND P450

INDUCTION FOLLOWING MATERNAL TCDD EXPOSURE IN DBA/2J MICE[1]

ABSTRACT

The increased prevalence of obesity associated with increased fat intake may

make obese individuals uniquely susceptible to the effects of lipophilic aryl hydrocarbon

receptor (AhR) ligands, like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 7,12-

dimethylbenz[a]anthracene (DMBA). TCDD exposure is known to increase risk of type

II diabetes and to alter mammary differentiation and cancer. To investigate the

consequences of dietary fat and maternal TCDD exposures on peripubertal body

composition and hepatic P450 expression, we examined the susceptibility of progeny

from DBA/2J (D2) dams exposed to TCDD on two diets. Pregnant D2 were dosed at

mid-gestation with 1 μg TCDD/kg body weight or vehicle, and at parturition placed on

high- (HFD) or low-fat diet (LFD). On post-natal day 35, female progeny were weighed

and body composition determined before dosing with DMBA, a rapidly metabolized AhR

ligand, or vehicle. Fasting blood glucose was measured and liver and mammary glands

collected for real time PCR and whole mount analyses. HFD increased body mass and

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[1] Bittu S. Kuruvilla preformed hepatic RNA isolation, rtPCR, and real time PCR.

body fat, induced precocious mammary gland development, and increased *ahr*, relative to LFD. Maternal TCDD exposure increased fat pad length, *Cyp1a1*, and *Cyp1b1* hepatic transcripts in the progeny. Maternal TCDD exposure decreased the size of terminal end buds, and pubertal DMBA exposure decreased their number. Diet modified the effects of maternal TCDD- and pubertal DMBA-exposures. Only in D2 progeny fed HFD did TCDD increase blood glucose and size of mammary fat pad, and decrease both branch elongation and the number of terminal end buds. DMBA depressed *Cyp1b1* only in D2 fed HFD. We conclude only D2 progeny fed HFD were at risk for developmental TCDD and DMBA exposure effects on pubertal blood glucose levels, mammary differentiation, and hepatic *Cyp1b1* at these doses and developmental stages. These diet by aryl hydrocarbon ligand exposure interactions impact major endocrine systems involved in pubertal maturation.

INTRODUCTION

Increased prevalence of childhood and adult obesity contributes to elevated risk for metabolic syndrome, precocious mammary development, and breast cancer (Carmichael 2006; Himes *et al*. 2004 Carmichael, 2006 #426; Hotamisligil 2006; Ogden *et al*. 2006). Total lifetime estrogen (E2) exposure increases mammary cancer risk (De Assis and Hilakivi-Clarke 2006). E2 produced by adipose tissue is an underlying mechanism by which obesity may contribute to early puberty, and if obesity and weight gain extends into adulthood, increased post-menopausal breast cancer risk (Britton *et al.*

2004; De Assis and Hilakivi-Clarke 2006; Hakkak *et al.* 2007; Simpson 2003; Stoll 1998).

In addition to contributing to obesity, consumption of animal-based foods, frequently associated with high fat diets, is correlated with human serum and milk levels of dioxins (Goldman *et al.* 2000; LaKind *et al.* 2004). Increased food intake, particularly from a high fat diet (HFD), may alter breast cancer risk through higher intake of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). There is some evidence of TCDD decreasing breast cancer risk *in vitro* (Wang *et al.* 1998; Wormke *et al.* 2003), and *in vivo* (Holcomb and Safe 1994). However, human data indicates that TCDD increases mammary cancer risk. Women in Seveso, Italy who have elevated TCDD in their serum due to an industrial accident have a dose-dependent increase in breast cancer incidence (Warner *et al*. 2002). Breast cancer incidence is also increased among Russians with elevated dioxin exposure (Revich *et al*. 2001).

Although infants are exposed to dioxins at low doses in breast milk worldwide, few studies have examined developmental toxicity, or evaluated incidences of adult cancer (Ayotte *et al.* 1996; Gladen *et al.* 2000; LaKind *et al.* 2004). However, several animal studies have explored this relationship. Sprague Dawley rats gestationally exposed to TCDD developed a higher frequency of 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary adenocarcinomas (Brown *et al.* 1998). Exposure to TCDD on post-natal day (PND) 18 also increases the incidence of N-nitroso-N-methylurea-induced mammary tumors in Sprague Dawley rats (Desaulniers *et al.* 2001).

Polyhalogenated aromatic hydrocarbons (PHAH) and polycyclic aromatic hydrocarbons (PAH), like TCDD and DMBA, respectively, bind the aryl-hydrocarbon

receptor (AhR) to induce cytochrome P450, polypeptide 1 (CYP1) enzymes (Nebert *et al.* 2004). Both CYP1B1 and CYP1A1 enzymes work in parallel to biotransform estradiol and DMBA into mutagenic metabolites (Cavalieri and Rogan 2004; DiGiovanni *et al.* 1986). These alterations in estrogen and DMBA metabolism have been proposed as a mechanism for carcinogen-induced mammary cancer initiation (Cavalieri and Rogan 2004; Zhu *et al.* 2008). Additionally, AhR has both pro- and anti-estrogenic cross talk with the estrogen receptor (ER) (Ohtake *et al.* 2003; Safe *et al.* 2000).

These AhR-ER actions are further complicated by greater TCDD stores in the adipose of obese- compared to lean- individuals. The elimination of TCDD in obese women is slowed significantly with their increasing adiposity (Michalek and Tripathi 1999) because of a larger volume of TCDD distribution (Emond *et al.* 2006). Treatment of C57BL/6J mice with a high fat diet resulted in elimination half-life of TCDD 2.4 and 1.4 times longer in liver and adipose respectively, compared to those on a normal diet (DeVito *et al.* 2003).

Endocrine disruption by dietary fat and AhR ligands, TCDD and DMBA, may also increase breast cancer risk through changes in mammary gland morphology. Dietary fat induces precocious breast development (Britton *et al.* 2004). This may occur through adipose-driven activity of ERα and EGFR, receptors involved with pubertal mammary morphogenesis (Ciarloni *et al.* 2007; Howlin *et al.* 2006). In 55-day-old female rats, DMBA increased the number of terminal end buds and their mitotic activity (Russo and Russo 1978). It was thought that increased proliferation in the terminal end buds decreased the effectiveness of DNA repair and checkpoint responses to result in the formation of adenocarcinomas (Russo and Russo 1978). Conversely, gestational exposure

to TCDD disrupts the differentiation of mammary glands in female rat progeny (Fenton *et al*. 2002; Vorderstrasse *et al*. 2004). Further, as serum dioxin concentrations increased in girls, their pubertal breast development was delayed (Den Hond *et al*. 2002). This may lengthen the temporal window in which glands are susceptible to insults, and ultimately lead to an increased instance of mammary tumor growth when adults (Birnbaum and Fenton 2003; Brown *et al*. 1998; Desaulniers *et al*. 2001).

In this study, we sought to examine how maternal TCDD exposure interacts with dietary fat to influence postnatal physiology of DBA/2J (D2), who have a low affinity for AhR which makes them less sensitive to TCDD and non-responsive to PAH induction (Chapman and Schiller 1985; Moriguchi *et al.* 2003). We determined the contributions of both maternal TCDD exposure and dietary fat on phenotypes such as alterations in body mass, percentage of body fat and fasting glucose levels. Maternal TCDD slows mammary epithelial growth in rat offspring, and HFD and pubertal DMBA are known to accelerate this process. We addressed to what extent dietary fat and developmental exposures impact mammary glands during puberty. These studies also explored whether maternal TCDD exposure, dietary fat and DMBA influence mRNA expression involved with mammary gland differentiation and AhR ligand- and estrogen- metabolism.

MATERIALS AND METHODS

Chemicals. TCDD (99.9% pure, Ultra Scientific, North Kingstown, RI) and DMBA (98% pure, Sigma-Aldrich, St. Louis, MO) were obtained commercially. Both chemicals were dissolved in 95%/5% olive oil/toluene by volume (Sigma-Aldrich).

DMBA and TCDD were dosed from 25 mg/ml and 500 ng/ml stock concentrations, respectively. These corresponded to 2.4 μl DMBA solution/g mouse and 1.8-1.9 μl TCDD solution/g mouse.

Mice and dosing. D2 nulliparous mice (Jackson Laboratory, Bar Harbor, ME) were time bred and dosed with 1 μg/kg of TCDD or vehicle control by gavage at 12.5 days post coitus, which corresponds to the time when fetal mammary fat pads are developing (n = 24 dams). On post-natal day (PND) 0, dams were changed from 5058 chow (Purina) to a high-fat diet (HFD; 45% of total kcal from fat and 35% of total kcal from carbohydrate; D12451, Research Diets, NJ; n = 12 dams) or a low-fat matched control diet (LFD; 10% of total kcal from fat and 70% kcal from carbohydrate; D12450B, Research Diets; n = 12 dams). Diets had the same percentage of protein, and diet differences were achieved by increased maltodextrin and lard, and decreased cornstarch and sucrose in HFD compared to LFD (400, 1598, 291, 691 kcal vs. 140, 180, 1260, 1400 kcal respectively). The LFD has fat levels that fall into the range often found in standard rodent chows. Mice had *ad libitum* access to feed and water. Female pups were weaned at PND 21, ending any lactational exposure to TCDD, but continuing their LFD and HFD exposures. Offspring were dosed with 60 mg/kg of DMBA (n = 12 litters) or vehicle (n= 12 litters) by gavage on PND 35, when developing pubertal mammary glands are known to be sensitive to carcinogen exposure. The experimental design in shown in Figure 2-1. Mice were euthanized 24 hours later by carbon dioxide asphyxiation. All animal experiments were approved by the UNC Institutional Animal Care and Use Committee and were performed in an AAALAC-accredited vivarium.

Metabolic endpoints. On PND 0, body weights were assessed by weighing the entire litter, and determining the average pup weight. Individual body weights were measured at PND 4, 7, 10, 14, 18, 21, and 35. On PND 35, percent body fat was measured with a Lunar PIXImus dual-energy X-ray absorptiometry (DEXA) scanner using isofluorane anesthesia (GE Lunar PIXImus Corporation, Madison, WI). Blood glucose levels were measured from tail blood on PND 36 using a FreeStyle blood glucose kit after a 24 hour fast (Abbott Laboratories, Abbott Park, Il).

Histology. Inguinal mammary glands from PND 36 were fixed and stained with Carmine Alum to detect terminal end buds and branch elongation according to published methods (Fenton *et al*. 2002).

Molecular analyses. At PND 36, median liver lobes and inguinal mammary glands were dissected, homogenized and RNA extracted. The High Capacity cDNA archive kit (ABI) was used to generate cDNA for PCR analysis (Applied Biosystems, Foster City, CA). Real time PCR was performed to assess relative transcript levels of *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Areg*, *Ereg*, *Ahr*, *Egfr* and *Esr1* using Assays-on-Demand (Applied Biosystems) with *Gusb* and *Actb* as endogenous controls in hepatic and mammary tissues, respectively.

Statistical analyses. All data analyses were performed using SAS 9.1.3 (Cary, NC). The litter median of female progeny traits was used as the unit of TCDD analyses

to control for potential maternal exposure bias, although the result trends were identical when analyzing data from each pup individually or for litter means. Three litters (LFD + vehicle + vehicle, LFD +vehicle + DMBA, and HFD+TCDD+DMBA) had no females. Because of this imbalance in the design, the generalized linear model (Proc GLM) was used to evaluate the effect of these relationships on phenotypes (e.g. body weight, percent body fat, fasting blood glucose, branch elongation, fat pad length, number of terminal end buds, size of terminal end buds, and fold change of *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Areg*, *Ereg*, *Ahr*, *Egfr* and *Esr1* levels) (Livak and Schmittgen 2001). All Proc GLM analyses modeled phenotypes with additive main effects (TCDD, diet, DMBA) along with all two-way interactions and the three-way interaction of the main effects. Any significant interactions were explored with stratified analyses. Using the *LSMEANS* option of the Proc GLM, the multivariate geometric means were determined to be significantly different at unadjusted $p < 0.05$ across a limited number of *a priori* contrasts. This conservative approach was taken because of small sample size and imbalance between some contrasts, e.g. fewer exposed to DMBA than vehicle.

RESULTS

Maternal TCDD exposure alters body composition and glucose of progeny.
TCDD did not have any effects on either body mass or adiposity (data not shown), and because these measurements were made prior to DMBA exposure, body mass and adiposity were evaluated on individual mice to examine diet effects. HFD differentially influenced post-natal growth compared to LFD as early as PND 4 ($p < 0.05$, n = 27 and

31 mice respectively, Fig. 2-2A). Concordantly, pubertal D2 adiposity was 28.9% higher among those fed HFD (25.6%, n = 26 mice) relative to LFD (19.9%, n = 28 mice; $p < 0.001$; Fig. 2-2B). Although D2 progeny weigh more and have more body fat on a HFD than LFD, HFD (n = 11 litters) did not significantly alter blood glucose compared to those progeny fed LFD (n = 10 litters). Yet among D2 on HFD, TCDD heightened fasting blood glucose 59.3% over vehicle-treated D2 ($p < 0.05$, n = 5 and 6 litters respectively, Fig. 2-2C).

Pubertal mammary gland growth is suppressed in female progeny by maternal TCDD exposure. D2 pubertal mammary glands were analyzed for susceptibility to maternal TCDD exposure and diet (Fig. 2-3). TCDD significantly increased fat pad length relative to mammary glands from progeny of vehicle-treated dams ($p < 0.05$, n = 11 and 10 litters respectively, Fig. 2-3A). Relative to maternal vehicle, TCDD significantly decreased branch elongation ($p < 0.05$, n = 10 and 11 litters respectively, Fig. 2-3B), the number of terminal end buds ($p < 0.05$, Fig. 3B), and the size of terminal end buds ($p < 0.01$, n = 10 and 11 litters respectively, Fig. 2-3C). Similar to maternal TCDD, pubertal DMBA exposure reduced the number of terminal end buds by 50% below the number of terminal end buds seen in vehicle exposed D2 (2.4 +/- 0.8 and 5.0 +/- 0.6 respective means +/- SE, n = 10 and 11 litters respectively, $p < 0.05$, data not shown). This was the only DMBA effect seen in mammary glands. D2 females maintained on HFD showed significant increases in branch elongation and number of terminal end buds compared to those maintained on LFD ($p < 0.01$, n = 11 and 10 litters

respectively, Fig. 2-3B-C). However relative to LFD (n = 10 litters), HFD (n = 11 litters) was not associated with any changes in terminal end buds size or fat pad length.

Branch elongation and the number of terminal end buds were also modified by TCDD and dietary fat interactions. Among D2 maintained on HFD, maternal TCDD decreased branch elongation and the number of terminal end buds significantly less than vehicle- predicted by an additive model (p < 0.05, n = 5 and 6 litters respectively, Fig. 2-3B-C). However among D2 maintained on LFD, maternal TCDD exposure had no different branch elongation and number of terminal end buds than vehicle (n = 6 and 4 litters respectively, Fig. 2-3B-C).

