Assessment of Breast Cancer Development and Aggression

with Heavy Metal Exposures in Illinois

BY

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THESIS

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This thesis is dedicated to my wife-to-be, Catherine Marie Bulka, who has helped me understand and reach my potential as a student, researcher and human. I also dedicate this thesis to my Mother, Janice Elaine Kilburn; Father, Stephen Kresovich; and Brother, Alexander Kilburn Kresovich who offered me unconditional love and support through good and bad times.

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LIST OF ABBREVIATIONS

5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcystine
AAS	Atomic Absorption Spectrophotometry
As	Arsenic
ATSDR	Agency for Toxic Substances and Disease Registry
BCCC	Breast Cancer Care in Chicago
Be	Beryllium
BMI	Body Mass Index
Cd	Cadmium
CI	Confidence Intervals
Co	Cobalt
Cr	Chromium
Cr(VI)	Hexavalent Chromium
DCT1	Divalent Cation Trasporter-1
DDR	DNA Damage Repair
EPA	Environmental Protection Agency
ER	Estrogen Receptor
ER/PR	Estrogen Receptor and Progesterone Receptor
FFPE	Formalin-Fixed, Paraffin-Embedded
FFQ	Food Frequency Questionnaires
H&E	Hematoxylin and Eosin
HER2	Human Epidermal Growth Factor Receptor 2

LIST OF ABBREVIATIONS (continued)

Hg	Mercury
IARC	International Agency for Research on Cancer
ICP-MS	Inductive Coupled Plasma-Mass Spectroscopy
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
IHC	Immunohistochemical
KHB	Karlson, Holm and Breen
LIDPBC	Long Island Database Project for Breast Cancer
MAPKs	Mitogen-Activated Protein Kinases
Mn	Manganese
MnSOD	Manganese Superoxide Dismutase
NATA	National-scale Air Toxics Assessment
NEI	National Emissions Inventory
nH	non-Hispanic
NHANES	National Health and Nutrition Examination Survey
Ni	Nickel
OR	Odds Ratio
Pb	Lead
PCBs	Polychlorinated Biphenyls
PM	Particular Matter
$PM_{2.5}$	Particular Matter less than 2.5 µm
PM_{10}	Particular Matter less than 10 µm
PR	Progesterone Receptor

ROS	Reactive Oxygen Species
RPF	Reproductive Factors
Sat2	Satalite2
Sb	Antimony
Sb(III)	Trivalent Antimony
Se	Selenium
SES	Socioeconomic Status
TCGA	The Cancer Genome Atlas

SUMMARY

Heavy metals are ubiquitous in the environment and are naturally found throughout the ecosystem. They are non-biodegradable, persistent and bioaccumulate making them a particularly serious class of environmental contaminants. The Agency for Toxic Substances and Disease Registry (ATSDR) ranked arsenic, lead and mercury as the top three substance priorities regarding public health. The International Agency for Research on Cancer (IARC) identified arsenic, beryllium, cadmium, chromium and nickel as 'known' carcinogens (Group 1) while lead has been identified as a 'probable' carcinogen (Group 2A). *In vitro* and *in vivo* studies have demonstrated heavy metals bind to estrogen receptor (ER)-alpha and mimic the effects of estrogen/estradiol suggesting exposure to them may serve as a risk factor for the development of breast cancer. This dissertation examined associations between airborne heavy metal exposures with aggressive breast cancer characteristics and examined DNA methylation as a potential intermediary.

Using the Breast Cancer Care in Chicago cohort, a population-based and ethnically diverse sample of women diagnosed with breast cancer in Chicago, we showed increasing exposure to antimony, arsenic, cobalt, manganese and selenium was associated with increased prevalence of estrogen receptor/progesterone receptor (ER/PR)-negative tumors. We additionally showed chronic exposure to antimony, cobalt, lead and manganese was associated with increased aberrant methylation of *GSTM2*, a gene responsible for the detoxification of environmental pollutants. We finally showed increased aberrant methylation of *GSTM2* was associated with increased prevalence of ER/PR-negative breast tumors. Together these results suggest exposure to airborne heavy metals are mechanistically involved in the development of aggressive breast cancer phenotypes through their effects on gene-specific methylation of cancer-associated genes. Non-Hispanic (nH) Black women are more likely to develop ER/PR-negative breast cancer compared with nH White women and are more likely to be

exposed to antimony and manganese; these results therefore suggest environmental remediation of these contaminants may result in the reduction of the racial disparity in aggressive breast cancer phenotypes.

I. INTRODUCTION

A. Heavy Metal Background

Heavy metals are ubiquitous in the environment and are naturally found throughout the ecosystem. They are defined as an elemental metal with a density of greater than five grams per cubic centimeter (1). This definition includes metals such as beryllium, cadmium, chromium, cobalt, mercury, nickel and lead. Furthermore, the term 'heavy metal' is used loosely and generally refers to metalloids such as arsenic, antimony and selenium.

Heavy metals originate from the Earth's crust and enter the air, water and soil through various natural processes and human activities (2). The main routes of human exposure are though inhalation and ingestion; occupational and agricultural workers have higher exposures than the general population. Moreover, smokers have increased exposure compared to nonsmokers (3). Regardless of occupational and smoking status, everyone is primarily exposed through diet as heavy metals are drawn into vegetables and grains from the soil (4).

Biologically, certain metals are required by living organisms for biochemical and physiological functions. These metals are required in small amounts and are referred to as 'trace' or 'essential' metals. Examples include chromium, cobalt, copper, iron, magnesium, manganese, molybdenum, nickel, selenium and zinc (5). Diseases that arise from deficiencies of these metals include anemia and Keshan disease (6). Although these metals are essential at specific amounts, they become toxic at high levels of exposure.

Almost all other heavy metals have no beneficial effects and become toxic when a certain threshold is met. Examples of these include arsenic, cadmium, mercury and lead. High exposure to these metals have been associated with a number of disorders and diseases--particularly

mental retardation (7), Parkinson's Disease (8), Blackfoot Disease (9), diseases of the nervous system (10) and various cancers including breast (11), lung (12), skin (9, 13) and bladder (14).

An important characteristic of heavy metals is they are non-biodegradable, persistent and bioaccumulate (6). These qualities make heavy metals a particularly serious class of environmental contaminants. The Agency for Toxic Substances and Disease Registry (ATSDR) ranks public health priorities for over two hundred identified hazardous materials every two years with a focus on substances that pose the most significant potential threat to human health. In 2013, the ATSDR ranked arsenic, lead and mercury as the top three substance priorities. Additionally, cadmium and chromium were ranked seventh and seventeenth, respectively (15). These results were unchanged for the 2015 rankings (16). The International Agency for Research on Cancer (IARC) has additionally identified arsenic, beryllium, cadmium, chromium and nickel as 'known' carcinogens (Group 1) (17) while lead has been identified as 'probable' carcinogen (Group 2A) (18, 19).

In vitro and *in vivo* studies have demonstrated heavy metals bind to ER-alpha and mimic the effects of estrogen in the body (20). These effects have been found for antimony, arsenite, barium, cadmium, copper, chromium, cobalt, lead, mercury, nickel, tin, selenite and vanadate (21-25) . As a result, these metals are often referred to as 'metalloestrogens.' An important implication from these studies is metals may have multiple pathways through which they may induce certain cancers, particularly ones that are driven by hormonal exposures. To date, very few epidemiologic studies have investigated associations between heavy metal exposure with breast cancer incidence and subtype. Table I summarizes the effects of a selected list of heavy metals.

TABLE I.

CHARACTERISTICS OF SELECTED HEAVY METALS

Heavy Metal	Known Carcinogen [a]	Probable Carcinogen [a]	Possible Carcinogen [a]	Non-classifiable [a]	Essential metal	Metalloestrogen
Antimony (Sb)			X			X
Arsenic (As)	X					X
Beryllium (Be)	X					
Cadmium (Cd)	X					X
Chromium (Cr)	X				X	X
Cobalt (Co)			X		X	X
Lead (Pb)		X				X
Manganese (Mn)				X	X	
Mercury (Hg)				X		X
Nickel (Ni)	X				X	X
Selenium (Se)				X	X	X

[a] As determined by the International Agency for Research on Cancer

B. Metal Bioavailability in the Body

An important consideration for the carcinogenicity of metals is that toxicity depends largely on bioavailability. Essential metals are often maintained within a narrow concentration range through various mechanisms in the human body, although these processes tend to be inadequate at controlling cellular concentrations of nonessential metals (26). There is additionally a large amount of variability among metals in the fraction that is absorbed into the bloodstream compared to the amount that is immediately excreted. This process is highly dependent on the route of exposure (6). Metals that are not excreted or controlled via mechanisms of homeostasis can exert adverse biological effects throughout the body.

Inhaled and ingested heavy metals move beyond the respiratory and gastrointestinal systems by binding to transport proteins, disbursing systemically throughout the body. Previous studies have shown human serum albumin is an important transporter of essential metals like copper and zinc. Divalent nickel, cobalt and cadmium also bind to this protein and are similarly transported throughout the body (27). Additionally, other heavy metals such as cadmium, lead and mercury have been shown to bind to metallothionein, a cysteine-rich class of proteins with the ability to affix to xenobiotic agents (28-31). This process results in the bioaccumulation of metals in the liver and kidneys (32).

Metal ions additionally bioaccumulate in cells by passing through membrane protein channels. Divalent cation trasporter-1 (DCT1) is a transmembrane protein responsible for cellular uptake of divalent iron and other essential metals. This protein also mediates the uptake of divalent cadmium, lead, and other toxic metals (26). Additionally, proteins from the SLC39 and ZIP family of transmembrane metal ion transporters are responsible for the cellular uptake of

iron, manganese and zinc. Evidence suggests that exposure to cadmium inhibits uptake of these metals, suggesting cadmium is an important substrate for these transporters as well (33, 34).

Metals also bioaccumulate in cells by mimicry of other endogenous molecules. Toxic metal ions compete for binding sites of essential metal ions if they are of similar charge and size (35, 36). Phosphate is a molecule responsible for normal cellular functions and skeletal mineralization. It shares an almost identical structure and physiochemical properties with arsenate, a toxic molecule (37). When arsenate accumulates in biological systems, it metabolizes into other forms of inorganic arsenic and affect organs in which it concentrates, such as the liver, lung and bladder (38). Sulfate is molecule that is important for normal cellular growth and function in the human body (39) and it has an identical structure as chromate. Chromate is toxic and will competitively inhibit cellular uptake of sulfate (40). Lead binds irreversibly to neurons through the mimicry of natural minerals such as calcium and zinc (41). Finally, certain heavy metals such as antimony, cadmium, chromium, cobalt, lead, mercury, and nickel exert estrogenic effects by binding to ER- alpha at its hormone binding complex (20, 21).

Heavy metals have the ability to become systematic toxicants and induce multiple organ damage, even at low levels of exposure. As heavy metals are highly bioavailable, they move beyond the respiratory and gastrointestinal tracks and contribute to the carcinogenicity of internal organ systems. This process is generally accomplished by binding to transport proteins, passage through non-specific transmembrane protein channels, and mimicry of other endogenous molecules.

C. **Studies of Metals in Breast Tissue**

Metals have the ability to move beyond their initial point of contact allowing them to affect various organ systems throughout the body. Metals additionally have the ability to exert

estrogenic effects by binding to cells expressing estrogen-receptor alpha. Therefore the presence of certain heavy metals in breast tissue is expected. Furthermore, concentrations of heavy metals are more likely to be higher in breast cancer patients as this disease is primarily hormonally driven and often includes high proliferation of cells expressing estrogen-receptor alpha.

The first study to investigate the association between heavy metal concentrations in breast cancer patients and cancer-free controls analyzed the concentration of cadmium in breast fat tissue from forty-three breast cancer patients and thirty-two healthy control subjects (42). The researchers did not find a significant difference between cases and controls, but did find unexpectedly high amounts of cadmium in in the breast cancer samples. Importantly, they found cadmium concentrations in breast tissue did not correlate with age, stage of cancer, or concentration of zinc, copper or selenium. The researchers did however identify associations between breast tissue cadmium concentration and smoking status ($p = 0.05$) and found higher concentrations of cadmium in the tissue of ER- positive compared to ER- negative patients ($p = 0.06$). While the data showed interesting associations, namely the differences in cadmium concentrations by breast cancer subtype, the authors concluded the results neither proved nor disproved a relationship between cadmium exposure and breast cancer (42).

Ionescu et al. (2006) investigated the concentrations of transition and heavy metals in breast tumor samples and hypothesized metals that contribute to free radical generation through Fenton- and Harber-Weiss-reactions would be higher in breast cancer biopsies compared with healthy breast tissue biopsies (43). Using twenty frozen breast cancer biopsies and eight healthy breast tissue samples, the researchers used atomic absorption spectrophotometry (AAS) to quantify the concentrations of iron, cadmium, lead, chromium, tin, nickel, copper, mercury, silver, gold, palladium and zinc in the tumor biopsies. They used inductive coupled plasma-mass

spectroscopy (ICP-MS) to measure the same metals in the control biopsies. Consistent with their hypothesis, the researchers found breast cancer biopsies had significantly higher amounts of iron, nickel, chromium, zinc, mercury and cadmium compared with the healthy breast tissue samples (all tests p-values < 0.005) (43).

Strumylaite et al. (2011) conducted a case-control study with fifty-seven incident breast cancer patients and fifty-one patients with benign breast tumors. Using AAS to quantify metal concentrations, it was found that among breast cancer patients, tumor tissue had 2.7 times the amount of cadmium compared with healthy tissue from the controls (44). Additionally, cadmium was 1.5 times higher in breast cancer patients' tumors compared with benign tumor samples from controls. Importantly, this study analyzed cadmium concentrations within subgroups of breast tumors—ER-positive tumors had significantly higher concentrations of cadmium compared with ER-negative tumors. The researchers also found a significant correlation between age and urinary cadmium, but no correlation between age and tissue cadmium concentration (44). This finding suggests that cadmium bioaccumulates in the kidneys but not in breast tissue, suggesting the metal may be involved in cellular processes localized specifically within the breast.

Romanowicz-Makowska et al. (2011) examined concentrations of cadmium, nickel and aluminum in paired samples of tumor and healthy tissues within the same patient. The researchers collected invasive ductal carcinoma samples from 67 affected women, and additionally collected non-cancerous normal tissue from as far from the neoplasia as possible from 16 women. Using AAS, the researchers found statistically higher amounts of cadmium and aluminum in tumor components compared with healthy tissue ($p < 0.05$). Additionally, they identified a marginally significant association with nickel concentrations ($p = 0.06$). The

researchers did not find any associations between metal concentration and tumor grade. The authors acknowledged that this study had a very small sample size and likely lacked power to sufficiently examine all research questions (45).

El-Harouny et al. (2011) employed a case-control design studying breast tissue metal content in an Egyptian population of women. A total of 75 breast cancer patients and 25 women with benign breast disease were enrolled. Metal concentrations were quantified using ICP-MS (46). In unadjusted analyses, the researchers found significantly higher amounts of cadmium in the urine and breast tissue of the breast cancer patients compared with controls, although they did not find any significant differences for iron, copper, lead or zinc. This study was likely confounded as the breast cancer patients were more likely to be postmenopausal and have three or more children. Moreover, the breast cancer patients were an average of ten years older than the women with benign breast disease, although this difference was not statistically significant (46).

Finally, Mohammadi et al. (2014) conducted a descriptive study investigating the concentrations of cadmium, lead, mercury and selenium in various breast tissue components. The sample included fourteen patients who underwent mastectomy surgery where tissue from the tegmen, tumor, tumor adiposity and tegmen adiposity was collected and frozen (47). Lead and cadmium concentrations were quantified using AAS while selenium concentration was measured using inductively coupled plasma optical emission spectroscopy (ICP-OES). Finally, total mercury concentration was estimated using the Leco AMA 254 Advanced Mercury Analyzer. The researchers found no significant differences in the concentrations of cadmium, lead, mercury or selenium across any of the breast tissue components (47). The authors argued this was likely due to the small sample size and the low power to detect differences. The differences between

the metal concentrations across the tissue components were non-significant for cadmium (p= 0.32), selenium (p= 0.24) or mercury (p= 0.14).

These previously mentioned studies were pioneers in examining heavy metal concentrations in the breast tissue of breast cancer patients and showed that these metals are present in all women. These studies additionally indicated that metals ions do not bioaccumulate in breast tissue as identified by the lack of correlations with age. These results suggest the metals are present for another reason. Two studies explicitly tested this hypothesis and showed no correlation between age and cadmium concentration in heathy or cancerous breast tissues, although significant correlations were identified with urinary cadmium (42, 44). When the researchers additionally stratified breast cancer patients by receptor status, they found higher amounts of cadmium in estrogen-receptor positive compared with estrogen-receptor negative samples. These findings suggest cadmium is acting as a metalloestrogen and is potentially mimicking the effects of estrogen in the breast tissue. Importantly, all of these studies utilized a small number of samples and were restricted to simple statistical tests to investigate differences. None of the studies accounted for potential confounders; therefore these findings need to be interpreted cautiously. Studies with larger sample sizes will be able to control for confounding variables and be able to better elucidate the relationship between breast tissue metal concentrations and associations with breast cancer risk and aggression.

D. **Xenobiotic Mechanisms of Carcinogenesis**

Environmental toxins, such as heavy metals, influence tumor development at various stages of carcinogenesis. Through different mechanisms, xenobiotic molecules affect the processes of tumor initiation, promotion, malignant conversion and progression (48).

Initiation of cancer development is carried out through the buildup of somatic mutations and aberrant DNA methylation at the cellular level. Mutations in biologically relevant areas of the genome affect tumor development in many ways. The most common are through inactivation of tumor suppressor, genomic stability, and DNA damage repair genes and conversion of proto-oncogenes into oncogenes. These processes are usually carried out by environmental exposures through the development of a DNA adducts or reactive oxygen species which mutate DNA (49). Aberrant hypermethylation of the promoter regions of tumor suppressor, genomic stability, and DNA damage repair genes additionally increase the probability of cancer initiation. Similarly the lack of DNA methylation, or hypomethylation, in certain genomic areas results in either up-regulation of oncogenes or decreased global genomic stability (50).

Tumor promotion refers to the process in which mutated cells divide uncontrollably into a larger population of cells, commonly referred to as cellular proliferation. This process is normally carried out through the activation of intracellular signaling pathways in which ligands bind to receptors on the cellular surface (51). For example, heavy metals bind to ER-alpha resulting in the up-regulation of estrogen-mediated pathways resulting in increased cellular division. Other important components of tumor promotion relate to the proinflammatory effects of carcinogenic agents and cellular resistance to apoptosis (52, 53).

Malignant conversion is the first stage in tumor progression and is indicated by the transformation of a pre-malignant lesion into a primary tumor with a cancerous phenotype. An example of malignant conversion is development of human epidermal growth factor receptor 2 (HER2)/neu receptors on the cellular membrane of proliferating breast or ovarian cells (54). This process is usually accompanied by the development of additional mutations which are the result of imprecise DNA replication mechanisms (55).

Tumor progression is the uncontrolled growth and development of aggressive features in cells showing a malignant phenotype (48). This is commonly caused by the accumulation of alternations in tumor suppressor and oncogenes. In this stage, malignant lesions develop the ability to metastasize to other organs beyond the primary site. A major mechanism in which malignant lesions develop metastatic characteristics is through the loss of heterozygosity of cellular adhesion genes (56). For example, loss of heterozygosity of the genomic region 14q has been consistently observed in metastatic colorectal carcinomas (57-59). Furthermore, researchers have identified a number of tumor suppressor genes located in the same region resulting in multiple pressures for tumor progression (60).

Xenobiotic agents such as heavy metals can affect carcinogenesis at any of these stages through a variety of mechanisms. Two major categories of how metals can accomplish this are through estrogen-independent and estrogen-dependent pathways. For example, estrogen independent pathways can refer to metals' ability to form DNA adducts, increase levels of inflammation, or to produce reactive oxygen species. Estrogen-dependent pathways involve certain metals' ability to bind to ER-alpha resulting in the exertion of estrogenic effects in the target tissue.

E. Mechanisms of Metal Carcinogenesis

1. Estrogen Independent Mechanisms

Investigations are currently underway to determine the etiologic role of heavy metal exposure in carcinogenesis. Studies that employ *in vitro* and *in vivo* methods shed light on the precise biological mechanisms and cellular pathways through which heavy metals induce toxicological properties.

Heavy metals affect carcinogenesis at the initiation phase through either direct or indirect mutational mechanisms. Hexavalent chromium (Cr(VI)) is the only heavy metal which directly mutates DNA by covalently binding to nucleotides and forming DNA adducts (61-63). Other metals are also genotoxic, but are generally considered weak mutagens and therefore have additional properties that affect rates of DNA mutations (3). In particular, metals bind to DNA damage repair (DDR) proteins inhibiting their effectiveness at correcting genomic errors. Over time, this process results in persistent DNA damage (63-65). Aluminum, nickel, cobalt, cadmium, copper, zinc and iron all inhibit the activity of NEIL1, a DNA glycosylase responsible for the repair of oxidative DNA lesions (66). Therefore, toxic metal ions generally do not directly cause DNA damage, but rather impair the ability of cells to respond to it. As a result, toxic metal ions are co-mutagenic and enhance the mutagenicity of other present genotoxic agents (64, 67).

Heavy metal ions also cause indirect DNA mutation through the generation of free radicals such as reactive oxygen species (ROS). Examples of ROS include peroxides, superoxide, singlet oxygen and hydroxyl radicals (68) and have been shown to damage DNA through the formation of DNA adducts and the creation of single- and double-strand breaks resulting in structural aberrations (69-71). Oxidative stress, a mechanism of carcinogenesis, refers to the cellular build-up of free radicals due to the inability to neutralize their harmful effects by way of anti-oxidant molecules (72, 73). Metals such as iron, copper, cobalt, chromium and nickel are known as redox active metals and contribute to mutagenesis via the direct generation of reactive oxygen species. These molecules are generated through known chemical reaction pathways including Fenton- and Haber-Weiss-type reactions (74, 75). Other metals, such as arsenic and cadmium do not directly form ROS through these reactions (76), rather they

form ROS through interactions with other systems. Cadmium interacts with electron transfer chains in the mitochondria to form ROS while arsenite interacts with superoxide dismutases to create excess levels of hydrogen peroxide and hydroxyl radicals (77, 78).

Heavy metals also have the ability to affect carcinogenesis at the tumor promotion stage by affecting gene expression, dysregulating apoptosis and disrupting intracellular signaling resulting in increased cellular proliferation. Heavy metals alter gene expression through their effects on epigenomic mechanisms such as DNA methylation and histone modifications. High heavy metal exposures are associated with increased gene promotor hypermethylation and genome-wide hypomethylation, both of which are a hallmark of cancer (79-81). Studies using animal models have shown high nickel exposure results in the hypermethylation of the promoters regions of tumor suppressors *TP16*, *RASSF1* and *RAR-β2* (82, 83). High nickel exposure affects histone modifications by decreasing levels of histone H4 acetylation and increasing H3K9 dimethylation, both of which are markers of transcriptional repression (84, 85). Exposure to high concentrations of arsenic is associated with promoter hypermethylation of *TP53* and *TP16*; this process is carried out through the up-regulation of methyltransferases (86, 87). Finally, low-level, long-term exposure to cadmium is associated with hypomethylation of the DNA repeat LINE1 (88).

Exposure to heavy metals also affects tumor promotion through the dysregulation of apoptosis. Cadmium-induced carcinogenicity is partially carried out through the development of cellular resistance to apoptosis (89). Cadmium transformed cells overexpress Nrf2 and p62 pathways, both of which result in resistance to apoptosis (90). Conversely, exposure to metals such as antimony, arsenic, beryllium, cadmium, chromium, cobalt, copper, lead, mercury and nickel induces apoptosis through the generation of reactive oxygen species (91, 92). More

specifically, chromium induces apoptosis through reduction of mitochondrial membrane potential, increases in p53 protein expression and development of ROS (93, 94).

Heavy metals affect carcinogenesis during the tumor promotion phase by disrupting intracellular signaling. Cadmium activates mitogen-activated protein kinases (MAPKs) which to regulate proliferation, mitosis and cellular survival mechanisms (95, 96). *In vivo* studies show arsenic and chromium promote tumorigenesis through the upregulation of the Wnt/β-catenin signaling pathway, a well-known pathway known to regulate proliferation, fate specification and differentiation (97, 98). Importantly, studies show that many cancers rely on Wnt signaling for proliferation and survival, even in the absence of mutations (99). Finally, heavy metals which contribute to the development of ROS affect cellular proliferation through redox regulation and ROS-mediated processes (100, 101).

2. **Estrogen Dependent Mechanisms**

Various heavy metals have the unique ability to exert estrogenic effects in living organisms. Metals such as antimony, arsenite, barium, cadmium, copper, chromium, cobalt, lead, mercury, nickel, tin, selenite and vanadate are often referred to as ‘metalloestrogens’ (20). Estrogen exposure is a well-known risk factor for the development of cancer, particularly in breast, ovary, cervix and endometrium tissues (102). This is especially true for hormonally driven cancers which overexpress ERs (103). Therefore, any xenobiotic agent which mimics the effect of estrogen, such as metalloestrogens, will also be a risk factor for the development of hormonally driven cancers.

a. **Metal Mimicry of Estrogen**

The first study to investigate the estrogenic properties of metals *in vitro* studied the cadmium and zinc exposure on an ER-alpha expressing breast cancer cell line. Garcia-Morales et

al. (1994) treated MCF-7 cells with one μM of cadmium and found similar cellular responses as if the cells had been treated with estradiol. These reactions included decreased levels of cellular ER concentrations and mRNA. Additionally, progesterone receptor (PR) levels were increased by 3.2 fold. These effects were blocked when anti-estrogens were introduced. Garcia-Morales et al. (1994) also found increased transcription of *PGR* and pS2 (*TFF1*) genes. Both of these genes have an estrogen response element which regulates gene transcription in the presence of estrogen. Furthermore, these effects were replicated in a basal breast cancer cell line (MDA-MB-231) transfected with cellular ERs. The cadmium exposure was also shown to induce the growth and proliferation of the MCF-7 cell line. Importantly, these findings were not identified for zinc, hinting the estrogen-mimicking effects were not applicable to all metals (25).

An additional study investigated the effects of the heavy metals of copper, chromium, cobalt, lead, mercury, nickel, tin and vanadate on the same breast cancer cell line. Martin et al. (2003) found the aforementioned heavy metals acted similar to estradiol by stimulating cellular proliferation. Furthermore, these metals decreased the cellular concentrations of ER-alpha and ER-alpha mRNA, while inducing expression of *PGR* and pS2 (*TFF1*). Again, these effects were blocked by an anti-estrogen, suggesting the activity of these compounds was mediated by ER-alpha. Martin et al. (2003) expanded their findings by investigating the locations the metals were binding to on the ER-alpha molecule. They found the metals were activating ER-alpha by forming a complex with the hormone-binding domain of the receptor. This is the same location estradiol binds to ER-alpha. Using binding assays, the researchers also found that the metals blocked estradiol from binding without directly affecting the binding affinity of estradiol. These results suggest the metals were in pure competition for the hormone-binding domain and not

chemically altering estradiol (21). Similar results were identified for antimony and barium in an additional study conducted using the same methods (22).

b. **Estrogen-Receptor Mediated Pathways**

Estrogen exposure is a risk factor for the development of many types of hormonally-associated cancers. One explanation is that estrogen enhances cellular proliferation through the activation of ER-alpha (104) . Furthermore, estradiol, a ligand which binds to the ER, has the ability to alter cell-cycle control processes resulting in the inhibition of apoptosis.

Uncontrolled cellular proliferation is associated with a number of human malignancies, but is most strongly associated with cancer. As the number of cellular divisions increases, there are more opportunities arise for errors during DNA replication (105). Furthermore, when cell divisions are rapid, DNA repair mechanisms do not work as effectively and DNA adducts and breaks are converted into fixed mutations, accumulating in daughter cells (106). This results in the inhibition of tumor suppressor genes or the conversion of proto-oncogenes into oncogenes. A recent study showed ER-alpha mediates proliferation of breast cancer cells via suppression of p53 and p21 pathways while upregulating PNCA and Ki-67 antigens (107). Estrogen exposure additionally upregulates cell division associated genes such as *CDC6*, *CDC2* and Cyclin D1 (*CCND1*) (108).

Exposure to estrogen also affects carcinogenesis through its ability to inhibit apoptosis through dysregulation of cellular pathways. Exposure increases the Bcl-2 expression in breast cancer cells (109, 110) and downregulates the proapoptopic genes *CASP9* and *BAK1* (108). These altered pathways result in increased cellular survival and inhibition of apoptotic signaling. These studies highlight estrogen-dependent mechanisms by which exposure to heavy metals may result in the development of hormonally-driven cancers. Importantly, these effects are also

shown with exposure to other xenobiotic agents such as dioxins, organochlorines and polychlorinated biphenyls (PCBs) (111, 112).

F. Literary Review of Heavy Metals and Breast Cancer

There exist numerous explanations for the carcinogenicity of various heavy metals in relation to hormonally driven cancers. *In vitro* and *in vivo* studies offer support that even non-carcinogenic metals can affect carcinogenesis through estrogen-independent and estrogen-dependent mechanisms. There is therefore a strong need to investigate associations between heavy metal exposures and cancer risk using an epidemiologic design. Furthermore, there is a need for these studies to focus on estrogen-driven cancers such as breast cancer. Nearly all studies examining the association between heavy metals and breast cancer have focused exclusively on cadmium, although the studies have relied on various cadmium exposure measurements. Generally these studies have quantified exposures differently; some studies have focused on the role of ingested cadmium using dietary metrics while others have focused on chronic exposure by using urinary measurements. Finally, a research gap exists on the role of inhaled cadmium on breast cancer incidence although a few studies have used unique methodologies to examine this question.

1. Dietary Cadmium Associations with Breast Cancer

Cadmium's main route of exposure is generally through ingestion. Low levels of cadmium can be found in shellfish, liver and kidney meats. Additionally, cadmium is naturally occurring in soil and water; therefore it is often absorbed into vegetables and grains, making diet a major route of exposure (113). Previously, six epidemiologic studies have investigated the association between dietary cadmium intake and breast cancer risk. These studies have employed

both cohort and case-control designs, and have collected information on confounding covariates such as age, body mass index (BMI), and various measures of estrogen exposure histories.

The first study to examine the association between dietary cadmium exposure and breast cancer risk employed a cohort design and focused exclusively in post-menopausal women (114). Using the Vitamins and Lifestyle cohort, food frequency questionnaires (FFQ) were conducted on 30,543 women. Dietary cadmium intake was estimated by combining information from the FFQ with the US Food and Drug Administration data on food cadmium content. The women were followed up between seven to nine years and incident invasive breast cancer diagnosis was ascertained through cancer registry linkage. Vegetables and grains accounted for 66% of estimated dietary cadmium exposure. After adjustment for age, total energy intake, education, race, hormone replacement therapy use, vegetable, potato and whole grain consumption, cigarette smoking, BMI, physical activity, alcohol consumption, age at first childbirth, multivitamin use, and mammography, the researchers did not identify any associations between dietary cadmium and breast cancer risk (highest vs lowest quartile HR: 1.00 (0.72-2.41), p-trend = 0.95). Furthermore, the researchers did not identify any interactions between cadmium and breast cancer risk by smoking habits or total intake of calcium, iron or zinc (114).

Julin et al. (2012) conducted a population-based prospective cohort study in Sweden exploring the association between dietary cadmium intake and the risk breast cancer in postmenopausal women. This study was unique in that it was the first to differentiate between breast cancer subtypes measured by ER status (115). A FFQ was given at baseline in 1987 to 55,987 postmenopausal women. Dietary cadmium content was estimated by combining the FFQ with the National Food Administration data regarding food cadmium content. Cross classification of FFQ-estimated dietary cadmium and urinary cadmium concentration resulted in

51% sensitivity and 58% specificity. Women were followed for an average of 12.2 years, and after adjustment for age, height, BMI, education, estrogen-exposure associated variables, alcohol consumption, glycemic load, total energy intake and intake of whole grains and vegetables, a positive association between dietary cadmium and breast cancer risk was identified (highest vs lowest tertile RR: 1.21, 95% CI: 1.07-1.36, p-trend = 0.02). In analyses focused on breast cancer subtypes, among lean and normal weight women, an increased risk of ER-positive breast cancer was significantly associated with cadmium intake (RR= 1.25, 95% CI: 1.03-1.52), although no significant increases in risk for ER-negative tumors were observed (RR= 1.22, 95% CI: 0.76-1.93) (115).

Sawada et al. (2012) conducted a population-based prospective cohort study to assess the association between long-term dietary cadmium intake and overall cancer risk. A self-administered FFQ and a 5-year follow-up survey were taken by 90,383 men and women, between the ages of 45 and 74 (116). Dietary cadmium was estimated from six food groups. A validation study was conducted in a subsample of 31 men and 57 women to assess the association between estimated dietary cadmium intake and urinary cadmium concentrations. The Spearman correlation coefficient for men and women was 0.38 and 0.45, respectively. The researchers found that among for premenopausal women and after adjustment for age, smoking status, BMI, and various estrogen exposure associated variables, no associations were identified (T2 vs T1 RR: 1.03, 95% CI: 0.59-1.77, T3 vs T1 RR: 0.66, 95% CI: 0.31-1.41). Similar results were identified among postmenopausal women (T2 vs T1 RR: 1.35, 95% CI: 0.95-1.90, T3 vs T1 RR: 0.95, 95% CI: 0.62-1.46) (116).

Itoh et al. (2014) conducted a hospital based case-control study to investigate the association between dietary cadmium intake and breast cancer risk in Japanese women. Using

405 matched pairs, dietary cadmium was measured via FFQ in combination with the Joint FAO/WHO Expert Committee on Food Additives and the Committee on Pharmaceutical and Food Sanitation of the Ministry of Health, Labour and Welfare in Japan. After adjustment for age, residential area, menopausal status, physical activity, smoking status, family history of breast cancer, parity, isoflavone, vegetable and total energy intake, the researchers found no associations between dietary cadmium intake and overall breast cancer risk, although they identified a significant positive association among postmenopausal women with ER-positive tumors (T3 vs T1 RR: 1.94, 95% CI: 1.04-3.63, continuous RR= 1.08, 95% CI: 1.03-1.14, p-trend= 0.03). Furthermore, this association was strongest among women with ER-positive and PR-negative tumors (T3 vs T1 RR: 3.41, 95% CI: 1.24-9.22, continuous RR= 1.17, 95% CI: 1.08-1.26, p-trend= 0.02) (117).

Eriksen et al. (2014) conducted a prospective cohort study in Denmark examining the association between dietary cadmium intake and the risk of breast, endometrial and ovarian cancers. The study design focused exclusively on postmenopausal women enrolled in the Diet, Cancer and Health cohort between 1993 and 1997. Dietary cadmium exposure was assessed by FFQ and combined with the cadmium content in food using The Danish Food Monitoring Programme for Nutrients and Contaminants to estimate dietary exposure. Among the 23,815 enrolled postmenopausal women, 1,390 cases of breast cancer were identified by December 31, 2010 using the Danish Cancer Registry. Overall, the researchers did not identify any significant associations between dietary cadmium and breast cancer risk, although after adjustment for education, smoking, parity, estrogen-exposure associated variables, BMI, height, physical activity and alcohol intake, a suggestive relationship was found among never smokers with a BMI less than 25 (IRR: 1.23, 95% CI: 0.94-1.60). The researchers did not identify any

associations between dietary cadmium and breast cancer characteristics, including ER status (118).

Finally, Adams et al. (2014) used the Women's Health Initiative to assess associations between dietary cadmium intake and the risk of breast, endometrial and ovarian cancers among postmenopausal women. A total of 155,069 women between the ages of 50 and 79 participated in the study. Dietary cadmium intake was estimated by FFQ and data on food cadmium content from the U.S Food and Drug Administration. The age adjusted Spearman rank partial correlation between energy-adjusted dietary cadmium and creatinine-corrected urinary cadmium was 0.09. After adjustment for total energy intake, age, study component, BMI, smoking, alcohol consumption, race, education, physical activity, estrogen-exposure associated variables and daily vegetable and grain servings, the researchers did not identify any associations between dietary cadmium and breast cancer risk (119).

While these studies hint at potential associations between dietary cadmium and breast cancer risk, additional studies are needed to better examine susceptible sub-groups, such as lean post-menopausal women. These studies additionally reflect the difficultly in determining dietary cadmium exposure from questionnaire data, offering one explanation for the inconsistent results across the studies. Finally, the implications of these studies are unclear; cadmium is traditionally found in vegetable and grains, which have been shown to be protective against a number of diseases and disorders. These studies potentially point to the importance of environmental remediation to limit the amount of cadmium uptake into vegetables and grains, thereby limiting dietary exposures to heavy metals.

2. Urinary Cadmium Associations with Breast Cancer

Urinary cadmium is a long-term marker of cadmium exposure and is not subject to traditional biases which commonly arise in survey data. Additionally, when investigating diseases with long latency periods such as cancer, a long-term marker of exposure is preferred. Researchers have recently relied on these considerations to examine association between breast cancer risk and long-term cadmium exposure through the use of urinary markers.

The first study to investigate the association between urinary cadmium and breast cancer risk employed a population-based case-control design of 246 women with breast cancer and 254 age-matched controls (11). Cadmium was measured in urine by ICP-MS and covariate information was collected via telephone interviews. After adjustment for age, estrogen-exposure associated variables, family history of cancer, alcohol consumption, BMI, education and marital status, the researchers found breast cancer patients were more likely to be in the highest quartile of cadmium exposure, compared to non-breast cancer patients (OR: 2.29, 95% CI: 1.3-4.2). When cadmium exposure was treated as a continuous exposure, a significant positive association remained (OR= 2.09, 95% CI: 1.2-3.8). The authors additionally commented that, in this study sample, the population attributable risk of cadmium exposure could account for 45, or approximately 36%, of the 124 annual breast cancer cases per 100,000 (95% CI: 0 to 77) (11).

Gallagher et al. (2010) conducted a case-control study using the Long Island Database Project for Breast Cancer (LIDPBC) to examine the association between urinary cadmium and breast cancer risk. The researchers further validated their results in a cross-sectional sample of U.S women using the National Health and Nutrition Examination Survey (NHANES). In both study samples, urinary cadmium was measured using ICP-MS and covariates were collected via questionnaire given by a trained interviewer. After adjustment for age, smoking status, alcohol

intake, menopausal status and either race (NHANES) or family history of breast cancer (LIDPBC), the researchers identified significant positive associations between urinary cadmium and breast cancer risk. In the NHANES sample, the researchers found breast cancer patients had twice the odds of being in the third and fourth quartiles of cadmium exposure compared with non-breast cancer patients (OR: 2.56, 95% CI: 1.13-5.78 for the third quartile and OR: 2.32, 95% CI: 0.92-5.84 for the fourth quartile, p-trend= 0.03). The LIDPBC sample had similar results—the researchers found breast cancer patients were more than three times more likely to be in the fourth quartile of exposure compared to non-breast cancer patients (OR: 2.81, 95% CI: 1.11-7.13, p-trend= 0.02) (120).