Pubertal hepatic Cyp1 *expression is elevated by maternal TCDD exposure.* Maternal TCDD (n = 11 litters) increased *Cyp1a1* and *Cyp1b1* mRNA at puberty relative to vehicle exposure (p < 0.01, n = 10 litters, Fig. 2-4A-B). HFD increased *Ahr* expression significantly over LFD (p < 0.05, n = 11 and 10 litters respectively, Fig. 2-5A). DMBA and diet had no significant main additive effects on *Cyp1b1*, but they significantly interacted with each other. When D2 were maintained on HFD, DMBA significantly reduced *Cyp1b1* induction relative to its vehicle (p < 0.05, n = 5 and 6 litters respectively, Fig. 2-5B). However, when D2 were maintained on LFD, DMBA had no effect on *Cyp1b1* induction. These diet-dependent differences of *Cyp1b1* induction were significant (p < 0.05, Fig. 2-5B).

Increased *Cyp1* induction is indicative of AhR activation. AhR, EGFR, and ER signaling pathways interact in mammary morphogenesis and carcinogenesis (Buters *et al*. 1999; Ciarloni *et al*. 2007; Patel *et al*. 2006), However, no effects of developmental

chemical exposures or diet were observed on the expression of *Areg, Ereg, Egfr, Esr1,*

Cyp1a1, or Cyp1b1 in D2 mammary glands (data not shown).

DISCUSSION

TCDD causes a broad range of toxic effects yet its mechanisms are only partially

understood and likely dependent upon many variables such as dose, developmental stage

of exposure, and possibly diet. In the present study we treated D2 with 1 μg/kg of TCDD,

which is a very low dose in D2 (Poland and Glover 1980). We focused on two variables

potentially affecting susceptibility to this early life TCDD exposure, DMBA and diet,

since it is likely that an individual's susceptibility to the effects of TCDD exposure is

influenced by interactions with other environmental factors (Han *et al.* 2004b; Hakkak *et*

al., 2007; Thomsen *et al.* 2006).

Transient increases and decreases in body weight have been seen in rodents

exposed to TCDD during gestation or adulthood, with typical- or high fat- diets

(Desaulniers *et al.* 2001; Thiel *et al.* 1994; Zhu *et al.* 2008). Further, paternal exposure to

Agent Orange had no effect on birth weight (Lawson *et al.* 2004), and we did not detect

any transient or prolonged depression of body mass or fat due to 1 μg TCDD/kg dam

exposure in D2.

The amount of TCDD actually accumulated in the mammary gland of D2 mice on

HFD is influenced by several findings. Increased adiposity slows TCDD elimination thus

extending its $T_{1/2}$ (DeVito *et al.* 2003; Michalek and Tripathi 1999). Also, the larger fat

pads of HFD- and TCDD-exposed D2 sequester a higher cumulative dose of TCDD than

those on LFD (Hoppe and Carey 2007). The higher target-organ dosage and slower elimination of 1 μg TCDD/kg dam in D2 progeny on HFD may exceed the minimum TCDD dose required to activate signaling of the growing mammary epithelium.

Maternal TCDD exposure and HFD interacted in a significantly non-additive manner on both fasting blood glucose and mammary development at puberty. HFD may have enhanced TCDD effects on glucose, branch elongation, and the number of terminal end buds. Alternatively, maternal TCDD could have enhanced HFD effects on fasting blood glucose while depressing HFD effects on mammary gland development. The simplest explanation is that HFD enhanced maternal TCDD effects through interaction on estrogen and its complex milieu of signaling. Evidence of TCDD depressing HFD diet effects on mammary toxicity is limited because the timing of exposure is so critical in endocrinology. TCDD is known to decrease adipogenesis in the high dose wasting syndrome (Phillips *et al.* 1995). This could indirectly reduce HFD-induced precocious gland development, but here maternal TCDD did not significantly change adiposity of D2. Instead maternal TCDD increased the size of the fat pad.

Obesity is frequently comorbid with type II diabetes. The risk of insulin resistance, incidence of type II diabetes, and diabetes-associated mortality are linked to low TCDD exposure in several epidemiological studies (Consonni *et al*. 2008; Cranmer *et al*. 2000; Henriksen *et al*. 1997). Only one study demonstrated an interaction between overweight and TCDD on type II diabetes risk (Fujiyoshi *et al.* 2006). Impaired glucose tolerance and adiposity are risk factors for type II diabetes in humans (Laspa *et al.* 2007). At 1 μg TCDD/kg dam, our impaired glucose tolerance data support the adiposity x TCDD interaction seen in Vietnam veterans (Fujiyoshi *et al.* 2006). It remains unclear

whether the interaction of HFD x maternal TCDD on fasting blood glucose reflects a TCDD- increase in HFD effects, or vice versa. The potential interaction of maternal TCDD and HFD on type II diabetes risk should be further investigated.

The increase in fasting blood glucose due to maternal TCDD and diet together may also increase risk for aberrant puberty through hyperinsulineaemia. Pubertal hyperinsulineaemia is common but can persist into adult life (Stoll 1998). Normally during puberty insulin stimulates the synthesis of E2 in ovaries. The IGFR hormone pathway may further stimulate mammary growth through crosstalk with the ER hormone pathway (Stoll 1998). However, hyperinsulineaemia alters steroidogeneis in ovaries to change the E2 balance (Stoll 1998). Pubertal breast development is enhanced in overweight girls who eat diets high in polyunsaturated fats (Britton *et al.* 2004). Consistent with these observations, we found HFD caused precocious mammary development. Yet the precocious gland development was substantially reduced by both HFD and maternal TCDD exposure, in the same female offspring that had elevated fasting blood glucose. While this reduction in mammary growth was no further than that seen in vehicle treated D2 mice on LFD, gestational exposure to TCDD imprints on mammary gland morphology into adulthood (Fenton *et al.* 2002). This is consistent with delayed adolescent breast development seen correlated to increased serum TCDD levels in peripubertal girls (Den Hond *et al.* 2002).

Estrogenic activity of adipose tissue also may be advancing the growth of the gland as well as enhancing signaling between the mammary epithelium, fat pad and TCDD (De Assis and Hilakivi-Clarke 2006). Consequently, adipose-dependent aromatase differences may be contributing to the mammary gland phenotypes we observed. In a

higher adipose environment, there is greater aromatization of estrogen in peripheral adipose tissue such as mammary fat pads (Cheshenko *et al*. 2007; Lorincz and Sukumar 2006; Simpson 2003). TCDD can downregulate aromatase in a high E2 environment, yet will not alter aromatase levels at basal E2 levels (Cheshenko *et al*. 2007). Based on these findings, a HFD-dependent action of TCDD on mammary development could occur through direct and indirect actions of HFD and TCDD on aromatase.

Ahr transcription is downregulated during adipogenesis (Shimba *et al.* 2003). The fact that basal *Ahr* was higher in D2 maintained on HFD indicates that elevation of *Ahr* expression is likely a consequence of weight gain, and not a casual mechanism. AHR signaling involves crosstalk with ERα and EGFR, receptors that are upregulated in overweight individuals (Lorincz and Sukumar 2006; Moral *et al*. 2003). Each of these AHR-mediated signaling pathways also interacts with mammary morphogenesis during puberty (Howlin *et al*. 2006). Although several groups demonstrated a role for EGFR and its ligands in both mammary morphogenesis and in AHR-mediated TCDD activity (Ciarloni *et al*. 2007; Howlin *et al*. 2006; Patel *et al*. 2006), we found no change in *Esr1*, *Egfr* or the EGFR ligand genes *Areg* and *Ereg* in the mammary gland.

In addition to cross talk with E2-mediated pathways, AHR facilitates *Cyp1a1* and *Cyp1b1* induction by TCDD. While neither *Cyp1a1* nor *Cyp1b1* was altered in the mammary gland, at 1 μg TCDD/kg dam we saw a modest increase in D2 hepatic *Cyp1a1* and *Cyp1b1* progeny at puberty. While this may not translate to changes in protein activity, TCDD may be altering the metabolism of DMBA and E2. Because D2 are non-responsive to PAH induction, without this maternal TCDD exposure, DMBA would not be metabolically activated (Chapman and Schiller 1985; Moriguchi *et al*. 2003).

However, if increased *Cyp1a1* and *Cyp1b1* message by maternal TCDD translates to their increased protein activity, DMBA could be metabolically activated (Chapman and Schiller 1985; Moriguchi *et al.* 2003). This implies that maternal exposure to TCDD could cause DMBA-induced mammary carcinogenesis in mice not typically susceptible to DMBA-induced mammary carcinogenesis.

Maternal TCDD could indirectly decrease E2 by increasing its metabolism. In the liver and mammary glands, CYPA1A1 and CYP1B1 generate the catechols 2-hydroxyestradiol (2OH-E2) and 4-hydroxyestradiol (4OH-E2) from E2, respectively (Tsuchiya *et al.* 2005). Recent evidence suggests that TCDD-stimulated production of these catechols is increased further in mice fed HFD (Zhu *et al.* 2008). By increasing body weight gain and adiposity, HFD can indirectly contribute to increased number of terminal end buds, local E2, and mammary tumor incidence (Hakkak *et al.* 2007; Hilakivi-Clarke *et al.* 1997; Mizukami *et al.* 1992). It may be that upregulation of hepatic *Cyp1a1* and *Cyp1b1* by maternal TCDD reduces the levels of E2 in the mammary gland, especially in mice fed HFD. Increased estrogen metabolism and less E2 may be a mechanism of TCDD-decreased mammary growth (Ciarloni *et al.* 2007; Howlin *et al.* 2006) and cancer risk (Zhu *et al.* 2008) in adult mice.

While maternal TCDD could reduce E2 levels and thus decrease risk of mammary cancer, upregulation of *Cyp1a1* and *Cyp1b1* in female progeny by 1 μg TCDD/kg dam may also increase risk of mammary carcinogenesis. If increased CYP1A1 and CYP1B1 levels are not met with concurrent increases in other E2-metabolizing enzymes, mutagenic-E2 metabolites may increase breast cancer risk (Cavalieri and Rogan 2004; Cavalieri *et al.* 1997). Upregulation of *Cyp1a1* and *Cyp1b1* in female progeny by

maternal TCDD may also increase risk of mammary carcinogenesis through interaction with pubertal DMBA exposure. CYP1A1 genotype-stratified analyses demonstrate a stronger association between exogenous AHR ligands and breast cancer when CYP1A1 is more active (Ambrosone *et al*. 1995; Li *et al*. 2005a). This hypothesis is consistent with the increased susceptibility to DMBA-carcinogenesis in rats after perinatal TCDD exposure (Brown *et al.* 1998; Desaulniers *et al.* 2004).

Since delayed breast development is closely associated with delayed menarche, a well-known protection against breast cancer (Kadlubar *et al*. 2003), one might expect delayed or slowed breast differentiation to protect against breast cancer. Yet evidence suggests that by disrupting mammary gland differentiation, early TCDD exposure extends the period that mammary glands are most susceptible to mutagenic insults (Birnbaum and Fenton 2003; Brown *et al.* 1998; Desaulniers *et al.* 2001; Fenton *et al.* 2002). In humans, rats, and now mice, evidence supports that some prepubertal TCDD exposures may delay mammary differentiation and enhance susceptibility to adult mammary carcinogenesis.

We saw a similar apparent delay in differentiation with DMBA treatment. Mammary tumors are correlated with the number of terminal end buds at the time of DMBA dosing (Russo and Russo 1978). If this DMBA is the only pubertal exposure in female progeny, TCDD-decreased number of terminal end buds may protect them from breast cancer. If they are exposed again, or perhaps chronically, it may increase risk of breast cancer (Birnbaum and Fenton 2003; Brown *et al*. 1998; Desaulniers *et al*. 2001). The potential interaction of maternal TCDD and HFD on the risk of mammary cancer in female progeny should be further investigated.

DMBA and TCDD represent PAHs and PHAHs, respectively, that are ubiquitous chemicals in the environment, and that frequently occur as mixtures in human and environmental samples. Because of the prevalence of TCDD exposure and elevated adiposity in humans, it is likely that everyone has some TCDD tissue burden. Women that have been exposed to TCDD may transfer TCDD to their children through maternofetal transfer and breast feeding, further increasing the risk of exposure to environmental mediators of breast cancer during developmental windows of susceptibility. As this study reports the third species to show TCDD inhibition of pubertal mammary growth, reduced mammary development in female progeny may be a generalized effect of maternal TCDD exposure.

FIGURE LEGENDS

Figure 2-1. Schematic diagram of treatment groups (top) and timeline (bottom). Impregnated D2 mice were treated with 1 µg/µl TCDD or 95%/% olive oil/toluene (vehicle) at 12.5 days post coitus. Litters were put on HFD or LFD at PND 0 and weaned at PND 21 continuing on same diets. On PND 35, mice were treated with 60 mg/kg DMBA or vehicle, fasted, weighed, and measured for percent body fat. Twenty-four hours later, blood glucose was measured and inguinal mammary and median liver lobes harvested.

Figure 2-2. Diet and maternal TCDD exposure effects metabolic phenotypes. (A) The effect of diet on post-natal D2 body weights. Symbols represent means +/- SEM, where at PND 4 and later, * indicates $p < 0.05$ between diets. D2 ranged from 27 and 31 mice at PND 0 to 26 and 28 mice at PND 35 for HFD and LFD respectively. (B) HFD (n = 26 mice) increases percent fat at PND 35 relative to LFD (n = 28 mice), where * indicates $p < 0.0001$. (C) Fasting blood glucose is increased by HFD and maternal TCDD (strips) in female progeny at PND 36. Vehicle is solid, Means +/- SEM, where * indicates $p < 0.05$

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for HFD and vehicle (n = 6 litters) vs. HFD + TCDD (n = 5 litters). Because diet, but not TCDD, changed body weight and percent body fat, these analyses were done on individual mice.

Figure 2-3. Mammary gland morphology is altered by maternal TCDD and HFD. Inguinal mammary whole mounts were made of glands removed from vehicle- and TCDD- treated D2 on HFD or LFD at PND 36.   Whole mounts were stained with carmine alum, blinded, and measured for (A) fat pad length, (B) branch elongation, (C) # of terminal end buds, and (D) largest terminal end bud. Means +/- SEM are shown for LFD and vehicle (n = 4 litters), LFD and TCDD (n = 6 litters, strips), HFD and vehicle (n = 6 litters), HFD and TCDD (n = 5 litters, strips) treatment groups. Significant diet- and TCDD x diet-effects are distinguished by * p < 0.05.

Figure 2-4. Maternal TCDD significantly increases pubertal hepatic *Cyp1* message. RNA was extracted from median liver lobes. Messages were assessed using quantitative real time PCR, with *Gusb* as an endogenous control. Means +/- SEM are shown, where levels are distinguished by p < 0.01. (A) Induction of *Cyp1a1* message by TCDD compared to vehicle (n = 11 and 10 litters respectively), pooled across diet and DMBA groups. (B) Induction of *Cyp1b1* messages by TCDD compared to vehicle (n= 11 and 10 litters respectively), pooled across diet and DMBA groups.

Figure 2-5. Diet and DMBA significantly alter hepatic gene expression. RNA was extracted from median liver lobes. Messages were assessed using quantitative real time PCR, with *Gusb* as an endogenous control. Means +/- SEM are shown, where levels are distinguished by * p < 0.05. (A) Induction of *Ahr* message is increased by HFD relative to LFD (n = 11 and 10 litters respectively), pooled across TCDD and DMBA groups. (B) Induction of Cyp1b1 message by DMBA decreases below vehicle in HFD-, but not LFD-fed D2, where groups are LFD and vehicle (n = 5 litters), LFD and DMBA (n = 5 litters), HFD and vehicle (n = 6 litters), HFD and DMBA (n = 5 litters) treatment groups.

TCDD

No TCDD

HFD

LFD

DMBA

No DMBA

		DMBA	
		Body fat	Blood glucose
	Diet	Pups	Body wt	Mammary glands
TCDD	Assigned	Weaned	Fast	Liver

12.5 dpc   PND 0   PND 21   PND 35   PND 36
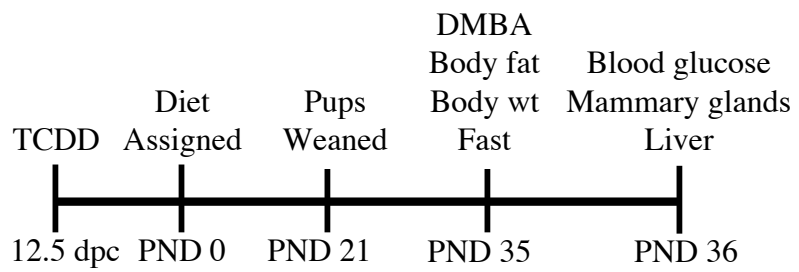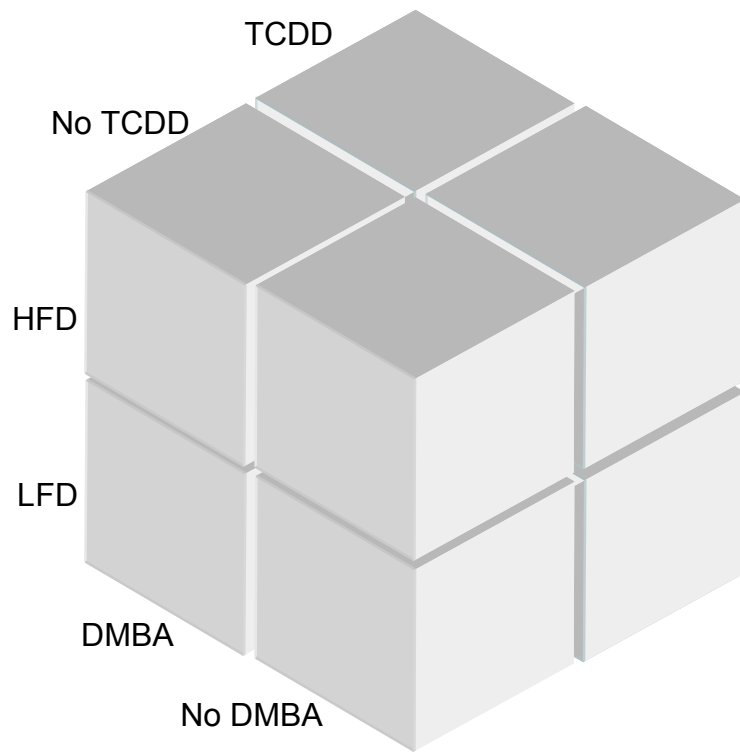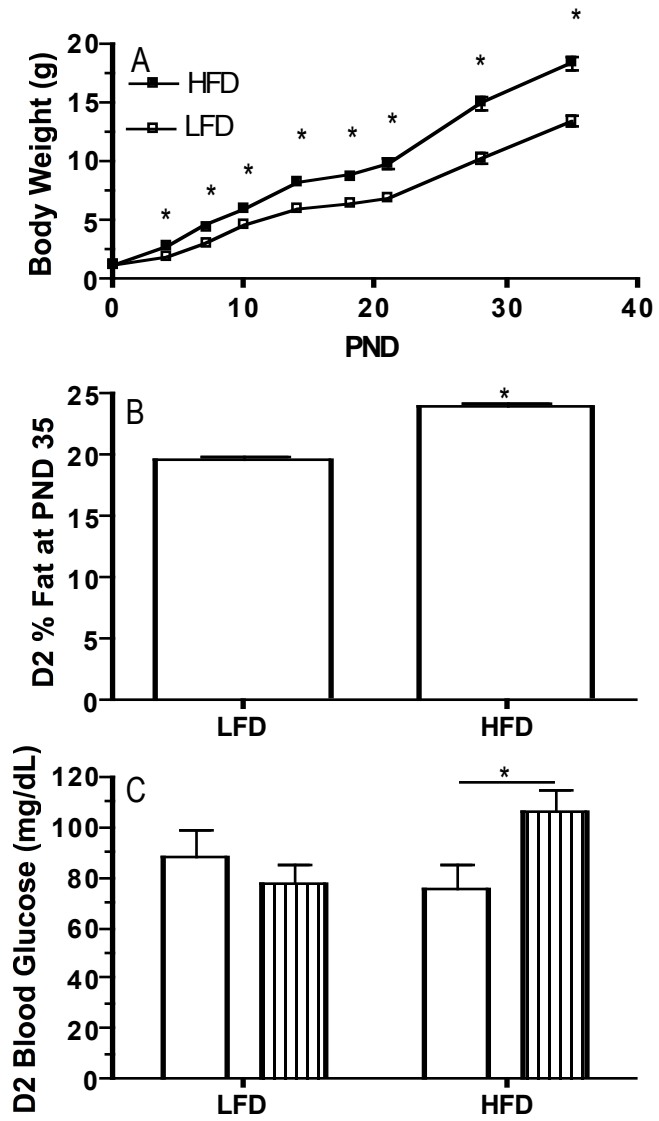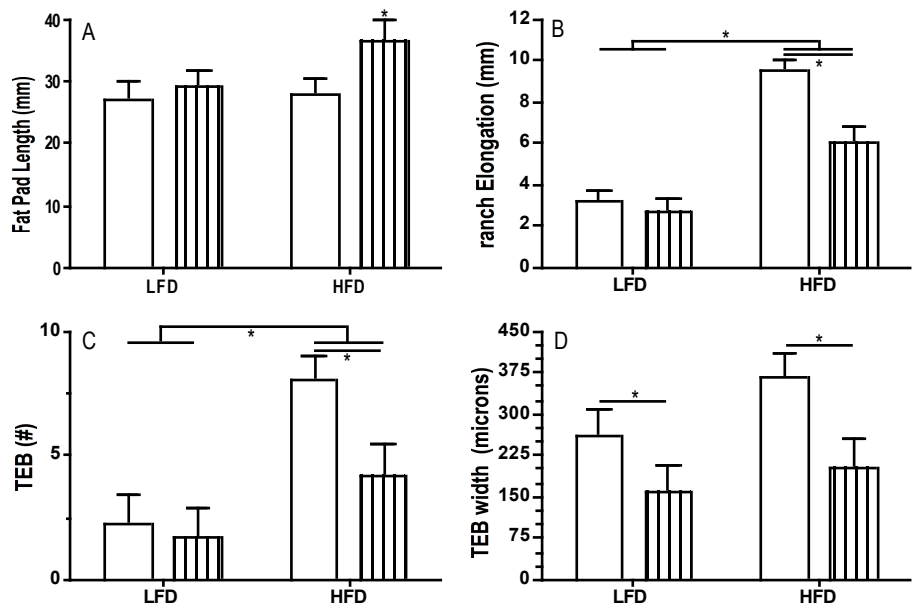
Figure 2-1.

Figure 2-2.

Figure 2-3.
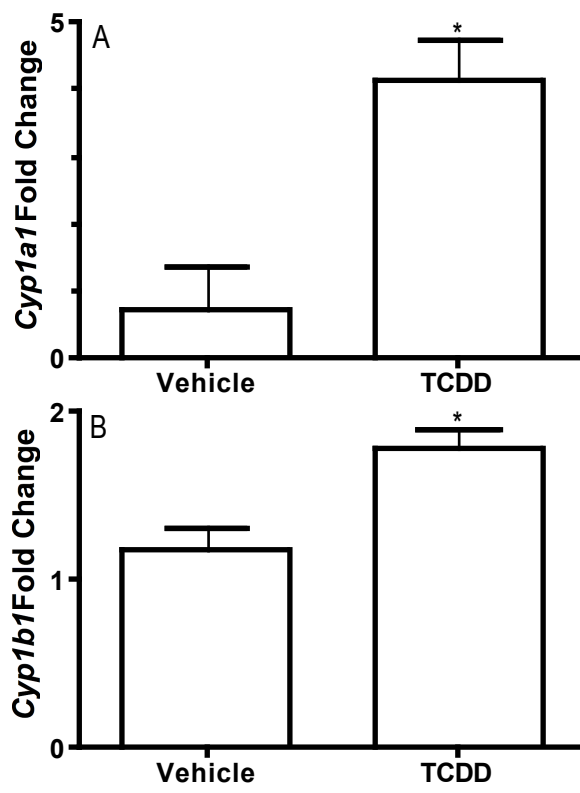
Figure 2-4.
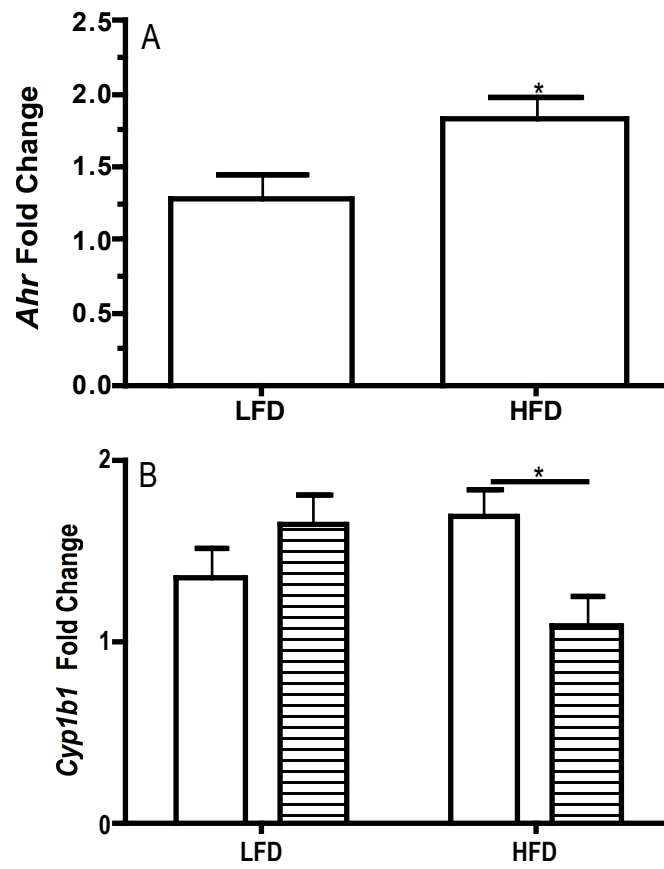
Figure 2-5.

CHAPTER 3

MOUSE BREAST CANCER-MODEL DEPENDENT CHANGES IN

METABOLIC SYNDROME-ASSOCIATED PHENOTYPES CAUSED BY

MATERNAL TCDD EXPOSURE AND DIETARY FAT[1]

ABSTRACT

Diets high in fat are associated with increased susceptibility to obesity and metabolic

syndrome. Increased adipose tissue caused by high fat diets (HFD) may result in altered

storage of lipophilic toxicants like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and

susceptibility to cancer. Since both TCDD and HFD are associated with increased breast

cancer risk, we examined their effects on metabolic syndrome-associated phenotypes in

three mouse models of breast cancer: 7,12-dimethylbenz[a]anthracene (DMBA),

Tg(MMTV-Neu)202Mul/J (HER2) and TgN(MMTV-PyMT)634Mul/J (PyMT), all on an

FVB/N genetic background. Pregnant mice were dosed with 1 μg/kg of TCDD or vehicle

on gestational day 12.5, and at parturition placed on HFD or low fat diet (LFD). Body

weights, percent body fat, and fasting blood glucose were measured longitudinally, and

triglycerides were measured at study termination. All cancer models reached the pubertal

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[1] David S. Baston preformed nuclear receptor assays.

growth spurt ahead of FVB controls. From puberty through adulthood, the HER2 model had increased body weight and adipose tissue compared to PyMT and DMBA. However, the DMBA model consistently had higher fasting blood glucose levels than PyMT and HER2. TCDD only impacted serum triglycerides in the PyMT model maintained on HFD. Since the estrogenic activity of the HFD was three times lower than that of the LFD, differential estrogenic activities did not drive the observed phenotypic differences. Rather, the HFD-dependent changes were cancer-model dependent.

INTRODUCTION

Metabolic syndrome represents a constellation of disease-associated physiological changes affecting over 47 million US residents (Ford *et al.* 2002). The key etiological component of metabolic syndrome is obesity, which can promote insulin insensitivity and type II diabetes. Additionally, excess adipose tissue results in increased storage of fatty acids and triglycerides in peripheral tissues (Roche *et al.* 2005). Abundant adipose tissue also causes excess endocrine signaling that increases insulin insensitivity and estrogen aromatization, which in turn increases adipose proliferation, body weight and fasting blood glucose insensitivity (Bunt 1990; Mayes and Watson 2004; Trayhurn and Wood 2004; Vidal *et al.* 1999). Peripheral estrogen aromatization by excess adipose tissue is also thought to contribute to the increased post-menopausal breast cancer risk among obese women (Hakkak *et al.* 2007; Simpson 2003).

Individuals that have metabolic syndrome or that are obese usually have higher consumption of fatty foods from animal origin, which is associated with a greater body

burden of lipophilic endocrine disruptors such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Harrad *et al*. 2003; Hayward *et al*. 1999; Hooper *et al*. 1998; Muntean *et al*. 2003). Elimination of TCDD is slowed substantially as body fat increases (Emond *et al*. 2006; Michalek and Tripathi 1999). Consequently, obese individuals may have unique susceptibilities to the effects of TCDD and other lipophilic endocrine disruptors.

Although the effects of high doses of TCDD are a depletion of both lean and adipose tissue that results in a wasting syndrome(Pohjanvirta and Tuomisto 1994), the effects of low doses of TCDD seem to shift away from body compositional changes to endocrine disruption and promotion of diseases associated with metabolic syndrome like type II diabetes. Human exposure to TCDD has resulted in an increase in serum insulin levels, diabetes, diabetes-associated mortality in females and an earlier diabetes onset (Consonni *et al*. 2008; Cranmer *et al*. 2000; Henriksen *et al*. 1997).

While there is evidence that TCDD exposure and diets high in fat (HFD) interact and effect metabolic syndrome, little is known about how these exposures impact breast cancer. Breast cancers can be partitioned into distinct subtypes that represent unique histopathologies, genetic alterations, treatment responses and perhaps risk factors (Herschkowitz *et al*. 2007). Because of human breast cancer diversity, multiple mouse models have been used to recapitulate clinically significant aspects of initiation, progression and invasion.