Nagata et al. (2013) employed a hospital-based case-control study design of non-occupationally exposed women to test the association between cadmium exposure and the risk of breast cancer in Japanese women. A total of one hundred fifty three women with newly diagnosed and histologically confirmed breast cancer were enrolled along with four hundred thirty one controls were individually matched to cases by age, menopausal status and timing of urine samples collection. Urinary cadmium was measured using flameless AAS and potential confounding covariates were collected via questionnaire. After adjustment for age, education, age at menarche, parity, age at first birth, BMI, smoking status, alcohol intake and family history of breast cancer, breast cancer patients were over two times more likely to be in the second tertile of cadmium exposure compared with cancer-free controls (OR= 2.25, 95% CI: 1.17-4.37). Furthermore, breast cancer patients had over six times the odds of being in the third tertile of cadmium exposure compared with cancer-free controls (OR= 6.05, 95% CI: 2.90-12.62). A significant positive association was observed when urinary cadmium was treated as a continuous variable (OR= 1.67, 95% CI: 1.39-2.01 per 1.0 μg/g creatinine increment of cadmium) (121).

Strumylaite et al. (2014) used a hospital-based case-control design to study the association between urinary cadmium and breast cancer risk. Importantly, this was the first study to investigate this relationship in the context of breast cancer subtype, with a focus on the presence of ER and HER2 receptors. In the largest case-control study to date, 585 cases and 1,170 controls were enrolled. Urinary cadmium was determined using AAS, while covariate data was collected via questionnaire. ER and HER2 receptor status was measured in breast tumor tissue using immunohistochemical analysis. After adjustment for age, estrogen-exposure associated variables, family history of breast cancer, alcohol use, smoking status, BMI, education, marital status, diabetes and thyroid disease, women in the third and fourth quartile of cadmium exposure had increased odds of being a breast cancer patient compared with the lowest quartile of exposure (OR= 1.60, 95% CI: 1.19-2.17 for the third quartile and OR= 1.62, 95% CI: 1.19-2.21 for the fourth quartile, p-trend= 0.001). Importantly, when stratified by breast cancer receptor status, the results were only significant among ER-positive (p-trend < 0.001), HER2-negative (p-trend < 0.001), and the combination of ER-positive and HER2-negative tumors (p-trend < 0.001) (122).

Wei et al. (2015) performed a case-control study to investigate the association between urinary cadmium on breast cancer risk and additionally examined modification of the relationship by selenium. The study enrolled 241 incidence cases of breast cancer along with 246 age-matched controls. Urinary cadmium and selenium were measured using ICP-MS. Covariates were collected via questionnaire. Using tertiles of exposure and after adjustment for age, BMI, age at menarche, marital status, education, parity, menopausal status and family history of breast cancer, the researchers found no association for the relationship between urinary cadmium and breast cancer risk. Interestingly, the researchers did identify a protective relationship between the

second tertile of selenium and breast cancer risk (OR= 0.50, 95% CI: 0.30-0.81). These associations were also consistent when stratified by menopausal status. Notably, a positive significant relationship with breast cancer risk was shown between women in the highest tertile of cadmium exposure and the lowest tertile of selenium exposure (OR= 2.83, 95% CI: 1.18-6.86). This association became more pronounced with further stratified by menopausal status; among postmenopausal women, those with the highest level of cadmium exposure and lowest level of selenium exposure had the greatest risk for breast cancer development (OR= 5.93, 95% CI: 1.13-31.01) (123).

The aforementioned studies investigating urinary cadmium exposure on breast cancer risk were part of a meta-analysis conducted by Larsson et al. (2015). They found odds ratios ranging between 1.35 and 6.05 comparing the highest and lowest categories of cadmium exposure, with a pooled odds ratio of 2.24 (95% CI: 1.50-3.34). When cadmium exposure was assessed as a continuous variable, a significant linear dose-response was shown (OR= 1.66, 95% CI: 1.23-2.25 per 0.5 μg/g creatinine increment of cadmium). Importantly, heterogeneity between these studies was observed; when stratified by geographical region, the odds ratio between urinary cadmium and breast cancer was 1.85 (95% CI: 1.44-2.38) for studies conducted in the United States and Europe, while the odds ratio was 2.78 (95% CI: 0.64-12.10) for studies conducted in Asia. The meta-analysis found no evidence of publication bias (Egger's test: P= 0.16) (124).

Most recently, the first cohort study investigating urinary cadmium exposure and breast cancer risk was conducted (125). Using the Women's Health Initiative, Adams et al. (2016) examined 12,701 postmenopausal women who were followed for an average of 13.2 years. Over this time period, 508 cases of invasive breast cancer were diagnosed. A total of 1,050 comparison women were selected to conduct a case-cohort analysis. No associations with breast

cancer risk were identified using the urinary cadmium variable in either quartiles (HR= 0.80, 95% CI: 0.56-1.14, p-trend = 0.20) or as a log-transformed continuous variable (HR= 0.94, 95% CI: 0.86-1.03 per 2-fold increase in urinary cadmium). No associations were observed when the sample was restricted to never smokers or women with a BMI less than twenty five. Furthermore, no associations were identified between different subtypes of breast tumors (125).

3. **Additional Studies**

Two additional studies have investigated the relationship between heavy metal exposure and breast cancer risk using novel or surrogate methods to estimate exposure to heavy metals via inhalation. One study approached this question using methodologies based in occupational epidemiology, and the other relied on publically-available, modelled census tract exposure estimates derived from the Environmental Protection Agency's (EPA) National-scale Air Toxics Assessment (NATA).

Brophy et al. (2012) employed a community based case-control study design to investigate the association between occupations with exposure to carcinogens and endocrine disruptors with breast cancer risk. A total of 1,005 breast cancer cases and 1,146 randomly selected community controls were enrolled in the study. Participants provided data regarding their occupational and reproductive histories. After adjustment for reproductive and demographic risk factors, women who worked in metalworking occupations such as foundry work, metal stamping, and fabrication had significantly increased odds of the breast cancer compared with community controls (OR= 1.73, 95% CI: 1.02-2.92). Furthermore, after stratification by estrogen-receptor subtype, among metalworkers, the increased odds of breast cancer was only identified for women with ER+/PR+ breast cancer (OR= 2.03, 95% CI: 1.11-3.71) (126).

Lui et al. (2015) conducted a prospective cohort study using The California Teachers Study. Participants were 112,379 women free of breast cancer and were living at a California address between 1995 and 1996. Environmental data on air exposures identified as endocrine disruptors were collected using the EPA's National-scale Air Toxics Assessment. The NATA estimates for census-tracts air concentration estimates from 2002 were assigned to the participants' baseline address. Potential confounding covariates were ascertained from the baseline questionnaire. Between 1995 and 2010, 5,361 incident breast cancer cases were identified. After adjustment for age, race/birthplace, family history of breast cancer, alcohol consumption, BMI, age at menarche, age at first first-term pregnancy, menopausal status and hormone therapy no significant associations were identified between census-tract heavy metal air concentration and breast cancer risk. Upon stratification by breast cancer subtype, among never-smoking and residentially stable participants, women in the third and fifth quintiles of arsenic air concentration showed increased risk of development of ER-negative/PR-negative breast tumors (OR= 1.5, 95% CI: 1.0-2.3 for the third quintile and OR= 1.17, 95% CI: 1.1-2.5 for the fifth quintile of arsenic exposure). Furthermore, among the same subset of women, individuals in the fourth and fifth quintile of cadmium exposure had significantly higher risk of development of ER-negative/PR-negative breast cancer (OR= 1.6, 95% CI: 1.1-2.4 for the fourth quintile and OR= 1.6, 95% CI: 1.1-2.5 for the fifth quintile of cadmium exposure) (127).

4. **Conclusions and Implications**

The previously mentioned studies suggest a relationship between heavy metals, particularly cadmium and arsenic, and breast cancer risk in women. Additionally, these investigations offer insights regarding the importance of the various routes of exposure. In particular, the dietary studies focused exclusively on ingested cadmium and identified weak to no

association between cadmium and breast cancer risk. One possible explanation for these findings is the benefits of eating vegetables and grains, the primary food groups with high cadmium concentrations, outweighs the harmful effects of their metal concentrations. It is worth noting these findings are less clear when restricted to ER-positive tumors, as some studies did identify an increased risk of this subtype of breast cancer, particularly among postmenopausal women (115, 117).

The studies which focused on urinary measurements consistently showed strong associations between body burden of cadmium and breast cancer risk, with the exception of the analysis conducted by Adams et al. (2016). Furthermore, these findings were consistently stronger among ER-positive breast cancers. An important limitation of these studies is urinary measurements of cadmium reflect body burden and cannot discriminate between various routes of exposure. Regardless, these investigations suggest exposure to cadmium may be influential to the development of breast cancer, with a particularly strong influence on estrogen-positive tumors.

Finally, the two additional studies which relied upon occupational and novel methods identified associations between inhaled heavy metals and breast cancer risk but had inconsistent findings regarding tumor subtype. The study which relied upon occupational methods found increased breast cancer risk, but only among ER-positive tumors (126). This was contradicted by the findings by Lui et al. (2015) who identified associations between cadmium and arsenic, but only among ER-negative tumors. An important component of these studies is that they focused exclusively on inhaled metals. Further studies regarding the associations between breast cancer risk and inhaled metals are warranted.

Future investigations should aim to develop more of an understanding the route of exposure plays on breast cancer risk. This area is generally understudied and deserves particular attention. Investigations should additionally focus exclusively on tumor subtype as increased risk has been consistently identified in studies of metal body burden and inhalation. Furthermore, studies focusing on tumor subtype development may suggest which mechanisms heavy metals rely upon for their carcinogenicity of hormonally driven cancers. It is possible that if, through epidemiologic designs, clearer associations between heavy metal exposure and breast cancer risk were identified mainly among ER-positive tumors, one may suggest the exposure to heavy metals is acting, at least in part, through estrogen-dependent mechanisms. Conversely, it is possible that if associations are mostly identified among ER-negative tumors, the findings would suggest heavy metals exert their carcinogenic properties mainly through estrogen-independent mechanisms.

The purpose of this dissertation is to explore associations between exposure to heavy metals and aggressive breast cancer characteristics, with a particular focus on breast cancer subtype, as defined by ER status, and tumor grade. This dissertation will expand on previous findings using proxy measurements for inhaled heavy metal exposure using the National-scale Air Toxics Assessment and will examine potential mechanisms through which heavy metals exert their effects on breast cancer subtype development.

G. Specific Aims

1. Residential Airborne Heavy Metal Concentrations and Breast Cancer Characteristics

The purpose of Aim 1 is to explore the associations between census tract level measures of heavy metals with various demographic, socioeconomic and breast cancer characteristics in Cook County, IL.

Exposure to heavy metals is ubiquitous in the general U.S population, and exposure routes can vary. Modelled census tract air concentrations and human exposure estimates are available from the EPA's National-scale Air Toxics Assessment. Using information on the residential histories of the Breast Cancer Care in Chicago (BCCC) population, we examined the association between exposure to heavy metals with demographic, socioeconomic and breast cancer characteristics. The main hypothesis is that higher levels of exposure to known carcinogenic metals available through NATA (IARC Group 1 metals: arsenic, beryllium, cadmium, chromium and nickel) will be associated with increased prevalence of ER-negative subtypes of breast cancer. Exposure to metals which are identified as probable or possible carcinogens (IARC Group 2 and 3 metals: antimony, cobalt, lead, manganese, mercury and selenium) are hypothesized to be associated with ER-positive subtypes of breast cancer. Additionally, we hypothesize that racial/ethnic minority women, women with less income and education, and women who resided in census tracts with high concentrated disadvantage and low affluence at diagnosis will tend to be exposed to greater levels of all heavy metals.

2. **Residential Airborne Heavy Metal Concentrations and Changes in Cancer-Associated Gene Methylation**

The purpose of Aim 2 is to examine associations between census tract level measures of heavy metals and DNA methylation of six previously selected cancer-associated genomic regions in cancer-adjacent normal, *in situ*, and invasive tissue components.

Evidence is emerging that heavy metal concentrations affect expression of DNA methylation enzymes and directly affect methylation of both DNA repeats and cancer-associated genes. To our knowledge, these potential effects have not been examined in breast tissue using epidemiologic designs. We examined associations of heavy metals exposures with hypomethylation of the DNA repeat Sat2 and the gene *TFF1* and hypermethylation of the cancer-associated genes *APC, EGFR, GSTM2,* and *RASSF1*. Our hypothesis is that greater exposure to heavy metals will be associated with DNA repeat and oncogene hypomethylation, and cancer-associated tumor suppressor gene hypermethylation.

3. **Cancer-Associated Gene Methylation and Breast Cancer Characteristics**

The purpose of Aim 3 is to examine associations between cancer-associated gene methylation with markers of breast cancer tumor aggression. More specifically, we are interested in examining the relationship between gene-specific methylation of *BRCA1*, *EGFR, GSTM2, RASSF1,* and *TFF1,* and global methylation marker Satalite2 (Sat2).

It is well known the underlying biology of ER/PR-negative and high-grade tumors are considerably different from other breast cancer subtypes. Using a candidate gene approach, we examined specific gene regions which have been associated with breast cancer incidence, but not tumor aggression. We hypothesize DNA methylation in selected gene regions will be associated with ER/PR tumor receptor status and tumor grade.

II. RESDENTIAL AIRBORNE HEAVY METAL CONCENTRATIONS AND BREAST CANCER CHARACTERISTICS

A. Background

Breast cancer is the most commonly diagnosed cancer in women worldwide (128). Biologically, it is a heterogeneous disease commonly classified by cellular receptor status. Luminal A and B (both ER/PR-positive) tumors are the most common subtype and tend to be less aggressive with good prognoses (129-131). Other breast cancer subtypes, such as basal-like and triple-negative (both ER/PR-negative), are less common, more aggressive, and have worse prognoses (131, 132). Importantly, strong racial and socioeconomic disparities exist in subtype incidence rates, with younger women and African-American women more likely to develop ER/PR-negative tumors (133). While differential risk factor distributions across age and ethnicities explain some of these disparities (134), the identification of additional, modifiable risk factors may help further reduce them.

A portion of these disparities is explained by differential exposure to estrogen. Previous studies have shown risk factors differ based on breast tumor molecular subtypes; exposure to estrogen has been identified as a major risk factor for ER-positive, but not ER-negative, breast tumors (135, 136). Endogenous exposure to estrogen is affected by reproductive factors including parity, breastfeeding, and age at menarche and menopause (137). Exogenous exposure to oral contraceptives and hormone replacement therapy may also affect risk (138, 139). Additional evidence suggests exposure to environmental agents with estrogenic properties, such as some heavy metals, may also contribute to breast cancer risk (111, 115, 140).

Exposures to heavy metals are presently understudied as a mechanism of breast cancer development. Everyone is exposed to heavy metals, as they are often ingested via contaminated

food or water, and are inhaled via occupational settings, ambient air, and tobacco smoke (3). Heavy metals are etiologically associated with many types of cancer, but their involvement in breast cancer is less clear. Arsenic (As), beryllium (Be), cadmium (Cd), chromium (Cr), and nickel (Ni) are identified as 'known' carcinogens by the International Agency for Research on Cancer (IARC) (17, 141, 142). They are believed to be carcinogenic through their generation of reactive oxygen species which form DNA adducts and increase cellular oxidative stress (69-71, 74-76). These metals have additionally been shown to bind to DNA damage repair proteins, limiting their effectiveness and exaggerating the effects of existing DNA mutations (63-66). Furthermore, several heavy metals including antimony (Sb), cobalt (Co), lead (Pb), mercury (Hg), and selenium (Se) have been shown to affect estrogenic pathways through their ability to bind with ER-alpha, presenting an additional pathway through which metals can induce carcinogenesis (20-22, 25).

Epidemiologic studies investigating the role of heavy metals in breast cancer development have focused almost exclusively on cadmium. Additionally these studies have focused on chronic exposures measured by urinary markers, or exposure via diet measured by food frequency questionnaire (11, 114-123, 125). The etiologic role of inhalation of metals has only recently come under investigation (127). Lui et al. (2015) identified an association between census tract level concentrations of cadmium and arsenic, and ER-negative breast tumors among residentially-stable, never-smoking women; they found no association between inhaled selenium and breast cancer incidence (127). The purpose of this study is to replicate and expand on previous findings by examining associations between airborne heavy metal concentrations with various demographic, socioeconomic and breast cancer characteristics.

B. Methods

1. Study Population

The Breast Cancer Care in Chicago (BCCC) study is a population-based sample of women who were diagnosed with breast cancer at one of the fifty-six Chicago-area hospitals. Women who were eligible for enrollment were between the ages of thirty and seventy-nine; self-identified as non-Hispanic (nH) White, nH Black, or Hispanic; resided in Chicago; had a first primary *in situ* or invasive breast cancer diagnosed between 2005 and 2008; and gave written, informed consent to participate in the study. Overall, 989 women were enrolled and completed a 90-minute interview on social, demographic, and healthcare-related factors. Of these, 812 (82%) consented to allow samples of diagnostic tissue to be obtained by research staff, of whom samples were obtained from 351 patients (35%); 723 (73%) participants consented to a blood draw, from which DNA was extracted and stored on 668 patients (68%). A total of 260 (27%) patients had both blood and tissue collected. The study population has been described in detail elsewhere (143). Participants were dropped from the analysis if they were missing exposure, outcome or any of the *a priori* covariate data. After this consideration, the final sample sizes were 664 and 661 when examining the tumor receptor status and tumor grade outcomes, respectively. The protocol for this study was approved by the University of Illinois at Chicago Institutional Review Board.

2. Outcome Assessment

Breast cancer characteristics of interest were hormone receptor status, as defined by ER/PR status, and tumor grade. Estrogen and progesterone receptor status was determined by immunohistochemical (IHC) analysis on women who consented to retrieval of clinical breast tissue samples. For those who consented to tissue donation, copies of pathology reports and

hematoxylin and eosin (H&E) slides were requested from the diagnosing institution. A single pathologist selected formalin-fixed, paraffin-embedded (FFPE) tumor blocks which generally represented the tumor. Recuts (4 µm each) of the selected tumor blocks were created for additional H&E staining. The recuts were examined to identify invasive components of the tumor. Cores of invasive tissue components were selected and tissue microarrays produced. Samples were stained using a monoclonal antibody for nuclear estrogen and progesterone receptor status (manufacturer: Ventana, product catalogue number: 790-4324 and 790-2223 for ER and PR antibodies, respectively). Stains were optimized on invasive breast tumor tissue before use in this study. ER and PR status were interpreted separately and given an H-score, which is the product of staining intensity (0, 1+, 2+, 3+) and the proportion of cells with the given intensity (possible range of H-score values: 0-300). A tumor sample was determined to be ER/PR-negative if it had an H-score < 10 for both receptor stains. For women who did not donate breast tissue, receptor status was determined by medical record abstraction. For patients with both IHC and medical record information, concordance was very high (<1% of patients were discordant on ER/PR-negative status).

Breast tumor grade was collected via medical record abstraction. Grade was determined by a trained pathologist at the diagnosing institution. Tumor grade was defined as either well differentiated (G1, low grade), moderately differentiated (G2, intermediate grade), or poorly differentiated (G3, high-grade).

3. Airborne Heavy Metal Exposure Assessment

a. National-Scale Air Toxics Assessment Database

The Environmental Protection Agency (EPA) conducts the National-Scale Air Toxics Assessment (NATA), which is an ongoing, comprehensive evaluation of air toxics in the United

States. Briefly, the process for air toxics estimates is as follows: emission data collected from countrywide anthropogenic sources (point, non-point, on-road mobile, non-road mobile, and background emissions) included in the National Emissions Inventory (NEI) are compiled and prepared for use as model inputs. Using these inputs in combination with meteorological and dispersion models, ambient concentrations of census tract-level air toxics are estimated (air concentration). Estimates of air toxics are available for four different time points (1999, 2002, 2005, and 2011). For the purposes of this analysis, we used data from the first three time points as the 2011 air concentrations were measured after the study period of interest. Minor methodological changes were made across the different time points. Ambient concentrations of antimony, arsenic, beryllium, cadmium, chromium, cobalt, lead, manganese, mercury, nickel, and selenium were recorded across all three time points. In 1999, NATA accounted for different chromium species (III vs VI). In 2002 and 2005, the air concentration estimates for chromium were combined into one variable. For the purposes of this analysis, the two chromium variables from 1999 were summed to generate a total chromium air concentration, consistent with the 2002 and 2005 measures.

b. **Residential Histories**

Patients' residential histories 15 years prior to breast cancer diagnosis were collected using the CLEAR database offered by Thomson Reuters. Briefly, this database is used to monitor individuals using information from public and credit gateways. Information on past and current addresses, along with dates of residence, was obtained via household listings, utility bills, phone records, and information from the Transunion and Experian credit databases and used to identify specific addresses. We conducted queries on BCCC participants using name and date of birth. Correct listings were determined if the record retrieved had a matching address to the one given

at the time of interview. One address was selected for each year of follow up. Priority was given to addresses and dates from household listing and utility sources. In instances where no household or utility information was available, we relied upon residential information from credit sources. For women with multiple addresses within a year, we selected the residence in which they lived the longest. In order to account for women with missing residential information, we imputed the last known address for women with at least one CLEAR residence record prior to 2000, between 2000 and 2004, and between 2005 and year of diagnosis. The extent of the missing data is shown in Appendix A.

c. **Computation of Airborne Heavy Metal Exposure Assessment**

Due to differences in methodology over the NATA time points (1999, 2002 and 2005), it was not possible to take a simple or weighted average of air concentration estimates over the study period. For this reason, we created an air concentration ranking for each metal for each exposure year.

Our strategy for combining NATA estimates with residential histories was as follows: (1) NATA 1999 estimates were applied to residences between 15 years prior to diagnosis and 1999; (2) NATA 2002 estimates were applied to residences between 2000 and 2003; (3) NATA 2005 estimates were applied to residences between 2004 and year of diagnosis. If a participant did not have at least one known residence in all three of the time periods, we were unable to calculate a 15-year chronic metal exposure ranking and the participant was assigned a missing value for the chronic metal exposures. After residential history imputation, we calculated exposure rankings by year for each individual metal, which were then summed across all 15 years of follow up. Rankings were shifted and scaled by dividing the exposure rank score by the maximum possible value so that each rank score ranged between 0 and 1.

We created combined metal exposure variables by summing the rankings for the following metal exposure combinations: (1) all metals, (2) International Agency for Research on Cancer (IARC) Group 1 metals (arsenic, beryllium, cadmium, chromium and nickel), and (3) IARC Group 2 and 3 metals (antimony, cobalt, lead, manganese, mercury and selenium). Finally, we employed principal component analysis to estimate eigenvectors representing combined exposure. This procedure converts the highly correlated airborne exposure variables into uncorrelated variables comprised of proportions of the airborne exposure estimates offering an additional approach to examine mixtures of heavy metals.

4. **Covariate Information**

Data on breast cancer risk factors (socioeconomic/demographic and reproductive factors) were collected from the 90-minute in-person interview conducted upon enrollment into the BCCC study. Information on age, race/ethnicity, BMI, comorbidities, education, income, parity, age at first and last birth, breastfeeding duration, duration of oral contraception use, age at menopause, duration of hormone replacement use, and family history of breast cancer were collected.

A biomarker for smoking status was measured retrospectively on a subsample of the BCCC participants using DNA methylation derived from peripheral blood monocyte samples at three pre-identified loci (Illumina IDs: cg06644428 (2q37), cg21566642 (2q37), cg06126421 (6p21.33)). Previous studies have identified strong inverse linear associations of these markers with smoking history and have tested their utility in predicting past tobacco exposure (144, 145).

Measures of neighborhood socioeconomic status (SES) were computed based on the 2000 census. Census tract affluence was measured by combining percentage of families with income of $75,000 or more, percentage of adults with college education or more, and percentage

of civilian labor force in professional and managerial occupations. Census tract disadvantage was measured by combining percentage of families with incomes below the poverty line, percentage of families receiving public assistance, percentage of persons unemployed, and percentage of female-headed households with children. Both census tract variables were defined by creating an equally weighted sum of the relevant variables, then standardizing that sum to have a mean of zero and standard deviation of one (146).

5. **Statistical Analysis**

The prevalences of ER/PR-negative breast cancer and high-grade disease were separately examined in bivariate analyses using chi-squared values to test for differences in the distribution of covariates across these outcomes. We additionally examined pairwise spearman correlations across the individual chronic metal exposure rankings. Wilcoxon rank sum tests were then used to assess differences in chronic exposure rankings for each metal between nH Blacks and Hispanics compared to nH Whites. Kruskal –Wallis tests were used to assess differences in chronic metal exposure ranking distributions by socioeconomic factors (income, education, and census tract affluence and disadvantage).

Chronic metal exposure rankings were categorized into quartiles and modeled in logistic regression to estimate odds ratios (ORs) and 95% confidence intervals (95% CIs) with respect to prevalences of ER/PR-negative breast cancer and high-grade disease. *A priori* confounders were age (continuous), BMI (categories: < 25 normal, 25-30 overweight, > 30 obese), socioeconomic status factors (race, education, income, and census tract affluence and disadvantage) (continuous), and reproductive factors (age at first birth and parity) (continuous). Participants were dropped from the analyses if they had missing values for the exposure, the outcome, or any *a priori* confounder (N=325 for ER/PR status; N= 328 for tumor grade). We assessed potential

confounding by comorbidities, breast feeding behaviors, family history and chronic exposure to tobacco smoke and included the covariate in the model if the estimates changed by greater than 10%. *A priori,* effect modification was assessed by stratifying analyses on menopausal status (yes, no) and by restricting analyses to individuals with chronic exposure to environmental tobacco smoke above the median (likely non-smokers).

In order to model both parity and age at first birth together, we employed a method which combined information into one variable which generally represented reproductive factors (RPF) by assigning nulliparous women a value corresponding to an age at first birth equal to 40 (146). Statistical significance was defined as a two-sided p-value < 0.05. All data analyses were conducted using Stata version 12 (Stata Corp., College Station, TX).

C. **Results**

The median age of participants at diagnosis was 55 years. Overall, the BCCC participant sample was approximately forty percent nH White, forty percent nH Black and twenty percent Hispanic. Generally, the women were overweight/obese (70%), educated (>60% completed high school), and made greater than $25,000 (80%). Among women with information on ER/PR status (664) a total of 143 (22%) were ER/PR-negative, while among women with information on tumor grade (661) a total of 269 (41%) had high-grade (G3, G4) tumors.

Table II presents the covariate distribution by tumor receptor status and grade. Briefly, older age at diagnosis was associated with low/intermediate grade but not with receptor status. Non-Hispanic Black women were more likely to have ER/PR-negative and high-grade tumors. Higher SES (higher education, income, and census tract affluence, and lower census tract disadvantage) was associated with ER/PR-positive tumors, and higher census tract affluence was associated with low/intermediate grade tumors. High BMI was associated with ER/PR-negative

TABLE II.

BREAST CANCER CARE IN CHICAGO SELECTED SAMPLE DISTRIBUTIONS WITH TUMOR RECEPTOR STATUS AND TUMOR GRADE

	Receptor status (n= 664)			Tumor grade (n= 661)		
	ER/PR + N= 521 (%)	ER/PR – N= 143 (%)	P-value	Low/Intermediate N= 392 (%)	High N= 269 (%)	P-value
Age at diagnosis			0.87			0.01
18-49	155 (30)	45 (31)		102 (26)	95 (35)	
50-59	157 (30)	44 (31)		115 (29)	85 (32)	
60-79	209 (40)	54 (38)		175 (45)	89 (33)	
Race/ethnicity			<0.001			0.06
nH White	238 (46)	36 (25)		176 (45)	96 (36)	
nH Black	193 (37)	84 (59)		156 (40)	128 (47)	
Hispanic	90 (17)	23 (16)		60 (15)	45 (17)	
Education			0.001			0.83
Less than HS	72 (14)	30 (21)		63 (16)	40 (15)	
HS diploma	100 (19)	41 (29)		78 (20)	58 (22)	
Greater than HS	349 (67)	72 (50)		251 (64)	171 (63)	
Income			<0.001			0.45
< $25,000	102 (19)	47 (33)		80 (20)	66 (25)	
$25,000-$87,499	259 (50)	72 (50)		200 (51)	130 (48)	
≥ $85,000	160 (31)	24 (17)		112 (29)	73 (27)	
CT affluence			0.02			0.01
< 1 SD	25 (5)	10 (7)		20 (5)	11 (4)	
± 1 SD	377 (72)	115 (80)		277 (71)	217 (81)	
> 1 SD	119 (23)	18 (13)		95 (24)	41 (15)	
CT disadvantage			0.01			0.29
< 1 SD	93 (18)	11 (8)		67 (17)	34 (13)	
± 1 SD	337 (64)	98 (68)		247 (63)	180 (67)	
> 1 SD	91 (17)	34 (24)		78 (20)	55 (20)	
BMI			0.02			0.59
≤ 25	163 (31)	36 (25)		117 (30)	79 (29)	
25-30	171 (33)	37 (26)		129 (33)	80 (30)	
> 30	187 (36)	70 (49)		146 (37)	110 (41)	
Number of births			0.01			0.27
0	123 (23)	17 (12)		92 (23)	47 (18)	
1	88 (17)	25 (17)		62 (16)	46 (17)	
2	131 (25)	33 (23)		95 (24)	76 (28)	
3+	179 (34)	68 (48)		143 (37)	100 (37)	
Age at first birth			<0.001			0.07
< 20	116 (22)	56 (39)		92 (23)	80 (30)	
20-29	203 (39)	59 (41)		153 (39)	109 (40)	
30+	202 (39)	28 (20)		147 (38)	29 (30)	

tumors but not tumor grade. Lower exposure to endogenous estrogen (younger age at first birth and greater parity) was associated with ER/PR-negative tumors. Comorbidities, breastfeeding behaviors, and exposure to tobacco smoke were not associated with either tumor receptor status or grade. Finally, having a family history of breast cancer was associated with higher tumor grade ($p= 0.03$).

Table III presents the Spearman's correlations between chronic metal exposure rankings. Importantly, all correlations are positive reflecting that exposure to one metal was associated with exposure to all other metals. Certain metal combinations had particularly strong correlations, including antimony with both cobalt ($r_s = 0.80$) and manganese ($r_s = 0.75$), arsenic with cobalt ($r_s = 0.77$), and lead with both mercury ($r_s = 0.75$) and nickel ($r_s = 0.77$).

Figure 1 depicts the distributions of chronic metal exposure concentrations by race/ethnicity. Non-Hispanic Blacks had significantly higher exposure to all of the metals examined compared to nH Whites, with the exceptions of beryllium and cobalt. Non-Hispanic Whites had higher exposure to beryllium than nH Blacks and there was no difference between races with regard to cobalt. Hispanics had significantly higher exposures to beryllium, chromium, lead, manganese, mercury, and nickel compared to nH Whites. No significant differences were detected for the other metals of interest. Lower income was associated with greater exposures to beryllium, chromium, lead, manganese, mercury, and nickel whereas lower education was associated with greater exposures to beryllium, chromium, lead, manganese, and nickel. Interestingly, census tract affluence was positively associated with exposures to cadmium and inversely associated with exposure to lead, manganese, mercury, and nickel. A U-shaped distribution was found for the relationship between census tract affluence and exposures to antimony, arsenic, beryllium, chromium and cobalt. Finally, census tract disadvantage was

TABLE III.

SPEARMAN'S CORRELATIONS BETWEEN CHRONIC METAL EXPOSURE RANKINGS

Metal	Sb	As	Be	Cd	Cr	Co	Pb	Mn	Hg	Ni	Se
Sb	1.00	------	------	------	------	------	------	------	------	------	------
As	0.58	1.00	------	------	------	------	------	------	------	------	------
Be	0.13	0.55	1.00	------	------	------	------	------	------	------	------
Cd	0.67	0.50	0.40	1.00	------	------	------	------	------	------	------
Cr	0.46	0.53	0.55	0.59	1.00	------	------	------	------	------	------
Co	0.80*	0.77*	0.39	0.63	0.50	1.00	------	------	------	------	------
Pb	0.46	0.61	0.55	0.36	0.55	0.49	1.00	------	------	------	------
Mn	0.75*	0.49	0.14	0.53	0.63	0.53	0.59	1.00	------	------	------
Hg	0.74	0.69	0.48	0.62	0.55	0.69	0.75*	0.73	1.00	------	------
Ni	0.45	0.56	0.74	0.52	0.70	0.51	0.77*	0.52	0.70	1.00	------
Se	0.67	0.65	0.07	0.39	0.12	0.73	0.31	0.44	0.58	0.13	1.00

Correlations greater than 0.75 are identified with an asterisk.

positively associated with exposures to chromium, lead, manganese, mercury, nickel, and selenium.

Table IV presents the adjusted results assessing the relationship between individual and combined air metal concentrations with ER/PR-negative tumor receptor status. After adjustment for socioeconomic and reproductive factors (i.e., age, education, income, race/ethnicity, BMI, RPF, and census tract affluence and disadvantage) antimony, cobalt, manganese, and selenium showed significant positive trends indicating that as exposure to these metals increased among breast cancer patients, the prevalence ER/PR-negative tumors also increased. Arsenic, chromium, lead, and mercury additionally showed marginally significant positive associations with prevalent ER/PR-negative breast tumors. Restricted cubic splines graphing the association

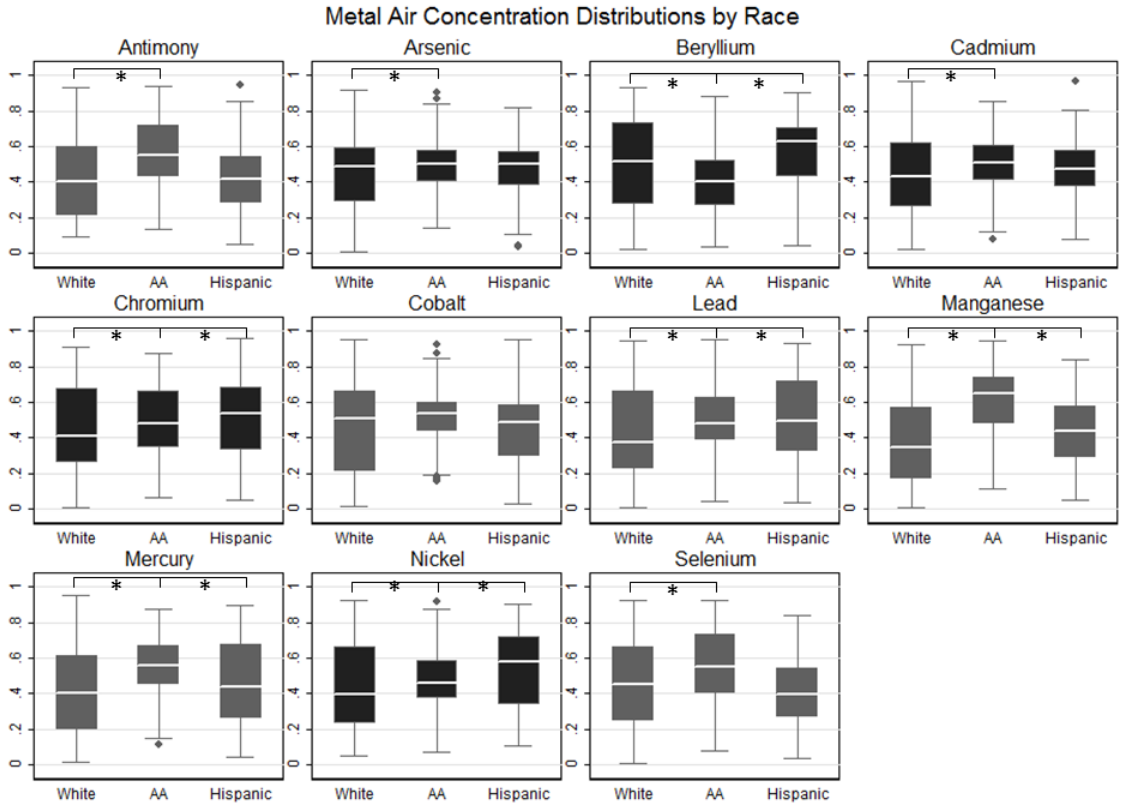

Figure 1. Standardized chronic metal air concentrations rankings by race.

between the chronic metal exposure ranking and prevalence of ER/PR-negative disease are shown in the Appendix B. Regarding the combined measurements, exposure to all metals, IARC Group 2 and 3 metals, and the first principal component eigenvector were all positively associated with prevalence of ER/PR-negative disease. Importantly, all metals loaded into the first principal component with fairly similar strengths explaining the similarity of results between the 'all metals combined' variable and the first principal component. The loading factors for the first two principal components are identified in the Appendix C. Upon stratification by menopausal status, the positive association between antimony air concentration

TABLE IV.

ADJUSTED MODELS[a] FOR ASSOCIATIONS BETWEEN AIR METAL CONCENTRATIONS AND PREVALENCE OF ER/PR-NEGATIVE TUMOR RECEPTOR STATUS

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P-trend
Single THM measures					
Antimony (Sb)	Ref.	1.13 (0.61-2.11)	1.47 (0.78-2.75)	1.81 (0.95-3.44)	0.04*
Arsenic (As)	Ref.	1.48 (0.79-2.75)	1.80 (0.99-3.27)*	1.76 (0.93-3.33)	0.06
Beryllium (Be)	Ref.	0.59 (0.34-1.04)	0.59 (0.33-1.04)	1.01 (0.56-1.80)	0.83
Cadmium (Cd)	Ref.	0.72 (0.40-1.30)	1.03 (0.58-1.84)	1.38 (0.76-2.52)	0.16
Chromium (Cr)	Ref.	1.06 (0.58-1.92)	1.31 (0.73-2.33)	1.58 (0.89-2.81)	0.08
Cobalt (Co)	Ref.	1.46 (0.79-2.69)	1.98 (1.09-3.59)*	2.37 (1.26-4.45)*	0.01*
Lead (Pb)	Ref.	1.14 (0.59-2.18)	1.27 (0.67-2.42)	1.65 (0.89-3.05)	0.08
Manganese (Mn)	Ref.	2.13 (1.07-4.23)*	1.99 (0.99-4.01)*	2.55 (1.24-5.24)*	0.04*
Mercury (Hg)	Ref.	1.25 (0.68-2.31)	1.15 (0.60-2.20)	1.76 (0.96-3.20)	0.07
Nickel (Ni)	Ref.	1.58 (0.86-2.91)	1.04 (0.55-1.96)	1.53 (0.82-2.87)	0.47
Selenium (Se)	Ref.	1.39 (0.77-2.52)	1.43 (0.78-2.61)	1.85 (1.03-3.29)*	0.05*
Combined measures					
All metals	Ref.	1.71 (0.89-3.30)	1.04 (0.52-2.09)	2.62 (1.36-5.05)*	0.02*
IARC Group 1[b]	Ref.	0.70 (0.37-1.32)	0.73 (0.40-1.36)	1.29 (0.70-2.39)	0.32
IARC Group 2 & 3[c]	Ref.	2.19 (1.15-4.18)*	1.83 (0.94-3.64)	2.48 (1.28-4.81)*	0.03*
PC 1	Ref.	1.71 (0.89-3.29)	0.99 (0.49-2.00)	2.57 (1.33-4.94)*	0.02*
PC 2	Ref.	0.63 (0.36-1.09)	0.49 (0.26-0.92)*	0.81 (0.47-1.40)	0.42

[a] Models adjusted for age, education, income, race/ethnicity, BMI, RPF, and census tract affluence and disadvantage (n=664).

[b] IARC Group 1 metals: Arsenic, Beryllium, Cadmium, Chromium, Nickel.

[c] IARC Group 2 & 3 metals: Antimony, Cobalt, Lead, Manganese, Mercury, Selenium.