Two human breast cancer subtypes are estrogen receptor (ER)-negative with poor prognosis (Sorlie *et al*. 2001; Sorlie *et al*. 2003). One of these subtypes has basal/myoepthilial cell characteristics (basal-like). Basal-like subtypes represent 5-10% of breast cancers and include those with *BRCA1* and *HRAS* mutations (Herschkowitz *et*

al. 2007), which are involved in proliferation and invasion (Moon 2006). The other ER-negative breast cancer subtype has high ERBB2 (HER2) expression and is associated with reduced patient survival (Witton *et al*. 2003). Similar to these human breast cancers, the 7,12-dimethylbenz[a]anthracene (DMBA) carcinogen-induced mouse model of breast cancer results in hyperactivation of *Hras* and heightened proliferation (Cardiff *et al*. 1988; Herschkowitz *et al*. 2007). Luminal-type breast cancers represent a third subtype, and prognosis for these can be good (luminal A) or bad (luminal B) (Sorlie *et al*. 2001; Sorlie *et al*. 2003). The PyMT transgenic mouse model has characteristics of the luminal subtype of breast cancer (Herschkowitz *et al*. 2007).

To elucidate consequences of the interaction between dietary fat and TCDD exposure on metabolic syndrome-associated phenotypes in different breast cancer subtypes, three mouse distinct models of breast cancer were exposed maternally to low-dose TCDD in combination with either a HFD or a lower-fat, matched control diet (LFD). To directly compare metabolic syndrome-associated phenotypes across different cancer types, all three models were maintained on a uniform FVB/NJ genetic background, which is susceptible to TCDD toxicity and diet-induced obesity (Cleary *et al*. 2004; Hakkak *et al*. 2007; Jones *et al*. 1991; Luijten *et al*. 2004; Martin *et al*. 2006).

MATERIALS AND METHODS

Chemicals. DMBA (98% pure), 17β-estradiol (98% pure E2), and 5-alpha-androstan-17beta-OL-3-one (99% pure DHT) were purchased from Sigma-Aldrich (St. Louis, MO). TCDD was purchased from Ultra Scientific (99.9% pure, North Kingstown,

RI). DMBA and TCDD were dissolved in 95%/5% olive oil/toluene by volume (Certified

ACS, Sigma-Aldrich, St. Louis, MO), and used at 25 mg/ml and 0.5 ng/μl stock

concentrations, respectively. Dosing was at 2.4 μl DMBA solution/g mouse and 1.8-1.9

μl TCDD solution/g mouse. In the diet activity assays, TCDD, E2 and DHT were

dissolved in dimethyl sulfoxide (DMSO, Certified ACS, Fisher Scientific, Pittsburg, PA)

and activity levels determined as described below.

Diets. Mice were fed a HFD (4.73 kcal/g, D12451, Research Diets, New

Brunswick, NJ) composed of 20% protein, 35% carbohydrate, and 45% fat by total kcal

or a LFD (3.85 kcal/g, D12450B, Research Diets) composed of 20% protein, 70%

carbohydrate, and 10% fat by total kcal. The primary differences between the matched

diets are increased maltodextrin and lard, and decreased cornstarch and sucrose in the

HFD compared to the LFD (400, 1598, 291, 691 kcal vs. 140, 180, 1260, 1400 kcal,

respectively). The LFD has fat levels that fall within the range commonly found in

standard rodent chows, but much lower than the typical human diet in the United States.

Mice and husbandry. Females from three mouse models of breast cancer, all on

the FVB/NJ (FVB) background, were used. Nulliparous female FVB mice were mated

with male homozygous FVB-Tg(MMTVNeu)202Mul/J mice (Jackson Laboratories, Bar

Harbor, ME) to generate female pups for the HER2 model, representing ER-negative,

HER2-positive breast cancers (Herschkowitz *et al.* 2007; Weinstein *et al.* 2000).

Nulliparous female FVB and male homozygous FVB-TgN(MMTV-PyMT)634Mul/J

(NCI MMHCC Repository, Frederick, MD) mice were crossed to generate female

offspring for the PyMT model, representing ER-negative luminal breast cancers (Guy *et al*. 1992; Herschkowitz *et al*. 2007; Qiu *et al*. 2004). Nulliparous female and male FVB mice were mated to produce female pups used in the DMBA model, representing basal-like breast cancers (Herschkowitz *et al*. 2007), as well as untreated FVB controls. , Six litters per treatment were used for the DMBA model because of the added variability associated with carcinogen models, while three litters were used for the Her2 and PyMT models and FVB controls.

Heterozygous PyMT mice were identified using primers 5'-AACGGCGGAGCGAGGAACTG and 5'-ATCGGGCTCAGCAACACAAG (Operon, Huntsville, AL) and a PCR protocol previously described (Jackson Laboratories, Bar Harbor, ME;(Laboratories 2008). Heterozygous HER2 mice were identified using PCR primers 5'-TTTCCTGCAGCAGCCTACGC and 5'-CGGAACCCACATCAGGCC (Qiu *et al.* 2004). Mice were housed in HEPA-filtered ventilated cages with food and water provided *ad libitum*. Care and treatment of the mice complied with the guidelines of the Animal Welfare Act under an Institutional Animal Care and Use Committee (IUCAC)-approved protocol. Carbon dioxide asphyxiation euthanasia was performed on mice that developed tumor of one cm in diameter or at 11 months of age, whichever came first. FVB control mice were euthanized by carbon dioxide asphyxiation at post-natal day (PND) 121.

Treatment and experimental groups. The effects of diet and TCDD were studied in a randomized 2 x 2 factorial design (Fig. 3-1). Noon on the day when females, housed with males representing one of the three breast cancer models, were observed with a

vaginal plug was designated at 0.5 days post coitus (dpc). On 12.5 dpc, 1 μg/kg of TCDD

was administered by oral gavage to pregnant FVB (n = 12), PyMT (n = 6) and HER2 (n =

3) dams, or the equivalent volume of vehicle to pregnant FVB (n = 18) and PyMT (n = 6)

dams. From parturition (PND 0), dams were fed one of the two standardized diets, and

their respective pups were weaned at PND 21 onto the same diet until euthanasia. FVB

and PyMT litters were assigned to HFD or LFD symmetrically with respect to TCDD

exposure status, while HER2 litters from TCDD-exposed dams were assigned to HFD

only. In total, 30 FVB, 12 PyMT and six HER2 litters were fed HFD or LFD. Litters

were culled to four mice at PND 4, maximizing the number of females with the desired

genotype. At PND 35, 49 and 63, 60 mg/kg of DMBA was administered by oral gavage

to 24 FVB litters. The remaining six FVB litters served as controls. DMBA and HER2

mice were palpated weekly for tumors beginning at PND 83; PyMT mice were palpated

for tumors three times per week beginning at PND 35.

Body composition. At PND 0, body mass was determined by dividing the total

litter mass by the number of pups within the litter. Individual body masses were

evaluated at PND 4, 7, 10, 14, 21, 35, 49, 63, 90, 120, 150, 180, 210, 240, 270, 300 and

330. At PND 35, 90 and 180, all mice were placed under isoflourane anesthesia for

approximately five minutes while undergoing dual energy x-ray absorptiometry (DEXA;

GE Lunar PIXImus Corp., Madison, WI) to evaluate percent body fat.

Blood chemistry. Following a 24-hour fast at PND 35, 120, 180, 240 and 300,

blood glucose levels were measured using a ThermaSense FreeStyle blood glucose kit

(Alameda, CA). Immediately following euthanasia, blood was drawn from the inferior vena cava and serum stored at -80°C for triglyceride analysis using StanBio Laboratories Enzymatic Triglycerides Procedure 2150 (Boerne, TX).

Nuclear receptor activity assays. Approximately 10 g of each diet were crushed with a mortar and pestle, and extracted three times with 20 ml of toluene (non-polar extract) or ethanol (polar extract). The extract was allowed to settle before passing the supernatant through a Celite filter column. The column was rinsed with 20 ml of toluene or ethanol. The toluene or ethanol extracts were dried and resuspended in 4 ml of the same solvent. Aliquots of polar (200 μl) and non-polar (300 μl) suspensions were mixed with 3.5 μl of DMSO, and the solvents were removed by vacuum centrifugation before the remaining DMSO was mixed with 350 μl of culture media. Each diet extract was analyzed for its ability to activate expression of an estrogen receptor (ER), androgen receptor (AR) or aryl hydrocarbon receptor (AhR)-dependent firefly luciferase reporter gene in stably transfected human ovarian (BG1), human breast cancer (T47D) and mouse hepatoma (Hepa1c1c7) cell lines, respectively (Han *et al.* 2004a; Rogers and Denison 2002; Ziccardi *et al.* 2000).

Concentration-dependent reporter gene induction was determined by analysis of the inducing potency of a serial dilution of each sample extract (100 μl/well). Cells (75,000) were plated into sterile COSTAR white clear-bottomed 96-well tissue culture microplates (Corning Inc., Corning NY) and allowed to attach for 24 hr prior to chemical treatment. For the ER bioassay, cells were maintained in estrogen-stripped media for 5 days before plating to reduce background estrogen activity. Cells were incubated with

diet extracts, carrier DMSO solvent (1% final solvent concentration) or increasing

concentrations of E2, DHT or TCDD standards for 24 hr at 37ºC. After 24 hr of

incubation, all microplate wells were washed twice with phosphate-buffered saline before

adding of 50 μl of cell lysis buffer (Promega Inc., Madison, WI) and shaking for 20 min

at room temperature to facilitate cell lysis. The plates were inserted into a Berthold

microplate luminometer (Spectranalyzed Berthold Detection Systems, Bleichstrasse 56-

68, Pforzheim, Germany), and luciferase activity in each well was measured, integrated

over 10 sec after a 2 sec delay, following automatic injection of 50 μl Promega stabilized

luciferase reagent (Ziccardi et al., 2000; Rogers et al., 2000; (Han *et al.* 2004a).

Luciferase activity in each well was expressed relative to the maximal inducing

concentration of the respective positive control (E2, DHT or TCDD).

Statistical analysis. All statistical analyses were performed with SAS software,

version 9.0 (SAS Institute, Cary, NC). Monophasic and diphasic sigmoidal models of

change in body mass over time were evaluated for all cancer models and controls

according to (1) using PROC NLIN (Koops 1986; Koops *et al.* 1987):

$$y_t = \sum i \, [a_i \, \{1 + \tanh (b_i \, (t - c_i))\}]$$

(1)

where y_t is the predicted body weight (g) at age t, i is the number of phases, a_i is half the

asymptotic value of y in phase i, b_i is a growth parameter in phase i, and c_1 is the age at

the inflection point of phase i. It follows that when $i = 1$, parameters a_1, b_1 and c_1

describe growth in prepubertal mice and when $i = 2$, parameters a_2, b_2 and c_2 describe

growth in postpubertal mice. a_1 is the body weight (g) at the maximum prepubertal

growth rate, $2a_1 + a_2$ is the body weight (g) at the maximum postpubertal growth rate, a_1b_1 is the maximum prepubertal growth rate (g/PND), a_2b_2 is the maximum postpubertal growth rate (g/PND), c_1 is the age at the maximum prepubertal growth rate (PND), and c_2 is the age at the maximum postpubertal growth rate (PND).

Changes in fasting blood glucose and percent body fat over time were modeled longitudinally with PROC MIXED. Because pregnant dams were exposed to TCDD, the litter was used as a random effect. Median triglycerides per litter were evaluated using ANOVA and student's two-way t-test for all pairwise comparisons at an alpha level of 0.05.

For nuclear receptor activity assays, mean relative light units were determined at each concentration in both toluene and ethanol extracts for standard and diet samples. From this, the effective extract concentration giving 50% of the maximal induction response was calculated (EC_{50}) for each standard in both toluene and ethanol extracts. The percent effective concentration ($EC_{[x]}$) of each diet was then determined in both toluene and ethanol extracts. ANOVA was used to assess significance of induction equivalents, as a ratio of the ECx value of the sample and the equivalent value from the standard curves, between diet samples for each standard and solvent combination.

RESULTS

HFD increases longitudinal body weight. The effect of dietary fat on body weight was determined by analyzing the interaction between body weight and age using individual mice. HFD increased the body weight and growth of mice from the DMBA

and PyMT models, but not of FVB control mice. Since mice from the HER2 model were only raised on HFD, no diet comparisons were performed with this model. Neither TCDD nor tumor presence had an effect on body weight for any model.

For mice from the DMBA model, the diphasic sigmoidal model of growth fit best ($R^2 = 0.945$) (Koops *et al.* 1987). At prepubertal time points before DMBA treatment, HFD increased the maximum growth rate and decreased the age at the maximum growth rate of mice from the DMBA model as compared to LFD ($p < 0.01$, Fig. 3-2a, Table 1). Similarly, HFD also increased the maximum growth rate of mice from the DMBA model at postpubertal time points as well as the weight at the maximum growth rate ($p < 0.001$). DMBA mice maintained on HFD had higher body weights compared to mice maintained on LFD from PND 4 until PND 300, when there were insufficient mice on HFD still surviving for comparison ($p < 0.01$).

The monophasic sigmoidal model of growth fit best for mice from the PyMT model ($R^2 = 0.989$) (Koops *et al.* 1987). Similar to mice from the DMBA model, prepubertal PyMT mice maintained on HFD had a faster maximum growth rate at a younger age than those maintained on LFD ($p < 0.01$ and 0.05, respectively; Fig. 3-3a, Table 2). Mice from the PyMT model maintained on HFD were also heavier at the maximum growth rate than those maintained on LFD ($p < 0.05$); there was a trend of increased body weight with HFD in PyMT model across all ages, though it was only significant between PND 14 and 35 ($p < 0.05$).

HFD increases longitudinal percent body fat. The effect of dietary fat on percent body fat was analyzed as an interaction between diet and PND for individual mice over

time with litter as a mixed effect. Similar to body weight, neither perinatal TCDD nor tumor presence caused a change in percent body fat.

Percent body fat was greater in mice from the DMBA model maintained on HFD compared to those maintained on LFD at all ages measured ($p < 0.01$, Fig. 3-2b), with the effect of HFD becoming more pronounced with aging ($p < 0.0001$). This trend was similar to that seen for the PyMT model, where percent body fat increased with time in mice maintained on HFD but not on LFD, causing a significant dietary-induced difference in percent body fat by PND 90 ($p < 0.05$, Fig. 3b). Relative to LFD, maintenance on HFD resulted in increased percent body fat in FVB control mice at PND 35 ($p = 0.05$) but not at PND 90 (data not shown). Unlike mice from the DMBA and PyMT breast cancer models, control FVB mice were resistant to diet-induced adiposity.

HFD increases longitudinal fasting blood glucose. Fasting blood glucose was modeled as an interaction between dietary fat and PND on individual mice using litter as a mixed effect. Consistent with elevated percent body fat, fasting blood glucose was significantly elevated in mice from the DMBA model maintained on HFD compared to those on LFD at all ages after PND 35 ($p < 0.05$). This difference between the effects of diet increased with age ($p < 0.0001$, Fig. 2c). Fasting blood glucose was not affected by diet in FVB control mice at PND 35 or 120 (data not shown), nor in the PyMT cancer model mice at PND 35 (Fig. 3c). Later time points were not evaluated in the PyMT cancer model mice due to the aggressive nature of the tumors in this model (mean age at death = PND 65).

HFD and TCDD depress serum triglycerides. Unlike body fat and fasting blood glucose, serum triglycerides were affected by TCDD, thus ANOVAs were performed on the medians of litter triglyceride values. Dietary fat and TCDD had no effect on triglycerides in mice from the DMBA model or FVB control mice (Fig. 2d). However, triglycerides were significantly depressed in mice from the PyMT model by a combined exposure of HFD and TCDD ($p < 0.01$, Fig. 3d).

Differences among models in the longitudinal effects of HFD. Mice from the DMBA and PyMT breast cancer models had faster maximum prepubertal growth rates than FVB control mice ($p < 0.05$, Table 3). Mice from the DMBA and HER2 models weighed the least and most, respectively, at their maximum prepubertal growth rates, possibly reflecting differences in their age of maximum prepubertal growth rate (Table 3). FVB control mice reached their maximum prepubertal growth rate later than mice from the breast cancer models . By PND 35, and continuing through adulthood at PND 180, mice from the HER2 model were heavier than all others ($p < 0.01$, Table 4). After PND 180, there was no difference between weights or growth trajectories of mice from the HER2 and DMBA models.

Trends for percent body fat in the three models of breast cancer were similar to trends in body mass. Mice from the HER2 model had a higher percent body fat early in life than mice from the DMBA and PyMT models, or FVB control mice ($p < 0.01$), but by PND 180 percent body fat was similar between mice from the DMBA and HER2 models (Table 4). Percent body fat did not differ at any age among mice from the DMBA and PyMT models, or the FVB control mice.

Mice from the DMBA model had higher fasting blood glucose than those from the PyMT and HER2 models at PND 35 ($p < 0.05$, Table 4). However, by PND 120 mice from both the DMBA model and FVB control had higher fasting blood glucose than those from the HER2 model ($p < 0.05$). This divergence increased throughout the duration of the study. Mice from the HER2 model maintained constant levels of fasting blood glucose over time, while mice from the DMBA model continued to increase until PND 300. There were no differences among the models of breast cancer or controls in their serum triglyceride levels, despite differences in age at euthanasia (Table 4).

LFD has higher nuclear receptor activity than HFD. Differences between the HFD and LFD with regards to the presence of endocrine disrupting chemicals could underlie their differential effects on metabolic syndrome-associated phenotypes (Thigpen *et al*. 2004). Accordingly, both diets were analyzed for their ability to activate ER, AR or AhR using luciferase reporter gene assays. Dose-response studies revealed that little or no AR- or AhR-dependent reporter gene expression was observed using polar and non-polar extracts from either diet (data not shown). In contrast, significant induction of ER-dependent reporter gene expression was observed using non-polar extracts from both diets (Table 5); little or no ER-dependent activity was observed using polar extracts. Comparison of ER-dependent reporter gene expression levels using dose-response analysis revealed that the overall estrogenic activity of the LFD was three times greater than that of the HFD ($p < 0.01$), counter to that expected if differences in diet-derived estrogenic activities were responsible for the metabolic syndrome-associated phenotype differences.

DISCUSSION

Metabolic syndrome and breast cancer are traits with complex etiologies driven in part by disrupted endocrine signaling. With the increasing prevalence of metabolic syndrome in humans, it is critical to characterize the susceptibility of those with metabolic syndrome to environmental endocrine disruptors and to cancer. Through a cross-model comparison using three mouse models of breast cancer on a uniform genetic background, we have provided insights into the contribution of different breast cancers to diet induced-metabolic syndrome-associated phenotypes.

Body composition. We observed age-dependent differences in percent body fat due to diet in all three models of breast cancer and the FVB controls, consistent with reports that DMBA, PyMT, and HER2 are sensitive to dietary fat and obesity (Gordon *et al*. 2008; Hakkak *et al*. 2007; Luijten *et al*. 2004).

The body weight and fat accumulation seen in the HER2 model, relative to all other mice, may be due to the biology of the model (Koops and Grossman 1991). HER2 is part of the receptor family involved in the MAPK proliferation pathway, and overexpression of HER2 may lead to greater mammary adiposity. Consistent with a link between HER2 and obesity, women with HER2-positive breast cancer are more likely to be overweight/obese than women without cancer (Jones *et al*. 2007). The increased adiposity in the HER2 model is also supported by results showing that higher ERBB2 (HER2) activity levels decrease preadipocyte differentiation, which is permissive of

clonal adipocyte expansion (Harrington *et al.* 2007). Since low transcript levels of the HER2 transgene have been detected in the thymus, it is also possible that the adiposity of this model was driven by thymic endocrine changes (Huang *et al.* 2003; Savino 2007).

Fasting blood glucose. We observed a general resistance to dietary effects on fasting blood glucose levels among the transgenic models of breast cancer. The PyMT and HER2 models as well as the FVB controls were resistant to HFD-induction of elevated blood glucose levels. In contrast, the DMBA model appeared to develop impaired glucose tolerance, a risk factor of both metabolic syndrome and type II diabetes (2001), in response to HFD.

The variation in fasting blood glucose levels among cancer models demonstrates the independent etiologies of their metabolic syndrome phenotypes. The HER2 model had greater adiposity than the DMBA model, yet the converse is true with respect to fasting blood glucose levels. These two metabolic syndrome-associated phenotypes are typically highly correlated (Eberhardt *et al.* 2004).

TCDD. Exposure to TCDD exposure in humans is associated with an increased risk for diseases linked to metabolic syndrome like type II diabetes. However, there are yet no animal models for low dose TCDD-associated changes in disease risk. Our results are consistent with previous observations that the TCDD wasting syndrome is only a high dose phenomena since we did not detect any influence of maternal TCDD exposure on longitudinal changes in body mass, percent body fat or fasting blood glucose. The only exception was serum triglycerides.

Triglycerides. Our low dose TCDD results clarify previous conflicting reports and suggest that serum triglycerides are decreased by acute TCDD exposure (Birnbaum *et al.* 1990; Chapman and Schiller 1985; Pelclova *et al.* 2001; Sweeney and Mocarelli 2000). We also found that increased dietary fat increases susceptibility to TCDD-induced changes in triglycerides. Interestingly, the interaction between dietary fat and TCDD on triglycerides was only observed with the PuMT model, underscoring the importance of defining susceptibility in terms of specific gene-by-environment interactions.

Summary. Here we show that three mouse models of breast cancer, driven by specific oncogenes or induced by a carcinogen, can differentially impact the presentation of metabolic syndrome-associated phenotypes. Exposure to HFD caused variation in metabolic syndrome-associated phenotypes among these widely used mouse models. In the DMBA model, HFD caused a modest change in body fat but a large change in fasting glucose level. Conversely, HFD in the HER2 model resulted in a large change in body fat without changing fasting glucose levels. In the PyMT model, HFD caused a large change in body fat and triglycerides without changing fasting glucose levels. The variation observed among different mouse models of breast cancer in response to HFD and maternal TCDD exposure may have utility in elucidating of the mechanisms of those metabolic syndrome-associated phenotypes that are etiologically linked to breast cancer risk.

Table 3-1. *Effects of dietary fat on body growth characteristics in the DMBA model.*

Growth phase	Parameter	Interpretation	LFD	HFD
1	a_1	Weight at maximum growth rate (g)	11.0 (0.27)	10.7 (0.45)
1	$a_1 \times b_1$	Maximum growth rate (g/PND)	**0.640 (0.02)**	**0.730 (0.02)**
1	d_1	Age at maximum growth rate (PND)	**18.8 (0.35)**	**16.7 (0.29)**
2	$2a_1 + a_2$	Weight at maximum growth rate (g)	**26.8 (0.28)**	**31.5 (0.52)**
2	$a_2 \times b_2$	Maximum growth rate (g/PND)	**0.062 (0.01)**	**0.116 (0.01)**
2	d_2	Age at maximum growth rate (PND)	164.3 (6.7)	154.2 (5.8)

Values are means (SE), with significance at $p < 0.05$ shown in bold. Parameters were estimated from a diphasic sigmoidal curve (Koops *et al*. 1987), where Growth phase = 1 denotes prepubertal, and = 2 postpubertal, respectively.

Table 3-2. *Effects of dietary fat on body growth characteristics in the PyMT model.*

Growth phase	Parameter	Interpretation	LFD	HFD
1	a_1	Weight at maximum growth rate (g)	**11.5 (0.16)**	**12.0 (0.18)**
1	$a_1 \times b_1$	Maximum growth rate (g/PND)	**0.639 (0.02)**	**0.721 (0.02)**
1	d_1	Age at maximum growth rate (PND)	**18.9 (0.45)**	**17.6 (0.41)**

Values are means (SE), with significance at $p < 0.05$ shown in bold. Parameters were estimated from a monophasic sigmoidal curve (Koops *et al*. 1987), growth phase = 1 denotes prepubertal growth.

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Table 3-3. *Comparison of body growth characteristics among breast cancer models maintained on HD.*

Growth phase	Parameter	Interpretation	FVB	PyMT	HER2	DMBA
1	a_1	Weight at maximum growth rate (g)	12.1 (0.12)	12.0 (0.18)	13.5 (1.6)	10.7 (0.45)
1	$a_1 \times b_1$	Maximum growth rate (g/PND)	0.681 (0.03)	0.721 (0.02)	0.761 (0.07)	0.729 (0.02)
1	d_1	Age at maximum growth rate (PND)	21.3 (0.43)	17.6 (0.41)	20.5 (1.3)	16.7 (0.29)
2	$2a_1 + a_2$	Weight at maximum growth rate (g)			44.8 (23.9)	31.5 (0.5)
2	$a_2 \times b_2$	Maximum growth rate (g/PND)			0.0947 (0.07)	0.116 (0.01)
2	d_2	Age at maximum growth rate (PND)			332.7 (233.7)	154.2 (5.8)

Values are means (SE), with intermodel significant differences at $p < 0.05$ noted by lines.

Parameters were estimated from monophasic and diphasic sigmoidal curves (Koops *et al.* 1987), where growth phase = 1 denotes prepubertal, and = 2 postpubertal, respectively.

Table 3-4. *Comparison of metabolic syndrome phenotypes among breast cancer models maintained on HD.*

Phenotype	FVB	DMBA	PyMT	HER2
BW at PND 35	20.4 (0.57)	20.7 (0.21)	21.0 (0.40)	23.7 (0.31)
Body fat at PND 35	21.0 (2.3)	22.0 (3.3)	20.8 (2.6)	26.2 (2.2)
Glucose at PND 35	104.9 (27.0)	111.4 (29.1)	83.9 (23.5)	92.1 (15.8)
BW at PND 90	25.2 (0.74)	25.1 (0.32)	20.1 (0.48)	29.4 (0.85)
Body fat at PND 90	27.3 (4.2)	25.0 (3.5)	29.4 (4.0)	31.9 (4.5)
BW at PND 120	27.8 (1.3)	28.1 (0.44)		29.5 (0.92)
Glucose at PND 120	118.2 (26.9)	129.3 (24.6)		93.8 (21.7)
TAG at sacrifice	128.6 (15.4)	193.5 (31.3)	172.6 (20.8)	179.8 (49.6)

Values are means (SE), with intermodel significant differences at $p < 0.05$ noted by lines. Means were estimated from monophasic and diphasic sigmoidal curves for BW (body weight, g) (Koops *et al*. 1987), from linear mixed models for fasting blood glucose (glucose, mg/dL) and body fat (%), and from ANOVA for serum triglycerides (TAG, mg/dL).

Table 3-5. *Estrogenic activity of organic fraction of diets.*

Diet	Extract Concentration (g/ml)	EC$_{50}$ Standards (pg E2)	EC$_{[x]}$ for Sample (g Diet Equivalents)	Induction Equivalent pg E2/ g Diet Equivalents
LD	2.500	0.07352	0.00705 [50.0%]	10.43*
HD	2.502	0.19172	0.05150 [18.6%]	3.72

Values are means (SE), based on triplicates with inter-diet significant differences at ANOVA $p < 0.05$ noted by *.

FIGURE LEGENDS

Figure 3-1. Schematic of DMBA, HER2, and PyMT breast cancer models and FVB controls. Numbers in 2 x 2 tables represent the number of litters per treatment group. Euth. represents euthansia of FVB. Cancer models are euthanized after palpation when lesion ≥ 1 cm or at PND 330.

Figure 3-2. Metabolic syndrome in DMBA mice. HFD increases a) body weight, b) percent body fat, and c) blood glucose over LFD. d) Triglycerides are not impacted by diet.

Figure 3-3. Metabolic syndrome in PyMT mice. HD increases a) body weight, b) percent body fat, but not c) blood glucose. d) Triglycerides are altered by HD and TCDD.

DMBA	Oil	TCDD
LFD	6	6
HFD	6	6

TCDD — Diet assigned — Cull litter to 4 pups — Pups weaned — DMBA — DMBA — DMBA — Palpate
GD 12.5 — PND 0 — PND 4 — PND 21 — PND 35 — PND 49 — PND 63 — PND 83

HER2	Oil	TCDD
LFD	0	0
HFD	3	3

TCDD — Diet assigned — Cull litter to 4 pups — Pups weaned — Palpate
GD 12.5 — PND 0 — PND 4 — PND 21 — PND 83

PyMT	Oil	TCDD
LFD	3	3
HFD	3	3

TCDD — Diet assigned — Cull litter to 4 pups — Pups weaned — Palpate
GD 12.5 — PND 0 — PND 4 — PND 21 — PND 35

FVB	Oil	TCDD
LFD	3	0
HFD	3	0

TCDD — Diet assigned — Cull litter to 4 pups — Pups weaned — Palpate — Euth.
GD 12.5 — PND 0 — PND 4 — PND 21 — PND 83 — PND 121

Figure 3-1.

63

Figure 3-2.

Fat	↑	↑	↓	↓
TCDD	-	+	-	+

CHAPTER 4

MATERNAL DIOXIN EXPOSURE COMBINE WITH A DIET
HIGH IN FAT IN FAT INCREASES MAMMARY CANCER
INCIDENCE THROUGH CYP1B1- AND COMT- MEDIATED
ESTROGEN METABOLISM[1]

ABSTRACT

Epidemiological studies show that breast cancer risk correlates with total lifetime

exposure to estrogens and that early life 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

exposure can also increase cancer risk. Because both TCDD and adipocytes impact the

estrogen pathway, we examined how TCDD and an obesegenic diet interact to alter

breast cancer susceptibility. At 12.5 days post coitus, we exposed pregnant FVB/NJ

female mice to 1 µg/kg of TCDD or vehicle and at parturition randomly assigned the

nursing dams to a low- or high-fat diet (HFD). Female offspring were maintained on the

same diets after weaning and exposed to 7,12-dimethyl-benz[a]anthracene (DMBA) at

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[1] Rachel Harper preformed mammary tissue rtPCR, as well as mammary tissue and tumor
real time PCR.

post-natal days (PND) 35, 49 and 63 to initiate mammary tumors. A second cohort of females was treated identically until PND 35 or 49, when mammary gland morphology was examined, or at PND 50, when mammary gland mRNA and ERBB2 was analyzed. We found that a combination of maternal TCDD exposure and HFD increases mammary tumor incidence. Among mice fed HFD, maternal TCDD exposure caused rapid mammary development and increased *Cyp1b1* expression and decreased *Comt* expression in mammary tissue. Mammary tumor *Cyp1b1* expression was also increased by maternal TCDD exposure. Our data suggest that HFD increases sensitivity to maternal TCDD exposure, resulting in increased breast cancer incidence through changes in the timing of mammary differentiation and estrogen metabolism during puberty.