Asterisk denotes significance at $p < 0.05$.

and ER/PR-negative disease was found only among premenopausal women in the highest quartile (OR = 5.66, 95% CI: 1.03-31.16). Upon restriction to likely non-smokers, only a marginally significant trend between cobalt and development of ER/PR-negative tumors existed.

Table V presents the adjusted results examining associations between individual and combined air metal concentrations and high tumor grade. After adjustment for socioeconomic and reproductive factors, no significant associations remained for any of the individual or combined metal measurements. Upon stratification by menopausal status, the highest quartile of antimony exposure was associated with high-grade tumors among premenopausal women (OR= 6.97, 95% CI: 1.61-30.19). Similarly, among premenopausal women the highest quartile of exposure to arsenic was associated with the development of high-grade tumors (OR= 5.10, 95% CI: 1.27-20.52). Among likely non-smokers, the highest quartile of exposure to antimony showed a positive association with high-grade disease (OR= 3.47, 95% CI: 1.01-11.91).

D. **Discussion**

Our study was the first to identify positive trends between airborne concentrations of antimony, cobalt, manganese and selenium with prevalent ER/PR-negative breast tumors. Previous epidemiologic studies identified positive associations between air pollution and breast cancer risk (127, 147-151). Lui et al. (2015) was the first study to specifically examine estrogen-disrupting hazardous air pollutants and found a positive relationship between arsenic and cadmium and development of ER/PR-negative breast tumors. We also found that among breast cancer patients, arsenic was positively associated with prevalence of ER/PR-negative tumors and although we did not find a significant association between cadmium and ER/PR-negative tumors, our data are suggestive of a similar relationship. Unlike Lui et al.'s (2015) study, we identified a positive relationship between selenium and prevalence of ER/PR-negative tumors. Finally, our

TABLE V.

ADJUSTED MODELS[a] FOR ASSOCIATIONS BETWEEN AIR METAL CONCENTRATIONS AND PREVALENCE OF HIGH TUMOR GRADE

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P-trend
Single THM measures					
Antimony (Sb)	Ref.	0.69 (0.42-1.12)	0.83 (0.51-1.36)	1.05 (0.63-1.76)	0.58
Arsenic (As)	Ref.	0.92 (0.57-1.47)	0.82 (0.52-1.31)	0.94 (0.58-1.54)	0.69
Beryllium (Be)	Ref.	0.94 (0.58-1.46)	0.76 (0.48-1.21)	0.80 (0.49-1.33)	0.27
Cadmium (Cd)	Ref.	1.39 (0.87-2.21)	0.91 (0.56-1.47)	1.18 (0.71-1.95)	0.97
Chromium (Cr)	Ref.	0.95 (0.59-1.52)	1.26 (0.79-2.01)	1.27 (0.78-2.05)	0.19
Cobalt (Co)	Ref.	0.70 (0.43-1.12)	0.78 (0.48-1.25)	1.07 (0.65-1.76)	0.75
Lead (Pb)	Ref.	1.08 (0.66-1.77)	1.12 (0.67-1.85)	1.21 (0.74-1.96)	0.44
Manganese (Mn)	Ref.	0.81 (0.49-1.34)	0.68 (0.40-1.15)	1.27 (0.73-2.20)	0.40
Mercury (Hg)	Ref.	0.74 (0.46-1.20)	0.82 (0.49-1.36)	0.96 (0.59-1.56)	0.92
Nickel (Ni)	Ref.	1.21 (0.74-1.94)	0.92 (0.57-1.50)	0.92 (0.56-1.51)	0.49
Selenium (Se)	Ref.	0.71 (0.44-1.14)	1.06 (0.65-1.71)	0.92 (0.57-1.47)	0.87
Combined measures					
All metals	Ref.	0.61 (0.37-1.01)	0.77 (0.46-1.29)	1.06 (0.64-1.76)	0.41
IARC Group 1[b]	Ref.	0.73 (0.44-1.22)	0.98 (0.61-1.60)	0.84 (0.51-1.39)	0.86
IARC Group 2 & 3[c]	Ref.	0.91 (0.56-1.46)	0.78 (0.47-1.29)	1.10 (0.67-1.82)	0.78
PC 1	Ref.	0.81 (0.45-1.47)	1.08 (0.57-2.05)	1.23 (0.65-2.33)	0.43
PC 2	Ref.	1.03 (0.55-1.93)	0.56 (0.30-1.05)	0.79 (0.42-1.50)	0.35

[a] Models adjusted for age, education, income, race/ethnicity, BMI, RPF, and census tract affluence and disadvantage (n=664).

[b] IARC Group 1 metals: Arsenic, Beryllium, Cadmium, Chromium, Nickel.

[c] IARC Group 2 & 3 metals: Antimony, Cobalt, Lead, Manganese, Mercury, Selenium.

Asterisk denotes significance at $p < 0.05$.

study was the first to examine associations between chronic exposure to heavy metals and tumor grade and found that exposure to antimony and arsenic were associated with prevalence of high-grade tumors among premenopausal women.

Although antimony, cobalt, manganese, and selenium are not identified as 'known' carcinogens by IARC, there is evidence that exposure to these metals may be mechanistically related to breast cancer risk. Trivalent antimony (Sb(III)) has been shown to be genotoxic in mammalian test systems (152, 153). Antimony has additionally been shown to inhibit DNA damage repair enzymes and affect the ability of cells to repair DNA double strand breaks (154, 155). One epidemiologic study found a positive trend between plasma antimony levels and breast cancer risk among *BRCA1* mutation carriers, such that women in the highest tertile of exposure exhibited a 2.4-fold increased risk of breast cancer (156). In combination with our finding that nH Blacks have greater antimony exposure to nH Whites, this finding suggests antimony exposure may be contributing to ethnic disparities in breast cancer subtype incidence.

Cobalt is an essential element and part of the chemical structure of vitamin B12. It has therefore has been associated with beneficial health effects in humans (157). Inhalation of cobalt has been associated with increased mortality from lung cancer among occupationally exposed individuals (158, 159), although there is very limited evidence that cobalt is genotoxic (160). Interestingly co-exposure to cadmium, cobalt and lead is highly associated with DNA single strand breaks and the inability to repair 8-oxoguanine, a marker of DNA damage due to oxidative stress (161). In the present study, chronic exposure to cobalt was strongly correlated with both cadmium (r_s= 0.63) and lead (r_s= 0.49). Therefore, co-exposure among three metals may offer a biological mechanism explaining the association with prevalent ER/PR-negative tumors.

Like cobalt, manganese is an essential element and is required for normal physiological processes. *In vitro* models of have shown manganese exhibits cytotoxic properties suggesting it may play a role in the etiology of breast cancer (162, 163). Few epidemiologic studies have examined the effects of airborne manganese exposure on breast cancer incidence. One ecological study found airborne manganese was associated with decreased lung, breast, and total cancer mortality at the county level (164). Two studies additionally found lower concentrations of manganese in the hair of breast cancer patients compared to controls (165, 166), although neither of the previous studies focused on breast cancer subtype. Interestingly, animal studies have shown manganese intake is associated with expression of manganese superoxide dismutase (MnSOD) (167); MnSOD expression has been shown to be higher in mesenchymal-like breast cancers (168). These breast cancers tend to be ER/PR-negative and exhibit increased migratory, invasive, and metastatic abilities. While the evidence is conflicting, it is possible manganese exerts both a protective effect on ER/PR-positive disease, and a harmful effect on ER/PR-negative disease.

This study also found a significant positive trend between airborne selenium concentrations and prevalent ER/PR-negative cases, with the highest quartile of exposure exhibiting a 1.9-fold increase in ER/PR-negative prevalence. Selenium is a micronutrient that is necessary in small doses. Generally, most studies have identified either no association or a protective association between selenium exposure and cancer risk (169). Interestingly, *in vitro* studies have shown that selenium binds to ER-alpha and disrupts downstream signaling (24, 170-172). These results have been interpreted as selenium possessing anti-estrogenic properties that likely decrease the risk of ER/PR-positive tumors. Therefore, our results suggest that greater

exposure to selenium results in an increased proportion of ER/PR-negative tumors among all female breast cancer patients, rather than a true increase in ER/PR-negative tumor risk.

Similar to Lui et al.'s (2015) study, we found an increased risk of ER/PR-negative tumors among women with greater exposure to arsenic. Arsenic is classified by IARC as a known carcinogen and has been implicated in the development of various cancers (17, 173). Although arsenic has been shown to demonstrate estrogenic properties (23), other studies have shown that it can induce basal-like phenotypes through other molecular pathways (174). One explanation which combines both mechanisms is that exposure to arsenic can induce ER/PR-negative phenotypes via estrogen-mediated pathways through the development of genotoxic estrogen metabolites resulting in the formation of DNA adducts which cause subsequent DNA damage (175).

This is the first study of which we are aware that examined associations of inhaled heavy metals with tumor grade. Importantly, we stratified by menopausal status as premenopausal breast cancer tends to be more aggressive in nature compared to postmenopausal breast cancer. The association of antimony with ER/PR-negative breast cancer and the associations of antimony and arsenic with high-grade tumors were found to be restricted among premenopausal patients. Associations observed for premenopausal patients, while unstable, nonetheless suggest that exposure to inhaled heavy metals may play a greater role in the etiology of more aggressive (e.g., ER/PR-negative, high-grade) rather than less aggressive breast cancer subtypes.

This study had a number of limitations worth noting. In particular, this study was conducted only among breast cancer cases—there were no cancer-free controls available so we were unable to examine associations with breast cancer incidence. Rather, we examined associations with respect to prevalence of specific aggressive subtypes. In addition, because we

used information from multiple NATA cycles and due to changes in NATA methodology over time, chronic estimates of inhaled heavy metal exposure used in these analyses were inevitably based on chronic exposure rankings rather than absolute measures. By creating rankings we were able to link patients' historical addresses to multiple cycles of NATA data—something no previous study of airborne exposures and breast cancer risk has done. Similarly, this study relied on using residential histories to create exposure estimates. Most large-scale environmental epidemiology studies rely on residential addresses while acknowledging participants are not confined to their homes, thus resulting in some exposure misclassification. We would expect this misclassification to be non-differential resulting in an attenuation of the observed effect estimates. More generally, even if an individual spent 100% of their time in a particular census tract, using modeled exposure estimates from NATA data as a measure of individual-level exposures would still generate a great deal of exposure misclassification, which would tend to attenuate our estimates. Finally we did not examine disease latency and exposure windows; our exposure marker was measured up to disease diagnosis. We were unable to incorporate an appropriate lag as cancer onset can begin more than 15 years prior to diagnosis.

Given the listed limitations, this study also had a number of strengths. Our study was population-based and included a great deal of patient risk factor information. Our findings indicated that chronic exposure to airborne heavy metals may be associated with the development of aggressive breast cancer subtypes, or at a minimum, are protective of developing ER/PR-positive tumors. Both situations would suggest exposure to airborne heavy metals is contributing to the ethnic disparities in breast cancer tumor aggression. This was the second study to date to associate airborne exposures to arsenic as an aggressive breast cancer risk factor. We additionally were the first to attempt to account for mixtures of metal air concentrations by

using principal component analysis, although this approach was limited due to high correlations between heavy metal exposures.

E. **Conclusions**

This study identified an elevated prevalence of ER/PR-negative breast cancer among Breast Cancer Care in Chicago participants who lived in areas characterized by higher ambient air concentrations of antimony, arsenic, cobalt, manganese, and selenium. This study was additionally the first to identify associations between antimony and arsenic exposure and high-grade tumors among premenopausal women. These results suggest that long-term, low-dose exposure to certain heavy metals may play a role in the etiology of aggressive breast cancer characteristics. With the exception of arsenic, the other identified heavy metals are not known carcinogens but do have genotoxic and cytotoxic properties. Airborne exposures have the ability to affect large populations of individuals and findings from this and similar studies may have large public health implications such as identifying additional environmental exposures which contribute to the ethnic and socioeconomic disparities in aggressive breast cancer incidence.

III. RESDENTIAL AIRBORNE HEAVY METAL CONCENTRATIONS AND CHANGES IN CANCER-ASSOCIATED GENE METHYLATION

A. Background

Heavy metals are ubiquitous in the environment and are naturally found throughout the ecosystem. They are non-biodegradable and bioaccumulate in humans and animals, making them a serious class of environmental contaminants (6). Generally, humans are exposed to heavy metals via inhalation of ambient air and ingestion of contaminated food and water (3). In 2015, the Agency for Toxic Substances and Disease Registry (ATSDR) prioritized over 200 environmental contaminants by their impacts on public health. Arsenic, lead and mercury were identified as the top three substances and cadmium and chromium were ranked 7th and 17th, respectively (16).

Exposures to heavy metals have been associated with the development of breast (11, 120), lung (12), skin (9, 13), and bladder cancers (14). Of these, the effect on the development of breast cancer is among the most pressing from a public health perspective. Breast cancer is the most commonly diagnosed cancer in women worldwide (128). Although the etiology of breast cancer is fairly well understood, there exist major gaps in our knowledge. Previous studies have found that exposure to airborne concentrations of arsenic and cadmium are associated with the development of aggressive breast cancer subtypes, although the underlying mechanisms for these associations remain unidentified (127).

Emerging evidence suggests that heavy metals dysregulate the epigenome, resulting in increased cancer risk. Previous studies have shown that exposure to heavy metals results in gene promotor hypermethylation and genome-wide hypomethylation, both of which are hallmarks of cancer (79-81). Additionally, studies have shown that exposure to nickel is associated with

promoter hypermethylation of the tumor suppressor genes *TP16*, *RASSF1*, and *RAR-β2* (82, 83). Likewise, exposure to arsenic has been associated with promoter hypermethylation of *TP53* and *TP16* through the up-regulation of methyltransferases (86, 87). Finally, low-level exposure to cadmium is associated with hypomethylation of the DNA repetitive element LINE-1 (88).

Our group previously reported that chronic, airborne residential exposures to antimony, arsenic, cobalt, manganese, and selenium were associated with increased prevalence of ER/PR-negative breast tumors. The purpose of this study is to examine the associations between chronic and short-term residential airborne heavy metal concentrations with patterns of tumor DNA methylation. Specifically, we will investigate eleven individual heavy metal concentrations in relation to DNA methylation of six pre-identified, cancer-associated genes in various breast tumor tissue components.

B. **Methods**

1. **Study Population**

The Breast Cancer Care in Chicago (BCCC) study is a population-based sample of women who were diagnosed with breast cancer at one of the 56 Chicago-area hospitals. Women who were eligible for enrollment were between the ages of 30 and 79; self-identified as non-Hispanic (nH) White, nH Black, or Hispanic; resided in Chicago; had a first primary *in situ* or invasive breast cancer diagnosed between 2005 and 2008; and gave written, informed consent to participate in the study. Overall, 989 women were enrolled and completed a 90-minute interview on social, demographic, and healthcare-related factors. Of these, 812 (82%) consented to allow samples of diagnostic tissue to be obtained by research staff, of whom samples were obtained from 351 patients (35%); 723 (73%) participants consented to a blood draw, from which DNA

was extracted and stored on 668 patients (68%). A total of 260 (27%) patients had both blood and tissue collected. The study population has been described in detail elsewhere (143). Participants were dropped from the analysis if they were missing heavy metal exposure, DNA methylation or any of the *a priori* covariate data. After this consideration, the maximum possible sample size was 316 samples. The protocol for this study was approved by the University of Illinois at Chicago Institutional Review Board.

Covariate information was generally collected via questionnaire or medical record abstraction, with the exception of smoking status. A biomarker for smoking status was measured retrospectively on a subsample of the BCCC participants using DNA methylation derived from peripheral blood monocyte samples at three pre-identified loci (Illumina IDs: cg06644428 (2q37), cg21566642 (2q37), cg06126421 (6p21.33)). Previous studies have identified strong inverse linear associations of these markers with smoking history and have tested their utility in predicting past tobacco exposure (144, 145).

2. **DNA Methylation Assessment**

a. **Source of Breast Tissue Component Samples**

For the subset of patients who donated tumor samples, hematoxylin and eosin (H&E) stained slides from FFPE tumor blocks were examined to determine representative component areas of invasive, *in situ*, and histologically and morphologically normal-appearing breast tissue adjacent to the tumor (adjacent normal). For lumpectomies, we selected adjacent breast tissue from the same block as the tumor. However, when available we used a separate block containing breast tissue and no tumor as the non-malignant, adjacent sample. We cut tissue core samples precisely from the selected area using a semi-automated tissue arrayer (Beecher Instruments,

Inc.). Because the tissue was fixed and sealed by paraffin, cells from the invasive tissue could not become dislodged and contaminate the *in situ* or adjacent tissue or vice versa (176).

b. **Choice of DNA Regions for Analysis**

We chose a diverse set of five genes and a DNA repeat to assay for DNA methylation in invasive, *in situ*, and adjacent normal tissue components. Information on the tested genomic regions is shown in Table VI. The DNA regions examined overlapped or were near regions previously reported to be aberrantly hypermethylated in breast cancer vs non-cancerous breast tissue namely *BRCA1 (177, 178)*, *EGFR* (179), and *RASSF1* (180); or aberrantly hypomethylated in breast cancer vs normal breast namely, *TFF1* (181) and DNA repeat, Satellite 2 (Sat2) (182, 183). We also examined a gene region from *GSTM2* found to display hypermethylation in more aggressive breast cancers (184).

c. **DNA Methylation Analysis**

Dissolution of paraffin was accomplished by the addition of 1 mL of clearing agent (Histochoice) and incubation at 65 °C for 30 min. Samples were digested by the addition of 100 μL of digestion buffer consisting of 10 μL 10X Target Retrieval Solution high pH (DAKO, Glostrup, Denmark), 75 μL of ATL Buffer (Qiagen), and 15 μL of proteinase K (Qiagen) and incubation at 65 °C overnight. They were then vortexed and checked for complete digestion. The sample volume was brought up to ~100 μL, and 20 μL of each sample was treated with bisulfite and purified using the Zymo EZ-96 DNA Methylation-Direct™ Kit, with a 15-minute denaturation step at 98 °C followed by a 3.5-h conversion at 64 °C, an additional 15-minute denaturation at 98 °C and a 60-minute incubation at 64 °C. DNA was eluted in 40 μL of elution buffer. Then, PCR was performed with 0.2 μM of each primer, one of which was biotinylated, and the final PCR product was purified (Streptavidin Sepharose HP, Amersham Biosciences,

TABLE XX.

LIST OF STUDIED DNA REGIONS AND NUMBER OF CPGS COVERED

Gene	Test Region	Test Region Coordinates (hg19)	Distance from TSS (bp)[a]	CGI[b]	CpGs[c]
BRCA1	Exon 1(extended promoter)	Chr17: 41277463-41277365	+37 to +135	No	11
EGFR	Intron 1 (extended promoter)	Chr7: 55088080-55088104	+1355 to +1379	Yes	4
GSTM2	Promoter	Chr1: 110210582-110210641	-62 to -3	Yes	8
RASSF1	Exon 1 (extended promoter)	Chr3: 50378294-50378232	+74 to +134	Yes	9
TFF1	Promoter	Chr21: 43786664-43786628	-20 to +16	No	5
Sat2	N.A.	DNA Repeat	N.A.		2

[a]TSS, transcription start site

[b]CGI, CpG island overlapping the test region

[c]The number of CpG dinucleotide pairs in the test region

Uppsala, Sweden), washed, alkaline-denatured, and rewashed (Pyrosequencing Vacuum Prep Tool, Qiagen). Then, the pyrosequencing primer (0.5 μM) was annealed to the purified single-stranded PCR product, and 10 μL of the PCR products were sequenced by Pyrosequencing PSQ96 HS System (Biotage AB) following the manufacturer's instructions. The methylation status of each locus was analyzed individually as a T/C SNP using Pyromark Q96 software (Qiagen, Germantown, Maryland).

3. Chronic and Short-Term Airborne Heavy Metal Exposure Assessments

a. National-Scale Air Toxics Assessment Database

The Environmental Protection Agency (EPA) conducts the National-Scale Air Toxics Assessment (NATA), which is an ongoing, comprehensive evaluation of air toxics in the United States. Briefly, the process for air toxics estimates is as follows: emission data collected from countrywide anthropogenic sources (point, non-point, on-road mobile, non-road mobile, and background emissions) included in the National Emissions Inventory (NEI) are compiled and prepared for use as model inputs. Using these inputs in combination with meteorological and

dispersion models, ambient concentrations of census tract-level air toxics are estimated (air concentration). Estimates of air toxics are available for four different time points (1999, 2002, 2005, and 2011). For the purposes of this analysis, we used data from the first three time points as the 2011 air concentrations were measured after the study period of interest. Minor methodological changes were made across the different time points. Ambient concentrations of antimony, arsenic, beryllium, cadmium, chromium, cobalt, lead, manganese, mercury, nickel, and selenium were recorded across all three time points. In 1999, NATA accounted for different chromium species (III vs VI). In 2002 and 2005, the air concentration estimates for chromium were combined into one variable. For the purposes of this analysis, the two chromium variables from 1999 were summed to generate a total chromium air concentration, consistent with the 2002 and 2005 measures.

b. **Residential Histories**

Patients' residential histories 15 years (chronic) and one year (short-term) prior to breast cancer diagnosis were collected using the CLEAR database offered by Thomson Reuters. Briefly, this database is used to monitor individuals using information from public and credit gateways. Information on past and current addresses, along with dates of residence, was obtained via household listings, utility bills, phone records, and information from the Transunion and Experian credit databases and used to identify specific addresses. We conducted queries on BCCC participants using name and date of birth. Correct listings were determined if the record retrieved had a matching address to the one given at the time of interview. One address was selected for each year of follow up. Priority was given to addresses and dates from household listing and utility sources. In instances where no household or utility information was available, we relied upon residential information from credit sources. For women with multiple addresses

within a year, we selected the residence in which they lived the longest. In order to account for women with missing residential information, we imputed the last known address for women with at least one CLEAR residence record prior to 2000, between 2000 and 2004, and between 2005 and year of diagnosis.

c. Computation of Airborne Heavy Metal Exposure Assessment

Due to differences in methodology over the NATA time points (1999, 2002 and 2005), it was not possible to take a simple or weighted average of air concentration estimates over the study period. For this reason, we created an air concentration ranking for each metal for each exposure year.

Our strategy for combining NATA estimates with residential histories was as follows: (1) NATA 1999 estimates were applied to residences between 15 years prior to diagnosis and 1999; (2) NATA 2002 estimates were applied to residences between 2000 and 2003; (3) NATA 2005 estimates were applied to residences between 2004 and year of diagnosis. If a participant did not have at least one known residence in all three of the time periods, we were unable to calculate a 15-year chronic metal exposure ranking and the participant was assigned a missing value for the chronic metal exposures. After residential history imputation, we calculated exposure rankings by year for each individual metal, which were then summed across all 15 years of follow up. Rankings were shifted and scaled by dividing the exposure rank score by the maximum possible rank so that each rank score ranged between 0 and 1. For the short-term marker of heavy metal exposure, we applied 2005 NATA measurements to the participant's residence at diagnosis.

For both the chronic and short-term exposure assessments, we created metal exposure variables by summing the rankings for the following metal exposure combinations: (1) all metals, (2) International Agency for Research on Cancer (IARC) Group 1 metals (arsenic,

beryllium, cadmium, chromium and nickel), and (3) IARC Group 2 and 3 metals (antimony, cobalt, lead, manganese, mercury and selenium). Finally, we employed principal component analysis to estimate eigenvectors representing combined exposure. This procedure converts the highly correlated airborne exposure variables into uncorrelated variables comprised of proportions of the airborne exposure estimates offering an additional approach to examine mixtures of heavy metals.

4. **Statistical Analysis**

We performed descriptive analyses to examine covariate distributions in the sample population. We treated chronic and short-term measurements of heavy metal exposure as continuous variables. Mean (and standard deviation) and median (and interquartile range) percent methylation values for each tested CpG site were computed to examine within-assay variability. We averaged percent DNA methylation across the individual genomic regions to compute a single methylation measurement for each assayed gene region. We used linear regression models to estimate beta values for the associations between chronic and short-term heavy metal concentrations with DNA methylation. We estimated 95% and 99% confidence intervals (CIs) via 1000 bootstrap replications with bias correction to account for the non-normality of the DNA methylation measurements. To construct the smoking variable, we combined the percent methylation values from each of the three CpG sites into one marker using principal component analysis. We used the first eigenvector to represent estimated cumulative exposure to environmental tobacco smoke, with lower values representing higher likelihood of prolonged exposure. Fully adjusted models included age, race, and body mass index (BMI). Additionally, we stratified models across the three types of tissue components. Finally, we produced restricted cubic splines to visualize the shape of the associations between chronic and

short-term heavy metal concentrations and DNA methylation. All analyses were conducted using Stata version 12 (Stata Corp., College Station, TX) and statistical significance was determined at a two-sided $p \leq 0.05$.

C. **Results**

Table VII shows the covariate distributions in the sample population. Briefly, the women had an average age of 56 at enrollment, were mostly non-Hispanic (nH) Black (42%), and had greater than 12 years of education (55%). Additionally, half (50%) the women earned between $25,000-$87,499 per year and over three-quarters (76%) were overweight or obese. Percent methylation distributions by CpG site are shown in Appendix D.

Tables VIII through XIII present the associations between chronic metal exposure rankings and DNA methylation of the six gene regions, stratified by tissue component. We identified significant inverse associations between exposure to ambient airborne chromium (β = -1.06, 95% CI: -2.13, -0.23) and manganese (β = -1.11, 95% CI: -2.29, -0.05) with *BRCA1* methylation in adjacent normal tissue. We additionally identified significant positive associations between exposures to ambient airborne antimony (β = 15.6, 95% CI: 0.11, 33.3), cobalt (β = 20.0, 95% CI: 4.60, 34.8), lead (β = 13.3, 95% CI: 0.10, 29.0), and manganese (β = 18.8, 95% CI: 4.78, 35.9), and the following combined exposure markers: all metals (β = 23.1, 95% CI: 4.26, 45.6) and IARC group 2 & 3 metals (β = 22.5, 95% CI: 6.52, 41.7) with *GSTM2* methylation in invasive tumor components. Furthermore, we identified significant inverse associations between exposure to ambient airborne antimony with *RASSF1* methylation in adjacent normal samples (β = -11.7, 95% CI: -20.8, -2.97) and in *in situ* samples (β = -22.5, 95% CI: -47.1, -0.82). Finally, we identified significant inverse associations between exposures to ambient airborne arsenic (β = -7.26, 95% CI: -15.7, -0.59), lead (β = -7.13, 95% CI: -13.6, -0.44),

TABLE VII.

PARTICIPANT CHARACTERISTICS
(N=316)

Variable	N	%
Age (mean [sd])	56.42 [10.8]	
Race		
nH White	115	36
nH Black	133	42
Hispanic	68	22
Education		
Less than HS	67	21
HS Diploma	75	24
Greater than HS	174	55
Income		
< $25,000	88	28
$25,000 - $87,499	154	50
≥ $85,000	67	22
BMI		
≤ 25	75	24
25-30	103	33
> 30	136	43
Tobacco Smoke Exposure		
Quartile 1	52	26
Quartile 2	49	24
Quartile 3	54	26
Quartile 4	49	24

and IARC group 2 & 3 metals (β = -6.90, 95% CI: -15.4, -0.06) with Sat2 methylation in adjacent normal samples. No other tested associations between chronic metal exposure and breast tissue DNA methylation met the threshold for significance.

Tables XIV through XIX present the associations between short-term heavy metal exposures and DNA methylation of the six gene regions, stratified by tissue component. We found inverse associations between exposures to ambient airborne nickel (β = -1.00, 95% CI: -

TABLE VIII.

BRCA1 ASSOCIATIONS WITH FIFTEEN YEAR AIRBORNE METAL EXPOSURES BY TISSUE TYPE

	Adjacent normal n= 208		In situ n= 152		Invasive n= 212	
	Beta	CI (95%)	Beta	CI (95%)	Beta	CI (95%)
Individual Metals						
Antimony	-0.24	-1.21, 0.88	-0.11	-1.36, 1.18	-2.80	-7.86, 1.36
Arsenic	0.06	-1.26, 1.50	0.14	-1.32, 1.70	2.25	-1.50, 8.50
Beryllium	0.05	-1.02, 1.24	-0.02	-0.95, 1.05	0.81	-2.76, 4.70
Cadmium	0.31	-1.04, 2.00	-0.14	-1.39, 1.38	-2.00	-7.48, 4.64
Chromium	-1.06	-2.13, -0.23*	0.28	-0.62, 1.28	-0.57	-3.79, 3.82
Cobalt	0.00	-1.02, 1.12	-0.12	-1.27, 1.01	0.21	-4.27, 3.95
Lead	-0.13	-1.14, 1.02	-0.06	-1.22, 1.09	4.58	-0.80, 11.7
Manganese	-1.11	-2.29, -0.05*	0.33	-0.96, 1.63	1.16	-2.57, 5.82
Mercury	0.23	-0.96, 1.99	-0.13	-1.16, 1.14	0.68	-3.02, 4.89
Nickel	0.25	-0.89, 1.31	0.37	-0.65, 1.61	0.89	-2.27, 4.78
Selenium	-0.00	-0.94, 1.06	-0.20	-1.21, 0.69	1.03	-3.43, 5.49
Combined Markers						
All Metals	-0.30	-1.55, 1.16	0.05	-1.64, 1.88	1.18	-2.19, 6.18
IARC Grp. 1	-0.22	-1.32, 1.04	0.21	-1.22, 1.92	0.43	-2.59, 4.16
IARC Grp. 2&3	-0.28	-1.49, 1.19	-0.09	-1.71, 1.53	1.37	-2.49, 6.80
PC1	0.09	-0.81, 1.23	-0.09	-1.12, 1.08	0.46	-1.84, 3.13
PC2	-0.08	-0.99, 0.85	0.32	-0.44, 1.10	0.63	-2.91, 5.11

*Denotes statistical significance at $p < 0.05$; **Denotes significance at $p < 0.01$

Models adjusted for age, race, and BMI.

TABLE IX.

EGFR ASSOCIATIONS WITH FIFTEEN YEAR AIRBORNE METAL EXPOSURES BY TISSUE TYPE

	Adjacent normal n= 254		In situ n= 169		Invasive n= 225	
	Beta	CI (95%)	Beta	CI (95%)	Beta	CI (95%)
Individual Metals						
Antimony	0.25	-3.19, 3.92	-3.74	-15.8, 10.9	-4.26	-15.6, 6.13
Arsenic	0.42	-3.10, 3.25	-3.61	-19.7, 11.4	-2.40	-15.0, 11.0
Beryllium	0.17	-2.40, 2.75	-1.04	-12.6, 9.94	-1.92	-11.8, 7.72
Cadmium	-2.48	-7.08, 1.09	3.67	-8.55, 16.9	-2.57	-15.5, 9.74
Chromium	-1.87	-6.47, 2.17	-1.28	-12.8, 10.6	-2.54	-12.3, 7.66
Cobalt	2.01	-0.59, 4.61	-4.98	-16.3, 6.75	-4.43	-15.8, 5.23
Lead	1.60	-0.89, 4.88	2.38	-9.33, 15.0	-4.39	-13.9, 5.73
Manganese	0.60	-2.98, 4.02	-0.29	-14.2, 13.6	-2.75	-14.2, 7.12
Mercury	0.77	-2.91, 4.35	-2.27	-14.9, 11.7	-3.76	-13.2, 5.79
Nickel	-0.17	-4.19, 3.07	-1.47	-13.5, 11.4	-3.00	-13.2, 6.86
Selenium	2.29	-0.40, 5.41	-1.07	-11.1, 8.52	-4.20	-12.9, 4.98
Combined Markers						
All Metals	0.75	-3.46, 4.79	-2.23	-19.7, 15.3	-6.07	-20.5, 7.68
IARC Grp. 1	-1.22	-6.13, 2.86	-1.06	-17.5, 15.7	-3.97	-18.2, 9.48
IARC Grp. 2&3	1.95	-1.35, 5.56	-2.53	-18.7, 12.9	-5.93	-19.7, 5.08
PC1	0.01	-3.11, 2.79	-3.68	-14.6, 7.96	-3.55	-12.6, 4.73
PC2	-1.31	-3.97, 1.34	1.87	-8.47, 10.6	1.42	-7.00, 9.67

*Denotes statistical significance at $p < 0.05$; **Denotes significance at $p < 0.01$

Models adjusted for age, race, and BMI.

TABLE X.

GSTM2 ASSOCIATIONS WITH FIFTEEN YEAR AIRBORNE METAL EXPOSURES BY TISSUE TYPE

	Adjacent normal n= 206		In situ n= 148		Invasive n= 212	
	Beta	CI (95%)	Beta	CI (95%)	Beta	CI (95%)
Individual Metals						
Antimony	-4.09	-12.6, 1.70	-4.14	-17.9, 12.7	15.6	0.11, 33.3*
Arsenic	-1.18	-6.11, 3.61	-2.10	-17.2, 14.5	13.2	-4.57, 31.1
Beryllium	0.14	-3.63, 4.29	-5.36	-18.1, 9.18	3.49	-12.0, 18.4
Cadmium	-5.58	-16.4, 0.70	-3.88	-16.0, 8.61	14.4	-3.83, 36.2
Chromium	-4.58	-13.8, 1.35	-5.58	-18.5, 8.02	8.00	-7.04, 24.2
Cobalt	0.97	-1.63, 4.01	-0.69	-12.6, 11.1	20.0	4.60, 34.8**
Lead	4.48	-0.04, 11.1	-3.99	-19.1, 14.2	13.3	0.10, 29.0*
Manganese	-0.66	-7.99, 4.38	-5.24	-19.8, 11.6	18.8	4.78, 35.9*
Mercury	0.58	-6.13, 6.25	-5.52	-18.4, 9.29	12.1	-1.63, 28.4
Nickel	-1.79	-9.42, 3.61	-7.24	-18.4, 4.86	13.2	-3.21, 31.2
Selenium	2.39	-0.59, 6.42	-1.01	-14.2, 11.8	12.7	-0.10, 26.3
Combined Markers						
All Metals	-1.05	-9.21, 4.54	-7.34	-22.7, 10.5	23.1	4.26, 45.6*
IARC Grp. 1	-3.85	-14.3, 2.20	-7.73	-22.1, 5.87	15.5	-5.29, 38.5
IARC Grp. 2&3	1.23	-3.52, 6.60	-5.02	-22.2, 13.2	22.5	6.52, 41.7**
PC1	-1.12	-7.55, 3.12	-5.49	-16.7, 6.03	12.2	-0.75, 27.2
PC2	-1.16	-5.37, 2.36	-1.23	-13.0, 11.7	-4.47	-17.5, 7.99

*Denotes statistical significance at $p < 0.05$; **Denotes significance at $p < 0.01$

Models adjusted for age, race, and BMI.

TABLE XI.

RASSF1 ASSOCIATIONS WITH FIFTEEN YEAR AIRBORNE METAL EXPOSURES BY TISSUE TYPE

	Adjacent normal n= 225		In situ n= 155		Invasive n= 219	
	Beta	CI (95%)	Beta	CI (95%)	Beta	CI (95%)
Individual Metals						
Antimony	-11.7	-20.8, -2.97*	-22.5	-47.1, -0.82*	-12.1	-29.9, 5.92
Arsenic	0.19	-9.33, 10.0	-11.5	-41.7, 14.9	-7.30	-31.2, 12.6
Beryllium	1.84	-7.11, 10.0	-0.31	-21.6, 21.5	3.36	-12.6, 18.5
Cadmium	-7.55	-18.9, 2.38	-10.9	-33.8, 17.6	-15.2	-36.4, 6.52
Chromium	-1.49	-9.62, 8.11	-13.1	-31.7, 6.45	-13.5	-30.2, 3.44
Cobalt	-3.82	-12.1, 5.11	-20.2	-44.0, 1.15	-7.13	-24.1, 10.6
Lead	4.66	-3.12, 13.8	-0.46	-21.7, 21.1	-1.99	-19.8, 16.5
Manganese	-3.15	-12.7, 5.71	-21.2	-43.5, 1.30	-14.0	-30.8, 5.04
Mercury	-2.48	-10.3, 5.44	-7.02	-30.5, 15.0	0.58	-16.3, 18.9
Nickel	-0.49	-9.53, 9.06	-5.08	-27.0, 15.8	1.02	-18.8, 19.4
Selenium	0.02	-7.87, 8.86	-9.84	-28.5, 7.83	-8.09	-22.8, 7.68
Combined Markers						
All Metals	-3.37	-13.0, 7.87	-17.4	-53.2, 11.1	-11.4	-38.3, 11.1
IARC Grp. 1	-1.94	-12.2, 9.11	-11.7	-40.9, 17.3	-8.99	-33.5, 14.2
IARC Grp. 2&3	-3.52	-12.9, 5.89	-20.2	-50.2, 7.25	-10.1	-32.1, 10.7
PC1	-2.46	-9.08, 5.62	-13.4	-33.8, 6.14	-5.61	-21.7, 9.74
PC2	4.35	-4.44, 12.8	9.55	-6.48, 24.7	6.05	-7.95, 19.4

*Denotes statistical significance at $p < 0.05$; **Denotes significance at $p < 0.01$

Models adjusted for age, race, and BMI.

TABLE XII.

TFF1 ASSOCIATIONS WITH FIFTEEN YEAR AIRBORNE METAL EXPOSURES BY TISSUE TYPE

	Adjacent normal n= 237		In situ n= 164		Invasive n= 221	
	Beta	CI (95%)	Beta	CI (95%)	Beta	CI (95%)
Individual Metals						
Antimony	8.27	-3.11, 20.1	-6.08	-23.3, 13.0	6.55	-8.35, 23.7
Arsenic	-7.28	-17.0, 3.61	4.49	-19.5, 26.8	5.43	-17.2, 24.5
Beryllium	-7.52	-16.1, 1.72	10.6	-4.57, 27.2	-2.46	-17.3, 12.8
Cadmium	-0.07	-12.4, 11.7	-8.30	-25.2, 13.1	10.7	-7.50, 29.5
Chromium	0.54	-9.31, 10.8	10.7	-5.62, 26.5	6.46	-9.05, 21.8
Cobalt	-0.00	-9.87, 10.2	6.44	-8.95, 24.8	6.02	-9.47, 22.3
Lead	-4.53	-15.2, 5.53	-0.87	-19.0, 19.0	2.19	-15.4, 16.7
Manganese	4.33	-6.31, 15.4	7.88	-10.7, 27.7	9.45	-7.34, 24.5
Mercury	-4.96	-14.5, 4.89	2.53	-15.1, 20.5	0.33	-16.2, 15.6
Nickel	-4.25	-15.0, 6.90	6.40	-10.4, 25.3	-1.67	-17.8, 16.0
Selenium	-3.10	-12.4, 5.72	-1.30	-16.3, 14.5	8.72	-5.33, 23.5
Combined Markers						
All Metals	-3.15	-15.3, 9.21	6.30	-15.7, 30.2	8.18	-14.6, 30.9
IARC Grp. 1	-5.59	-17.5, 6.62	8.91	-11.6, 32.4	5.03	-17.2, 27.8
IARC Grp. 2&3	-0.64	-11.9, 11.4	2.21	-18.4, 24.5	8.21	-11.1, 26.7
PC1	-1.99	-10.9, 6.79	5.20	-9.90, 20.9	4.55	-9.26, 19.3
PC2	-3.35	-13.4, 6.09	6.02	-8.72, 20.0	-6.11	-18.9, 7.80

*Denotes statistical significance at $p < 0.05$; **Denotes significance at $p < 0.01$

Models adjusted for age, race, and BMI.

TABLE XIII.

Sat2 ASSOCIATIONS WITH FIFTEEN YEAR AIRBORNE METAL EXPOSURES BY TISSUE TYPE

	Adjacent normal n= 235		In situ n= 163		Invasive n= 218	
	Beta	CI (95%)	Beta	CI (95%)	Beta	CI (95%)
Individual Metals						
Antimony	-2.59	-11.2, 3.50	5.11	-3.37, 13.8	1.78	-7.95, 11.2
Arsenic	-7.26	-15.7, -0.59*	0.50	-9.34, 10.6	4.14	-6.62, 15.3
Beryllium	-2.54	-8.25, 3.44	4.81	-4.16, 12.0	4.46	-4.00, 12.6
Cadmium	-1.77	-10.3, 5.62	-0.11	-10.7, 8.64	5.47	-5.76, 16.2
Chromium	-0.78	-7.04, 5.68	1.23	-5.47, 9.65	5.01	-4.06, 13.5
Cobalt	-5.83	-13.1, 0.01	3.63	-3.89, 11.6	1.48	-6.65, 11.2
Lead	-7.13	-13.6, -0.44*	7.07	-1.83, 14.8	1.08	-7.04, 9.37
Manganese	-4.52	-12.0, 1.08	8.52	-0.82, 17.8	3.34	-5.91, 12.1
Mercury	-4.06	-10.9, 1.35	6.02	-1.34, 14.3	1.97	-6.29, 10.3
Nickel	-4.80	-11.5, 1.49	6.29	-3.21, 15.0	3.41	-6.65, 12.5
Selenium	-3.77	-9.54, 1.14	2.11	-6.21, 9.27	1.19	-6.45, 8.91
Combined Markers						
All Metals	-7.02	-16.8, 1.15	7.54	-3.04, 18.3	5.25	-7.03, 17.5
IARC Grp. 1	-4.74	-13.4, 3.16	4.22	-4.99, 14.4	7.33	-4.58, 19.1
IARC Grp. 2&3	-6.90	-15.4, -0.06*	8.11	-2.07, 18.5	2.63	-8.12, 12.9
PC1	-4.34	-10.7, 1.18	4.08	-2.67, 12.0	1.37	-6.87, 9.71
PC2	0.01	-5.21, 6.17	0.44	-6.69, 7.66	2.68	-4.07, 9.61

*Denotes statistical significance at $p < 0.05$; **Denotes significance at $p < 0.01$

Models adjusted for age, race, and BMI.

2.17, -0.00) with *BRCA1* methylation in adjacent normal samples. Furthermore, we found inverse associations between exposures to ambient airborne antimony (β = -1.13, 95% CI: -2.02, -0.13), nickel (β = -1.12, 95% CI: -1.77, -0.45) and the first principle component (β = -2.74, 95% CI: -4.72, -0.01) with *BRCA1* methylation in *in situ* samples. We additionally identified an inverse relationship between exposure to ambient airborne cadmium (β = -3.59, 95% CI: -6.45, -0.25) with *GSTM2* methylation in adjacent normal samples. For *RASSF1*, we found inverse

associations between exposure to ambient airborne antimony (β = -7.38, 95% CI: -13.9, -0.21) in adjacent normal samples. Furthermore, we identified inverse associations with exposures to ambient airborne chromium (β = -16.4, 95% CI: -30.6, -1.76), manganese (β = -15.5, 95% CI: -30.0, -2.08), and the following combined markers of exposure: all metals (β = -21.9, 95% CI: -42.4, -1.27), IARC group 1 (β = -18.3, 95% CI: -37.6, -0.97), IARC group 2 & 3 (β = -20.9, 95% CI: -39.4, -2.51), and the first principle component (β = -35.5, 95% CI: -67.0, -3.38) in invasive samples. We identified positive associations between exposures to nearly all ambient airborne heavy metals, and the following combined markers of exposure: all metals (β = 16.1, 95% CI: 5.44, 28.0), IARC group 1 (β = 12.9, 95% CI: 3.16, 23.8), IARC group 2 & 3 (β = 15.9, 95% CI: 4.90, 27.7), and the first principle component (β = 27.7, 95% CI: 9.87, 47.2) with *TFF1* methylation in adjacent normal samples. We identified similar relationships, albeit stronger, between the previously mentioned combined markers of exposure with *TFF1* methylation in invasive samples. Finally, we found inverse associations between exposures to ambient airborne cobalt (β = -5.61, 95% CI: -10.6, -0.42), manganese (β = -5.43, 95% CI: -11.0, -0.82) and the first principle component (β = -11.6, 95% CI: -22.2, -2.20) with Sat2 methylation in adjacent normal samples, and positive associations with exposures to ambient airborne antimony (β = 9.87, 95% CI: 2.84, 16.8), lead (β = 9.35, 95% CI: 2.50, 16.2) and IARC group 2 & 3 (β = 9.98, 95% CI: 1.13, 18.6) with Sat2 methylation in *in situ* samples. No other significant associations between ambient airborne metal exposure and methylation were identified.

Restricted cubic splines visualizing the associations between chronic and short-term heavy metal exposures with DNA methylation by tissue component types are shown in Appendix E.

TABLE XIV.

BRCA1 ASSOCIATIONS WITH ONE YEAR AIRBORNE METAL EXPOSURES BY TISSUE TYPE

	Adjacent normal n= 241		In situ n= 168		Invasive n= 245	
	Beta	CI (95%)	Beta	CI (95%)	Beta	CI (95%)
Individual Metals						
Antimony	-0.83	-1.87, 0.07	-1.13	-2.02, -0.13*	-1.94	-5.76, 1.70
Arsenic	0.02	-1.13, 1.27	-0.88	-1.67, 0.16	3.17	-1.15, 9.27
Beryllium	0.02	-1.03, 1.19	-0.84	-1.61, 0.28	2.98	-1.16, 9.75
Cadmium	0.18	-0.95, 1.48	-0.74	-1.77, 0.46	-1.81	-6.88, 3.08
Chromium	-0.47	-1.53, 0.69	-0.59	-1.47, 0.66	0.39	-2.88, 4.57
Cobalt	0.19	-0.86, 1.55	-0.47	-1.32, 0.45	1.01	-4.08, 6.87
Lead	-0.31	-1.30, 0.72	-0.92	-1.77, 0.13	3.31	-1.14, 9.43
Manganese	-0.65	-1.58, 0.29	-0.26	-1.15, 0.98	0.04	-4.41, 5.36
Mercury	-0.03	-0.95, 1.23	-0.75	-1.48, 0.26	4.11	-0.73, 10.9
Nickel	-1.00	-2.17, -0.00*	-1.12	-1.77, -0.45**	2.07	-1.98, 9.26
Selenium	-0.09	-1.09, 1.11	-0.79	-1.49, 0.05	2.49	-2.40, 7.49
Combined Markers						
All Metals	-0.54	-2.00, 1.15	-1.50	-2.79, 0.22	3.01	-0.99, 9.22
IARC Grp. 1	-0.46	-1.90, 1.19	-1.40	-2.48, 0.24	2.47	-1.45, 9.37
IARC Grp. 2&3	-0.51	-1.87, 1.04	-1.33	-2.60, 0.24	2.89	-1.40, 8.78
PC1	-0.84	-3.53, 1.75	-2.74	-4.72, -0.01*	6.86	-0.70, 17.6
PC2	1.48	-0.93, 4.53	0.39	-1.32, 1.98	1.29	-13.2, 13.3
PC3	-1.11	-3.01, 1.08	-0.61	-2.62, 1.24	-8.87	-23.1, 3.22

*Denotes statistical significance at $p < 0.05$; **Denotes significance at $p < 0.01$

Models adjusted for age, race, and BMI.

TABLE XV.

EGFR ASSOCIATIONS WITH ONE YEAR AIRBORNE METAL EXPOSURES BY TISSUE TYPE

	Adjacent normal n= 296		In situ n= 191		Invasive n= 265	
	Beta	CI (95%)	Beta	CI (95%)	Beta	CI (95%)
Individual Metals						
Antimony	-0.92	-3.80, 1.77	2.67	-8.28, 13.0	2.54	-7.35, 10.5
Arsenic	-1.05	-3.23, 1.15	-2.95	-12.3, 7.29	-3.39	-13.0, 5.76
Beryllium	-1.37	-3.48, 0.52	-2.92	-12.2, 6.89	-0.09	-9.78, 9.17
Cadmium	-0.57	-2.97, 1.64	2.45	-7.03, 12.0	-1.55	-11.2, 6.84
Chromium	-1.59	-4.52, 1.04	1.54	-8.72, 10.7	0.12	-9.66, 8.37
Cobalt	2.11	-0.98, 6.27	-0.22	-10.2, 10.7	-5.91	-13.1, 1.54
Lead	-0.78	-3.39, 1.55	-1.41	-10.4, 8.33	-4.15	-12.1, 3.83
Manganese	-1.24	-4.36, 1.10	-0.24	-10.7, 10.7	-4.02	-12.7, 3.90
Mercury	-0.43	-3.28, 2.03	-0.91	-10.6, 9.59	-6.10	-13.3, 2.80
Nickel	-1.10	-3.31, 0.66	-1.62	-10.5, 6.98	-3.84	-11.4, 4.98
Selenium	1.34	-1.33, 4.94	0.81	-8.41, 11.6	-4.45	-11.9, 3.81
Combined Markers						
All Metals	-0.93	-4.49, 2.22	-0.54	-14.8, 13.2	-5.78	-18.6, 5.37
IARC Grp. 1	-1.92	-5.34, 0.80	-1.20	-13.6, 10.8	-3.07	-15.6, 7.69
IARC Grp. 2&3	0.07	-3.34, 3.58	0.22	-14.5, 14.0	-6.93	-17.7, 3.83
PC1	-2.09	-8.23, 3.23	0.22	-22.9, 21.8	-8.29	-28.1, 9.41
PC2	5.26	-0.28, 13.2	3.53	-19.7, 27.9	-6.31	-25.5, 11.2
PC3	-3.50	-12.3, 3.24	12.8	-8.50, 33.3	10.0	-10.2, 27.9

*Denotes statistical significance at $p < 0.05$; **Denotes significance at $p < 0.01$

Models adjusted for age, race, and BMI.

TABLE XVI.

GSTM2 ASSOCIATIONS WITH ONE YEAR AIRBORNE METAL EXPOSURES BY TISSUE TYPE

	Adjacent normal n= 235		In situ n= 164		Invasive n= 247	
	Beta	CI (95%)	Beta	CI (95%)	Beta	CI (95%)
Individual Metals						
Antimony	-2.11	-8.06, 1.99	-2.78	-14.5, 10.6	-2.91	-15.8, 9.90
Arsenic	1.60	-1.60, 5.74	1.21	-11.0, 12.2	-0.43	-11.3, 12.5
Beryllium	-0.32	-3.05, 2.26	2.11	-9.64, 14.1	-3.96	-16.0, 8.16
Cadmium	-3.59	-6.45, -0.25*	3.07	-7.92, 15.5	-0.94	-13.5, 13.2
Chromium	-2.63	-9.29, 0.98	5.26	-5.39, 19.0	-3.55	-15.7, 9.32
Cobalt	5.04	-0.62, 13.6	12.4	-0.29, 27.6	4.02	-7.00, 16.0
Lead	0.81	-3.35, 4.87	5.64	-5.88, 20.4	-2.90	-14.5, 7.19
Manganese	-1.74	-7.48, 1.61	7.55	-3.39, 23.0	1.08	-11.8, 13.8
Mercury	0.12	-4.09, 3.53	6.87	-4.39, 20.2	-0.34	-12.6, 11.3
Nickel	-0.35	-2.59, 1.84	-3.71	-15.3, 9.50	-7.51	-19.2, 4.99
Selenium	4.39	-0.37, 11.5	5.66	-6.75, 19.3	7.79	-3.82, 19.9
Combined Markers						
All Metals	0.36	-4.40, 4.40	6.95	-6.79, 25.9	-1.39	-18.1, 15.1
IARC Grp. 1	-1.66	-6.69, 1.54	2.32	-10.4, 18.2	-5.88	-22.3, 9.61
IARC Grp. 2&3	2.07	-2.20, 7.28	10.3	-4.31, 29.3	2.41	-13.0, 19.3
PC1	1.06	-7.40, 8.41	12.9	-12.1, 40.5	-0.42	-28.6, 25.3
PC2	7.79	-0.48, 21.6	15.1	-14.6, 45.1	19.9	-8.29, 48.1
PC3	-11.4	-30.0, 1.24	-7.86	-34.9, 23.2	-6.46	-35.7, 22.5

*Denotes statistical significance at $p < 0.05$; **Denotes significance at $p < 0.01$

Models adjusted for age, race, and BMI.

TABLE XVII.

RASSF1 ASSOCIATIONS WITH ONE YEAR AIRBORNE METAL EXPOSURES BY TISSUE TYPE

	Adjacent normal n= 255		In situ n= 172		Invasive n= 253	
	Beta	CI (95%)	Beta	CI (95%)	Beta	CI (95%)
Individual Metals						
Antimony	-7.38	-13.9, -0.21*	0.90	-17.8, 17.4	-5.88	-22.4, 7.32
Arsenic	-2.25	-7.48, 4.19	1.12	-18.4, 18.7	-10.2	-26.2, 4.84
Beryllium	-1.16	-6.84, 5.84	-1.24	-19.6, 17.0	-9.63	-24.7, 3.42
Cadmium	-7.32	-15.4, 0.43	1.90	-14.9, 19.4	-11.1	-26.6, 3.72
Chromium	-0.75	-7.36, 7.35	-2.17	-21.9, 16.3	-16.4	-30.6, -1.76*
Cobalt	-2.05	-8.89, 5.30	2.07	-16.8, 19.6	-14.0	-27.6, 0.09
Lead	-0.90	-6.60, 4.89	0.16	-18.0, 18.2	-11.2	-24.1, 1.41
Manganese	-6.00	-12.8, 1.06	-5.74	-26.1, 15.7	-15.5	-30.0, -2.08*
Mercury	-5.74	-11.7, 1.09	2.05	-18.2, 20.7	-11.2	-26.0, 3.99
Nickel	0.96	-5.61, 7.97	2.50	-13.3, 18.4	-6.65	-21.2, 7.26
Selenium	-1.72	-7.30, 5.27	3.60	-15.2, 19.9	-10.9	-24.3, 3.28
Combined Markers						
All Metals	-5.85	-13.5, 3.39	1.07	-25.4, 24.7	-21.9	-42.4, -1.27*
IARC Grp. 1	-3.22	-10.8, 6.13	0.76	-23.1, 23.0	-18.3	-37.6, -0.97*
IARC Grp. 2&3	-6.96	-14.3, 1.78	1.20	-25.0, 26.0	-20.9	-39.4, -2.51*
PC1	-7.98	-21.7, 8.63	5.60	-40.5, 44.0	-35.5	-67.0, -3.38*
PC2	-8.26	-25.9, 10.2	-1.27	-43.4, 40.0	-9.49	-40.4, 26.1
PC3	-15.0	-33.0, 3.00	2.81	-36.3, 42.1	-1.71	-34.4, 28.1

*Denotes statistical significance at $p < 0.05$; **Denotes significance at $p < 0.01$

Models adjusted for age, race, and BMI.

TABLE XVIII.

TFF1 ASSOCIATIONS WITH ONE YEAR AIRBORNE METAL EXPOSURES BY TISSUE TYPE

	Adjacent normal n= 277		In situ n= 184		Invasive n= 259	
	Beta	CI (95%)	Beta	CI (95%)	Beta	CI (95%)
Individual Metals						
Antimony	9.75	0.77, 19.2*	-0.04	-13.7, 13.3	11.6	-1.41, 24.4
Arsenic	7.50	0.11, 15.7*	0.80	-14.3, 15.0	20.3	7.92, 32.8**
Beryllium	8.40	1.33, 17.0*	7.42	-6.03, 21.2	12.9	0.03, 26.1*
Cadmium	8.84	0.17, 18.4*	-9.11	-23.6, 5.17	14.2	1.05, 27.5*
Chromium	6.33	-1.63, 14.3	3.36	-10.5, 17.5	13.4	0.62, 26.0*
Cobalt	7.75	-1.85, 16.9	-0.03	-16.7, 15.2	19.8	8.20, 32.1**
Lead	8.58	1.10, 15.2*	7.68	-7.58, 21.9	12.3	0.65, 23.0*
Manganese	11.0	2.62, 19.9*	3.73	-13.1, 19.8	16.2	4.15, 28.4**
Mercury	10.5	2.36, 18.7**	8.43	-8.13, 23.8	15.1	3.96, 26.8*
Nickel	7.57	0.71, 14.1*	5.49	-8.43, 18.7	10.6	-1.87, 21.2
Selenium	6.55	-1.85, 15.4	0.88	-16.4, 14.7	19.9	8.88, 31.7**
Combined Markers						
All Metals	16.1	5.44, 28.0**	5.02	-14.3, 24.4	29.9	11.9, 46.2**
IARC Grp. 1	12.9	3.16, 23.8**	2.92	-14.3, 19.6	24.4	7.69, 41.7**
IARC Grp. 2&3	15.9	4.90, 27.7**	6.24	-14.2, 24.8	29.0	12.5, 44.8**
PC1	27.7	9.87, 47.2**	12.5	-20.5, 43.1	47.8	19.1, 74.1**
PC2	0.82	-20.8, 20.6	-9.38	-45.6, 24.2	19.5	-7.60, 47.3
PC3	8.73	-10.4, 29.5	-24.3	-58.7, 9.69	-5.76	-35.6, 24.5

*Denotes statistical significance at $p < 0.05$; **Denotes significance at $p < 0.01$

Models adjusted for age, race, and BMI.

TABLE XIX.

Sat2 ASSOCIATIONS WITH ONE YEAR AIRBORNE METAL EXPOSURES BY TISSUE TYPE

	Adjacent normal n= 277		In situ n= 182		Invasive n= 256	
	Beta	CI (95%)	Beta	CI (95%)	Beta	CI (95%)
Individual Metals						
Antimony	2.72	-1.75, 7.17	9.87	2.84, 16.8*	0.89	-7.34, 9.66
Arsenic	-4.31	-9.48, 0.95	0.94	-7.20, 8.26	2.68	-4.92, 10.4
Beryllium	-2.77	-7.57, 1.42	4.62	-3.01, 12.0	3.79	-4.01, 11.6
Cadmium	-1.05	-6.72, 4.03	3.31	-3.61, 10.6	1.52	-6.45, 9.11
Chromium	-1.46	-6.32, 2.78	4.65	-3.83, 11.7	3.80	-4.21, 12.3
Cobalt	-5.61	-10.6, -0.42*	1.55	-5.53, 8.31	2.03	-4.44, 8.78
Lead	-3.24	-8.13, 1.10	9.35	2.50, 16.2**	3.84	-2.68, 11.5
Manganese	-5.43	-11.0, -0.82*	6.85	-0.59, 13.5	3.61	-3.30, 11.9
Mercury	-2.84	-8.32, 1.48	6.02	-0.54, 13.0	1.03	-5.54, 7.92
Nickel	-1.15	-5.29, 3.00	6.38	-0.87, 12.5	3.08	-3.79, 10.0
Selenium	-4.25	-9.57, 0.59	-0.49	-7.83, 6.84	2.55	-3.63, 9.05
Combined Markers						
All Metals	-5.22	-11.5, 0.48	9.13	-0.07, 18.2	5.31	-4.92, 16.7
IARC Grp. 1	-3.62	-9.09, 1.89	6.67	-2.93, 15.3	5.28	-5.66, 15.7
IARC Grp. 2&3	-5.62	-12.2, 0.73	9.98	1.13, 18.6*	4.36	-4.93, 14.8
PC1	-11.6	-22.2, -2.20*	11.2	-3.70, 26.4	8.64	-8.34, 27.0
PC2	-5.73	-17.6, 6.54	-6.67	-21.6, 12.4	-2.10	-17.3, 15.1
PC3	9.60	-1.96, 20.6	12.4	-1.69, 27.4	2.69	-14.8, 18.0

*Denotes statistical significance at $p < 0.05$; **Denotes significance at $p < 0.01$

Models adjusted for age, race, and BMI.

D. **Discussion**

Our results identified statistically significant relationships between chronic and short-term exposures to airborne heavy metals and breast tissue DNA methylation in numerous tissue components. Generally, chronic metal exposures to the combined all metal marker was positively associated with *GSTM2* methylation in invasive tumor tissue. Short-term metal exposures to the

combined all metals marker was inversely associated with *RASSF1* methylation in invasive tissue. Finally, short-term metal exposure to the combined all metals marker was positively associated with *TFF1* methylation in adjacent normal and invasive tissue. Qualitatively, most of the observed associations tended to be such that greater metal exposure was associated with less, not more, aberrant DNA methylation. The notable exception to this was chronic metal exposure to the combined all metals marker and increased aberrant methylation of *GSTM2*. These novel findings suggest ambient exposure to airborne heavy metals may affect breast cancer risk through dysregulation of DNA methylation in cancer-associated genes.

No epidemiologic studies have assessed the relationship between long-term exposure to airborne heavy metals and DNA methylation in the general population. However, studies of occupationally exposed individuals showed chronic exposure to metal fumes were associated with greater promoter (185), but lower gene body (186), methylation of *iNOS*. Additionally, welders with low-to-moderate occupational exposures showed greater *APC* methylation compared to unexposed controls (187). While these previous studies showed effects of metal exposure on DNA methylation measured in peripheral blood leukocytes, this study is the first to show these effects can also be identified in solid tissue.

We identified chronic exposure to antimony, cobalt, lead, manganese, the combined all metal exposure marker, and the combined IARC group 2 & 3 metal marker were positively associated with *GSTM2* promoter methylation in invasive breast tumor samples. *GSTM2* encodes Glutathione S-Transferase Mu 2 which functions in the detoxification of electrophilic compounds including carcinogens, therapeutic drugs, and environmental toxins (188). Previous studies have shown that promoter hypermethylation of *GSTM2* is associated with high-grade breast tumors (184). Importantly, the associations between airborne metal exposure and *GSTM2*

methylation were not found in adjacent normal or *in situ* tumor components, suggesting that hypermethylation of *GSTM2* may have a mechanistic role in the malignant conversion of breast tissue.

In addition to examining chronic effects of airborne metal exposure, we examined associations between short-term heavy metal exposures and DNA methylation. Previous research has shown that exposure to metal-rich particular matter (PM) less than 10 μm (PM_{10}) and less than 2.5 μm ($PM_{2.5}$) was associated with decreases in methylation of blood leukocytes of DNA repeats LINE-1, Alu, and SATα, all markers of global methylation (189, 190). Conversely, a repeated-measures study found that metal-rich $PM_{2.5}$ was associated with hypermethylation of LINE-1 (191). In studies of gene-specific methylation, exposure to metal-rich PM_{10} was associated with hypermethylation of *APC* while exposure to chromium, cadmium and lead were associated with hypomethylation of *RASSF1* (192).

Generally, we showed short-term exposure to the combined all metals marker was inversely associated with *RASSF1* methylation and positively associated with *TFF1* methylation. This study additionally replicated the finding that short-term chromium exposure was inversely associated with *RASSF1* methylation (192). Interestingly, all of the methylation signatures identified with short-term metal exposure confer reduced breast cancer risk. Hypermethylation of the tumor suppressor *RASSF1* are common events in breast carcinogenesis (180, 193, 194), while hypomethylation of *TFF1* is associated with increased breast cancer risk (181).

While this study identified novel associations between airborne heavy metal exposures and tissue DNA methylation, there are a number of limitations worth noting. First, our study was underpowered as only approximately one-third of the BCCC participants donated breast tissue for DNA methylation analysis. Therefore, our interpretations focused mainly on the combined

metal exposure markers. Additionally, due to the small sample size we were only able to adjust for common covariates such as age, race, and BMI. After adjustment for the smoking biomarker variable, we identified the same direction of effects, albeit with slightly weaker associations. Additionally, we did not correct for multiple comparisons increasing the likelihood of false-positive findings. We felt that this was acceptable as we selected the gene regions with *a priori* knowledge and our interpretations were focused on airborne metal exposures as a whole. Importantly, our finding for *GSTM2* is the only result that suggested increased aggressive breast cancer risk with greater airborne metal exposure. Although these findings would not have been statistically significant after correction for multiple testing, considering how the other identified associations suggested less aberrant methylation patterns, our findings for *GSTM2* justify follow-up investigation. Finally, most large-scale environmental epidemiology studies rely on residential addresses while acknowledging participants are not confined to their homes, thus resulting in some exposure misclassification. Generally, we would expect this misclassification to be non-differential resulting in an attenuation of the observed effect estimates.

E. **Conclusions**

This is the first study to identify associations between chronic and short-term airborne metal exposures with breast tissue DNA methylation. Importantly, these findings suggest heavy metal effects can be detected beyond methylation of blood leukocytes. We showed chronic exposure to airborne heavy metals were associated with hypermethylation of *GSTM2* in invasive breast tissue components. Further, we showed that short-term metal exposures were associated with lower methylation of *RASSF1* and greater methylation of *TFF1* in adjacent normal and invasive tissue. These results suggest that the duration of airborne heavy metal exposures results in competing breast cancer risk profiles. While chronic exposures are associated with markers of

increased risk of aggressive tumors, short-term exposures are associated with protective methylation signatures. Airborne exposures have the ability to affect large populations of individuals and findings from this and similar studies will have large public health implications. Additional research is needed to examine the burden through which airborne heavy metals affect breast cancer incidence rates.

IV. CANCER-ASSOCIATED GENE METHYLATION AND BREAST CANCER CHARACTERISTICS

A. Background

Breast cancer is the most commonly diagnosed cancer in women worldwide (128). It is heterogeneous by nature and is commonly classified based on tumor receptor status. Luminal A and B (both ER/PR-positive) tumors are the most common subtypes and tend to be less aggressive with good prognoses (129-131). Other breast cancer subtypes, such as basal-like and triple-negative (both ER/PR-negative), are less common, more aggressive, and have worse prognoses (131, 132). Racial and socioeconomic disparities exist in subtype incidence rates, with younger women and African-American women more likely to develop ER/PR-negative tumors (133). While differential risk factor distributions across age and ethnicities explain some of these disparities (134), underlying epigenomic differences, driven by environmental determinants, are also likely a contributing factor.

Emerging evidence suggests heavy metals dysregulate biological processes related to DNA methylation resulting in aberrant methylation patterns (80-83, 86, 88, 187, 192). DNA methylation is a mechanism that influences transcription, resulting in cellular differentiation and therefore is a potential mechanism for carcinogenesis (195). Aberrant DNA methylation is a hallmark of cancer (196); in comparison studies of DNA methylation patterns in tumor and adjacent normal tissue, tumor samples exhibit reductions in global methylation (197-199), and increased methylation at CpG islands in the promotor regions of tumor suppressor genes (200-203). Additionally these epigenome-wide patterns are distinct between ER/PR-positive and ER/PR-negative tumors (204-207).

Our group has previously identified associations between exposures to heavy metals with breast cancer characteristics. More specifically, we showed an elevated prevalence of ER/PR-negative breast cancer among women who lived in areas characterized by higher ambient concentrations of antimony, arsenic, cobalt, manganese, and selenium. We additionally showed associations between chronic exposures to ambient heavy metal concentrations with aberrant patterns of DNA methylation, particularly in the promoter region of *GSTM2*. The purpose of this study is to examine the relationship between patterns of DNA methylation and breast tumor characteristics. We hypothesize aberrant patterns of DNA methylation will be associated with ER/PR-negative and high-grade tumors.

B. **Methods**

1. **Study Population**

The Breast Cancer Care in Chicago (BCCC) study is a population-based sample of women who were diagnosed with breast cancer at one of the 56 Chicago-area hospitals. Women who were eligible for enrollment were between the ages of 30 and 79; self-identified as non-Hispanic (nH) White, nH Black, or Hispanic; resided in Chicago; had a first primary *in situ* or invasive breast cancer diagnosed between 2005 and 2008; and gave written, informed consent to participate in the study. Overall, 989 women were enrolled and completed a 90-minute interview on social, demographic, and healthcare-related factors. Of these, 812 (82%) consented to allow samples of diagnostic tissue to be obtained by research staff, of whom samples were obtained from 351 patients (35%); 723 (73%) participants consented to a blood draw, from which DNA was extracted and stored on 668 patients (68%). A total of 260 (27%) patients had both blood and tissue collected. The study population has been described in detail elsewhere (143, 146). Participants were dropped from the analysis if they were missing information DNA methylation,

breast cancer characteristics or any of the *a priori* covariate data. After this consideration, the sample sizes were 337 for tumor receptor status and 304 for tumor grade. The protocol for this study was approved by the University of Illinois at Chicago Institutional Review Board.

2. DNA Methylation Assessment

a. Source of Breast Tissue Component Samples

For the subset of patients who donated tumor samples, hematoxylin and eosin (H&E) stained slides from FFPE tumor blocks were examined to determine representative component areas of invasive, *in situ*, and histologically and morphologically normal-appearing breast tissue adjacent to the tumor (adjacent normal). For lumpectomies, we selected adjacent breast tissue from the same block as the tumor. However, when available we used a separate block containing breast tissue and no tumor as the non-malignant, adjacent sample. We cut tissue core samples precisely from the selected area using a semi-automated tissue arrayer (Beecher Instruments, Inc.). Because the tissue was fixed and sealed by paraffin, cells from the invasive tissue could not become dislodged and contaminate the *in situ* or adjacent tissue or vice versa (176).

b. Choice of DNA Regions for Analysis

We chose a diverse set of five genes and a DNA repeat to assay for DNA methylation in invasive, *in situ*, and adjacent normal tissue components. Information on the tested genomic regions is shown in Table XX. The DNA regions examined overlapped or were near regions previously reported to be aberrantly hypermethylated in breast cancer vs non-cancerous breast tissue, namely *BRCA1 (177, 178)*, *EGFR* (179), and *RASSF1A* (180); or aberrantly hypomethylated in breast cancer vs normal breast namely, *TFF1* (181) and DNA repeat, Satellite 2 (Sat2) (182, 183). We also examined a gene region from *GSTM2* found to display hypermethylation in more aggressive breast cancers (184). Based on prior literature, we defined

TABLE XX.

LIST OF STUDIED DNA REGIONS AND NUMBER OF CPGS COVERED

Gene	Test Region	Test Region Coordinates (hg19)	Distance from TSS (bp)[a]	CGI[b]	CpGs[c]
BRCA1	Exon 1(extended promoter)	Chr17: 41277463-41277365	+37 to +135	No	11
EGFR	Intron 1 (extended promoter)	Chr7: 55088080-55088104	+1355 to +1379	Yes	4
GSTM2	Promoter	Chr1: 110210582-110210641	-62 to -3	Yes	8
RASSF1	Exon 1 (extended promoter)	Chr3: 50378294-50378232	+74 to +134	Yes	9
TFF1	Promoter	Chr21: 43786664-43786628	-20 to +16	No	5
Sat2	N.A.	DNA Repeat	N.A.		2

[a]TSS, transcription start site

[b]CGI, CpG island overlapping the test region

[c]The number of CpG dinucleotide pairs in the test region

aberrant methylation as increasing methylation of *BRCA1*, *EGFR*, *GSTM2*, and *RASSF1* and decreasing methylation of *TFF1* and Sat2.

c. **DNA Methylation Analysis**

Dissolution of paraffin was accomplished by the addition of 1 mL of clearing agent (Histochoice) and incubation at 65 °C for 30 min. Samples were digested by the addition of 100 μL of digestion buffer consisting of 10 μL 10X Target Retrieval Solution high pH (DAKO, Glostrup, Denmark), 75 μL of ATL Buffer (Qiagen), and 15 μL of proteinase K (Qiagen) and incubation at 65 °C overnight. They were then vortexed and checked for complete digestion. The sample volume was brought up to ~100 μL, and 20 μL of each sample was treated with bisulfite and purified using the Zymo EZ-96 DNA Methylation-Direct™ Kit, with a 15-minute denaturation step at 98 °C followed by a 3.5-h conversion at 64 °C, an additional 15-minute denaturation at 98 °C and a 60-minute incubation at 64 °C. DNA was eluted in 40 μL of elution buffer. Then, PCR was performed with 0.2 μM of each primer, one of which was biotinylated,

and the final PCR product was purified (Streptavidin Sepharose HP, Amersham Biosciences, Uppsala, Sweden), washed, alkaline-denatured, and rewashed (Pyrosequencing Vacuum Prep Tool, Qiagen). Then, the pyrosequencing primer (0.5 μM) was annealed to the purified single-stranded PCR product, and 10 μL of the PCR products were sequenced by Pyrosequencing PSQ96 HS System (Biotage AB) following the manufacturer's instructions. The methylation status of each locus was analyzed individually as a T/C SNP using Pyromark Q96 software (Qiagen, Germantown, Maryland).

3. **Outcome Assessment**

Breast cancer characteristics of interest were hormone receptor status, as defined by ER/PR status, and tumor grade. Estrogen and progesterone receptor status was determined by immunohistochemical (IHC) analysis on women who consented to retrieval of clinical breast tissue samples. For those who consented to tissue donation, copies of pathology reports and hematoxylin and eosin (H&E) slides were requested from the diagnosing institution. A single pathologist selected FFPE tumor blocks which generally represented the tumor. Recuts (4 μm each) of the selected tumor blocks were created for additional H&E staining. The recuts were examined to identify invasive components of the tumor. Cores of invasive tissue components were selected and tissue microarrays produced. Samples were stained using a monoclonal antibody for nuclear estrogen and progesterone receptor status (manufacturer: Ventana, product catalogue number: 790-4324 and 790-2223 for ER and PR antibodies, respectively). Stains were optimized on invasive breast tumor tissue before use in this study. ER and PR status were interpreted separately and given an H-score, which is the product of staining intensity (0, 1+, 2+, 3+) and the proportion of cells with the given intensity (possible range of H-score values: 0-300). A tumor sample was determined to be ER/PR-negative if it had an H-score < 10 for both receptor

stains. For women who did not donate breast tissue, receptor status was determined by medical record abstraction. For patients with both IHC and medical record information, concordance was very high (<1% of patients were discordant on ER/PR-negative status).

Breast tumor grade was collected via medical record abstraction. Grade was determined by a trained pathologist at the diagnosing institution. Tumor grade was defined as either well differentiated (G1, low grade), moderately differentiated (G2, intermediate grade), or poorly differentiated (G3, high-grade).

4. **Covariate Information**

Data on breast cancer risk factors (socioeconomic/demographic and reproductive factors) were collected from the 90-minute in-person interview conducted upon enrollment into the BCCC study. Information on age, race/ethnicity, BMI, comorbidities, education, income, parity, age at first and last birth, breastfeeding duration, duration of oral contraception use, age at menopause, duration of hormone replacement use, and family history of breast cancer were collected (143, 146).

Measures of neighborhood socioeconomic status (SES) were computed based on the 2000 census. Census tract affluence was measured by combining percentage of families with income of $75,000 or more, percentage of adults with college education or more, and percentage of civilian labor force in professional and managerial occupations. Census tract disadvantage was measured by combining percentage of families with incomes below the poverty line, percentage of families receiving public assistance, percentage of persons unemployed, and percentage of female-headed households with children. Both census tract variables were defined by creating an equally weighted sum of the relevant variables, then standardizing that sum to have a mean of zero and standard deviation of one (146).

A biomarker for smoking status was measured retrospectively on a subsample of the BCCC participants using DNA methylation derived from peripheral blood monocyte samples at three pre-identified loci (Illumina IDs: cg06644428 (2q37), cg21566642 (2q37), cg06126421 (6p21.33)). Previous studies have identified strong inverse linear associations of these markers with smoking history and have tested their utility in predicting past tobacco exposure (144, 145).

5. **Statistical Analysis**

The prevalences of ER/PR-negative breast cancer and high-grade disease were separately examined in bivariate analyses using chi-squared values to test for differences in the distribution of covariates across these outcomes. Mean (and standard deviation) and median (and interquartile range) percent methylation values for each tested CpG site were computed to examine within-assay variability. We averaged percent DNA methylation across the individual genomic regions to compute a single methylation measurement for each assayed gene region. We conducted Wilcoxon rank sum tests for differences between median percent methylation values across tumor receptor status (ER/PR-negative vs ER/PR-positive) and grade (low/intermediate vs high-grade) of the six genomic regions by tissue component. Using a non-parametric test for trend across ordered groups, an extension from the Wilcoxon rank sum test developed by Cuzick et al. (208) (Stata command: nptrend), we examined differences in percent methylation values across the three tissue components within gene regions, and by tumor receptor status and grade. We explored heterogeneity of the trends across ER/PR status and grade using an interaction term derived from linear regression.

In covariate adjusted analyses, we stratified by tissue components and employed logistic regression to estimate odds ratios (ORs) and 95% confidence intervals (95% CIs) with respect to prevalences of ER/PR-negative breast cancer and high-grade disease. *A priori* confounders were

age, BMI, socioeconomic status factors (race, education, income, and census tract affluence and disadvantage), and reproductive factors (age at first birth and parity). In order to model both parity and age at first birth together, we employed a method which combined information into one variable which generally represented reproductive factors (RPF) by assigning nulliparous women a value corresponding to an age at first birth equal to 40 (146). We additionally examined potential confounding by the biomarker of chronic exposure to tobacco smoke. To construct the smoking variable, we combined the percent methylation values from each of the three CpG sites into one marker using principal component analysis. We used the first eigenvector to represent estimated cumulative exposure to environmental tobacco smoke, with lower values representing higher likelihood of prolonged exposure. All analyses were conducted using Stata version 12 (Stata Corp., College Station, TX) and statistical significance was determined at a two-sided $p \leq 0.05$.

C. **Results**

The median age of the participants at diagnosis was 56 years. Overall, the BCCC participants with information on tumor DNA methylation were approximately thirty percent nH White and Hispanic, and forty percent nH Black. Generally, the women were overweight/obese (76%), educated (74% completed high school), and made greater than $25,000 (66%). Among the 337 women with ER/PR status, 86 (26%) were diagnosed with ER/PR-negative; among the 304 women with information on tumor grade, 132 (43%) were diagnosed with high-grade (G3) tumors.

Table XXI presents the covariate distributions by tumor receptor status and grade. Briefly, younger age at diagnosis was marginally associated with high-grade tumors, but not ER/PR status. Race was associated with tumor receptor status but not tumor grade, with nH

Black women being more likely to be diagnosed with ER/PR-negative tumors. Census tract disadvantage was associated with ER/PR status; women residing in census tracts with greater disadvantage were more likely to be diagnosed with ER/PR-negative tumors. Finally, women with an earlier age at first birth were more likely to be diagnosed with ER/PR-negative tumors and marginally more likely to be diagnosed with higher grade tumors. Estrogen and progesterone receptor-negative tumors were marginally less likely to be diagnosed in nulliparous women than in parous women (Table XXI).