Introduction

Total lifetime exposure to estrogen (E2) is the single greatest environmental risk factor for breast cancer (Dunn *et al.* 2005). The classic pathway of E2-mediated carcinogenesis is through the estrogen receptor (ER), where E2 alters gene expression to increase cell proliferation (Currier *et al.* 2005). Consequently, it has been hypothesized that E2 metabolism acts to decrease breast cancer risk (Holcomb and Safe 1994). Yet, some estrogen metabolites may increase breast cancer risk through DNA damage (Cavalieri and Rogan 2004; Cavalieri *et al.* 1997). E2 is metabolized into reactive catechols primarily by CYP1B1 (Lee and Zhu 2006). The catechols undergo redox cycling resulting in oxidative stress, DNA adduct formation, and DNA mutations (Cavalieri and Rogan 2004; Chakravarti *et al.* 2001; Mitrunen and Hirvonen 2003). The

phase II enzyme catechol-o-methyltransferase (COMT) can mitigate this genotoxicity by inactivating the E2-catechol by *O*-methylation. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and other aryl hydrocarbon receptor (AhR) agonists can modulate estrogen activity through induction of estrogen-metabolizing CYP1 enzymes (Mitrunen and Hirvonen 2003; Tsuchiya *et al.* 2005). Thus TCDD and other AhR agonists have the potential to modify breast cancer risk through alteration of ER-mediated proliferation or CYP1-mediated DNA damage (Mitrunen and Hirvonen 2003; Ohtake *et al.* 2003; Safe *et al.* 2000). The influence of TCDD exposure may be more pronounced at younger ages since early life estrogenic exposures contribute to a greater risk of breast cancer than exposures later in life (Dolinoy *et al.* 2007b; Hilakivi-Clarke *et al.* 2000; Hilakivi-Clarke *et al.* 1999; Hilakivi-Clarke *et al.* 1997).

An industrial accident in Seveso, Italy supports the link between early TCDD exposure and cancer risk. TCDD exposure was positively associated with breast cancer risk only in women who were infants to 40 years of age at the time of the industrial accident (Warner *et al.* 2002). Early life TCDD exposure, particularly perinatally, has also been associated with increased mammary cancer in several rodent models (Brown *et al.* 1998; Desaulniers *et al.* 2001). Paradoxically, while greater estrogen exposure seen in adolescents with early menses contributes to increased breast cancer risk (Vihko and Apter 1984), delayed pubertal breast differentiation may also increase breast cancer risk. Rodent models have shown that perinatal TCDD exposure increases mammary cancer risk through altered mammary differentiation (Brown *et al.* 1998; Fenton *et al.* 2002), which extends the period in which the rapidly proliferating progenitor cells of the terminal end buds (TEB) are susceptible to carcinogenic insult (Birnbaum and Fenton

2003). A similar delay is seen in humans. As premenarcheal serum TCDD

concentrations increase, the timing of pubertal breast development is delayed (Den Hond

et al. 2002).

Obesity, like TCDD and E2 exposures, may have age- or developmental stage-

specific effects on breast cancer risk (De Assis and Hilakivi-Clarke 2006). Childhood

obesity appears to protect against breast cancer despite synthesis of E2 in adipose tissue

(De Assis and Hilakivi-Clarke 2006). The adipose synthesis of E2 likely contributes to

precocious pubertal mammary development, and if obesity and weight gain extend into

adulthood, increased post-menopausal breast cancer risk (Britton *et al.* 2004; De Assis

and Hilakivi-Clarke 2006; Hakkak *et al.* 2007; Stoll 1998). Obesity may also modify

breast cancer risk through increased persistence of lipophilic TCDD in adipose tissue,

including mammary stroma (Emond *et al*. 2006). Mammary glands of obese individuals

are likely exposed to greater TCDD levels than lean counterparts because of increased

dietary TCDD concentrations in animal products and increased TCDD persistence

(Emond *et al*. 2006; Harrad *et al*. 2003; Hooper *et al*. 1998; Michalek and Tripathi 1999).

Because obese individuals retain more TCDD, maternal TCDD exposure may result in

altered susceptibility to breast cancer among their offspring. Here the 7,12-dimethyl-

benz[a]anthracene (DMBA) mouse model of breast cancer was used to examine the

etiological basis of how maternal TCDD exposure and high-fat diet (HFD) increase

cancer risk. We report that the combined effect of maternal TCDD exposure and HFD

increases mammary cancer risk through alterations in E2 metabolism and mammary

gland remodeling.

RESULTS

Maternal TCDD exposure and HFD increase mammary tumor incidence.
Palpable lesions of both dermal and mammary origin were found after DMBA exposure,
with the majority of DMBA-treated mice developing dermal lesions. The palpable lesion
latency was shorter for DMBA fed HFD than those fed LFD (HR = 0.0075, $p < 0.01$) and
the lesions grew faster (HR = 0.24827, $p < 0.05$). Consistent with previous rat studies
(Brown *et al.* 1998; Desaulniers *et al.* 2001), DMBA-treated mice fed LFD after maternal
TCDD exposure had shortened lesion latency compared to those treated with vehicle (HR
= 2.01081, $p < 0.05$), although the number of mammary tumors was not effected.

Because of the increased mammary tumor incidence with either obesity or
perinatal TCDD exposure seen in previous studies ((Brown *et al.* 1998; Desaulniers *et al.*
2001; Hakkak *et al.* 2007), it was anticipated that DMBA fed HFD might have
heightened susceptibility to maternal TCDD exposure. Consistent with this expectation,
there was a significant increase in mammary lesions of mice fed HFD after maternal
TCDD exposure ($p < 0.0001$, Fig. 4-1A). DMBA fed HFD had substantially more
mammary tumors than DMBA fed LFD. Only two mammary lesions occurred in DMBA
on LFD, and neither of these mice were treated with TCDD. While no mammary tumors
arose in litters exposed to TCDD and LFD, and one third of unexposed litters fed HFD
had mammary tumors, every litter exposed to both TCDD and HFD developed mammary
tumors. Among DMBA treated mice fed HFD, TCDD exposure appeared to double
mammary tumor incidence (Fig. 4-1A). Mammary lesions were primarily squamous cell
carcinomas, typical of the DMBA model (Fig. 4-1B), and this was not altered by

treatment. However, several lesion types were clustered with respect to treatment. Three adenomyoepitheliomas, a rare lesion in the DMBA models, were found exclusively in DMBA treated mice fed HFD whose dams were treated with TCDD (Fig. 4-1C). In DMBA fed HFD without TCDD exposure, two solid nodular ERBB2-positive tumors were found with zonation (Fig. 4-1D; (Rosner *et al.* 2002).

Maternal TCDD exposure and HFD transiently alter pubertal mammary gland morphology. Previous reports demonstrated a correlation between perinatal TCDD exposure, altered pubertal mammary differentiation, and increased mammary tumors (Brown *et al.* 1998; Fenton *et al.* 2002). Maternal TCDD and HFD exposures had significantly non-additive effects on the number of TEB at PND 35 ($p < 0.001$, Fig 4-2). Among DMBA treated mice fed LFD, there were 22 TEB more with maternal TCDD exposure compared to vehicle, whereas among DMBA treated mice fed HFD, there were 18 TEB less with maternal TCDD exposure ($p < 0.01$, Fig. 4-2). While mean fat pad length, TEB number, and branch elongation were greater among DMBA treated mice fed HFD relative to LFD, only branch elongation was significantly increased in the HFD group at PND 35 ($p < 0.05$, data not shown). Though perinatal TCDD exposure in rats prevented the full differentiation of pubertal mammary glands (Fenton *et al.* 2002), we found the effects to be transient. By PND 49, neither HFD- nor maternal TCDD-exposure impacted the fat pad length, number of TEB, or ductal elongation (data not shown). Further, at PND 50, there were no treatment-associated changes in mammary morphology regulators *Egf* and *Ereg* mRNA (data not shown).

Maternal TCDD exposure alters E2 metabolism in mice fed HFD. At PND 50, we explored what transcriptional changes contributed to the progression of mammary carcinogenesis. Because mammary carcinogenesis involves either E2-ER mediated proliferation or E2-metabolite mediated genetic instability (Bolton and Thatcher 2008) (Fig. 1-1), we surveyed the genome for indications of these mechanisms. *Cyp1a1*, *Cyp1b1*, and *Comt* were examined as indicators of E2 metabolism. Exposure to HFD and maternal TCDD increased epithelial *Cyp1b1-* and decreased *Comt-* mRNA in mammary glands, though TCDD had no effect on expression in DMBA treated mice fed LFD ($p < 0.05$, Fig. 4-3A-B). *Cyp1b1* was also increased in mammary tumors compared to matched, normal mammary glands ($p < 0.05$, Fig. 4-3C). Among mammary tumors, *Cyp1b1* was elevated by maternal TCDD exposure relative to vehicle ($p < 0.05$, Fig. 4-3C). Our model suggested this was more than an additive interaction between TCDD and tumor tissue on *Cyp1b1* expression ($p = 0.08$, Fig. 4-3C). There was no change in *Cyp1a1*, *Ccdm1*, *Cmyc*, *Egfr*, *Esr1* or *Esr2* mRNA in the mammary glands. Together this suggests that genomic instability caused by E2-metabolites, and not E2-ER mediated proliferation, could be the mechanism underlying cancer progression (Fig. 1-1). Global gene expression analysis demonstrated a subtle role for pubertal onset of obesity in modifying metabolism of carbohydrates, lipids and proteins (data not shown). Microarray analysis indicated significantly reduced *Comt* and *Insig* in the PND 50 mammary glands of DMBA treated mice fed HFD (FDR $\delta < 0.05$) and these trends were confirmed by real time PCR. TCDD exposure was not associated with any global changes in gene expression at the time-point examined.

DISCUSSION

Human and rat studies suggest early life TCDD exposure increases mammary cancer incidence (Brown *et al.* 1998; Desaulniers *et al.* 2001; Warner *et al.* 2002), and this study extends these observations to mice fed HFD. Though the TCDD dose was higher than most acute human exposures, it is nonetheless considered very low for mice. There is substantial evidence that maternal estrogenic exposures increase E2-responsive cancer incidence in adult offspring (Brown *et al.* 1998; Ho *et al.* 2006; Li *et al.* 1997; Prins *et al.* 2008). Several studies implicate a role of maternal COMT and CYP1B1 in daughter's breast cancer risk (Inoue *et al.* 1980; Sata *et al.* 2006) (Ahsan *et al.* 2004). Thus TCDD-induced *Comt* and *Cyp1b1* changes *in utero* could be partially responsible for genetic instability leading to mammary tumors seen here.

This study addressed whether HFD and maternal TCDD exposure increases breast cancer risk through the E2-ER proliferation pathway, the E2-metabolism pathway, or through mammary structural remodeling (Fig. 1-1). TCDD modestly upregulated epithelial *Cyp1b1* and decreased *Comt* expression in mammary glands from DMBA fed HFD, supporting a role of the E2-metabolism pathway in cancer etiology. While modest changes are common in constitutive *Cyp1b1*, environmental exposures infrequently change *Comt* expression. In one rat model aryl hydrocarbon exposure decreased hepatic *Comt* expression (Desaulniers *et al.* 2005). Human studies have sometimes shown that high activity *Cyp1b1* alleles and low activity *Comt* alleles are associated with increased risk of breast cancer (Wen *et al.* 2007), and of other E2- responsive cancers (Nock *et al.* 2006; Sellers *et al.* 2005; Zimarina *et al.* 2004). Interactions between E2 exposures

during key life stages and E2-regulating genes, CYP1B1 and COMT, may modify breast cancer risk (Mitrunen *et al.* 2001).

In this study, DMBA treated mice fed HFD had increased mammary lesion incidence and shortened latency relative to those fed LFD. We have shown previously that HFD increased adiposity and fasting blood glucose from puberty throughout life (Chapter 3). Consistent with their elevated glucose, we demonstrate that these mice had decreased *Insig1* during carcinogenesis (Nakagawa *et al*. 2006). Thus the DMBA treated mice fed HFD had greater adiposity and type II diabetes risk than those fed LFD. Obesity increases post-menopausal breast cancer in women (Lorincz and Sukumar 2006), which may occur because of heightened E2 production in mammary adipose tissue (Hakkak *et al*. 2007; Simpson 2003). Further, several studies have found that increased adiposity interacts with alleles of E2 metabolizing enzymes to increase breast cancer risk (Kocabas *et al.* 2005; Kocabas *et al.* 2002; Thompson *et al.* 1998). Thus the doubled mammary tumor incidence in HFD fed mice exposed to maternal TCDD here may reflect higher steady state levels of both E2 and its toxic metabolites (Fig. 1-1).

Maternal TCDD substantially increased *Cyp1b1* expression in mammary tumors, suggesting that changes in mammary progenitor cells resulted in increased *Cyp1b1* that persisted through their expansion to tumors (Brown *et al.* 1998; Feinberg *et al.* 2006; Fenton *et al.* 2002). Unlike what was seen in mammary lesion incidence, DMBA treated mice fed HFD and exposed to TCDD had reduced TEB number during can initiation. Since TEB number correlates to carcinogenicity of DMBA, this TCDD effect was unexpected (Russo and Russo 1978). Mammary gland morphology was unchanged by diet and maternal TCDD at subsequent ages. The low *Comt* seen in these mice supports

this increase in the speed of their pubertal breast development (Eriksson *et al.* 2005), which could increase risk of DNA damage (Cavalieri and Rogan 2004; Chakravarti *et al.* 2001; Russo and Russo 1978). Together our data suggests rapid compensatory growth in mammary glands exposed to HFD and maternal TCDD, and when *Comt* was decreased and epithelial *Cyp1b1* was increased, increased susceptibility to mammary carcinogenesis resulted.

In summary, maintenance of mice on HFD increased the effects of maternal TCDD on offspring breast cancer risk through alterations in mammary gland-morphology and gene expression. E2-like exposures, such as an obesegenic diet and a low dose of TCDD during gestation, may increase the risk of association between *Cyp1b1*, *Comt*, and adult breast cancer. The inconsistent relationships between CYP1B1, COMT, and breast cancer risk seen across epidemiology studies may reflect divergent risk between those who have had substantial environmental E2 exposure and those who have not (Justenhoven *et al.* 2007; Le Marchand *et al.* 2005; McGrath *et al.* 2004). This may understate the susceptibility to TCDD among obese, genetically- susceptible women. This highlights the importance of using experimental animal models to evaluate the unique susceptibilities of varying subpopulations to breast cancer risk, and to ensure risk assessments in humans do not fail to take susceptible subpopulations into account.

MATERIALS AND METHODS

Mice and experimental design. We studied the effects of HFD and maternal TCDD exposure in a randomized 2 x 2 factorial design using the TCDD-responsive

mouse strain, FVB/NJ (FVB, Jackson Laboratories, Bar Harbor, ME; (Jones *et al*. 1991).

We have shown previously that FVB are responsive to HFD, gaining body weight and

adiposity along with elevated fasting blood glucose levels. At 12.5 days post coitus

(DPC), 1 μg/kg of TCDD (p. o., n = 13 dams, 1.8-1.9 μl TCDD solution/g mouse, 99.9%

purity, Ultra Scientific, North Kingstown, RI), or equivalent volume of vehicle (p. o.,

95%/5% olive oil/toluene by volume, n = 15 dams, 99.9% purity, Sigma-Aldrich, St.

Louis, MO) was administered to nulliparous FVB females. FVB litters were assigned to

HFD or matched control low fat diet (LFD; D12451 n = 14 litters and D12450B n = 15

respectively, Research Diets, New Brunswick, NJ) from post-natal day (PND) 0

(parturition) until euthanasia. At PND 4, all litters were culled to 4 pups, maximizing the

number of female pups per litter. At PND 21, all dams and any male offspring were

removed from the cage. At PND 35, 49 and 63, 60 mg/kg of DMBA (p. o., 2.4 μl DMBA

solution/g mouse, 95%/5% olive oil/toluene by volume, 98% purity, Sigma-Aldrich, St.

Louis, MO) was administered. A second cohort of FVB were treated identically until

euthanizing to examine mammary gland morphology (PND 35, 49), mRNA (PND 50),

and protein (PND 50). DMBA mice were palpated for mammary lesions biweekly

beginning at PND 83. All mice were given water *ad libitum* in sterile ventilated cages in

an American Association for the Accreditation of Laboratory Animal Care-approved

facility. Euthanasia was performed with CO_2 asphyxiation on PND 35, 49 and 50, and

when lesions were ≥ 1 cm, or at 11 months of age, whichever came first.

Histological analyses. Mammary lesions were bisected at necropsy. One lesion

half was fixed overnight at 4C in 4% paraformaldehyde before dehydrating, embedding

in paraffin, and sectioning. Hematoxylin and eosin (H&E) staining was used to diagnose lesion pathology. Immunohistochemical analysis of ERBB expression was preformed according standard methods. Briefly, 4-μm paraffin sections were placed onto Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA), deparaffinized, and cleared. IHC was performed after inhibition of endogenous peroxidase activity in a solution of 3% hydrogen peroxide in methanol and hydration in graded alcohol to distilled water. Antigen retrieval was performed by high temperature and pressure incubation in 0.01 mol/L of citric acid buffer (pH 6.0) for 3 x 4 minutes. Slides were allowed to cool for 30 minutes in citric acid buffer then transferred to phosphate-buffered saline (pH 7.4) (2 x 5 minutes each). Universal blocking reagent (BioGenex, San Ramon, CA) was applied to sections and incubated for 30 minutes in a humidified chamber at room temperature. ErbB2 and secondary antibodies (Neomarkers, Fremont, CA) diluted in PBS –Ova (albumin) were incubated for one hour each, and rinsed in PBS. Inguinal mammary glands from mice at PND 35 and 49 were weighed, fixed and Carmine Alum stained to evaluate fat pad length, number of TEB, and branch elongation according to published methods (Fenton *et al*. 2002).

Gene expression. The other half of each mammary lesion and a grossly normal gland from the same mouse were flash frozen and pulverized for RNA extraction (TRIzol Reagent, Invitrogen, Carlsbad, CA). PND 50 mammary glands were flash frozen, pulverized, and pooled within litter for RNA extraction (TRIzol Reagent, Invitrogen). The High Capacity cDNA archive kit (ABI, Foster City, CA) was used to generate cDNA for PCR analysis. Real time PCR was performed to assess relative transcript levels of

Cyp1a1, *Cyp1b1*, *Insig*, *Ccdm1*, *Cmyc*, *Egf*, *Ereg*, *Esr1* and *Esr2* using Assays-on-Demand (ABI) with *Gusb* as the endogenous control and *K18* as the epithelial marker in PND 50 mammary glands. Real time PCR was also preformed on *Cyp1b1* and *Comt* to determine relative transcript levels using *Tbp* as the endogenous control in mammary tumors and matched controls (ABI). Global gene expression changes in PND 50 mammary glands were assessed using a 4 x 44k chip (Agilent, Santa Clara, CA)(Syed and Threadgill 2006).

Statistics. Lesion latency, defined as time (PND) to reach palpable lesion, and lesion aggression, defined as the time from first palpable mass to time of mass ≥ 1 cm (PND), were evaluated using a mixed survival model in SUDAAN 9.0 software, with litter as a random effect. Lesion incidence, defined as the proportion of mice with mammary lesions per litter, was analyzed with Fisher's exact test (SAS 9.1.3, Cary, NC). ANOVA was used to evaluate the fixed effects of HFD and maternal TCDD and their interaction on branch elongation, fat pad length, number of TEB in PND 35 and 49 mammary glands, and on *Comt*, *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Areg*, *Ereg*, *Ahr*, *Egfr*, *Esr1* and *Esr2* levels in PND 50 mammary glands (Proc GLM). The fixed effects of tissue status (tumored or normal) and maternal TCDD, their interaction, and litter as a random effect were model on *Comt* and *Cyp1b1* levels in mammary tumors and matched tissue (Proc GLM). Microarrays were scanned on an Agilent scanner and analyzed using default settings of Feature Extraction version 9.1 (Agilent technologies, Santa Clara, CA). Microarray raw data were uploaded into the UNC Microarray Database and Log2 R/G Lowess normalization was performed on the Cy3 and Cy5 channels. The microarray data

are available at UNC Microarray Database (UNC 2008). The Lowess normalized data was quantile normalized (Barbacioru *et al.* 2006; Yang and Thorne 2003). Differentially expressed genes were identified using SAM software (Stanford, CA; (Tusher *et al.* 2001). Significance of IHC florescence was determined according to published methods (Rosner *et al.* 2002).

FIGURE LEGENDS

Fig. 4-1. HFD and maternal TCDD altered mammary tumor incidence and pathology. (A) HFD and maternal TCDD increase mammary tumor incidence ($p < 0.0001$, n = 28 litters). (B) Squamous cell adenocarcinoma typical of the DMBA model. (C) Adenomyoepithelioma in DMBA fed HFD and exposed to TCDD. (D) ErbB2-positive tumor in DMBA fed HFD.

Fig. 4-2. Interaction of HFD and maternal TCDD on terminal end bud numbers at PND 35. Means + SE are shown ($p < 0.01$, n = 20 litters).

Fig. 4-3. HFD and maternal TCDD increase E2 metabolism. Fold change of real time PCR data determined by $\partial\partial$CT method (Livak and Schmittgen 2001). (A) *Comt* mRNA is decreased in mammary glands exposed to HFD and maternal TCDD ($p < 0.05$, n = 20 mRNA pooled within litter). (B) Epithelial *Cyp1b1* mRNA is increased in mammary glands exposed to HFD and maternal TCDD ($p < 0.05$, n = 20 mRNA pooled within

litter). (C) *Cyp1b1* is elevated in mammary tumors by maternal TCDD exposure ($p <$ 0.05, n = 10 mammary tumors and 9 matched normal glands).
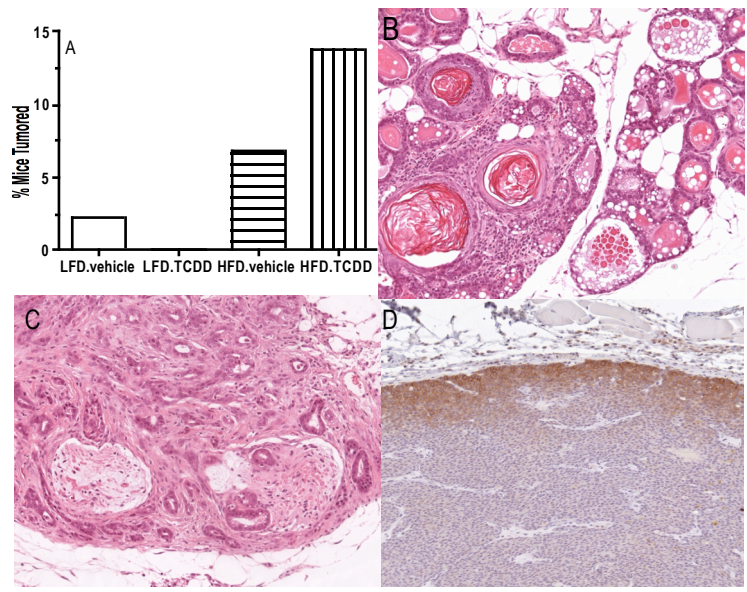
Figure 4-1.
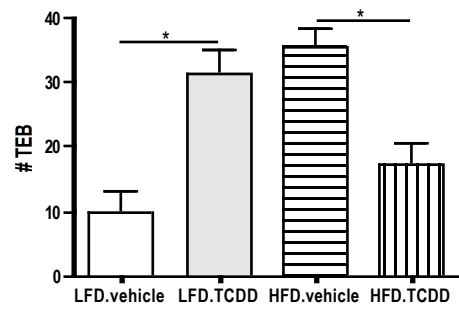
Figure 4-2.
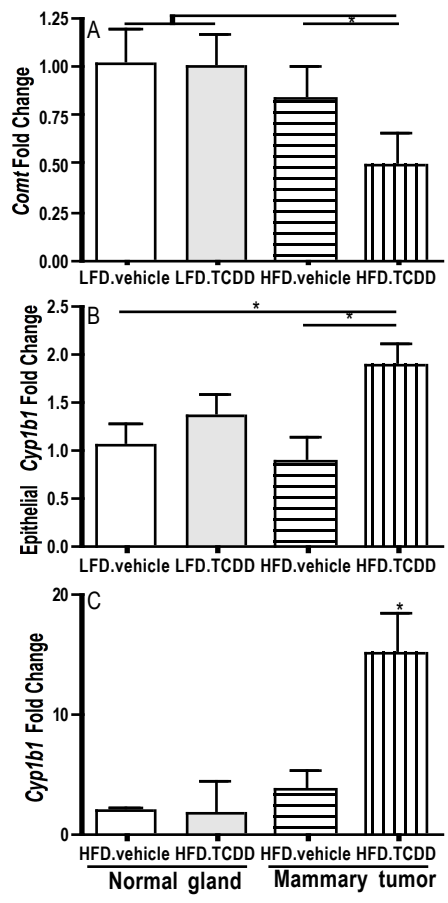
Figure 4-3.

CHAPTER 5

CONCLUSION

Total lifetime estrogen (E2) exposure increases mammary cancer risk. TCDD indirectly affects E2 signaling through many mechanisms, and early life TCDD exposures are also implicated in breast cancer. E2 produced by adipose tissue is the underlying mechanism by which obesity contributes to early puberty, and if obesity and weight gain extends into adulthood, increased post-menopausal breast cancer risk (Britton *et al.* 2004; De Assis and Hilakivi-Clarke 2006; Stoll 1998). Greater TCDD stores in the adipose of obese women have the potential to further increase cancer risk.

I examined how environmental exposures can modify the molecular and physical structure of the mammary gland. The treatments examined were maternal exposure to TCDD, lifelong exposure to high fat diet (HFD) and pubertal exposure to DMBA. The prediction was that high fat diet and gestational exposure to TCDD would interact to increase mammary cancer risk, through both structural remodeling of the pubertal gland and through direct molecular actions (Fig. 1-1). Molecular changes potentially underlying these phenotypes were evaluated with particular emphasis on E2 (Fig. 1-1). The physiological effects, *e.g.* growth trajectories, adiposity, blood glucose levels, and mammary gland development, of HFD and maternal TCDD were examined as potential causal intermediates leading to altered mammary gland phenotypes. Interactions of these exposures and physiological effects were explored in several mouse strains and mouse models of breast cancer. However, these objectives were primarily pursued using the

DMBA model of mouse mammary carcinogenesis. Here links between puberty, metabolic syndrome, environmental chemicals, and breast cancer etiology are examined.

FINDINGS

The expression of an oncogene or exposure to a carcinogen increased the adiposity and speed of the pubertal growth spurt relative to controls. Consistent with this observation, the speed of the pubertal growth spurt is a risk factor for breast cancer (Forman *et al.* 2005). In the strains and breast cancer models we examined, HFD increased adiposity and body weight gain. In breast cancer models, HFD-induced increased body weights were seen from pre-weaning throughout life. Moreover, there were breast cancer model-specific physiological responses to HFD. The DMBA breast cancer model had more highly impaired glucose tolerance as compared to the other breast cancer models maintained on HFD. The quantity of blood sugar in fasting DMBA were above the threshold for increased risk of metabolic syndrome, making them at risk for HFD-induced type II diabetes (2001).

Compared to other breast cancer models, the DMBA model had a relatively slow gain in HFD-induced adiposity. However, the DMBA model had greater adiposity and body weight gain through adulthood. This is consistent with human data, where obesity increases postmenopausal breast cancer risk. In the DMBA model mice fed HFD, mammary lesion incidence was doubled and latency was shortened relative to DMBA mice on LFD. Very few DMBA mice maintained on LFD had mammary lesions,

regardless of TCDD exposure. However, maternal TCDD exposure doubled mammary lesion incidence among DMBA fed HFD.

Analysis of pubertal mammary gland morphological changes provided insight into the mechanisms of DMBA carcinogenesis. Similar to the diet effect on mammary lesion incidence, DMBA fed HFD had more TEB than DMBA fed HFD at the time of initiation. However HFD and maternal TCDD exposure caused significantly non-additive changes in TEB number. In DMBA fed LFD, maternal TCDD exposure tripled the number of TEB. In contrast, in DMBA mice of the same age and fed HFD, TCDD cuts the number of TEB in half. Since TEB number correlates with the carcinogenicity of mutagens, such as DMBA, this TCDD effect was an unexpected result (Russo and Russo 1978). To test the hypothesis that by reducing TEB number, TCDD delays the window of susceptibility to carcinogenic insult, we looked at gland morphology at the time of the second DMBA dose (Birnbaum and Fenton 2003). Mammary gland morphology was unchanged by HFD and maternal TCDD at post-natal day (PND) 49. This rapid mammary gland compensatory growth in DMBA fed HFD with maternal TCDD exposure may have increased their mammary lesion incidence.