1. **Trends in Aberrant DNA Methylation by Tissue Component, Overall and by Tumor Receptor Status**

Table XXII and presents the distributions of percent methylation values of the six selected genomic regions and corresponding trends in aberrant methylation by tissue component (adjacent normal, *in situ* and invasive tissue). Aberrant methylation increased between adjacent normal, *in situ* and invasive components with significant positive trends in methylation for *BRCA1* ($p= 0.03$), *EGFR* ($p= 0.02$), *GSTM2* ($p < 0.001$), and *RASSF1* ($p < 0.001$), and significant negative trends in methylation for *TFF1* ($p < 0.001$) and Sat2 ($p < 0.001$). When restricting analyses to ER/PR positive tumors, we observed significant positive trends in methylation for *EGFR* ($p < 0.001$), *GSTM2* ($p < 0.001$), and *RASSF1* ($p < 0.001$), and significant negative trends in methylation again for *TFF1* ($p < 0.001$) and Sat2 ($p < 0.001$). We did not identify a significant trend for *BRCA1*. When restricting analyses to ER/PR negative tumors, we observed significant positive trends in methylation for *BRCA1* ($p = 0.02$), *GSTM2* ($p < 0.001$), and *RASSF1* ($p < 0.001$), and significant negative trends in methylation again for *TFF1* ($p = 0.04$) and Sat2 ($p = 0.001$). We did not identify a significant trend for *EGFR*.

TABLE XXI.

BREAST CANCER CARE IN CHICAGO SELECTED SAMPLE DISTRIBUTIONS WITH TUMOR RECEPTOR STATUS AND TUMOR GRADE

	Receptor status (n= 337)			Tumor grade (n= 304)		
	ER/PR + N= 251 (%)	ER/PR – N= 86 (%)	P-value [a]	Low/ Intermediate N= 172 (%)	High N= 132 (%)	P-value [b]
Age at diagnosis			0.50			0.06
18-49	67 (27)	27 (31)		39 (23)	45 (34)	
50-59	79 (31)	29 (34)		56 (33)	42 (32)	
60-79	105 (42)	30 (35)		77 (45)	45 (34)	
Race/ethnicity			0.01			0.49
nH White	87 (35)	20 (23)		59 (34)	37 (28)	
nH Black	90 (36)	48 (56)		71 (41)	58 (44)	
Hispanic	74 (29)	18 (21)		42 (24)	37 (28)	
Education			0.12			0.56
Less than HS	59 (24)	27 (31)		39 (23)	37 (28)	
HS diploma	61 (24)	25 (29)		43 (25)	31 (23)	
Greater than HS	131 (52)	34 (40)		90 (52)	64 (48)	
Income			0.43			0.43
< $25,000	83 (34)	32 (38)		51 (30)	49 (38)	
$25,000-$87,499	112 (46)	41 (48)		83 (50)	59 (45)	
≥ $85,000	50 (20)	12 (14)		33 (20)	22 (17)	
CT affluence			0.83			0.81
< 1 SD	21 (8)	9 (11)		13 (8)	10 (8)	
± 1 SD	206 (82)	68 (80)		140 (82)	111 (84)	
> 1 SD	24 (10)	8 (9)		18 (10)	11 (8)	
CT disadvantage			0.01			0.26
< 1 SD	37 (15)	3 (4)		21 (12)	13 (10)	
± 1 SD	168 (67)	58 (68)		119 (70)	85 (64)	
> 1 SD	46 (18)	24 (28)		31 (18)	34 (26)	
BMI			0.10			0.81
≤ 25	58 (23)	21 (24)		42 (24)	28 (21)	
25-30	91 (37)	21 (24)		57 (33)	44 (34)	
> 30	100 (40)	44 (51)		73 (42)	59 (45)	
Live births			0.09			0.53
0	46 (18)	6 (7)		32 (19)	17 (13)	
1	40 (16)	15 (17)		28 (16)	20 (15)	
2	61 (24)	23 (27)		41 (24)	37 (28)	
3+	104 (41)	42 (49)		71 (41)	58 (44)	

TABLE XXI (continued).

BREAST CANCER CARE IN CHICAGO SELECTED SAMPLE DISTRIBUTIONS WITH TUMOR RECEPTOR STATUS AND TUMOR GRADE

	Receptor status (n= 337)			Tumor grade (n= 304)		
	ER/PR + N= 251 (%)	ER/PR – N= 86 (%)	P-value [a]	Low/ Intermediate N= 172 (%)	High N= 132 (%)	P-value [b]
Age at first birth			< 0.001			0.07
< 20	65 (26)	39 (45)		44 (26)	49 (37)	
20-29	112 (45)	37 (43)		78 (45)	55 (42)	
30+	74 (29)	10 (12)		50 (29)	28 (21)	

[a] P-value determining the differences in covariate distribution between tumor receptor status.

[b] P-value determining the differences in covariate distribution between tumor grade.

TABLE XXII.

ASSOCIATIONS BETWEEN DNA METHYLATION AND TUMOR RECEPTOR STATUS BY TISSUE COMPONENT

	Overall		ER/PR Positive		ER/PR Negative		
Gene	N	Median [IQR]	N	Median [IQR]	N	Median [IQR]	p-value [a]
BRCA1							
Normal	219	0.4 [0.0-1.3]	163	0.3 [0.0-1.4]	56	0.4 [0.0-1.0]	0.73
In Situ	151	0.8 [0.3-1.6]	123	0.8 [0.3-1.6]	28	0.7 [0.3-1.0]	0.32
Invasive	239	0.6 [0.0-1.4]	175	0.6 [0.0-1.3]	64	0.9 [0.2-1.6]	0.06
p-trend[b]		0.03		0.24		0.02	
EGFR							
Normal	272	6.2 [4.3-8.4]	207	6.1 [4.3-8.6]	65	6.2 [4.3-7.9]	0.60
In Situ	172	16 [8.3-29]	140	18 [11-31]	32	8.7 [5.1-17]	< 0.001
Invasive	264	20 [8.1-34]	195	25 [14-36]	69	6.1 [4.3-17]	< 0.001
p-trend[b]		0.02		< 0.001		0.17	
GSTM2							
Normal	213	0.7 [0.0-3.3]	159	0.7 [0.0-2.8]	54	0.8 [0.0-4.2]	0.30
In Situ	148	2.0 [0.4-16]	119	1.5 [0.3-6.0]	29	14 [2.5-41]	0.002
Invasive	247	5.0 [1.1-35]	183	2.6 [0.7-19]	64	35 [11-54]	< 0.001
p-trend[b]		< 0.001		< 0.001		< 0.001	
RASSF1							
Normal	231	6.3 [3.2-16]	171	7.0 [3.5-17]	60	5.1 [2.7-13]	0.11
In Situ	155	49 [24-71]	128	53 [28-72]	27	31 [7.2-46]	0.005
Invasive	251	45 [17-64]	184	50 [28-68]	67	26 [4.6-52]	< 0.001
p-trend[b]		< 0.001		< 0.001		< 0.001	
TFF1							
Normal	254	73 [59-81]	191	70 [57-79]	63	78 [66-84]	0.001
In Situ	166	39 [25-60]	134	35 [23-53]	32	65 [46-79]	< 0.001
Invasive	258	41 [29-61]	193	36 [25-49]	65	68 [46-85]	< 0.001
p-trend[b]		< 0.001		< 0.001		0.04	
Sat2							
Normal	255	59 [54-64]	187	59 [55-64]	68	59 [52-64]	0.40
In Situ	164	52 [44-58]	133	53 [45-58]	31	49 [44-58]	0.45
Invasive	256	53 [42-59]	189	53 [41-59]	67	53 [45-59]	0.72
p-trend[b]		< 0.001		< 0.001		0.001	

[a] Wilcoxon rank sum test for differences

[b] Derived from non-parametric test for trend across ordered groups

The trend in increased aberrant methylation was stronger for ER/PR positive (vs ER/PR negative) tumors for *EGFR* ($p < 0.01$), *RASSF1* ($p < 0.01$) and *TFF1* ($p < 0.01$), whereas the trend in increased aberrant methylation was stronger for ER/PR negative (vs ER/PR positive) tumors for *BRCA1* ($p < 0.05$) and *GSTM2* ($p < 0.01$). There were no apparent differences in trends by subtype for Sat2 ($p > 0.05$). Figure 2 depicts the distributions of percent methylation values for the six gene regions, stratified by tissue component and tumor receptor group.

2. **Trends in Aberrant DNA Methylation by Tissue Component, Overall and by Tumor Grade**

Table XXIII presents the distributions of percent methylation values of the six selected genomic regions overall and by tumor grade. We again showed significant positive trends in percent methylation between adjacent normal, *in situ* and invasive components for *BRCA1* ($p= 0.04$), *EGFR* ($p < 0.001$), *GSTM2* ($p < 0.001$), and *RASSF1* ($p < 0.001$), and significant negative trends for *TFF1* ($p < 0.001$) and Sat2 ($p < 0.001$). When restricting analyses to high-grade tumors, we again observed significant positive trends in methylation for *BRCA1* ($p= 0.04$), *EGFR* ($p < 0.001$), *GSTM2* ($p < 0.001$), and *RASSF1* ($p < 0.001$) and significant negative trends in methylation again for *TFF1* ($p < 0.001$) and Sat2 ($p < 0.001$). When restricting analyses to low/moderate grade tumors, we observed significant positive trends in methylation for *EGFR* ($p < 0.001$), *GSTM2* ($p < 0.001$), and *RASSF1* ($p < 0.001$), and significant negative trends in methylation again for *TFF1* ($p < 0.001$) and Sat2 ($p < 0.001$). We did not identify any trends of DNA methylation across tissue components for *BRCA1* in low and intermediate grade tumors ($p= 0.30$).

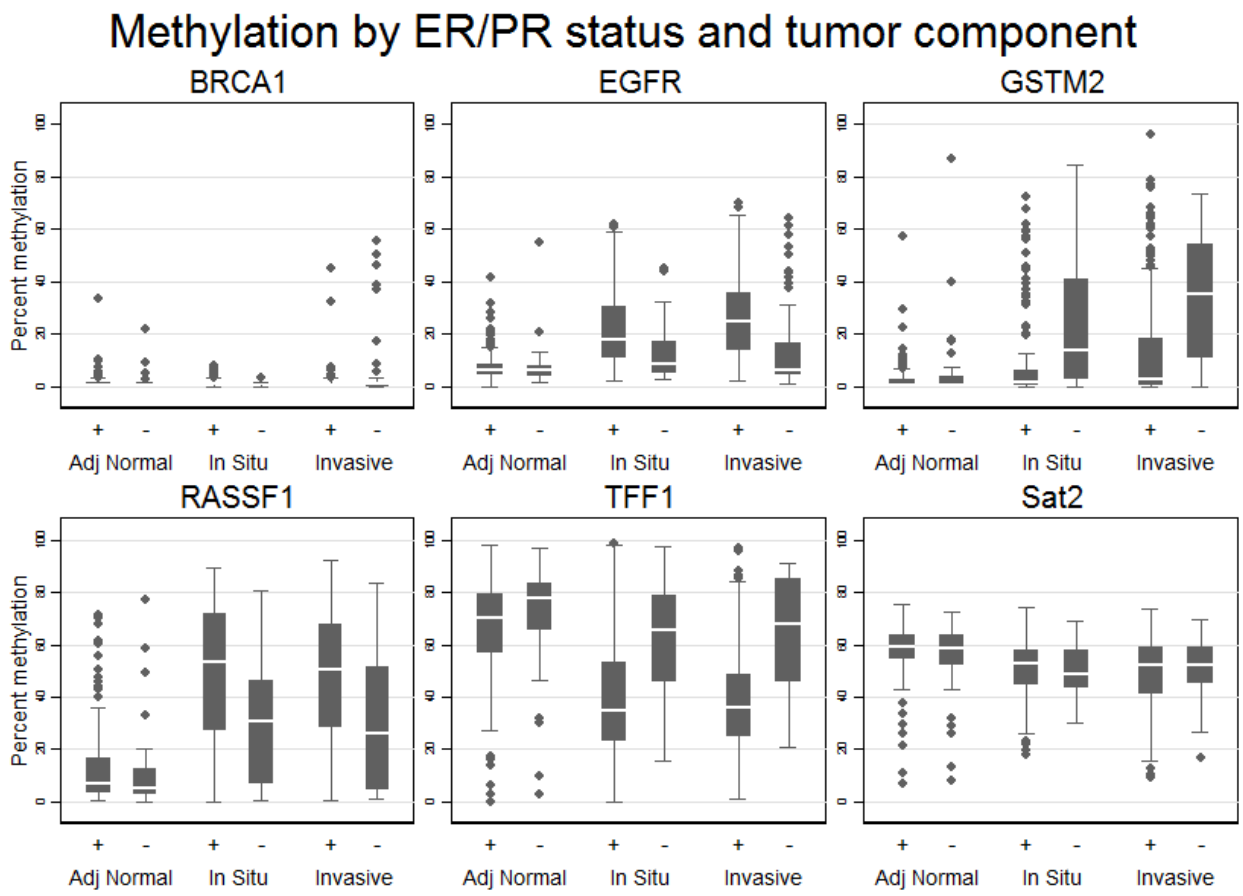

Figure 2. Distributions of gene methylation by ER/PR positive (+) and negative (-) status, stratified by breast tissue type.

With respect to heterogeneity in aberrant DNA methylation by tumor grade, the trend in increased aberrant methylation was stronger for high-grade (vs low/moderate grade) tumors for *BRCA1* ($p < 0.05$) and *GSTM2* ($p < 0.01$). We also identified stronger decreases in aberrant DNA methylation for low/intermediate (vs high-grade) tumors for *TFF1* ($p < 0.05$). There were no other apparent differences for trends in aberrant *EGFR* ($p > 0.05$), *RASSF1* ($p > 0.05$), and Sat2.

TABLE XXIII.

ASSOCIATIONS BETWEEN DNA METHYLATION AND TUMOR GRADE BY TISSUE COMPONENT

	Overall		Low/Intermediate Grade		High-Grade		
Gene	N	Median [IQR]	N	Median [IQR]	N	Median [IQR]	p-value [a]
BRCA1							
Normal	200	0.3 [0.0-1.1]	124	0.2 [0.0-1.4]	76	0.4 [0.0-1.0]	0.67
In Situ	141	0.8 [0.3-1.5]	91	0.8 [0.3-1.7]	50	0.7 [0.3-1.3]	0.47
Invasive	213	0.6 [0.0-1.3]	125	0.4 [0.0-1.3]	88	0.6 [0.1-1.4]	0.12
p-trend[b]		0.04		0.30		0.04	
EGFR							
Normal	245	6.2 [4.3-8.6]	149	6.4 [4.2-9.1]	96	6.1 [4.4-8.1]	0.70
In Situ	158	17 [8.5-29]	98	18 [9.0-31]	60	16 [7.8-28]	0.39
Invasive	235	20 [8.1-33]	136	22 [12-34]	99	18 [6.1-31]	0.15
p-trend[b]		< 0.001		< 0.001		< 0.001	
GSTM2							
Normal	193	0.7 [0.0-3.3]	117	0.6 [0.0-2.9]	76	0.9 [0.0-3.7]	0.51
In Situ	136	2.0 [0.5-19]	88	1.4 [0.3-6.2]	48	6.2 [0.7-33]	0.02
Invasive	219	6.2 [1.1-36]	129	2.5 [0.5-28]	90	23 [3.3-45]	< 0.001
p-trend[b]		< 0.001		< 0.001		< 0.001	
RASSF1							
Normal	210	6.8 [3.4-16]	128	7.5 [3.9-18]	82	6.1 [2.7-12]	0.05
In Situ	142	49 [24-70]	91	53 [21-73]	51	40 [24-64]	0.31
Invasive	223	45 [18-64]	133	45 [25-61]	90	45 [8.0-70]	0.90
p-trend[b]		< 0.001		< 0.001		< 0.001	
TFF1							
Normal	229	73 [59-81]	140	70 [57-80]	89	75 [63-83]	0.04
In Situ	154	39 [25-61]	95	36 [23-51]	59	55 [32-70]	< 0.001
Invasive	229	41 [31-61]	135	39 [28-53]	94	49 [33-80]	< 0.001
p-trend[b]		< 0.001		< 0.001		< 0.001	
Sat2							
Normal	229	59 [55-64]	139	60 [55-65]	90	58 [54-63]	0.12
In Situ	151	43 [44-58]	95	53 [45-58]	56	50 [44-56]	0.16
Invasive	228	52 [42-59]	133	54 [43-60]	95	51 [37-57]	0.04
p-trend[b]		< 0.001		< 0.001		< 0.001	

[a] Wilcoxon rank sum test for differences

[b] Derived from non-parametric test for trend across ordered groups

Figure 3 depicts the distributions of percent methylation values for the six gene regions, stratified by tissue component and tumor grade.

3. Adjusted Associations between Aberrant Methylation and Tumor Aggression Markers

Table XXIV shows the adjusted associations between a 10-percentage point change in aberrant gene methylation and ER/PR subtype. Based on prior literature, we defined aberrant methylation as increasing methylation of *BRCA1*, *EGFR*, *GSTM2*, and *RASSF1* and decreasing methylation of *TFF1* and Sat2. Models were adjusted *a priori* for age, education, income, race, BMI, reproductive factors, and census tract advantage and disadvantage. For invasive tissue components, hypermethylation of *BRCA1* (OR= 1.44, 95% CI: 1.25, 1.66) and *GSTM2* (OR= 1.72, 95% CI: 1.09, 2.70) were associated with increased prevalence of ER/PR-negative subtype, whereas hypermethylation of *EGFR* (OR= 0.58, 95% CI: 0.46, 0.75) and *RASSF1* (OR= 0.74, 95% CI: 0.65, 0.85) and hypomethylation of *TFF1* (OR= 0.95, 95% CI: 0.93, 0.96) were each associated with increased prevalence of ER/PR-positive subtype. For *in situ* tissue components, hypermethylation of *GSTM2* (OR= 1.48, 95% CI: 1.14, 1.93) was again associated with ER/PR-negative subtype, and hypermethylation of *EGFR* (OR= 0.55, 95% CI: 0.36, 0.86) and *RASSF1* (OR= 0.72, 95% CI: 0.57, 0.90) and hypomethylation of *TFF1* (OR= 0.95, 95% CI: 0.93, 0.97) were each once again associated with ER/PR-positive subtype. For adjacent normal tissue components, hypermethylation of *GSTM2* (OR= 1.67, 95% CI: 1.06, 2.64) was again associated with ER/PR-negative subtype and was the only gene region for which aberrant methylation was associated with subtype; Sat2 hypomethylation in adjacent normal tissue was marginally associated with increased prevalence of ER/PR-negative subtype (OR= 1.29, 95% CI: 0.97, 1.71).

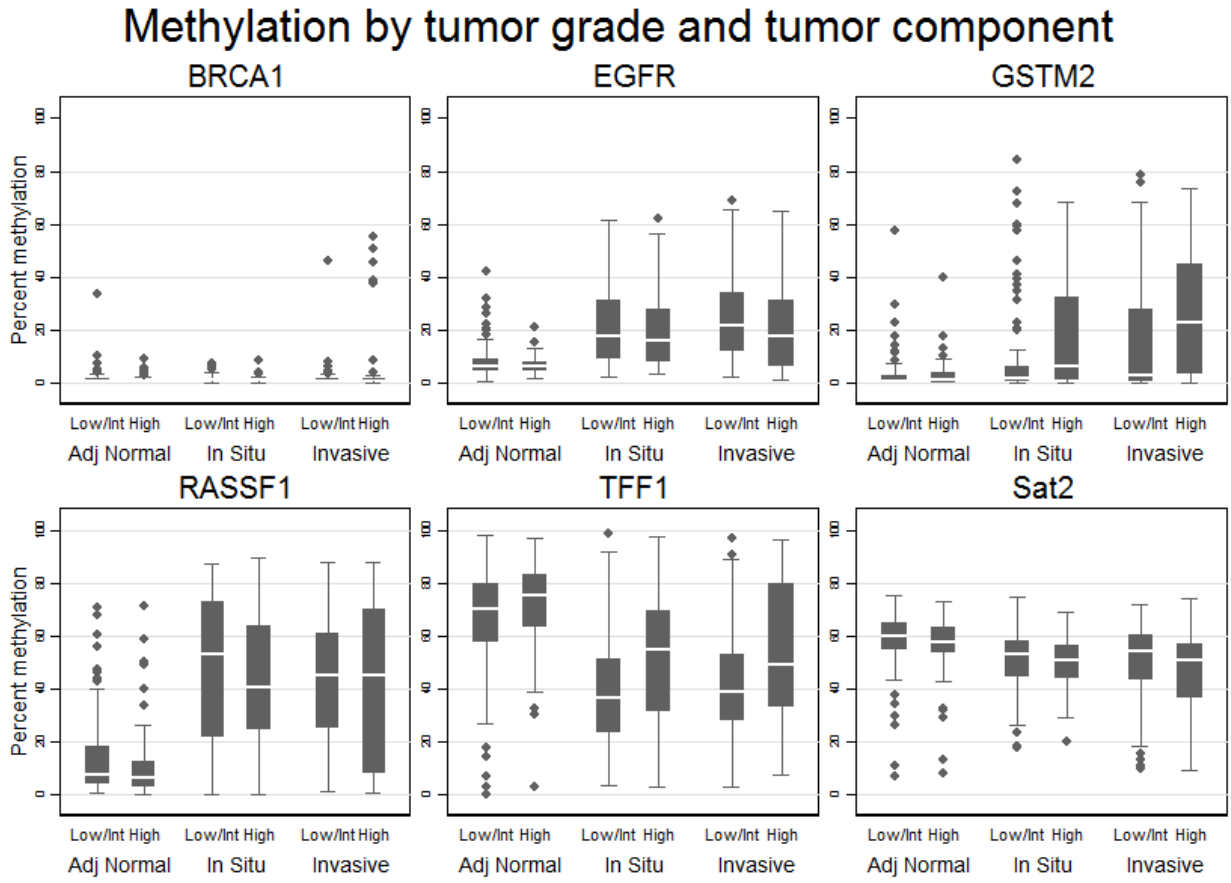

Figure 3. Distributions of gene methylation by tumor grade, stratified by breast tissue type.

TABLE XXIV.

ADJUSTED ASSOCIATIONS BETWEEN 10 PERCENTAGE POINT CHANGE IN ABERRANT [a] GENE METHTLATION AND ER/PR NEGATIVE TUMORS

	Breast Cancer Tissue Component								
	Adjacent Normal			In Situ			Invasive		
	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value
BRCA1	1.54	0.58-4.15	0.39	0.11	0.00-12.84	0.36	1.72	1.09-2.70	0.02
EGFR	1.09	0.63-1.89	0.76	0.55	0.36-0.86	0.01	0.58	0.46-0.75	< 0.001
GSTM2	1.67	1.06-2.64	0.03	1.48	1.14-1.93	0.004	1.44	1.25-1.66	< 0.001
RASSF1	0.84	0.66-1.07	0.17	0.72	0.57-0.90	0.005	0.74	0.65-0.85	< 0.001
TFF1	0.98	0.96-0.99	0.01	0.95	0.93-0.97	< 0.001	0.95	0.93-0.96	< 0.001
Sat2	1.29	0.97-1.71	0.08	1.11	0.72-1.72	0.64	0.84	0.66-1.05	0.13

Models adjusted for age, education, income, race, BMI, reproductive factors, and census tract advantage and disadvantage.

[a] Aberrant methylation is defined as increasing methylation of *BRCA1*, *EGFR*, *RASSF1*, and *GSTM2* and decreasing methylation of *TFF1* and Sat2.

Table XXV shows the adjusted associations between a 10-percentage point change in aberrant gene methylation and tumor grade. Models were again adjusted *a priori* for age, education, income, race, BMI, reproductive factors, and census tract advantage and disadvantage. For invasive tissue components, hypermethylation of *GSTM2* was associated with increased prevalence of high-grade tumors (OR= 1.24, 95% CI: 1.09, 1.42), whereas hypomethylation of *TFF1* was associated with increased prevalence of low/intermediate grade tumors (OR= 0.98, 95% CI: 0.96, 0.99). For *in situ* tissue components, only hypomethylation of *TFF1* was again associated with low/intermediate tumor grade (OR= 0.98, 95% CI: 0.96, 0.99). No associations between aberrant methylation and tumor grade were observed in adjacent normal tissue components.

D. **Discussion**

Our results indicate significant relationships between aberrant DNA methylation and breast tumor characteristics. After adjustment for age, race, socioeconomic and reproductive factors, hypermethylation of *BRCA1* and *GSTM2* were associated with prevalent ER/PR-negative tumors. We additionally showed hypermethylation of *GSTM2* was associated with prevalent high-grade tumors. Aberrant methylation of the other gene regions, namely *EGFR*, *RASSF1*, *TFF1* and Sat2 were generally associated with less aggressive breast cancer characteristics. These findings suggest underlying biological differences affect aggressive tumor development.

We found hypermethylation of the extended promoter region of *BRCA1* was associated with prevalent ER/PR-negative breast cancer. *BRCA1* encodes a nuclear protein that acts as a tumor suppressor by maintaining genomic stability (209). *BRCA1* promoter methylation is common in sporadic breast cancer and ovarian tumors (177, 178) and studies have demonstrated dysfunction of the *BRCA1* pathway in sporadic ER/PR-negative tumors (210-212). High-grade

TABLE XXV.

ADJUSTED ASSOCIATIONS BETWEEN 10 PERCENTAGE POINT CHANGE IN ABERRANT [a] GENE METHTLATION AND HIGH-GRADE TUMORS

	Breast Cancer Tissue Component								
	Adjacent Normal			In Situ			Invasive		
	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value
BRCA1	0.64	0.14-2.96	0.56	0.38	0.02-6.52	0.50	1.43	0.89-2.30	0.14
EGFR	0.67	0.34-1.30	0.24	0.96	0.76-1.23	0.76	0.98	0.82-1.18	0.86
GSTM2	1.20	0.76-1.91	0.43	1.16	0.95-1.42	0.15	1.24	1.09-1.42	0.002
RASSF1	0.85	0.68-1.08	0.19	0.94	0.81-1.08	0.37	0.98	0.88-1.09	0.69
TFF1	0.99	0.97-1.00	0.12	0.98	0.96-0.99	0.01	0.98	0.96-0.99	< 0.001
Sat2	1.15	0.87-1.52	0.32	1.18	0.84-1.66	0.34	1.16	0.95-1.41	0.16

Models adjusted for age, education, income, race, BMI, reproductive factors, and census tract advantage and disadvantage.

[a] Aberrant methylation is defined as increasing methylation of *BRCA1*, *EGFR*, *RASSF1*, and *GSTM2* and decreasing methylation of *TFF1* and Sat2.

and ER/PR-negative tumors also show lower levels of *BRCA1* protein expression (213). Additionally, the promoter region of *BRCA1* is methylated in over 60% of breast tumors exhibiting a basal-like phenotype (178, 210, 214). The findings from our study comport with the previous literature that has already examined patterns of *BRCA1* promoter methylation in ER/PR-negative tumor samples.

We also showed a relationship between aberrant hypermethylation of the *GSTM2* promoter region and aggressive breast cancer characteristics. Importantly, we identified increased prevalence of ER/PR-negative subtype across all tissue components, including adjacent normal, offering support that hypermethylation of *GSTM2* may be mechanistically involved in carcinogenesis. *GSTM2* encodes Glutathione S-Transferase Mu 2 which functions in the detoxification of electrophilic compounds including carcinogens, therapeutic drugs, and environmental toxins (188). *GSTM2* promoter methylation has previously been associated with high-grade tumors (184). To our knowledge, our group is the first to explicitly test the association between *GSTM2* promoter with methylation with ER/PR status; we previously reported hypermethylation of this gene region was associated with ER/PR-negative tumors using breast tumor samples from The Cancer Genome Atlas (TCGA) (176). Considering these results, there is growing support for the identified relationships between aberrant promoter hypermethylation of *GSTM2* and development of aggressive breast cancer characteristics.

While this study has a number of unique findings, there are some limitations worth noting. First, approximately one-third of the BCCC participants donated breast tissue, limiting our sample size for these analyses. As a result, we were unable to adjust for the smoking biomarker variable, although in a sensitivity analysis, we found adjustment for smoking exposure history did not appreciably change our findings. Another limitation was our study was that it was

cross-sectional in nature, so we cannot comment on temporality. It is possible aberrant methylation of *BRCA1* and *GSTM2* is a consequence of aggressive tumor characteristics, rather than a cause of them. Although for *GSTM2*, we showed aberrant methylation was associated with ER/PR-negative tumors in all three tissue components suggesting a causal role. An additional limitation of our study was the use of bisulfate conversion for the assessment of DNA methylation. This method alone lacks the ability to discriminate between 5-methylcytosine (5-mC) and 5-hydroxymethylcystine (5-hmC). Generally, promoter 5-mC content is a repressive marker and is associated with decreased transcription and gene expression. Conversely, 5-hmC is generally associated with increased transcription and 'poised' chromatin signatures (215). We therefore examined the associations between DNA methylation of these gene regions with gene expression using data from TCGA and found significant inverse associations for all five gene regions suggesting detection of 5-mC in our analysis. Another limitation was our use of FFPE-derived DNA; this type of DNA tends to be partly degraded and has greater issues with cross-linking compared to frozen tissue-derived DNA (216). Fortunately, the use of pyrosequencing allows for the accurate quantification of this type of DNA (217). Finally, although we examined regions previously used in other studies of breast cancer, our study population was ethnically diverse adding generalizability and implicating these regions as potential drivers of racial disparities in the incidence of aggressive breast cancer.

E. **Conclusions**

This study identified associations between aberrant tumor methylation and breast cancer characteristics. More specifically, we showed aberrant hypermethylation of the promoter regions of *BRCA1* and *GSTM2* are associated with aggressive breast cancer characteristics. Our findings with *BRCA1* methylation are unsurprising as this gene serves as a tumor suppressor, and down-

regulation is strongly associated with increased risk of breast cancer. Interestingly, *GSTM2* appears to be a candidate as an etiologic driver of tumor subtype given that aberrant methylation was associated with ER/PR-negative breast cancer in all three tissue components indicating hypermethylation of *GSTM2* is an early event in progression to ER/PR-negative breast cancer. The role of *GSTM2* is understudied in relation to breast cancer, but hypermethylation has been associated with high-grade breast tumors. As *GSTM2* functions in the detoxification of carcinogens and environmental toxins, we hypothesize aberrant methylation results in toxins having prolonged cellular effects contributing to the development of ER/PR-negative and high-grade tumors. Additional studies will be required to confirm these findings. Finally, the results from this study have wide-ranging population health implications. If modifiable environmental exposures, such as exposure to ambient airborne concentrations to heavy metals, are driving these genomic differences, it is possible racial disparities in aggressive breast cancer incidence can be reduced through environmental remediation.

V. CONCLUSIONS

In the previous chapters, our group identified associations linking exposure to ambient airborne concentrations of various heavy metals to prevalent aggressive breast cancer characteristics. We additionally identified a potential mechanism to explain these findings; namely, we found evidence that exposures to ambient airborne concentrations of heavy metals is associated with aberrant patterns of DNA methylation which are themselves associated with aggressive breast cancer phenotypes. Considering we found nH Blacks are generally more likely than nH Whites to be exposed to these metals, our findings suggest these exposures are contributing to the racial disparities in the development of aggressive breast cancer phenotypes.

Chapter II describes our identification of positive trends between airborne concentrations of antimony, arsenic, cobalt, manganese and selenium with prevalent ER/PR-negative breast tumors. Previous epidemiologic studies identified positive associations between air pollution and breast cancer risk. Lui et al. (2015) was the first study to specifically examine estrogen-disrupting hazardous air pollutants and found a positive relationship between arsenic and cadmium and development of ER/PR-negative breast tumors. We also found that among breast cancer patients, arsenic was positively associated with prevalence of ER/PR-negative tumors and although we did not find a significant association between cadmium and ER/PR-negative tumors, our data are suggestive of a similar relationship. Unlike Lui et al.'s (2015) study, we identified a positive relationship between selenium and prevalence of ER/PR-negative tumors. Additionally, this study was the first to examine associations between chronic exposure to heavy metals and tumor grade; we found that exposures to antimony and arsenic were associated with prevalence of high-grade tumors among premenopausal women.

Chapter III describes our identification of relationships between chronic and short-term exposures to airborne heavy metals and breast tissue DNA methylation across breast tumor tissue components. Generally, chronic metal exposures were positively associated with *GSTM2* methylation in invasive tumor tissue. More specifically, we showed positive associations between exposure to antimony, cobalt, lead and manganese with greater methylation of the promoter region of *GSTM2* in invasive tumor components. We additionally showed short-term metal exposures were inversely associated with *RASSF1* methylation in invasive tissue and was positively associated with *TFF1* methylation in adjacent normal and invasive tissue. Qualitatively, most of the observed associations tended to be such that greater metal exposure was associated with less, not more, aberrant DNA methylation; aberrant patterns were defined as increases in methylation of *BRCA1*, *EGFR*, *GSTM2*, and *RASSF1*, and decreases in methylation of *TFF1* and Sat2. The notable exceptions to this pattern were the associations identified between chronic metal exposure to antimony, cobalt, lead and manganese and increased aberrant methylation of *GSTM2*. These novel findings suggest ambient exposure to airborne heavy metals may affect breast cancer risk through dysregulation of DNA methylation in cancer-associated genes.

Finally, Chapter IV describes our identification of relationships between aberrant DNA methylation and breast tumor characteristics. We found hypermethylation of *BRCA1* and *GSTM2* were associated with prevalent ER/PR-negative tumors. We additionally showed hypermethylation of *GSTM2* was associated with prevalent high-grade tumors. Aberrant methylation of the other gene regions, namely *EGFR*, *RASSF1*, *TFF1* and Sat2 were generally associated with less aggressive breast cancer characteristics. These findings suggest underlying biological differences affect aggressive tumor development.

Taken together, these studies identified novel metals that affect breast cancer aggression and identified a potential mechanism through which these associations can be explained. More specifically, exposure to antimony, cobalt and manganese are associated with aggressive breast cancer phenotypes and aberrant methylation of *GSTM2*. Strong racial and socioeconomic disparities exist in subtype incidence rates, with younger women and African-American women more likely to develop ER/PR-negative tumors. Additionally, these women tend to have greater exposures to ambient airborne concentrations of antimony and manganese. Therefore, the findings from this study suggest exposures to airborne concentrations of these two metals are driving the racial disparity in aggressive breast cancer phenotypes.

While we discovered a number of novel findings, there are some limitations worth noting. Most importantly, because the heavy metal exposure metric was designed using multiple cycles from NATA, and due to changes in NATA methodology over time, the chronic estimates of ambient airborne heavy metal exposures were based on rankings, rather than absolute measures. Therefore our study alone cannot inform policy on allowable limits for airborne heavy metal concentrations. Similarly, this study relied on using residential histories to create exposure estimates. Most large-scale environmental epidemiology studies rely on residential addresses while acknowledging participants are not confined to their homes, thus resulting in some exposure misclassification, although we would expect this misclassification to be non-differential resulting in an attenuation of the observed effect estimates. Furthermore, we did not have any information on heavy metal exposure from food and water sources. With this information we would have been able to adjust for other sources of exposures, allowing for a more precise examination of the biological effects of ambient concentrations of airborne heavy metals. Other limitations included our inability to examine interactions due to low sample sizes.

We did, however, attempt to measure heavy metal mixtures via principle component analysis. Unfortunately, due to the high correlations between the metal exposures, particularly between antimony, cobalt and manganese, the principle component methodology was not informative. Additionally this study was based on a population of breast cancer patients; we were therefore unable to examine associations with breast cancer incidence. Similarly, only about one-third the population donated breast tumor tissue limiting our sample size further for the aims examining tissue DNA methylation. Lastly, we had difficultly controlling for potential confounding by smoking history as it was not collected during the patient interview. We attempted to estimate and adjust for smoking history via a novel method using DNA methylation patterns measured in whole blood samples on a sub-set of the population. Generally after adjustment for smoking history in our sensitivity analyses, our results were slightly attenuated but the overall implications remained consistent.

While this study had a number of limitations, it also had numerous strengths. Only one previous study has examined associations between airborne heavy metals and breast cancer characteristics in a large population. Similar to the Lui et al study, our findings implicate arsenic and cadmium in the development of ER/PR-negative subtypes. In addition, we also found evidence implicating other metals which are generally considered non-carcinogenic, particularly antimony, cobalt and manganese. Although our heavy metal exposure metrics were relative in nature, our methodology allowed for the combination of information from multiple NATA cycles, namely from 1999, 2002 and 2005. This, in turn, allowed us to link exposures with residential histories giving us the most complete chronic exposure metric to date. We additionally expanded on prior research by identifying a potential mechanism to explain our findings. Aberrant DNA methylation of cancer associated genes, particularly *GSTM2*, is strongly

associated with aggressive breast cancer phenotypes. This study also benefited from its patient population; the Breast Cancer Care in Chicago sample is population-based and ethnically diverse allowing for the generalization of our first study's findings. Lastly, our study benefitted from cutting-edge DNA methylation measurement techniques. Our breast tissue samples were formalin-fixed and paraffin embedded limiting the potential methods for methylation quantification. As chip-based assays were too expensive and the DNA quality was too poor, our use of pyrosequencing allowed for the accurate quantification of methylation at the selected genomic regions.

The findings from this study have a number of implications. Our findings suggest exposure to airborne heavy metals, particularly antimony and manganese are potentially responsible for the large racial disparity in the development of aggressive breast cancer subtypes. We showed exposure to these metals is associated with aggressive breast cancer subtypes after adjustment for traditional risk factors such as socioeconomic status and reproductive history. Additionally, nH Black women tend to reside in census-tracts characterized by greater airborne exposures to these heavy metals. In post-hoc analyses, using the Karlson, Holm and Breen (KHB) mediation method, we found approximately 24% of the racial disparity of ER/PR-negative breast cancer is due to exposures to airborne manganese after adjustment for age (data/results not shown). Airborne exposures have the ability to affect large populations and the remediation of these exposures are likely to have a large public health impact. Future studies should examine which metals are responsible for driving these racial disparities. Due to the high correlations between antimony, cobalt and manganese, we were unable to identify the responsible contaminant or explore interactions. Additionally, future examination is needed to further explore the role of aberrant DNA methylation in the development of aggressive

phenotypes. Breast cancer is a heterogeneous disease and is multifaceted in its development, but our study suggests hypermethylation of the promoter region of *GSTM2* could provide insight to carcinogenesis of breast cancer and may offer to be a therapeutic target for the protection against development of ER/PR-negative subtypes. Finally, this study adds to the growing literature that breast cancer aggression disparities could be reduced through environmental remediation such as limiting concentrations of heavy metals in exhaust-producing activities.

CITED LITERATURE

1. Srivastava S, Goyal P. Novel biomaterials decontamination of toxic metals from wastewater. *Environmental science and engineering Environmental engineering*. Heidelerg: Springer, 2010:1 online resource (xiii, 140 p.).

2. Nriagu JO, Davidson CI. *Toxic metals in the atmosphere*. New York: Wiley; 1986.

3. Tchounwou PB, Yedjou CG, Patlolla AK, et al. Heavy metal toxicity and the environment. *EXS* 2012;101:133-64.

4. Mahaffey KR, Corneliussen PE, Jelinek CF, et al. Heavy metal exposure from foods. *Environ Health Perspect* 1975;12:63-9.

5. World Health Organization. Trace elements in Human Health and Nutrition. Geneva, Switzerland: World Health Organization, 1996.

6. Nordberg G. *Handbook on the toxicology of metals*. 3rd ed. Amsterdam ; Boston: Academic Press; 2014.

7. Beattie AD, Moore MR, Goldberg, et al. Role of chronic low-level lead exposure in the aetiology of mental retardation. *Lancet* 1975;1(7907):589-92.

8. Gorell JM, Johnson CC, Rybicki BA, et al. Occupational exposure to manganese, copper, lead, iron, mercury and zinc and the risk of Parkinson's disease. *Neurotoxicology* 1999;20(2-3):239-47.