Molecular changes in the progressing mammary gland were also examined. Diet only had a modest effect on gene expression, though *Insig* and *Comt* were altered moderately. Further, maternal TCDD exposure caused no obvious global gene expression changes during progression. This small change in mRNA suggests that the proportion of epithelium to adipose in the progressing pubertal mammary gland did not shift due to these treatments, and that maternal TCDD did not alter epithelial morphology after an acute DMBA dose. More sensitive analyses demonstrated that among DMBA mice fed

HFD, maternal TCDD increased epithelial *Cyp1b1* and decreased *Comt* expression in progressing mammary glands. The effect of maternal TCDD on *Cyp1b1* expression persisted in the mammary tumors.

In summary, work described in this dissertation shows that maternal TCDD increases mammary lesions in mice fed HFD with high obesity and type II diabetes risk. This is consistent with the central hypothesis of this work, that high fat diet and gestational exposure to TCDD would interact to increase mammary cancer risk. Maternal TCDD exposure also caused diet-dependent effects in mammary gland pubertal development, blood glucose and percent body fat across models. In the DMBA model, these effects were transient and did not have the same HFD by TCDD interaction seen in mammary lesion incidence. Instead we found that maternal TCDD caused decreased *Comt* and increased epithelial and tumor *Cyp1b1* in mammary glands of DMBA fed HFD.

WHAT IS THE SIGNIFICANCE?

It may be that susceptibility to obesity and breast cancer is determined by genetic responses to environmental risk factors for obesity and breast cancer (Han *et al.* 2004b; Hewitt 1997). Several studies have shown that AhR ligands such as polycyclic aromatic hydrocarbons (PAHs) and polycyclic halogenated aromatic hydrocarbons (PHAHs) interact with the genotype of downstream transcription products to increase breast cancer risk (Ambrosone *et al.* 1995; Li *et al.* 2005b; Li *et al.* 2004). TCDD can induce or inhibit

estrogenic effects because of AhR-estrogen receptor alpha (Erα) crosstalk or E2 metabolism (Cavalieri and Rogan 2004; Ohtake *et al.* 2003; Safe *et al.* 2000)Fig. 1-1). If the E2-catechols formed by CYP1B1 during E2 metabolism are not conjugated by COMT, oxidative stress, DNA adducts and mutations may increase cancer risk (Cavalieri and Rogan 2004; DiGiovanni *et al.* 1986) Fig. 1-1). In this study genotoxic E2 metabolites may have increased mammary cancer incidence.

Timing is important for endocrine signals. E2 has both pro- and anti-estrogenic consequences depending on the age of the E2 exposure. Both *in utero* and preweaning E2 exposures accelerates puberty onset, but whereas *in utero* E2 exposure increases carcinogen-induced mammary tumorigenesis, preweaning E2 exposures protect against carcinogen-induced mammary tumorigenesis (Cabanes *et al.* 2004; De Assis and Hilakivi-Clarke 2006; Hilakivi-Clarke *et al.* 1997).

TCDD exposure can also negatively and positively correlate with breast cancer incidence (Brown *et al.* 1998; Holcomb and Safe 1994). While TCDD reduces mammary tumor incidence and even size when given to adult female rodents (Holcomb and Safe 1994), similar to E2, *in utero* TCDD exposure increases carcinogen-induced mammary tumorigenesis (Brown *et al.* 1998). Increased terminal end buds and decreased lobules suggest delayed mammary maturation in these same rats exposed to TCDD at mid-gestation (Brown *et al.* 1998), however in this study *accelerated* puberty mammary growth appeared linked to cancer incidence. This heightened mammary tumor susceptibility may result from more rapidly developing pubertal mammary glands or from mammary developmental reprogramming (Fenton *et al.* 2002; Russo and Russo 1978). As early as PND 4, mammary glands from mice gestationally exposed to TCDD

had reduced mammary gland development, and delayed mammary puberty and permanently altered gland morphology (Fenton *et al.* 2002).

Obesity may also extend the period of pubertal mammary development(Simpson 2003), although prepuberty adiposity makes inconsistent predictions of early onset breast development (Britton *et al.* 2004; Forman *et al.* 2005). These inconsistencies may be due to subpopulations of obese girls whose genetic susceptibility shifts the timing of pubertal breast development. For instance, the lower COMT activity allele likely causes inefficient metabolism of E2, which results in greater levels of active E2 systemically and an increase in the speed of puberty breast development in girls (Eriksson *et al.* 2005) Fig. 1-1). Because of auto-amplification of E2 levels, this systemic E2 elevation could occur throughout early pubertal mammary development (Eriksson *et al.* 2005). Here we saw TCDD reduce TEB and ductal growth in high fat fed DMBA mice during cancer initiation, but no TEB changes were seen during cancer promotion/progression. This suggests that maternal TCDD exposure depressed carcinogen initiation, but did not cause an increase of DMBA susceptibility due to delayed mammary development later. The increase in E2 would result in increased E2-derived mutagenic metabolites in mammary epithelium with elevated CYP1B1 (Fig. 1-1).

Altered E2 homeostasis is a potential mechanistic link between menarche and breast cancer (Fig. 1-1). However, caution is needed in linking pubertal mammary development to the risk of development of mammary cancer later in life. Accelerated, delayed or persistently altered mammary gland development may alter susceptibility to carcinogenic insult, and perinatal reprogramming of the gland may be the mechanism through which maternal TCDD exposure influences carcinogenesis in progeny. Such

reprogramming must involve more than altered gland morphology in obese DMBA mice (Brown *et al.* 1998; Fenton *et al.* 2002; Russo and Russo 1978). Results in this dissertation are nevertheless consistent with an increased susceptibility to later carcinogenic insult by *in utero* estrogenic exposures (Brown *et al.* 1998; Cabanes *et al.* 2004; De Assis and Hilakivi-Clarke 2006; Hilakivi-Clarke *et al.* 1997).

Multiple lines of evidence suggest that perinatal reprogramming of the mammary gland can increase susceptibility to mammary cancer, and this may be the mechanism of increased mammary tumor incidence in offspring fed HFD and maternally exposed to TCDD observed in present studies. First, the WNT pathway is involved in fetal mammary programming, puberty and in the DMBA model. WNT-dependent signals are involved in mammary bud formation at gestational day (GD)12.5, when dams were exposed to TCDD or vehicle (Robinson 2007). Bud formation is a critical imprinting period, therefore it is possible that on GD12.5, TCDD imprinted WNT. The mammary gland continues to develop postnatally, and at the next stage of mammary gland development, puberty, is again dependent on the canonical WNT/β-catenin pathway, including orchestration with ERα transcriptional regulation. The DMBA mouse model of breast cancer activates the canonical WNT/β-catenin pathway during carcinogenesis (Currier *et al.* 2005).

Epigenetic changes in *Cyp1b1* and *Comt* due to maternal TCDD exposure are possible additional mechanisms of fetal mammary programming could be (Fig. 1-1). There is substantial evidence to suggest that adult endocrine disease risk can be modified by environmental factors early in development, particularly through fetal epigenomic changes (Anderson *et al.* 2000; Dolinoy *et al.* 2007a; Junien and Nathanielsz 2007).

Maternal exposure can shift methylation patterns causing decreased reproductive health across generations and increased incidence of endocrine diseases such as obesity and cancer (Anway *et al.* 2005; Brown *et al.* 1998; Cropley *et al.* 2006; Dolinoy *et al.* 2007b; Ho *et al.* 2006; Li *et al.* 2003; Li *et al.* 1997; Prins *et al.* 2008). Further, *CYP1B1* and *COMT* are implicated in mammary lesion incidence here, and their promoter methylation impacts endocrine cancers. *CYP1B1* promoter methylation is associated with decreased survival of breast cancer patients, and its effect on E2 steady states profoundly impacts E2-targeted chemotherapy (Widschwendter *et al.* 2004). *COMT* methylation inactivates *COMT* in endometrial tumors, but not in control endometrium (Sasaki *et al.* 2003). Presently there was suggestive interaction ($p < 0.2$) between maternal TCDD and tissue status (normal vs. tumor) on *Comt* induction. Thus, it is possible that TCDD increases mammary cancer in DMBA mice because of interactions between TCDD-induced fetal programming and DMBA-induced WNT receptor signaling at puberty.

The rapid compensatory growth during pubertal mammary development and the *Comt* and *Cyp1b1* expression changes occurred only when DMBA offspring were exposed to maternal TCDD and fed HFD. However, the above hypotheses regarding maternal TCDD exposure do not fully explain the TCDD- increased incidence of mammary cancer in obese DMBA mice. To grasp this relationship between maternal TCDD and obesity, we must also consider the unique susceptibilities to maternal TCDD among the obese, possibly via differences in pharmacokinetic behaviors and estrogenic activities.

Obese mice have less TCDD-binding protein in the liver, thus relatively more TCDD in fat compared to liver (Emond *et al.* 2006). Because the larger size of the fat

compartment in DMBA fed HFD and maternally exposed to TCDD, the concentration of TCDD in the mammary fat pad may be lower. Offspring with increased body fat also eliminate TCDD at slower rates, making this acute dose more persistent in the mammary fat pads (DeVito *et al.* 2003; Michalek and Tripathi 1999). Since TCDD is in the mammary glands of DMBA fed HFD for longer times, this may result in greater TCDD effects in the mammary glands despite its lower concentration. These TCDD pharmacokinetic behaviors provide more opportunity for maternal TCDD to modify development and to postnatally imprint permanent changes on the mammary gland. Further, infants receive much of their mothers' TCDD body burden through maternofetal transfer and breastfeeding. This exacerbates the potential risk of TCDD exposure because of the increased sensitivity at these developmental stages.

These obese by TCDD interactions on mammary cancer places focus on E2 and its receptor, ERα. TCDD, a potent AhR activator, could indirectly interact with ER in mammary fat pads and thereby increase mammary tumor risk. The activated AhR complex directly associates with ER, recruiting ER to estrogen response elements (ERE) on E2 promoters (Ohtake *et al.* 2003). This results in transcriptional- dependent downstream estrogenic-like activity in ovariectomized mice, but not in ovarierectomized mice with deficiency in either AhR or ER (Ohtake *et al.* 2003).

It is commonly thought that extragonadal production of E2 poses an insignificant risk on breast cancer until menopause, but new lines of evidence are beginning to suggest otherwise. Adipose tissue produces E2, and excess adipose increases peripheral E2 levels (Simpson 2003). The decrease in *Comt* seen in DMBA fed HFD suggests that systemic E2 levels can increase also, at least during puberty (Eriksson *et al.* 2005; Fig.1-1). This

could cause greater proliferation through increased binding to ER, or if that saturates, increased genotoxicity through E2 metabolites (Fig 1-1). Obese mice and women have greater adiposity of their breasts and thus greater concentrations of E2 within their breasts. The role of adipocyte-produced E2 in mammary carcinogenesis has been recently demonstrated in several breast cancer models. HER2 breast cancer models are ER negative, and neither maternal TCDD nor obesity modifies their breast cancer risk (Cleary *et al.* 2004). However ER positive tumor incidence is increased in response to obesity (Cleary *et al.* 2004). Further, obese ovariectomized rats have increased mammary tumors compared to lean ovariectomized rats (Hakkak *et al.* 2007). Since obesity can increase cancer risk without central E2 production, it is feasible that the interaction between obesity and TCDD may increase steady state E2 levels in mammary tissue and tumors. This is noteworthy in part because obese women with ER positive breast cancers may be at a disproportional risk of breast cancer mortality because of the interaction of increased ERα and its ligand, E2 (Lorincz and Sukumar 2006).

REMAINING QUESTIONS AND FUTURE DIRECTIONS

TCDD and HFD might interact to increase mammary lesion incidence through a number of plausible explanations involving pharmacokinetics and endocrine disruption. To expand this finding to account for TCDD exposure variation among natural populations would first require a dose response analysis. It may be that individual genetic susceptibilities to obesity is influenced by the genetic response to such environmental factors (Hewitt 1997), and this should be further explored in the adipose of both pubertal

and tumor-bearing mice. Investigating other endocrine disrupting lipophilic toxicants would shed further light on the existence of an 'obese by endocrine disruptor *in utero* causes mammary cancer' paradigm (Birnbaum and Fenton 2003; De Assis and Hilakivi-Clarke 2006). The diet by TCDD relationships in fasting blood glucose and in mammary gland structure also needs further probing. Did the DMBA model develop type II diabetes and could this have influenced the mammary tumor etiology? Did the pubertal morphology of the gland influence its later mammary lesions and if so, how?

TCDD exposure affected with pubertal blood glucose, pubertal mammary development and mammary lesion incidence by differing mechanisms; in each of these three phenotypes, the effect of maternal TCDD uniquely interacted with diet. Will we consistently find endocrine disruptors uniquely interact with obesity on endocrine systems that control type II diabetes risk, mammary puberty and mammary lesions? More mechanistic data would help to estimate the justification of any interspecies comparisons. How and when is TCDD altering *Cyp1b1* and *Comt* expression? What physiological consequences ensue from this change? Additional studies are needed to examine the role of fetal TCDD exposure on epigenetic changes, particularly in the promoters of *Cyp1b1* and *Comt*.

We find that HFD contributes to an increase in adiposity, blood sugar, speed of pubertal mammary gland development and mammary lesion incidence. Here we show that TCDD doubles mammary lesion incidence among the obese. The interaction of HFD and TCDD on mammary tumor incidence is driven by alterations in the homeostasis of the one molecule consistently implicated in breast cancer risk: E2 (Fig. 1-1). This is likely due to increased mammary E2 production and increased E2-genoxicity in DMBA

mice that are maternally exposed to TCDD and fed HFD. TCDD-induced *Cyp1b1* expression in both mammary epithelia during carcinogenesis and in mammary tumors. These effects may further result from AhR-ERα receptor crosstalk (Ohtake *et al.* 2003), *in utero* gland programming (Fenton *et al.* 2002), and prolonged half-life of low dose TCDD in obese mice (Emond *et al.* 2006). These possibilities should be carefully examined in the future.

This study examined how environmental estrogen exposures, such as obesegenic diet- and maternal TCDD exposure, impact breast cancer. A better understanding of the biological and molecular history of breast cancer among the obese will support more informed choices for risk assessment and therapy. Obesity is on the rise in children and adults, therefore we can expect health care expenditures relating to obesity to rise dramatically if intervention aimed at curtailing obesity fails (Popkin and Doak 1998; Popkin and Udry 1998). Because of the ubiquity of TCDD exposure in humans, it is likely that everyone carries some TCDD burden, with those exposed to higher levels of TCDD during the 1960s and 1970s currently at the age where latent breast cancers will begin to develop. Although TCDD levels are likely low in most people, among obese women there is greater TCDD persistency (Emond *et al.* 2006). Obesity and maternal TCDD appear to be playing an etiological role in mammary cancer susceptibility through endocrine disruption. This may influence estrogen-targeted therapies. DMBA and TCDD represent PAHs and PHAHs, respectively, and are ubiquitous chemicals in the environment that occur as complex mixtures in human and environmental samples. These mixtures are overlapped by the increasing prevalence of obesity. Given the potential interactions of the obese phenotype with PAHs and PHAHs in altering breast cancer risk,

this study is highly relevant, as it has begun the evaluation of the patterns of

carcinogenesis ensuing from exposures among the obese.

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