9. Tseng WP. Effects and dose--response relationships of skin cancer and blackfoot disease with arsenic. *Environ Health Perspect* 1977;19:109-19.

10. Woimant F, Trocello JM. Disorders of heavy metals. *Handb Clin Neurol* 2014;120:851-64.

11. McElroy JA, Shafer MM, Trentham-Dietz A, et al. Cadmium exposure and breast cancer risk. *J Natl Cancer Inst* 2006;98(12):869-73.

12. Subramanian J, Govindan R. Lung cancer in never smokers: a review. *J Clin Oncol* 2007;25(5):561-70.

13. Baudouin C, Charveron M, Tarroux R, et al. Environmental pollutants and skin cancer. *Cell Biol Toxicol* 2002;18(5):341-8.

14. Marshall G, Ferreccio C, Yuan Y, et al. Fifty-year study of lung and bladder cancer mortality in Chile related to arsenic in drinking water. *J Natl Cancer Inst* 2007;99(12):920-8.

15. Agency for Toxic Substances and Disease Registry. 2013 Substance Priority List. 2013 (http://www.atsdr.cdc.gov/spl/index.html). (Accessed 21 OCT 2015).

16. Agency for Toxic Substances and Disease Registry. 2015 Substance Priority List. 2015. (http://www.atsdr.cdc.gov/spl/index.html). (Accessed 13 JAN 2016).

17. International Agency for Research on Cancer. Arsenic, Metals, Fibres and Dust. *IARC monographs on the evaluation of carcinogenic risks to humans*. Lyon, France, 2012.

18. International Agency for Research on Cancer. Inorganic and organic lead compounds. *IARC monographs on the evaluation of carcinogenic risks to humans*. Lyon, France, 2006.

19. International Agency for Research on Cancer. Cobalt in Hard Metals and Cobalt Sulfate, Gallium Arsenide, Indium Phosphide and Vanadium Pentoxide. *IARC monographs on the evaluation of carcinogenic risks to humans*. Lyon, France, 2006.

20. Darbre PD. Metalloestrogens: an emerging class of inorganic xenoestrogens with potential to add to the oestrogenic burden of the human breast. *J Appl Toxicol* 2006;26(3):191-7.

21. Martin MB, Reiter R, Pham T, et al. Estrogen-like activity of metals in MCF-7 breast cancer cells. *Endocrinology* 2003;144(6):2425-36.

22. Choe SY, Kim SJ, Kim HG, et al. Evaluation of estrogenicity of major heavy metals. *Sci Total Environ* 2003;312(1-3):15-21.

23. Stoica A, Pentecost E, Martin MB. Effects of arsenite on estrogen receptor-alpha expression and activity in MCF-7 breast cancer cells. *Endocrinology* 2000;141(10):3595-602.

24. Stoica A, Pentecost E, Martin MB. Effects of selenite on estrogen receptor-alpha expression and activity in MCF-7 breast cancer cells. *J Cell Biochem* 2000;79(2):282-92.

25. Garcia-Morales P, Saceda M, Kenney N, et al. Effect of cadmium on estrogen receptor levels and estrogen-induced responses in human breast cancer cells. *J Biol Chem* 1994;269(24):16896-901.

26. Ballatori N. Transport of toxic metals by molecular mimicry. *Environ Health Perspect* 2002;110 Suppl 5:689-94.

27. Bal W, Sokołowska M, Kurowska E, et al. Binding of transition metal ions to albumin: sites, affinities and rates. *Biochim Biophys Acta* 2013;1830(12):5444-55.

28. Zalups RK. Molecular interactions with mercury in the kidney. *Pharmacol Rev* 2000;52(1):113-43.

29. Vesey DA. Transport pathways for cadmium in the intestine and kidney proximal tubule: focus on the interaction with essential metals. *Toxicol Lett* 2010;198(1):13-9.

30. Nolan CV, Shaikh ZA. Lead nephrotoxicity and associated disorders: biochemical mechanisms. *Toxicology* 1992;73(2):127-46.

31. Coyle P, Philcox JC, Carey LC, et al. Metallothionein: the multipurpose protein. *Cell Mol Life Sci* 2002;59(4):627-47.

32. Onosaka S, Tanaka K, Cherian MG. Effects of cadmium and zinc on tissue levels of metallothionein. *Environ Health Perspect* 1984;54:67-72.

33. Eide DJ. The SLC39 family of metal ion transporters. *Pflugers Arch* 2004;447(5):796-800.

34. Gaither LA, Eide DJ. Functional expression of the human hZIP2 zinc transporter. *J Biol Chem* 2000;275(8):5560-4.

35. Hartwig A. Zinc finger proteins as potential targets for toxic metal ions: differential effects on structure and function. *Antioxid Redox Signal* 2001;3(4):625-34.

36. Beyersmann D. Physiochemical aspects of the interference of detrimental metal ions with normal metal metabolism. In: Berthon G, ed. *Handbook on metal-ligand interactions in biological fluids*. New York: Marcel Dekker, 1995:813-26.

37. Dani SU. The arsenic for phosphorus swap is accidental, rather than a facultative one, and the question whether arsenic is nonessential or toxic is quantitative, not a qualitative one. *Sci Total Environ* 2011;409(22):4889-90.

38. Kenyon EM, Hughes MF, Adair BM, et al. Tissue distribution and urinary excretion of inorganic arsenic and its methylated metabolites in C57BL6 mice following subchronic exposure to arsenate in drinking water. *Toxicol Appl Pharmacol* 2008;232(3):448-55.

39. Markovich D. Physiological roles and regulation of mammalian sulfate transporters. *Physiol Rev* 2001;81(4):1499-533.

40. Shennan DB. Selenium (selenate) transport by human placental brush border membrane vesicles. *Br J Nutr* 1988;59(1):13-9.

41. Check L, Marteel-Parrish A. The fate and behavior of persistent, bioaccumulative, and toxic (PBT) chemicals: examining lead (Pb) as a PBT metal. *Rev Environ Health* 2013;28(2-3):85-96.

42. Antila E, Mussalo-Rauhamaa H, Kantola M, et al. Association of cadmium with human breast cancer. *Sci Total Environ* 1996;186(3):251-6.

43. Ionescu JG, Novotny J, Stejskal V, et al. Increased levels of transition metals in breast cancer tissue. *Neuro Endocrinol Lett* 2006;27 Suppl 1:36-9.

44. Strumylaite L, Bogusevicius A, Abdrachmanovas O, et al. Cadmium concentration in biological media of breast cancer patients. *Breast Cancer Res Treat* 2011;125(2):511-7.

45. Romanowicz-Makowska H, Forma E, Bryś M, et al. Concentration of cadmium, nickel and aluminium in female breast cancer. *Pol J Pathol* 2011;62(4):257-61.

46. El-Harouny M, El-Morsi D, Ahmed B, et al. Chronic Toxicity of Some Heavy Metals and Breast Cancer in Egyptian Females. *J Clinic Toxicol* 2011;1(109).

47. Mohammadi M, Riyahi Bakhtiari A, Khodabandeh S. Concentration of cd, pb, hg, and se in different parts of human breast cancer tissues. *J Toxicol* 2014;2014:413870.

48. Kufe DW, Holland JF, Frei E, et al. Cancer Medicine 6. Hamilton, Ont. ; Lewiston, NY distributor: BC Decker,, 2003:1 online resource (2 v.).

49. Yuspa SH, Poirier MC. Chemical carcinogenesis: from animal models to molecular models in one decade. *Adv Cancer Res* 1988;50:25-70.

50. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002;3(6):415-28.

51. Cerutti PA. Prooxidant states and tumor promotion. *Science* 1985;227(4685):375-81.

52. Wright SC, Zhong J, Larrick JW. Inhibition of apoptosis as a mechanism of tumor promotion. *FASEB J* 1994;8(9):654-60.

53. Colotta F, Allavena P, Sica A, et al. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* 2009;30(7):1073-81.

54. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989;244(4905):707-12.

55. Loeb LA, Cheng KC. Errors in DNA synthesis: a source of spontaneous mutations. *Mutat Res* 1990;238(3):297-304.

56. Yokota J. Tumor progression and metastasis. *Carcinogenesis* 2000;21(3):497-503.

57. Ookawa K, Sakamoto M, Hirohashi S, et al. Concordant p53 and DCC alterations and allelic losses on chromosomes 13q and 14q associated with liver metastases of colorectal carcinoma. *Int J Cancer* 1993;53(3):382-7.

58. Ishimaru G, Ookawa K, Yamaguchi N, et al. Allelic losses associated with the metastatic potential of colorectal-carcinoma. *Int J Oncol* 1994;5(2):267-73.

59. Young J, Leggett B, Ward M, et al. Frequent loss of heterozygosity on chromosome 14 occurs in advanced colorectal carcinomas. *Oncogene* 1993;8(3):671-5.

60. Thorstensen L, Qvist H, Nesland JM, et al. Identification of two potential suppressor gene regions on chromosome arm 14q that are commonly lost in advanced colorectal carcinomas. *Scand J Gastroenterol* 2001;36(12):1327-31.

61. O'Brien T, Mandel HG, Pritchard DE, et al. Critical role of chromium (Cr)-DNA interactions in the formation of Cr-induced polymerase arresting lesions. *Biochemistry* 2002;41(41):12529-37.

62. Nickens KP, Patierno SR, Ceryak S. Chromium genotoxicity: A double-edged sword. *Chem Biol Interact* 2010;188(2):276-88.

63. Beyersmann D, Hartwig A. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. *Arch Toxicol* 2008;82(8):493-512.

64. Hartwig A, Asmuss M, Ehleben I, et al. Interference by toxic metal ions with DNA repair processes and cell cycle control: molecular mechanisms. *Environ Health Perspect* 2002;110 Suppl 5:797-9.

65. Witkiewicz-Kucharczyk A, Bal W. Damage of zinc fingers in DNA repair proteins, a novel molecular mechanism in carcinogenesis. *Toxicol Lett* 2006;162(1):29-42.

66. Grin IR, Konorovsky PG, Nevinsky GA, et al. Heavy metal ions affect the activity of DNA glycosylases of the fpg family. *Biochemistry (Mosc)* 2009;74(11):1253-9.

67. Hartwig A. Current aspects in metal genotoxicity. *Biometals* 1995;8(1):3-11.

68. Feig DI, Reid TM, Loeb LA. Reactive oxygen species in tumorigenesis. *Cancer Res* 1994;54(7 Suppl):1890s-4s.

69. Cooke MS, Evans MD, Dizdaroglu M, et al. Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J* 2003;17(10):1195-214.

70. De Bont R, van Larebeke N. Endogenous DNA damage in humans: a review of quantitative data. *Mutagenesis* 2004;19(3):169-85.

71. Wiseman H, Halliwell B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem J* 1996;313 (Pt 1):17-29.

72. Sies H. Oxidative stress: oxidants and antioxidants. *Exp Physiol* 1997;82(2):291-5.

73. Cadenas E, Sies H. Oxidative stress: excited oxygen species and enzyme activity. *Adv Enzyme Regul* 1985;23:217-37.

74. Reid TM, Feig DI, Loeb LA. Mutagenesis by metal-induced oxygen radicals. *Environ Health Perspect* 1994;102 Suppl 3:57-61.

75. Kasprzak KS. Possible role of oxidative damage in metal-induced carcinogenesis. *Cancer Invest* 1995;13(4):411-30.

76. Valko M, Jomova K, Rhodes CJ, et al. Redox- and non-redox-metal-induced formation of free radicals and their role in human disease. *Arch Toxicol* 2015.

77. Wang Y, Fang J, Leonard SS, et al. Cadmium inhibits the electron transfer chain and induces reactive oxygen species. *Free Radic Biol Med* 2004;36(11):1434-43.

78. Shi H, Hudson LG, Ding W, et al. Arsenite causes DNA damage in keratinocytes via generation of hydroxyl radicals. *Chem Res Toxicol* 2004;17(7):871-8.

79. Ehrlich M. DNA methylation in cancer: too much, but also too little. *Oncogene* 2002;21(35):5400-13.

80. Arita A, Costa M. Epigenetics in metal carcinogenesis: nickel, arsenic, chromium and cadmium. *Metallomics* 2009;1(3):222-8.

81. Martinez-Zamudio R, Ha HC. Environmental epigenetics in metal exposure. *Epigenetics* 2011;6(7):820-7.

82. Zhang J, Li M, Wu Y, et al. Methylation of RAR-β2, RASSF1A, and CDKN2A genes induced by nickel subsulfide and nickel-carcinogenesis in rats. *Biomed Environ Sci* 2011;24(2):163-71.

83. Govindarajan B, Klafter R, Miller MS, et al. Reactive oxygen-induced carcinogenesis causes hypermethylation of p16(Ink4a) and activation of MAP kinase. *Mol Med* 2002;8(1):1-8.

84. Broday L, Peng W, Kuo MH, et al. Nickel compounds are novel inhibitors of histone H4 acetylation. *Cancer Res* 2000;60(2):238-41.

85. Chen H, Ke Q, Kluz T, et al. Nickel ions increase histone H3 lysine 9 dimethylation and induce transgene silencing. *Mol Cell Biol* 2006;26(10):3728-37.

86. Chanda S, Dasgupta UB, Guhamazumder D, et al. DNA hypermethylation of promoter of gene p53 and p16 in arsenic-exposed people with and without malignancy. *Toxicol Sci* 2006;89(2):431-7.

87. Goering PL, Aposhian HV, Mass MJ, et al. The enigma of arsenic carcinogenesis: role of metabolism. *Toxicol Sci* 1999;49(1):5-14.

88. Hossain MB, Vahter M, Concha G, et al. Low-level environmental cadmium exposure is associated with DNA hypomethylation in Argentinean women. *Environ Health Perspect* 2012;120(6):879-84.

89. Hartwig A. Cadmium and cancer. *Met Ions Life Sci* 2013;11:491-507.

90. Son YO, Pratheeshkumar P, Roy RV, et al. Nrf2/p62 signaling in apoptosis resistance and its role in cadmium-induced carcinogenesis. *J Biol Chem* 2014;289(41):28660-75.

91. Franco R, Sánchez-Olea R, Reyes-Reyes EM, et al. Environmental toxicity, oxidative stress and apoptosis: ménage à trois. *Mutat Res* 2009;674(1-2):3-22.

92. Chen F, Vallyathan V, Castranova V, et al. Cell apoptosis induced by carcinogenic metals. *Mol Cell Biochem* 2001;222(1-2):183-8.

93. Ye J, Wang S, Leonard SS, et al. Role of reactive oxygen species and p53 in chromium(VI)-induced apoptosis. *J Biol Chem* 1999;274(49):34974-80.

94. Bagchi D, Bagchi M, Stohs SJ. Chromium (VI)-induced oxidative stress, apoptotic cell death and modulation of p53 tumor suppressor gene. *Mol Cell Biochem* 2001;222(1-2):149-58.

95. Pearson G, Robinson F, Beers Gibson T, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 2001;22(2):153-83.

96. Chen L, Liu L, Huang S. Cadmium activates the mitogen-activated protein kinase (MAPK) pathway via induction of reactive oxygen species and inhibition of protein phosphatases 2A and 5. *Free Radic Biol Med* 2008;45(7):1035-44.

97. Wang X, Mandal AK, Saito H, et al. Arsenic and chromium in drinking water promote tumorigenesis in a mouse colitis-associated colorectal cancer model and the potential mechanism is ROS-mediated Wnt/β-catenin signaling pathway. *Toxicol Appl Pharmacol* 2012;262(1):11-21.

98. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* 2009;17(1):9-26.

99. Barker N, Clevers H. Mining the Wnt pathway for cancer therapeutics. *Nat Rev Drug Discov* 2006;5(12):997-1014.

100. Trachootham D, Lu W, Ogasawara MA, et al. Redox regulation of cell survival. *Antioxid Redox Signal* 2008;10(8):1343-74.

101. Martínez-Reyes I, Cuezva JM. The H(+)-ATP synthase: a gate to ROS-mediated cell death or cell survival. *Biochim Biophys Acta* 2014;1837(7):1099-112.

102. International Agency for Research on Cancer. Combined estrogen-progestogen contraceptives and combined estrogen-progestogen menopausal therapy. *IARC monographs on the evaluation of carcinogenic risks to humans,*. Lyon, France, 2007:1 online resource (ix, 528 p.).

103. Anderson E. The role of oestrogen and progesterone receptors in human mammary development and tumorigenesis. *Breast Cancer Res* 2002;4(5):197-201.

104. Ciocca DR, Fanelli MA. Estrogen receptors and cell proliferation in breast cancer. *Trends Endocrinol Metab* 1997;8(8):313-21.

105. Preston-Martin S, Pike MC, Ross RK, et al. Increased cell division as a cause of human cancer. *Cancer Res* 1990;50(23):7415-21.

106. Lodish H, Berk A, Zipursky S. *Section 12.4, DNA Damage and Repair and Their Role in Carcinogenesis*. 4th edition ed. New York; 2000.

107. Liao XH, Lu DL, Wang N, et al. Estrogen receptor α mediates proliferation of breast cancer MCF-7 cells via a p21/PCNA/E2F1-dependent pathway. *FEBS J* 2014;281(3):927-42.

108. Frasor J, Danes JM, Komm B, et al. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. *Endocrinology* 2003;144(10):4562-74.

109. Gompel A, Somaï S, Chaouat M, et al. Hormonal regulation of apoptosis in breast cells and tissues. *Steroids* 2000;65(10-11):593-8.

110. Borner C. Diminished cell proliferation associated with the death-protective activity of Bcl-2. *J Biol Chem* 1996;271(22):12695-8.

111. Starek A. Estrogens and organochlorine xenoestrogens and breast cancer risk. *Int J Occup Med Environ Health* 2003;16(2):113-24.

112. Shekhar PV, Werdell J, Basrur VS. Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines. *J Natl Cancer Inst* 1997;89(23):1774-82.

113. Thornton I. Sources and pathways of cadmium in the environment. *IARC Sci Publ* 1992(118):149-62.

114. Adams SV, Newcomb PA, White E. Dietary cadmium and risk of invasive postmenopausal breast cancer in the VITAL cohort. *Cancer Causes Control* 2012;23(6):845-54.

115. Julin B, Wolk A, Bergkvist L, et al. Dietary cadmium exposure and risk of postmenopausal breast cancer: a population-based prospective cohort study. *Cancer Res* 2012;72(6):1459-66.

116. Sawada N, Iwasaki M, Inoue M, et al. Long-term dietary cadmium intake and cancer incidence. *Epidemiology* 2012;23(3):368-76.

117. Itoh H, Iwasaki M, Sawada N, et al. Dietary cadmium intake and breast cancer risk in Japanese women: a case-control study. *Int J Hyg Environ Health* 2014;217(1):70-7.

118. Eriksen KT, Halkjær J, Sørensen M, et al. Dietary cadmium intake and risk of breast, endometrial and ovarian cancer in Danish postmenopausal women: a prospective cohort study. *PLoS One* 2014;9(6):e100815.

119. Adams SV, Quraishi SM, Shafer MM, et al. Dietary cadmium exposure and risk of breast, endometrial, and ovarian cancer in the Women's Health Initiative. *Environ Health Perspect* 2014;122(6):594-600.

120. Gallagher CM, Chen JJ, Kovach JS. Environmental cadmium and breast cancer risk. *Aging (Albany NY)* 2010;2(11):804-14.

121. Nagata C, Nagao Y, Nakamura K, et al. Cadmium exposure and the risk of breast cancer in Japanese women. *Breast Cancer Res Treat* 2013;138(1):235-9.

122. Strumylaite L, Kregzdyte R, Bogusevicius A, et al. Association between cadmium and breast cancer risk according to estrogen receptor and human epidermal growth factor receptor 2: epidemiological evidence. *Breast Cancer Res Treat* 2014;145(1):225-32.

123. Wei XL, He JR, Cen YL, et al. Modified effect of urinary cadmium on breast cancer risk by selenium. *Clin Chim Acta* 2015;438:80-5.

124. Larsson SC, Orsini N, Wolk A. Urinary Cadmium Concentration and Risk of Breast Cancer: A Systematic Review and Dose-Response Meta-Analysis. *Am J Epidemiol* 2015;182(5):375-80.

125. Adams SV, Shafer MM, Bonner MR, et al. Urinary Cadmium and Risk of Invasive Breast Cancer in the Women's Health Initiative. *Am J Epidemiol* 2016.

126. Brophy JT, Keith MM, Watterson A, et al. Breast cancer risk in relation to occupations with exposure to carcinogens and endocrine disruptors: a Canadian case-control study. *Environ Health* 2012;11:87.

127. Liu R, Nelson DO, Hurley S, et al. Residential exposure to estrogen disrupting hazardous air pollutants and breast cancer risk: the California Teachers Study. *Epidemiology* 2015;26(3):365-73.

128. Key TJ, Verkasalo PK, Banks E. Epidemiology of breast cancer. *Lancet Oncol* 2001;2(3):133-40.

129. Howlader N, Altekruse SF, Li CI, et al. US incidence of breast cancer subtypes defined by joint hormone receptor and HER2 status. *J Natl Cancer Inst* 2014;106(5).

130. Metzger-Filho O, Sun Z, Viale G, et al. Patterns of Recurrence and outcome according to breast cancer subtypes in lymph node-negative disease: results from international breast cancer study group trials VIII and IX. *J Clin Oncol* 2013;31(25):3083-90.

131. Voduc KD, Cheang MC, Tyldesley S, et al. Breast cancer subtypes and the risk of local and regional relapse. *J Clin Oncol* 2010;28(10):1684-91.

132. Millar EK, Graham PH, O'Toole SA, et al. Prediction of local recurrence, distant metastases, and death after breast-conserving therapy in early-stage invasive breast cancer using a five-biomarker panel. *J Clin Oncol* 2009;27(28):4701-8.

133. Kohler BA, Sherman RL, Howlader N, et al. Annual Report to the Nation on the Status of Cancer, 1975-2011, Featuring Incidence of Breast Cancer Subtypes by Race/Ethnicity, Poverty, and State. *J Natl Cancer Inst* 2015;107(6):djv048.

134. Chlebowski RT, Chen Z, Anderson GL, et al. Ethnicity and breast cancer: factors influencing differences in incidence and outcome. *J Natl Cancer Inst* 2005;97(6):439-48.

135. Yang XR, Sherman ME, Rimm DL, et al. Differences in risk factors for breast cancer molecular subtypes in a population-based study. *Cancer Epidemiol Biomarkers Prev* 2007;16(3):439-43.

136. Althuis MD, Fergenbaum JH, Garcia-Closas M, et al. Etiology of hormone receptor-defined breast cancer: a systematic review of the literature. *Cancer Epidemiol Biomarkers Prev* 2004;13(10):1558-68.

137. Henderson BE, Ross RK, Pike MC, et al. Endogenous hormones as a major factor in human cancer. *Cancer Res* 1982;42(8):3232-9.

138. Gray S. Breast cancer and hormone-replacement therapy: the Million Women Study. *Lancet* 2003;362(9392):1332; author reply

139. Kumle M, Weiderpass E, Braaten T, et al. Use of oral contraceptives and breast cancer risk: The Norwegian-Swedish Women's Lifestyle and Health Cohort Study. *Cancer Epidemiol Biomarkers Prev* 2002;11(11):1375-81.

140. Darbre PD. Environmental oestrogens, cosmetics and breast cancer. *Best Pract Res Clin Endocrinol Metab* 2006;20(1):121-43.

141. International Agency for Research on Cancer., World Health Organization., IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. *Chromium, nickel and welding*. Lyon: International Agency for Research on Cancer : Distributed for the International Agency for Research on Cancer by the Secretariat of the World Health Organization; 1990.

142. International Agency for Research on Cancer., IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Beryllium, cadmium, mercury, and exposures in the glass manufacturing industry. *IARC monographs on the evaluation of carcinogenic risks to humans,*. Geneva: IARC, World Health Organization,, 1993.

143. Dookeran KA, Silva A, Warnecke RB, et al. Race/ethnicity and disparities in mastectomy practice in the Breast Cancer Care in Chicago study. *Ann Surg Oncol* 2015;22(1):66-74.

144. Shenker NS, Polidoro S, van Veldhoven K, et al. Epigenome-wide association study in the European Prospective Investigation into Cancer and Nutrition (EPIC-Turin) identifies novel genetic loci associated with smoking. *Hum Mol Genet* 2013;22(5):843-51.

145. Shenker NS, Ueland PM, Polidoro S, et al. DNA methylation as a long-term biomarker of exposure to tobacco smoke. *Epidemiology* 2013;24(5):712-6.

146. Rauscher GH, Campbell RT, Wiley EL, et al. Mediation of Racial and Ethnic Disparities in Estrogen/Progesterone Receptor-Negative Breast Cancer by Socioeconomic Position and Reproductive Factors. *Am J Epidemiol* 2016;183(10):884-93.

147. Garcia E, Hurley S, Nelson DO, et al. Hazardous air pollutants and breast cancer risk in California teachers: a cohort study. *Environ Health* 2015;14(1):14.

148. Crouse DL, Goldberg MS, Ross NA, et al. Postmenopausal breast cancer is associated with exposure to traffic-related air pollution in Montreal, Canada: a case-control study. *Environ Health Perspect* 2010;118(11):1578-83.

149. Hystad P, Villeneuve PJ, Goldberg MS, et al. Exposure to traffic-related air pollution and the risk of developing breast cancer among women in eight Canadian provinces: a case-control study. *Environ Int* 2015;74:240-8.

150. Nie J, Beyea J, Bonner MR, et al. Exposure to traffic emissions throughout life and risk of breast cancer: the Western New York Exposures and Breast Cancer (WEB) study. *Cancer Causes Control* 2007;18(9):947-55.

151. Lewis-Michl EL, Melius JM, Kallenbach LR, et al. Breast cancer risk and residence near industry or traffic in Nassau and Suffolk Counties, Long Island, New York. *Arch Environ Health* 1996;51(4):255-65.

152. De Boeck M, Kirsch-Volders M, Lison D. Cobalt and antimony: genotoxicity and carcinogenicity. *Mutat Res* 2003;533(1-2):135-52.

153. Asakura K, Satoh H, Chiba M, et al. Genotoxicity studies of heavy metals: lead, bismuth, indium, silver and antimony. *J Occup Health* 2009;51(6):498-512.

154. Takahashi S, Sato H, Kubota Y, et al. Inhibition of DNA-double strand break repair by antimony compounds. *Toxicology* 2002;180(3):249-56.

155. Schaumlöffel N, Gebel T. Heterogeneity of the DNA damage provoked by antimony and arsenic. *Mutagenesis* 1998;13(3):281-6.

156. Kotsopoulos J, Sukiennicki G, Muszyńska M, et al. Plasma micronutrients, trace elements, and breast cancer in BRCA1 mutation carriers: an exploratory study. *Cancer Causes Control* 2012;23(7):1065-74.

157. Yamada K. Cobalt: Its Role in Health and Disease. In: Sigel A, Sigel H, Sigel RKO, eds. *Interrelations between Essential Metal Ions and Human Diseases* Springer, 2013: 295–320.

158. Wild P, Perdrix A, Romazini S, et al. Lung cancer mortality in a site producing hard metals. *Occup Environ Med* 2000;57(8):568-73.

159. Moulin JJ, Wild P, Romazini S, et al. Lung cancer risk in hard-metal workers. *Am J Epidemiol* 1998;148(3):241-8.

160. United States. Agency for Toxic Substances and Disease Registry. Toxicological profile for cobalt. Atlanta, GA: U.S. Dept. of Health and Human Services, Public Health Service, Agency for Toxic Substances Disease Registry,, 2004:1 online resource (486 p.).

161. Hengstler JG, Bolm-Audorff U, Faldum A, et al. Occupational exposure to heavy metals: DNA damage induction and DNA repair inhibition prove co-exposures to cadmium, cobalt and lead as more dangerous than hitherto expected. *Carcinogenesis* 2003;24(1):63-73.

162. Pascal LE, Tessier DM. Cytotoxicity of chromium and manganese to lung epithelial cells in vitro. *Toxicol Lett* 2004;147(2):143-51.

163. Lima PD, Vasconcellos MC, Bahia MO, et al. Genotoxic and cytotoxic effects of manganese chloride in cultured human lymphocytes treated in different phases of cell cycle. *Toxicol In Vitro* 2008;22(4):1032-7.

164. Spangler JG, Reid JC. Environmental manganese and cancer mortality rates by county in North Carolina: an ecological study. *Biol Trace Elem Res* 2010;133(2):128-35.

165. Kilic E, Saraymen R, Demiroglu A, et al. Chromium and manganese levels in the scalp hair of normals and patients with breast cancer. *Biol Trace Elem Res* 2004;102(1-3):19-25.

166. Joo NS, Kim SM, Jung YS, et al. Hair iron and other minerals' level in breast cancer patients. *Biol Trace Elem Res* 2009;129(1-3):28-35.

167. Li S, Lu L, Hao S, et al. Dietary manganese modulates expression of the manganese-containing superoxide dismutase gene in chickens. *J Nutr* 2011;141(2):189-94.

168. Loo SY, Hirpara JL, Pandey V, et al. Manganese Superoxide Dismutase Expression Regulates the Switch Between an Epithelial and a Mesenchymal-Like Phenotype in Breast Carcinoma. *Antioxid Redox Signal* 2016;25(6):283-99.

169. United States. Agency for Toxic Substances and Disease Registry. Toxicological profile for selenium. Atlanta, GA: U.S. Dept. of Health and Human Services, Public Health Service, Agency for Toxic Substances Disease Registry,, 2003:1 online resource (457 p.).

170. IEH. Chemicals purported to be endocrine disrupters. A compilation of published lists. (Web Report W20). Leicester, UK: MRC Institute for Environment and Health, 2005.

171. Shah YM, Al-Dhaheri M, Dong Y, et al. Selenium disrupts estrogen receptor (alpha) signaling and potentiates tamoxifen antagonism in endometrial cancer cells and tamoxifen-resistant breast cancer cells. *Mol Cancer Ther* 2005;4(8):1239-49.

172. Lee SO, Nadiminty N, Wu XX, et al. Selenium disrupts estrogen signaling by altering estrogen receptor expression and ligand binding in human breast cancer cells. *Cancer Res* 2005;65(8):3487-92.

173. United States. Agency for Toxic Substances and Disease Registry. Toxicological profile for arsenic. Atlanta, Ga.: U.S. Dept. of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry,, 2007:1 online resource (xx, 500 p.).

174. Xu Y, Tokar EJ, Waalkes MP. Arsenic-induced cancer cell phenotype in human breast epithelia is estrogen receptor-independent but involves aromatase activation. *Arch Toxicol* 2014;88(2):263-74.

175. Yue W, Yager JD, Wang JP, et al. Estrogen receptor-dependent and independent mechanisms of breast cancer carcinogenesis. *Steroids* 2013;78(2):161-70.

176. Rauscher GH, Kresovich JK, Poulin M, et al. Exploring DNA methylation changes in promoter, intragenic, and intergenic regions as early and late events in breast cancer formation. *BMC Cancer* 2015;15:816.

177. Dobrovic A, Simpfendorfer D. Methylation of the BRCA1 gene in sporadic breast cancer. *Cancer Res* 1997;57(16):3347-50.

178. Esteller M, Silva JM, Dominguez G, et al. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *J Natl Cancer Inst* 2000;92(7):564-9.

179. Montero AJ, Díaz-Montero CM, Mao L, et al. Epigenetic inactivation of EGFR by CpG island hypermethylation in cancer. *Cancer Biol Ther* 2006;5(11):1494-501.

180. Pasquali L, Bedeir A, Ringquist S, et al. Quantification of CpG island methylation in progressive breast lesions from normal to invasive carcinoma. *Cancer Lett* 2007;257(1):136-44.

181. Martin V, Ribieras S, Song-Wang XG, et al. Involvement of DNA methylation in the control of the expression of an estrogen-induced breast-cancer-associated protein (pS2) in human breast cancers. *J Cell Biochem* 1997;65(1):95-106.

182. Jackson K, Yu MC, Arakawa K, et al. DNA hypomethylation is prevalent even in low-grade breast cancers. *Cancer Biol Ther* 2004;3(12):1225-31.

183. Cho YH, Yazici H, Wu HC, et al. Aberrant promoter hypermethylation and genomic hypomethylation in tumor, adjacent normal tissues and blood from breast cancer patients. *Anticancer Res* 2010;30(7):2489-96.

184. Christensen BC, Kelsey KT, Zheng S, et al. Breast cancer DNA methylation profiles are associated with tumor size and alcohol and folate intake. *PLoS Genet* 2010;6(7):e1001043.

185. Kile ML, Fang S, Baccarelli AA, et al. A panel study of occupational exposure to fine particulate matter and changes in DNA methylation over a single workday and years worked in boilermaker welders. *Environ Health* 2013;12(1):47.

186. Searles Nielsen S, Checkoway H, Criswell SR, et al. Inducible nitric oxide synthase gene methylation and parkinsonism in manganese-exposed welders. *Parkinsonism Relat Disord* 2015;21(4):355-60.

187. Li H, Hedmer M, Wojdacz T, et al. Oxidative stress, telomere shortening, and DNA methylation in relation to low-to-moderate occupational exposure to welding fumes. *Environ Mol Mutagen* 2015;56(8):684-93.

188. Tew KD, Ronai Z. GST function in drug and stress response. *Drug Resist Updat* 1999;2(3):143-7.

189. Guo L, Byun HM, Zhong J, et al. Effects of short-term exposure to inhalable particulate matter on DNA methylation of tandem repeats. *Environ Mol Mutagen* 2014;55(4):322-35.

190. Baccarelli A, Wright RO, Bollati V, et al. Rapid DNA methylation changes after exposure to traffic particles. *Am J Respir Crit Care Med* 2009;179(7):572-8.

191. Fan T, Fang SC, Cavallari JM, et al. Heart rate variability and DNA methylation levels are altered after short-term metal fume exposure among occupational welders: a repeated-measures panel study. *BMC Public Health* 2014;14:1279.

192. Tarantini L, Hou L, Bonzini M, et al. Effects of Exposure to Metal-Rich Air Particles on DNA Methylation. Epidemiology, 2009:S127-S8.

193. Li Y, Wei Q, Cao F, et al. Expression and promoter methylation of the RASSF1A gene in sporadic breast cancers in Chinese women. *Oncol Rep* 2008;19(5):1149-53.

194. Burbee DG, Forgacs E, Zöchbauer-Müller S, et al. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst* 2001;93(9):691-9.

195. Ehrlich M, Lacey M. DNA methylation and differentiation: silencing, upregulation and modulation of gene expression. *Epigenomics* 2013;5(5):553-68.

196. Heichman KA, Warren JD. DNA methylation biomarkers and their utility for solid cancer diagnostics. *Clin Chem Lab Med* 2012;50(10):1707-21.

197. Gama-Sosa MA, Slagel VA, Trewyn RW, et al. The 5-methylcytosine content of DNA from human tumors. *Nucleic Acids Res* 1983;11(19):6883-94.

198. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 1983;301(5895):89-92.

199. Varley KE, Gertz J, Bowling KM, et al. Dynamic DNA methylation across diverse human cell lines and tissues. *Genome Res* 2013;23(3):555-67.

200. Berman BP, Weisenberger DJ, Aman JF, et al. Regions of focal DNA hypermethylation and long-range hypomethylation in colorectal cancer coincide with nuclear lamina-associated domains. *Nat Genet* 2012;44(1):40-6.

201. Costello JF, Frühwald MC, Smiraglia DJ, et al. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat Genet* 2000;24(2):132-8.

202. Ehrlich M, Jiang G, Fiala E, et al. Hypomethylation and hypermethylation of DNA in Wilms tumors. *Oncogene* 2002;21(43):6694-702.

203. Esteller M, Corn PG, Baylin SB, et al. A gene hypermethylation profile of human cancer. *Cancer Res* 2001;61(8):3225-9.

204. Fackler MJ, Umbricht CB, Williams D, et al. Genome-wide methylation analysis identifies genes specific to breast cancer hormone receptor status and risk of recurrence. *Cancer Res* 2011;71(19):6195-207.

205. Li L, Lee KM, Han W, et al. Estrogen and progesterone receptor status affect genome-wide DNA methylation profile in breast cancer. *Hum Mol Genet* 2010;19(21):4273-7.

206. Rønneberg JA, Fleischer T, Solvang HK, et al. Methylation profiling with a panel of cancer related genes: association with estrogen receptor, TP53 mutation status and expression subtypes in sporadic breast cancer. *Mol Oncol* 2011;5(1):61-76.

207. Benevolenskaya EV, Islam AB, Ahsan H, et al. DNA methylation and hormone receptor status in breast cancer. *Clin Epigenetics* 2016;8:17.

208. Cuzick J. A Wilcoxon-type test for trend. *Stat Med* 1985;4(1):87-90.

209. Zhang J, Powell SN. The role of the BRCA1 tumor suppressor in DNA double-strand break repair. *Mol Cancer Res* 2005;3(10):531-9.

210. Turner NC, Reis-Filho JS, Russell AM, et al. BRCA1 dysfunction in sporadic basal-like breast cancer. *Oncogene* 2007;26(14):2126-32.

211. Turner N, Tutt A, Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. *Nat Rev Cancer* 2004;4(10):814-9.

212. Turner NC, Reis-Filho JS. Basal-like breast cancer and the BRCA1 phenotype. *Oncogene* 2006;25(43):5846-53.

213. Abd El-Rehim DM, Ball G, Pinder SE, et al. High-throughput protein expression analysis using tissue microarray technology of a large well-characterised series identifies biologically distinct classes of breast cancer confirming recent cDNA expression analyses. *Int J Cancer* 2005;116(3):340-50.

214. Osin P, Lu YJ, Stone J, et al. Distinct genetic and epigenetic changes in medullary breast cancer. *Int J Surg Pathol* 2003;11(3):153-8.

215. Pastor WA, Pape UJ, Huang Y, et al. Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. *Nature* 2011;473(7347):394-7.

216. von Ahlfen S, Missel A, Bendrat K, et al. Determinants of RNA quality from FFPE samples. *PLoS One* 2007;2(12):e1261.

217. Tost J, Gut IG. DNA methylation analysis by pyrosequencing. *Nat Protoc* 2007;2(9):2265-75.

APPENDICES

APPENDIX A. MISSING RESIDENTIAL DATA BY YEAR

TABLE XXVI.

MISSING RESIDENTIAL DATA BY YEAR

Year	Total Possible	Number missing	Percent
1994	989	245	24.8
1995	989	212	21.7
1996	989	180	18.2
1997	989	164	16.5
1998	989	150	15.2
1999	989	132	13.3
2000	989	120	12.1
2001	989	112	11.3
2002	989	105	10.6
2003	989	60	6.1
2004	989	77	7.8
2005	989	30	3.0
2006	823	31	3.7
2007	381	14	3.7
2008	25	0	0

APPENDIX B. SPLINED METAL ASSOCIATIONS WITH ER/PR-NEGATIVE BREAST CANCER

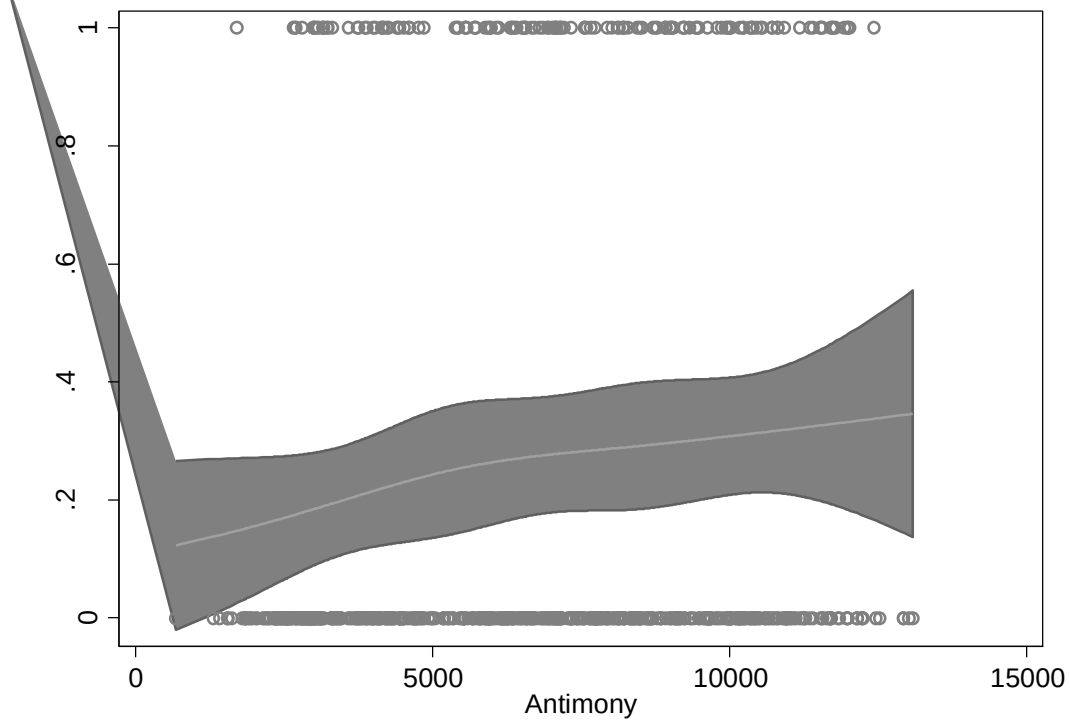

Figure 1. Restricted cubic spline mapping the shape of the association between antimony exposure and the prevalence of ER/PR negative breast tumors.

APPENDIX B (continued). SPLINED METAL ASSOCIATIONS WITH ER/PR-NEGATIVE BREAST CANCER

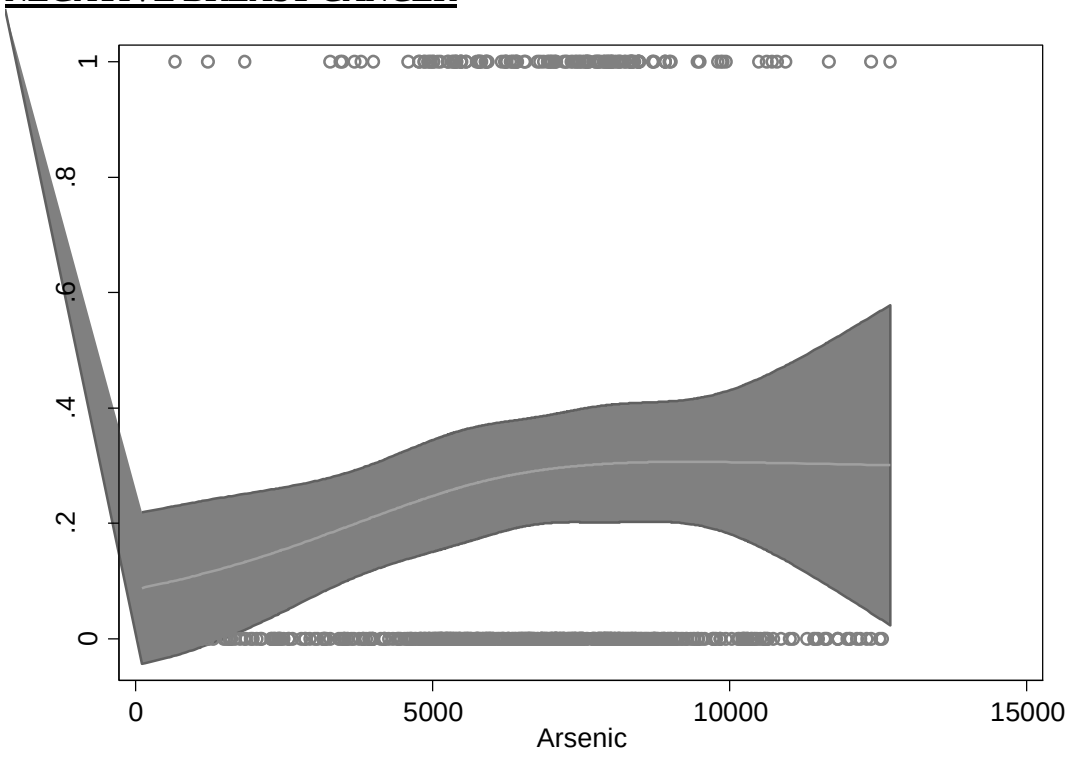

Figure 2. Restricted cubic spline mapping the shape of the association between arsenic exposure and the prevalence of ER/PR negative breast tumors.

APPENDIX B (continued). SPLINED METAL ASSOCIATIONS WITH ER/PR-NEGATIVE BREAST CANCER

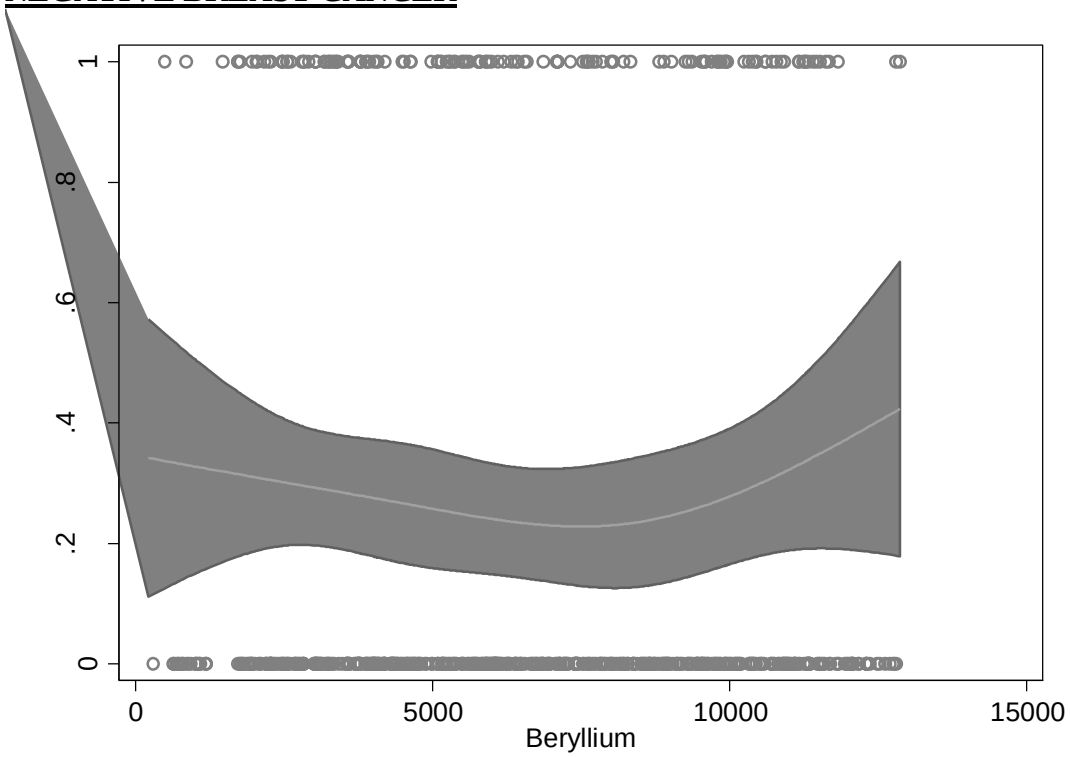

Figure 3. Restricted cubic spline mapping the shape of the association between beryllium exposure and the prevalence of ER/PR negative breast tumors.

APPENDIX B (continued). SPLINED METAL ASSOCIATIONS WITH ER/PR-NEGATIVE BREAST CANCER

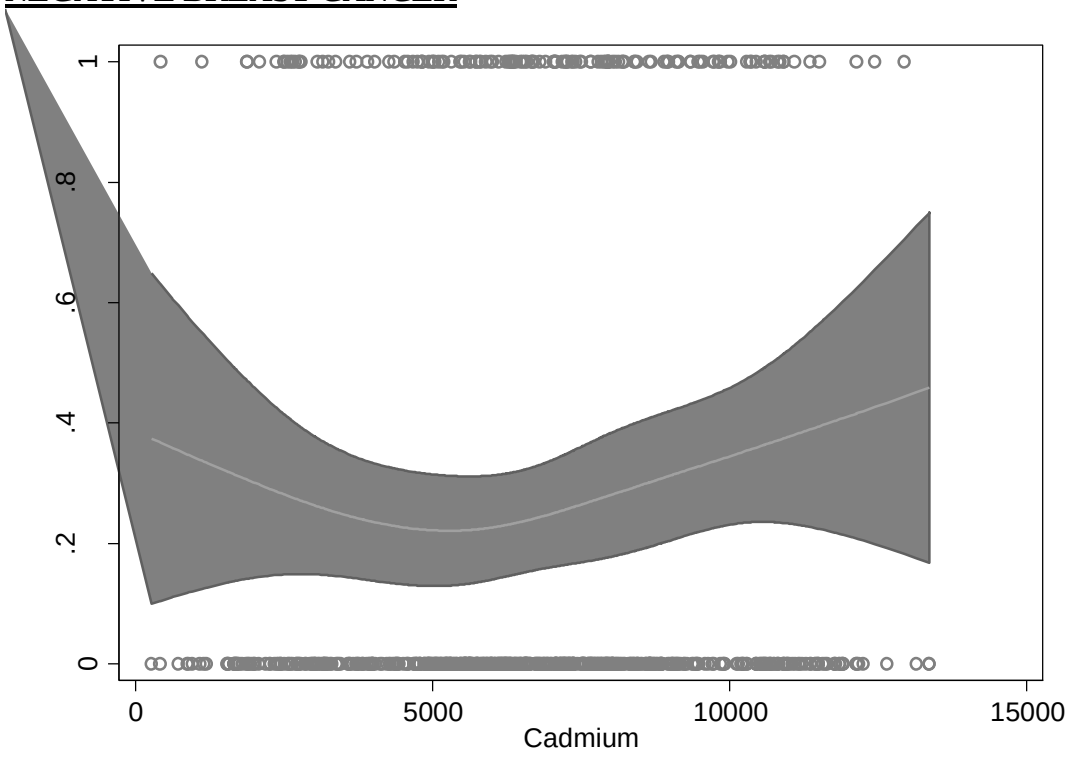

Figure 4. Restricted cubic spline mapping the shape of the association between cadmium exposure and the prevalence of ER/PR negative breast tumors.

APPENDIX B (continued). SPLINED METAL ASSOCIATIONS WITH ER/PR-NEGATIVE BREAST CANCER

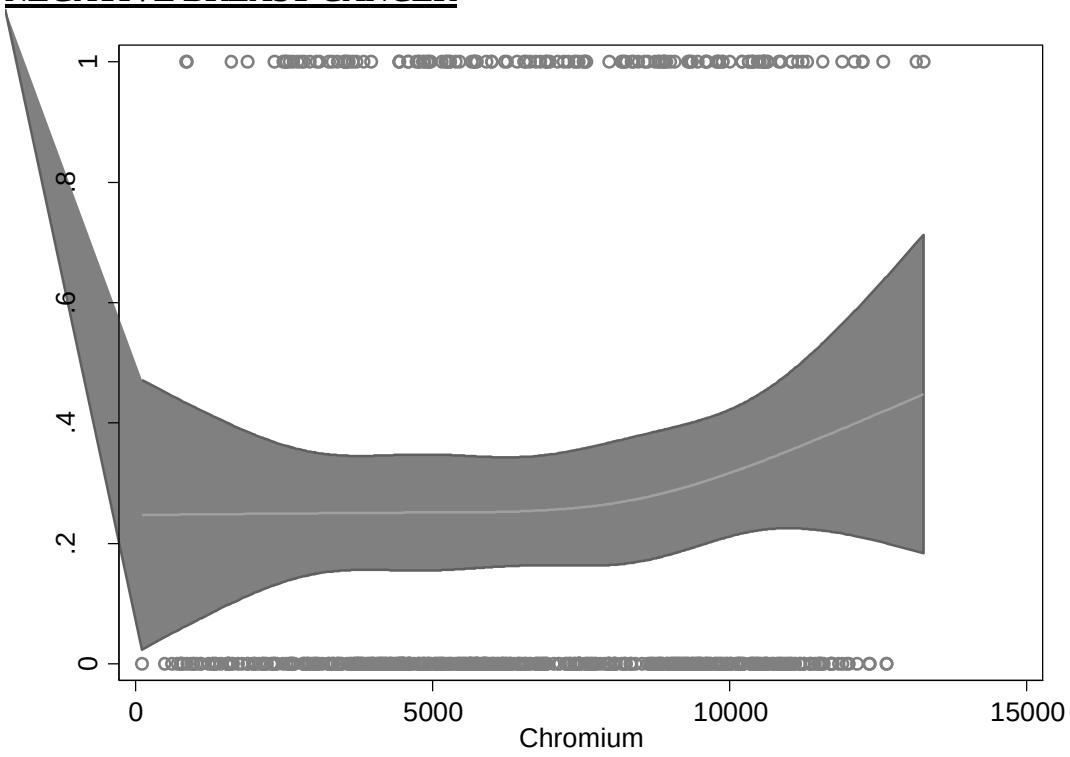

Figure 5. Restricted cubic spline mapping the shape of the association between chromium exposure and the prevalence of ER/PR negative breast tumors.

APPENDIX B (continued). SPLINED METAL ASSOCIATIONS WITH ER/PR-NEGATIVE BREAST CANCER

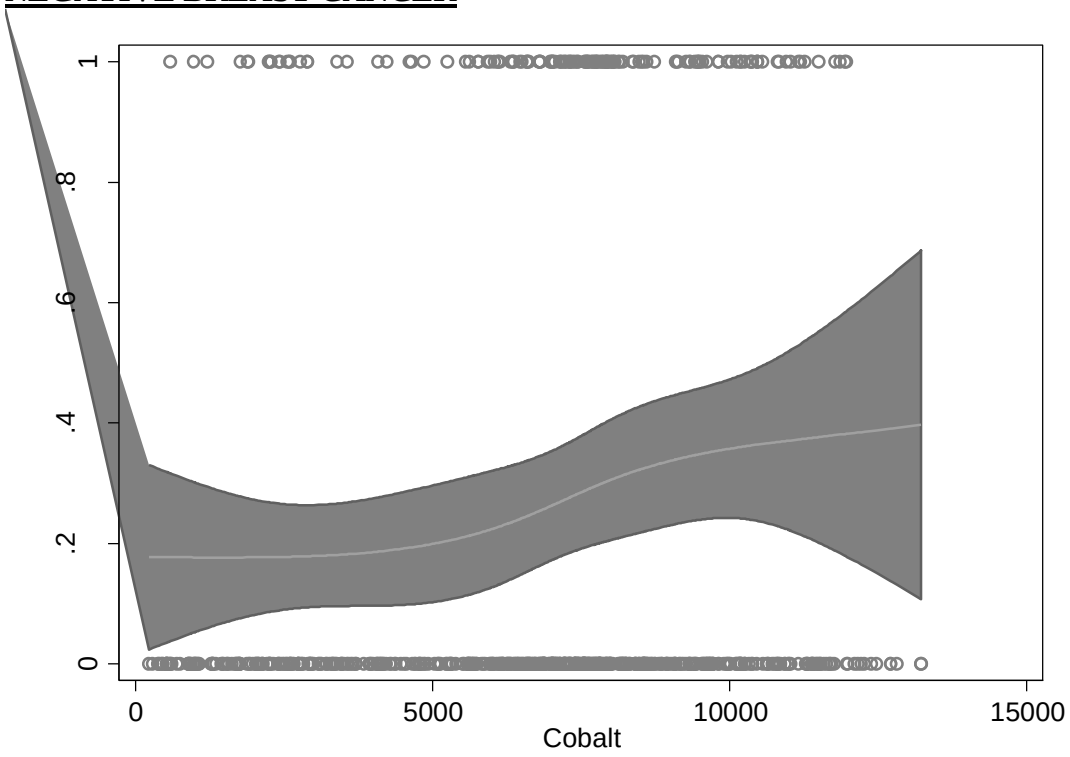

Figure 6. Restricted cubic spline mapping the shape of the association between cobalt exposure and the prevalence of ER/PR negative breast tumors.

APPENDIX B (continued). SPLINED METAL ASSOCIATIONS WITH ER/PR-NEGATIVE BREAST CANCER

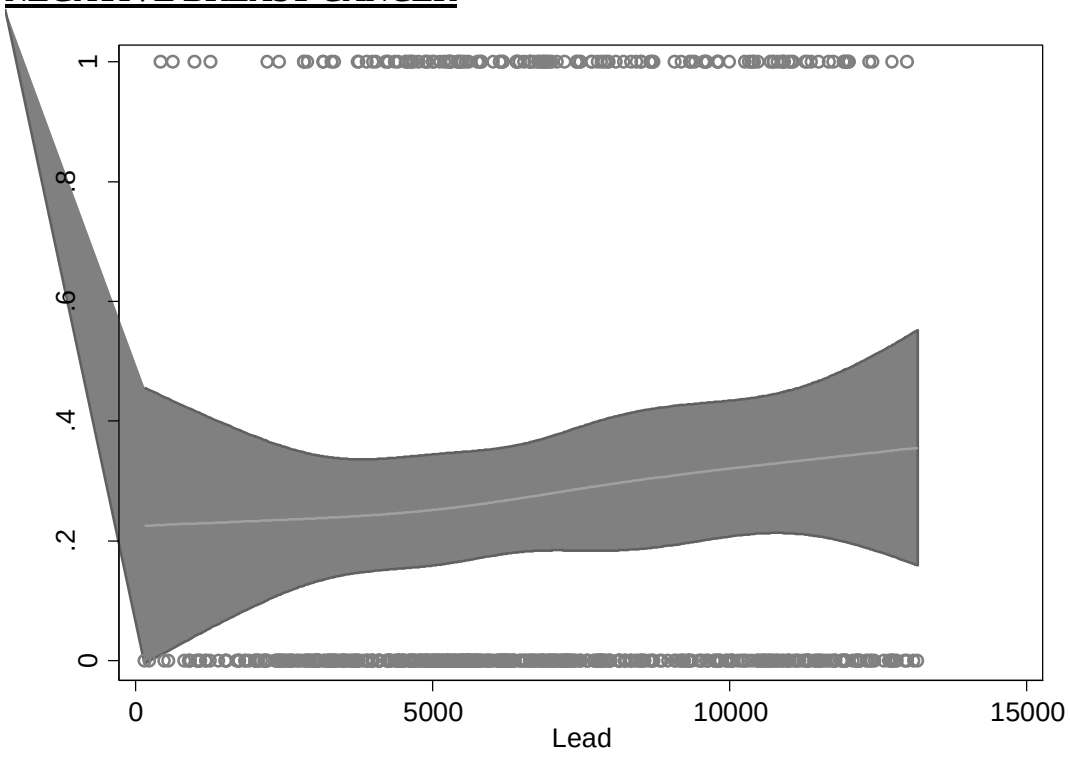

Figure 7. Restricted cubic spline mapping the shape of the association between lead exposure and the prevalence of ER/PR negative breast tumors.

APPENDIX B (continued). SPLINED METAL ASSOCIATIONS WITH ER/PR-NEGATIVE BREAST CANCER

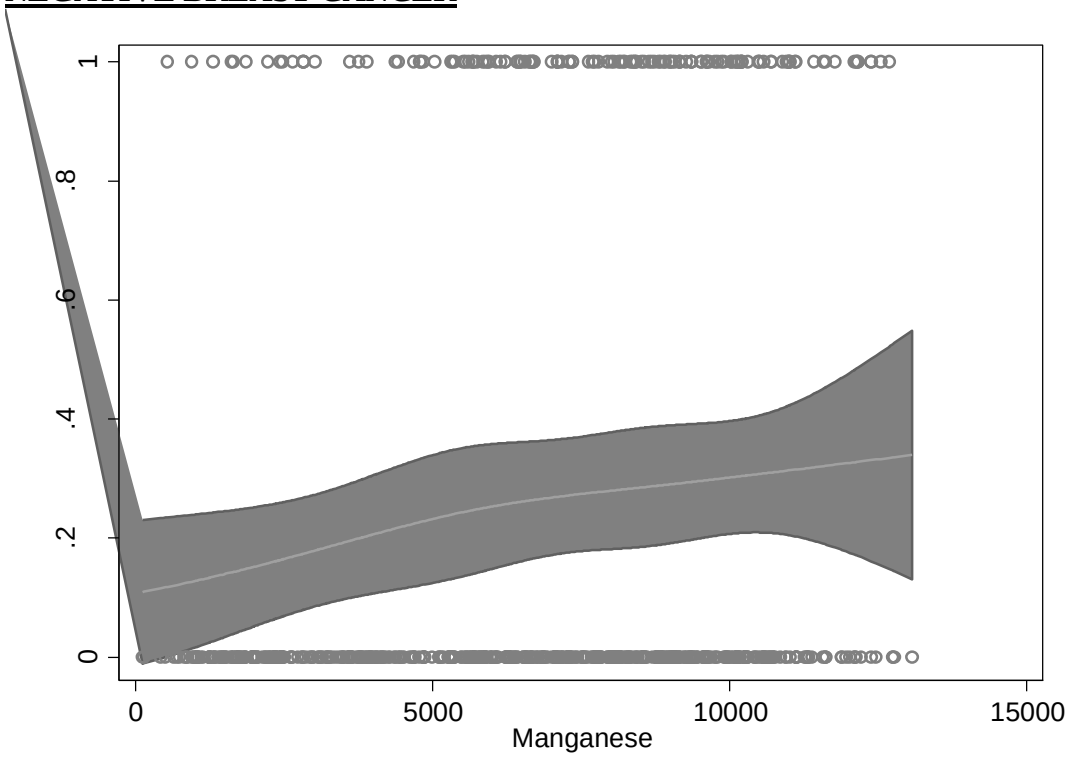

Figure 8. Restricted cubic spline mapping the shape of the association between manganese exposure and the prevalence of ER/PR negative breast tumors.

APPENDIX B (continued). SPLINED METAL ASSOCIATIONS WITH ER/PR-NEGATIVE BREAST CANCER

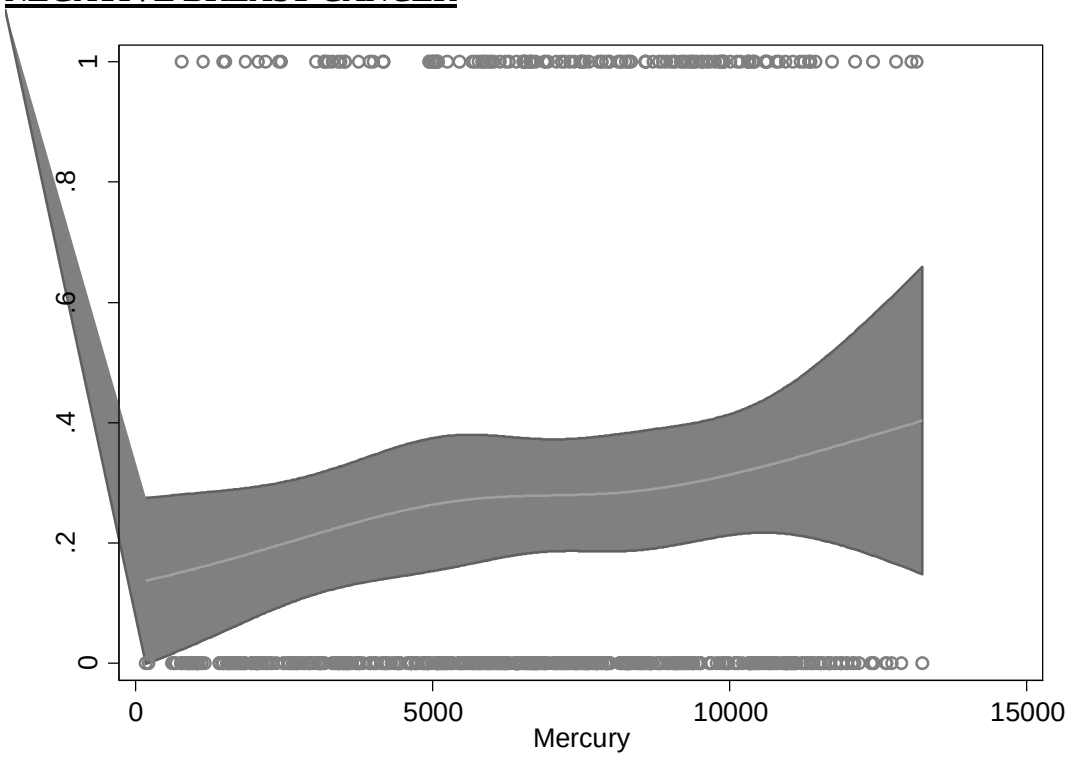

Figure 9. Restricted cubic spline mapping the shape of the association between mercury exposure and the prevalence of ER/PR negative breast tumors.

APPENDIX B (continued). SPLINED METAL ASSOCIATIONS WITH ER/PR-NEGATIVE BREAST CANCER

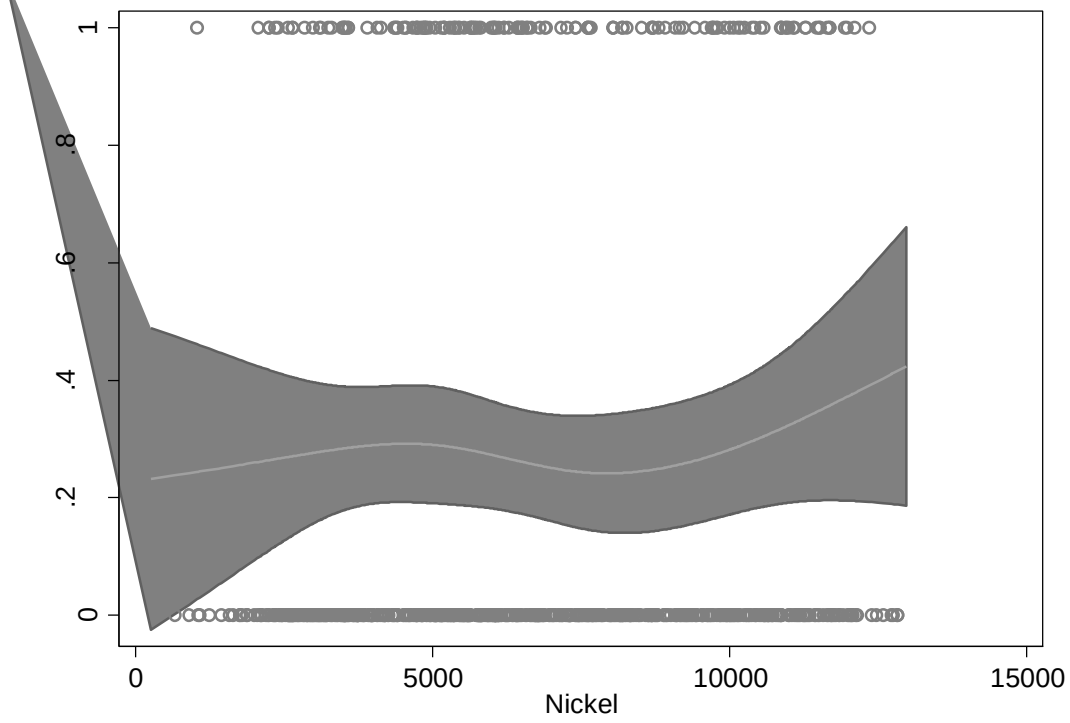

Figure 10. Restricted cubic spline mapping the shape of the association between nickel exposure and the prevalence of ER/PR negative breast tumors.

APPENDIX B (continued). SPLINED METAL ASSOCIATIONS WITH ER/PR-NEGATIVE BREAST CANCER

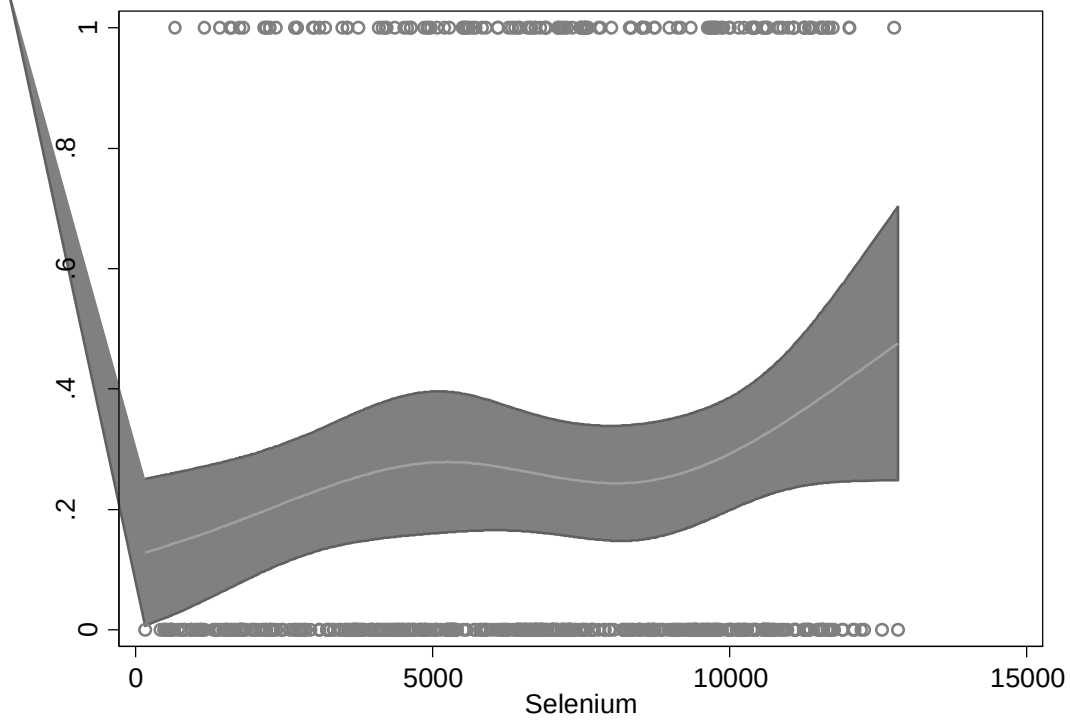

Figure 11. Restricted cubic spline mapping the shape of the association between selenium exposure and the prevalence of ER/PR negative breast tumors.

APPENDIX C. LOADING FACTORS FOR PRINCIPAL COMPONENTS

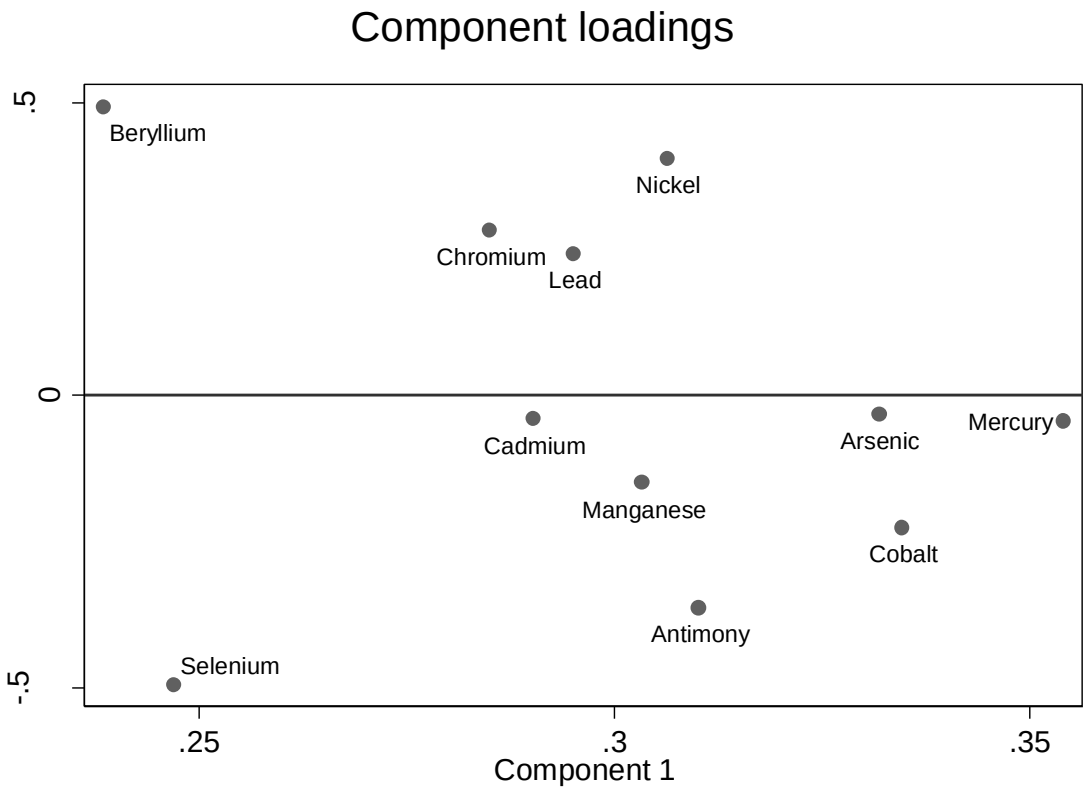

Figure 12. Loading factors for the first two principal components used in Chapter II.

APPENDIX D. METHYLATION VALUES BY INDIVIDUAL CpG SITE

TABLE XXVII.

BRCA1 CPG SITE MEAN AND MEDIAN VALUES BY TISSUE TYPE

Adjacent normal					In situ					Invasive				
Site	Mean	SD	Median	IQR	Site	Mean	SD	Median	IQR	Site	Mean	SD	Median	IQR
CpG 466	2.21	6.1	0.00	0.0-3.4	CpG 466	2.93	6.7	1.50	0.0-3.6	CpG 466	3.11	6.9	0.00	0.0-3.7
CpG 467	1.57	3.9	0.00	0.0-1.7	CpG 467	1.77	2.9	0.00	0.0-2.3	CpG 467	3.86	12.7	0.00	0.0-2.3
CpG 468	0.70	2.7	0.00	0.0-0.0	CpG 468	0.93	4.9	0.00	0.0-0.0	CpG 468	2.00	7.5	0.00	0.0-0.0
CpG 469	0.82	3.9	0.00	0.0-0.0	CpG 469	0.58	4.8	0.00	0.0-0.0	CpG 469	2.04	9.5	0.00	0.0-0.0
CpG 470	1.16	3.2	0.00	0.0-1.2	CpG 470	1.16	3.5	0.00	0.0-1.4	CpG 470	2.85	9.3	0.00	0.0-1.9
CpG 471	1.95	6.2	0.00	0.0-2.0	CpG 471	1.63	2.2	1.40	0.0-2.2	CpG 471	3.46	9.9	0.00	0.0-2.7
CpG 472	0.81	3.7	0.00	0.0-0.0	CpG 472	0.49	2.4	0.00	0.0-0.0	CpG 472	2.16	8.7	0.00	0.0-0.0
CpG 473	0.74	3.1	0.00	0.0-0.0	CpG 473	0.48	1.7	0.00	0.0-0.0	CpG 473	1.93	7.9	0.00	0.0-0.7
CpG 474	1.17	4.9	0.00	0.0-0.0	CpG 474	0.43	1.1	0.00	0.0-0.0	CpG 474	2.31	9.4	0.00	0.0-0.0
CpG 475	1.03	3.3	0.00	0.0-1.1	CpG 475	0.81	1.6	0.00	0.0-1.2	CpG 475	2.61	8.7	0.00	0.0-1.7
CpG 476	0.84	4.0	0.00	0.0-0.0	CpG 476	1.01	6.0	0.00	0.0-0.0	CpG 476	2.20	9.4	0.00	0.0-0.0
Mean	1.18	2.9	0.37	0.0-1.3	Mean	1.10	1.4	0.65	0.3-1.4	Mean	2.52	8.5	0.61	0.0-1.4

APPENDIX D (continued). METHYLATION VALUES BY INDIVIDUAL CpG SITE

TABLE XXVIII.

EGFR CPG SITE MEAN AND MEDIAN VALUES BY TISSUE TYPE

Adjacent normal				
Site	Mean	SD	Median	IQR
CpG 134	6.46	5.8	5.40	3.6-7.7
CpG 135	5.38	5.7	4.35	2.4-6.6
CpG 136	9.79	6.8	8.45	5.8-11.8
CpG 137	7.67	6.3	6.40	4.5-9.1
Mean	7.32	5.6	6.2	4.3-8.6
In situ				
Site	Mean	SD	Median	IQR
CpG 134	19.01	16.1	12.30	6.3-29.5
CpG 135	16.49	15.8	9.10	5.2-26.0
CpG 136	27.02	16.5	24.30	13.5-38.6
CpG 137	21.31	16.3	15.00	8.5-32.6
Mean	20.96	15.0	16.27	9.0-30.3
Invasive				
Site	Mean	SD	Median	IQR
CpG 134	20.83	17.4	15.60	6.2-31.6
CpG 135	16.78	16.9	8.50	4.3-26.3
CpG 136	29.85	18.0	31.10	12.6-42.7
CpG 137	23.82	18.0	19.80	7.9-36.6
Mean	22.82	16.1	20.1	8.1-34.0

APPENDIX D (continued). METHYLATION VALUES BY INDIVIDUAL CpG SITE

TABLE XXIX.

GSTM2 CPG SITE MEAN AND MEDIAN VALUES BY TISSUE TYPE

Adjacent normal				
Site	Mean	SD	Median	IQR
CpG 1	3.41	11.0	0.00	0.0-1.8
CpG 2	2.80	9.4	0.00	0.0-0.0
CpG 3	3.15	9.3	0.00	0.0-3.0
CpG 4	3.99	9.8	0.00	0.0-4.6
CpG 5	4.04	9.5	0.00	0.0-5.2
CpG 6	2.72	8.5	0.00	0.0-1.9
CpG 7	3.28	9.4	0.00	0.0-2.6
CpG 8	2.20	8.8	0.00	0.0-0.0
Mean	3.20	8.3	0.75	0.0-3.3

In situ				
Site	Mean	SD	Median	IQR
CpG 1	13.11	21.8	1.70	0.0-17.4
CpG 2	12.33	21.7	0.00	0.0-15.0
CpG 3	8.92	17.3	2.10	0.0-7.7
CpG 4	12.60	21.3	1.70	0.0-15.3
CpG 5	13.72	21.4	2.60	0.0-20.2
CpG 6	10.79	19.4	0.00	0.0-12.6
CpG 7	13.54	23.3	0.00	0.0-18.9
CpG 8	10.73	20.1	0.00	0.0-12.6
Mean	11.97	19.6	1.82	0.3-14.5

Invasive				
Site	Mean	SD	Median	IQR
CpG 1	21.15	26.8	4.70	0.0-43.5
CpG 2	19.93	26.0	3.70	0.0-41.7
CpG 3	13.04	18.3	3.70	0.0-18.5
CpG 4	19.26	24.5	4.05	0.0-39.3
CpG 5	21.61	25.1	7.70	0.0-42.1
CpG 6	16.71	22.5	4.50	0.0-31.2
CpG 7	21.11	26.7	5.50	0.0-40.7
CpG 8	17.12	23.9	1.85	0.0-32.5
Mean	18.74	22.9	4.81	1.1-35.2

APPENDIX D (continued). METHYLATION VALUES BY INDIVIDUAL CpG SITE

TABLE XXX.

RASSF1 CPG SITE MEAN AND MEDIAN VALUES BY TISSUE TYPE

Adjacent normal				
Site	Mean	SD	Median	IQR
CpG 24	13.42	17.1	7.05	1.7-18.5
CpG 25	11.34	16.2	4.85	0.0-15.2
CpG 26	9.99	13.8	4.75	1.2-14.0
CpG 27	10.71	15.8	4.30	0.0-15.0
CpG 28	12.68	15.2	7.00	2.9-6.8
CpG 29	12.24	14.4	8.15	2.3-16.4
CpG 30	11.38	15.7	6.00	1.1-14.5
CpG 31	15.96	17.4	9.50	4.4-20.1
CpG 32	12.33	17.4	6.30	2.3-14.7
Mean	12.24	14.4	3.60	3.4-15.5

In situ				
Site	Mean	SD	Median	IQR
CpG 24	49.49	29.7	51.25	27.1-77.5
CpG 25	48.40	29.5	51.65	22.6-74.1
CpG 26	46.71	29.6	50.90	21.8-73.0
CpG 27	45.91	28.0	46.40	23.7-72.1
CpG 28	45.15	26.1	46.15	24.6-69.9
CpG 29	35.35	21.7	37.65	17.5-53.9
CpG 30	44.45	28.3	48.55	21.9-68.3
CpG 31	49.38	25.2	50.15	27.6-72.6
CpG 32	46.60	30.1	47.80	22.3-74.3
Mean	45.72	26.8	48.09	24.4-70.2

Invasive				
Site	Mean	SD	Median	IQR
CpG 24	44.31	29.6	46.90	18.0-66.8
CpG 25	45.02	29.4	48.83	19.2-68.2
CpG 26	43.35	29.5	45.00	15.7-65.1
CpG 27	40.97	28.1	42.60	15.8-61.3
CpG 28	38.51	25.8	40.10	15.0-58.2
CpG 29	36.38	22.6	38.75	15.0-57.0
CpG 30	40.83	27.4	42.60	15.5-63.1
CpG 31	45.65	27.3	49.20	22.6-68.0
CpG 32	45.92	30.7	47.55	17.2-71.8
Mean	42.33	27.3	45.14	16.8-63.6

APPENDIX D (continued). METHYLATION VALUES BY INDIVIDUAL CpG SITE

TABLE XXXI.

TFF1 CPG SITE MEAN AND MEDIAN VALUES BY TISSUE TYPE

Adjacent normal				
Site	Mean	SD	Median	IQR
CpG 1	66.10	18.3	69.10	56.6-77.6
CpG 2	71.01	20.1	74.55	60.5-84.5
CpG 3	69.81	19.1	73.45	61.2-82.5
CpG 4	71.48	20.6	76.80	61.0-85.4
CpG 5	65.22	20.9	69.40	54.7-78.1
Mean	68.72	18.2	72.70	59.8-80.4

In situ				
Site	Mean	SD	Median	IQR
CpG 1	40.81	23.8	36.85	23.4-57.7
CpG 2	47.99	23.6	44.80	30.3-64.9
CpG 3	43.48	24.5	40.50	24.4-59.8
CpG 4	45.03	25.2	41.45	26.3-62.8
CpG 5	41.81	24.5	38.50	23.7-58.3
Mean	43.82	23.5	40.25	25.9-61.2

Invasive				
Site	Mean	SD	Median	IQR
CpG 1	41.01	23.6	37.00	24.4-56.6
CpG 2	48.31	24.3	44.10	31.7-63.8
CpG 3	44.74	25.2	39.90	27.0-61.2
CpG 4	45.48	25.5	41.30	27.9-60.5
CpG 5	42.82	23.7	39.20	26.5-58.9
Mean	44.47	23.9	40.15	27.9-60.0

APPENDIX D (continued). METHYLATION VALUES BY INDIVIDUAL CpG SITE

TABLE XXXII.

SAT2 CPG SITE MEAN AND MEDIAN VALUES BY TISSUE TYPE

Adjacent normal				
Site	Mean	SD	Median	IQR
CpG 1	67.85	11.0	69.00	64.0-75.0
CpG 2	47.51	10.5	48.8	43.1-53.9
Mean	57.68	10.3	59.05	54.2-63.8

In situ				
Site	Mean	SD	Median	IQR
CpG 1	59.49	12.3	61.75	53.2-67.4
CpG 2	42.70	11.6	43.75	35.4-50.7
Mean	51.09	11.5	52.55	44.1-59.1

Invasive				
Site	Mean	SD	Median	IQR
CpG 1	58.63	16.0	63.80	50.2-70.0
CpG 2	39.96	13.2	41.60	32.5-49.6
Mean	49.30	14.1	52.45	42.3-59.3

APPENDIX E. SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

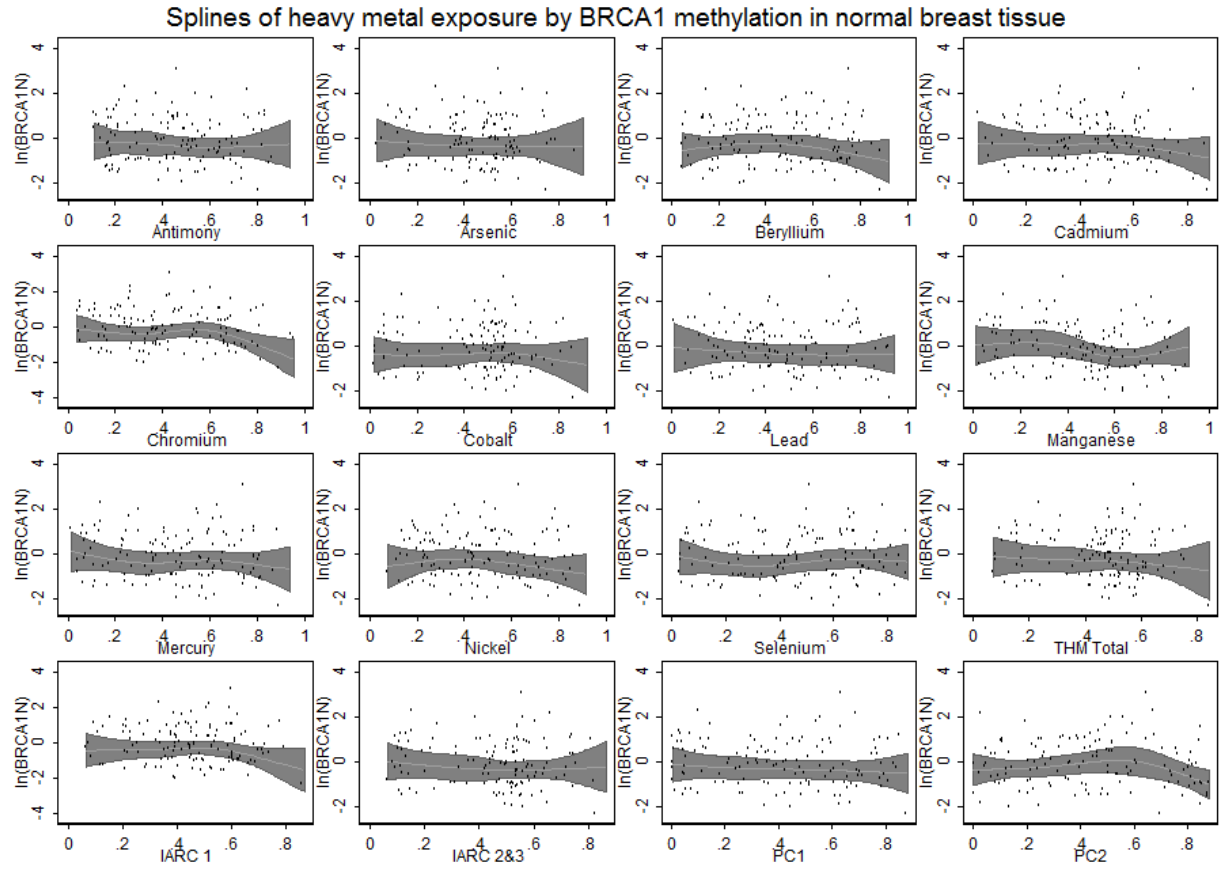

Figure 13. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *BRCA1* methylation in adjacent normal samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

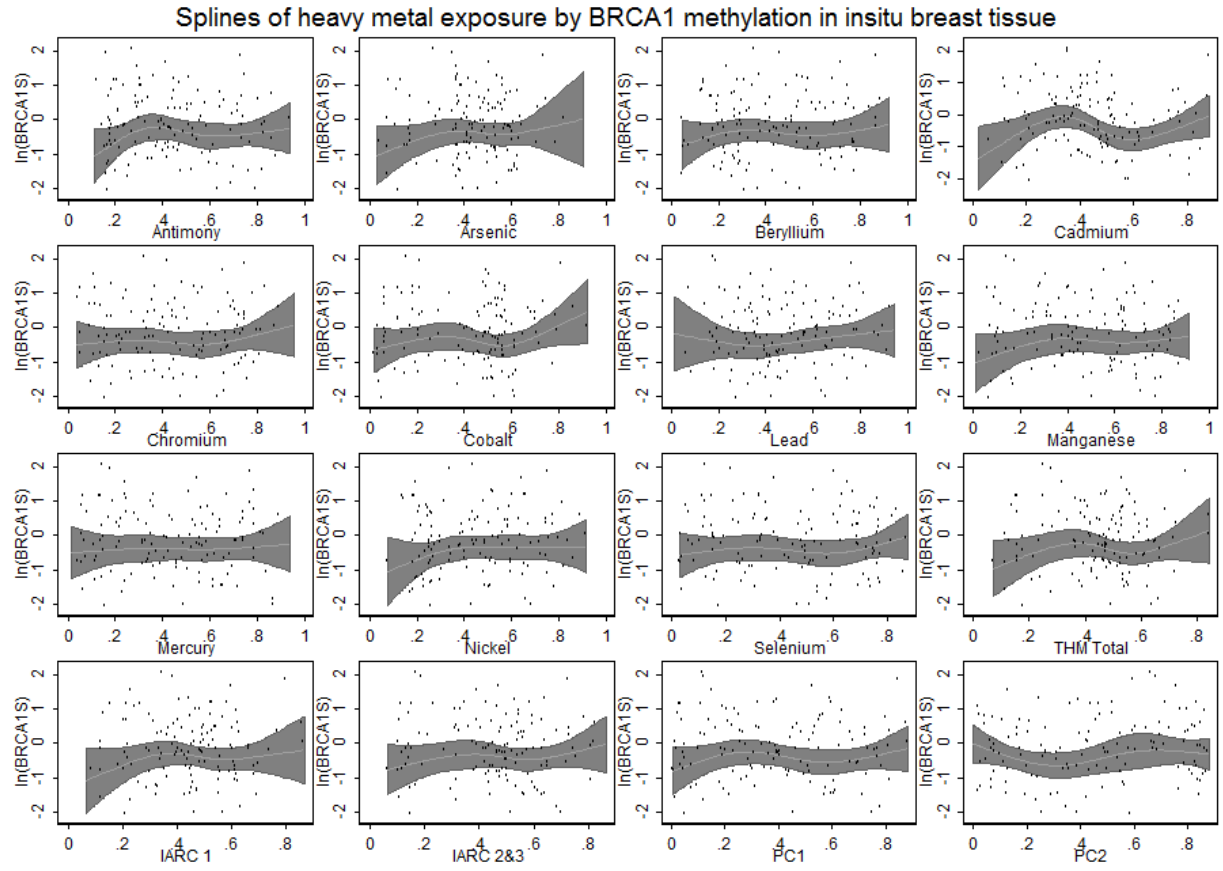

Figure 14. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *BRCA1* methylation in *in situ* samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

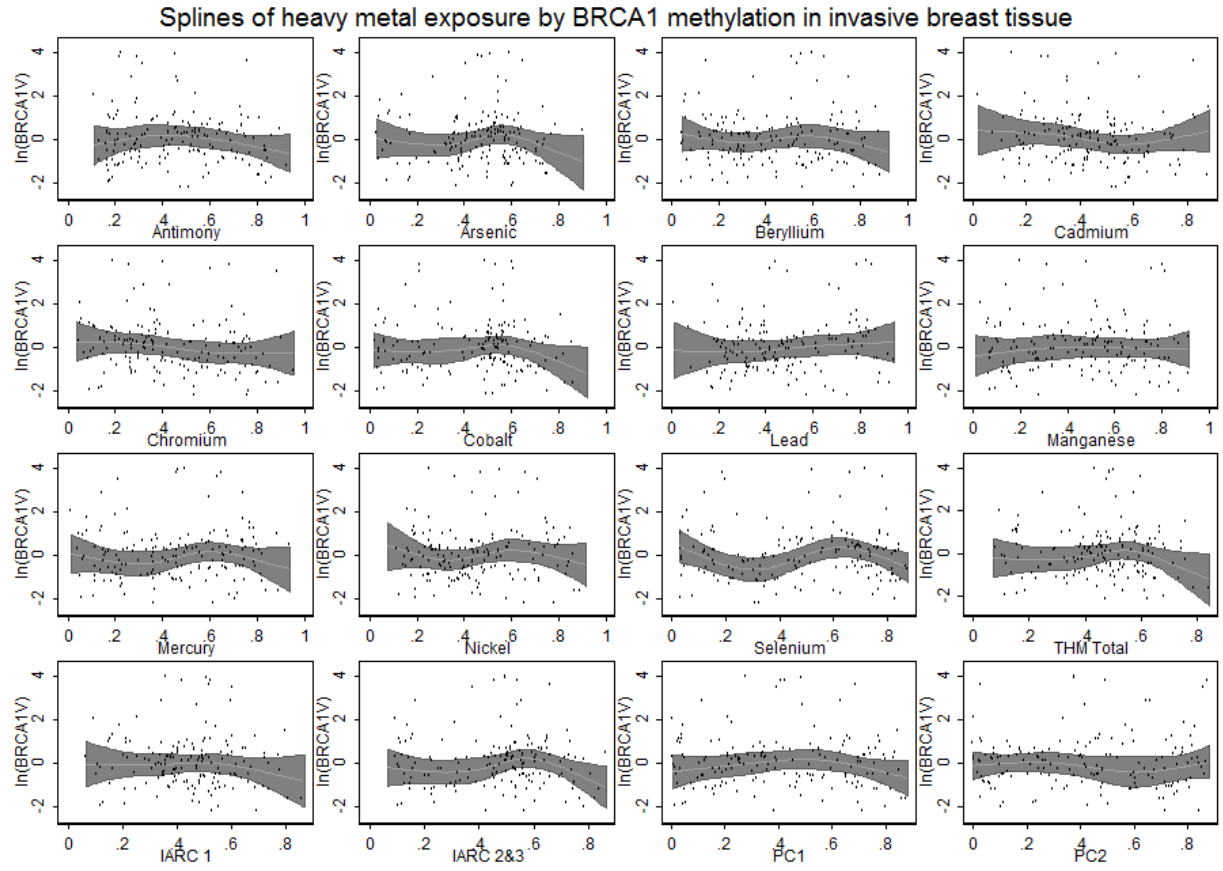

Figure 15. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *BRCA1* methylation in invasive samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

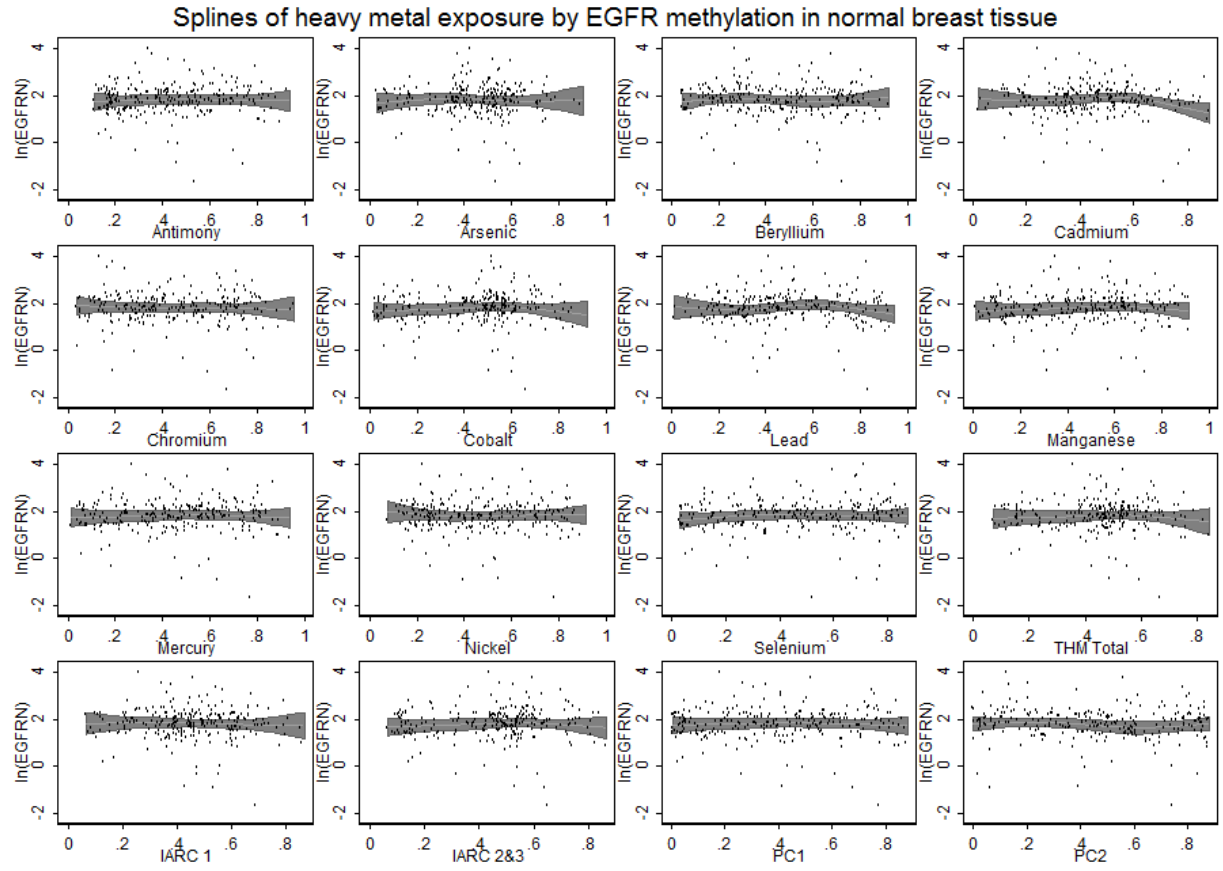

Figure 16. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *EGFR* methylation in adjacent normal samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

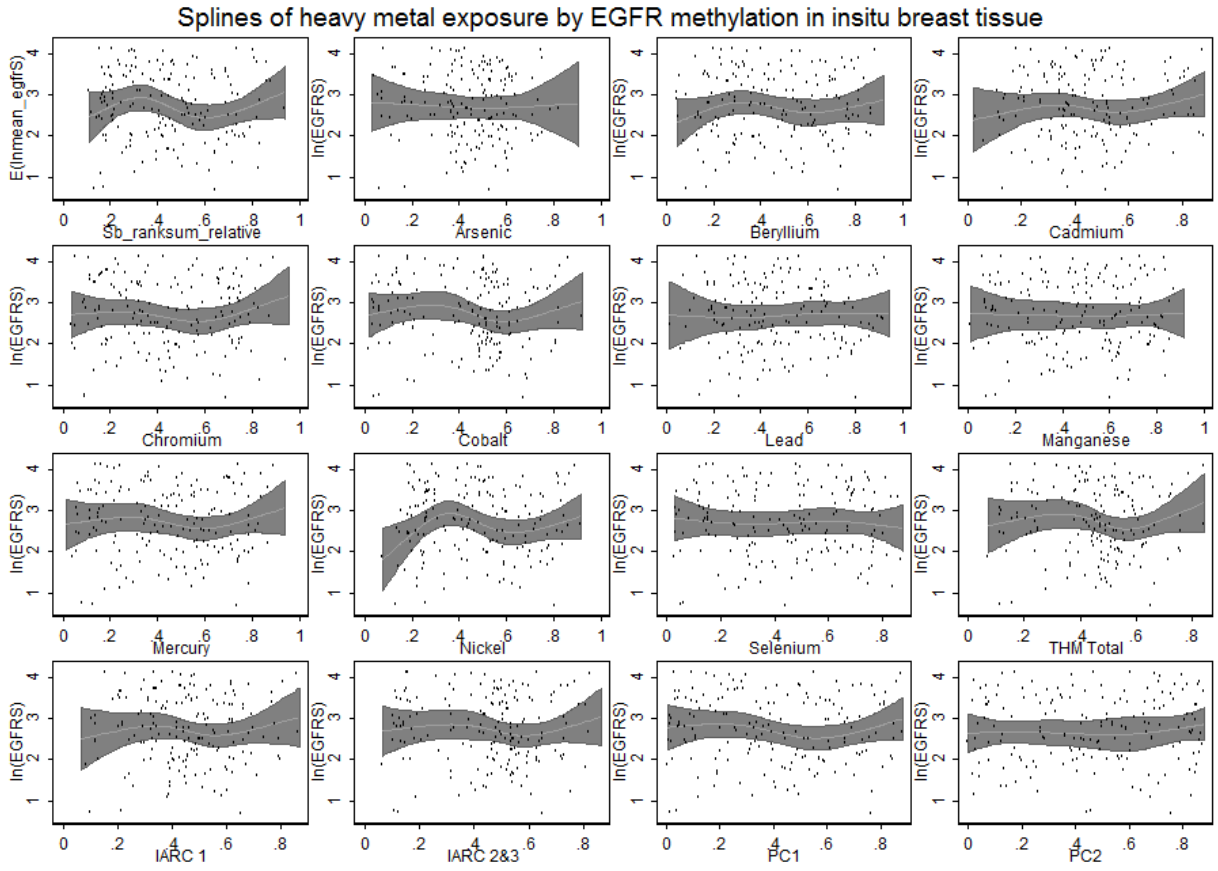

Figure 17. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *EGFR* methylation in *in situ* samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

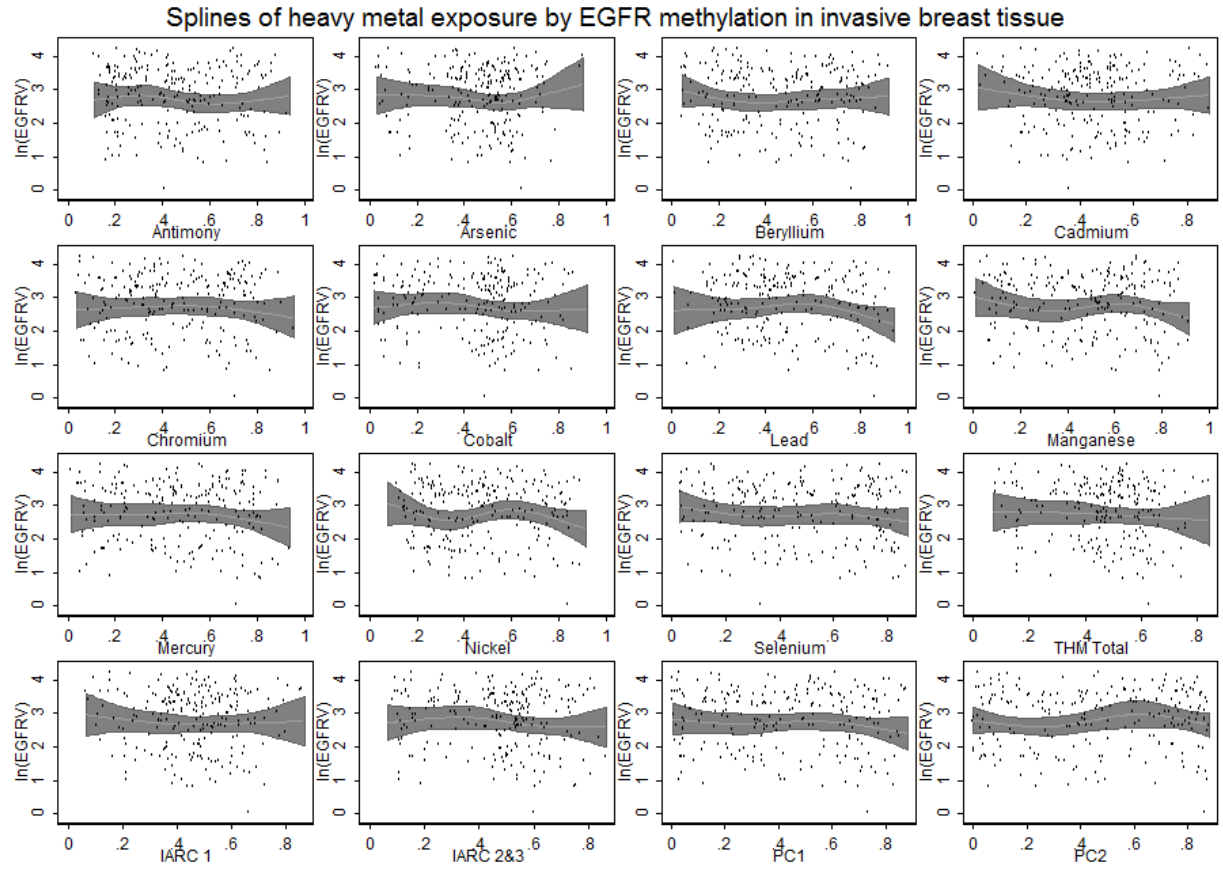

Figure 18. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *EGFR* methylation in invasive samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

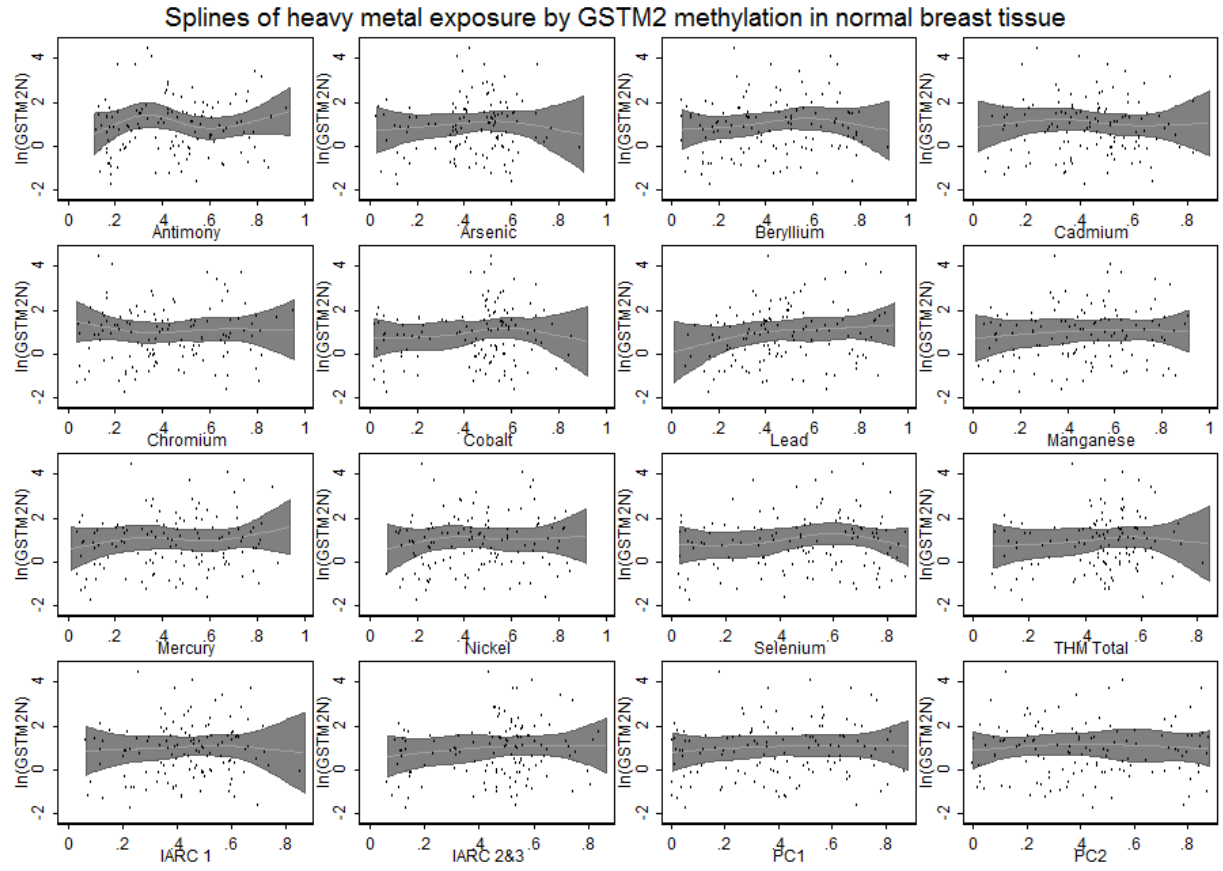

Figure 19. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *GSTM2* methylation in adjacent normal samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

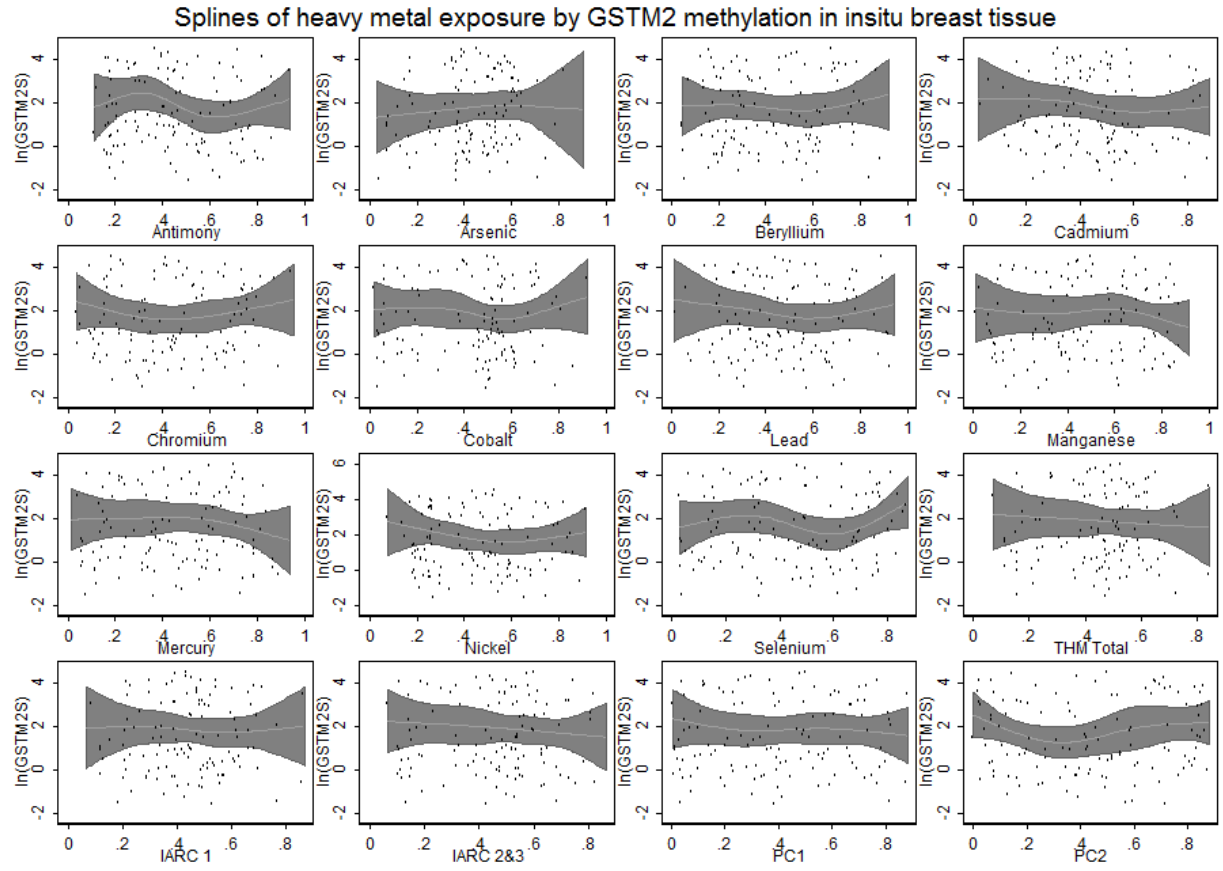

Figure 20. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *GSTM2* methylation in *in situ* samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

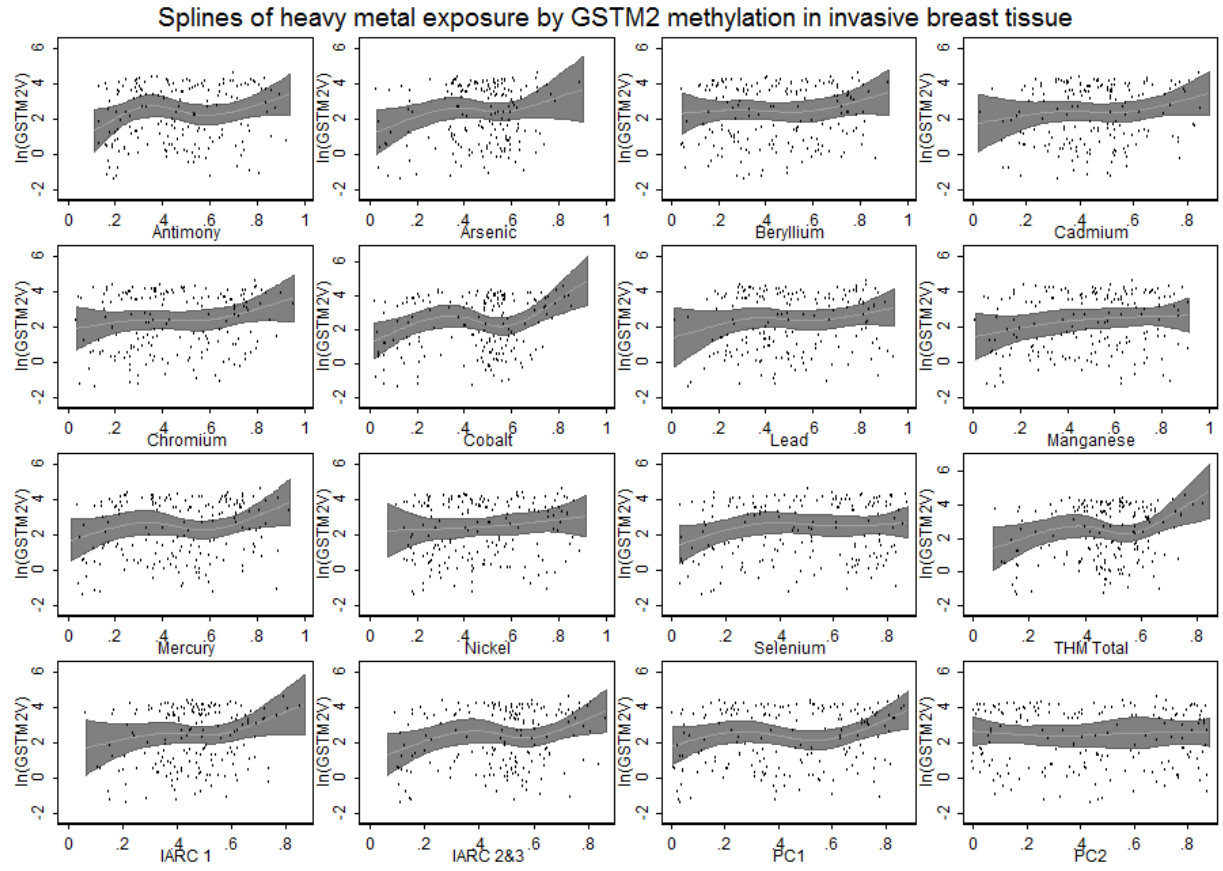

Figure 21. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *GSTM2* methylation in invasive samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

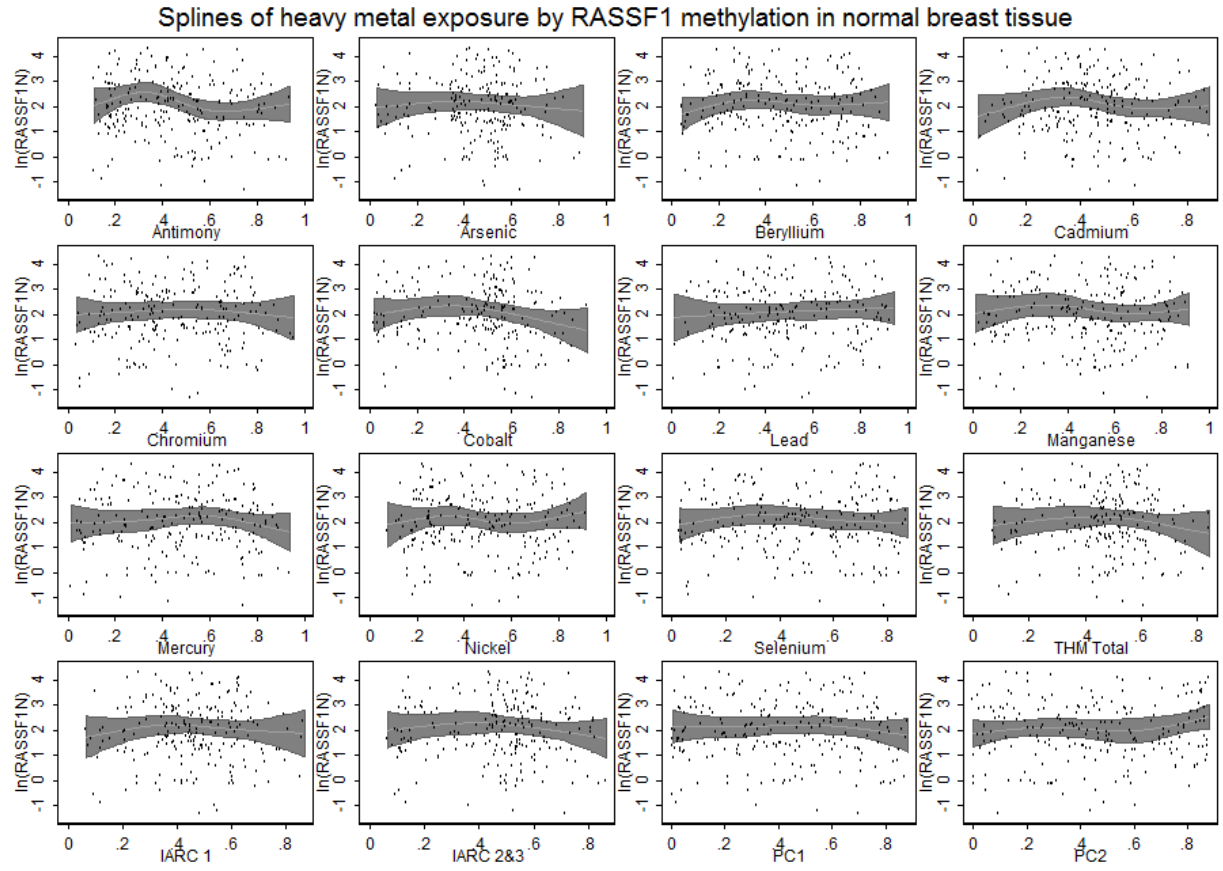

Figure 22. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *RASSF1* methylation in adjacent normal samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

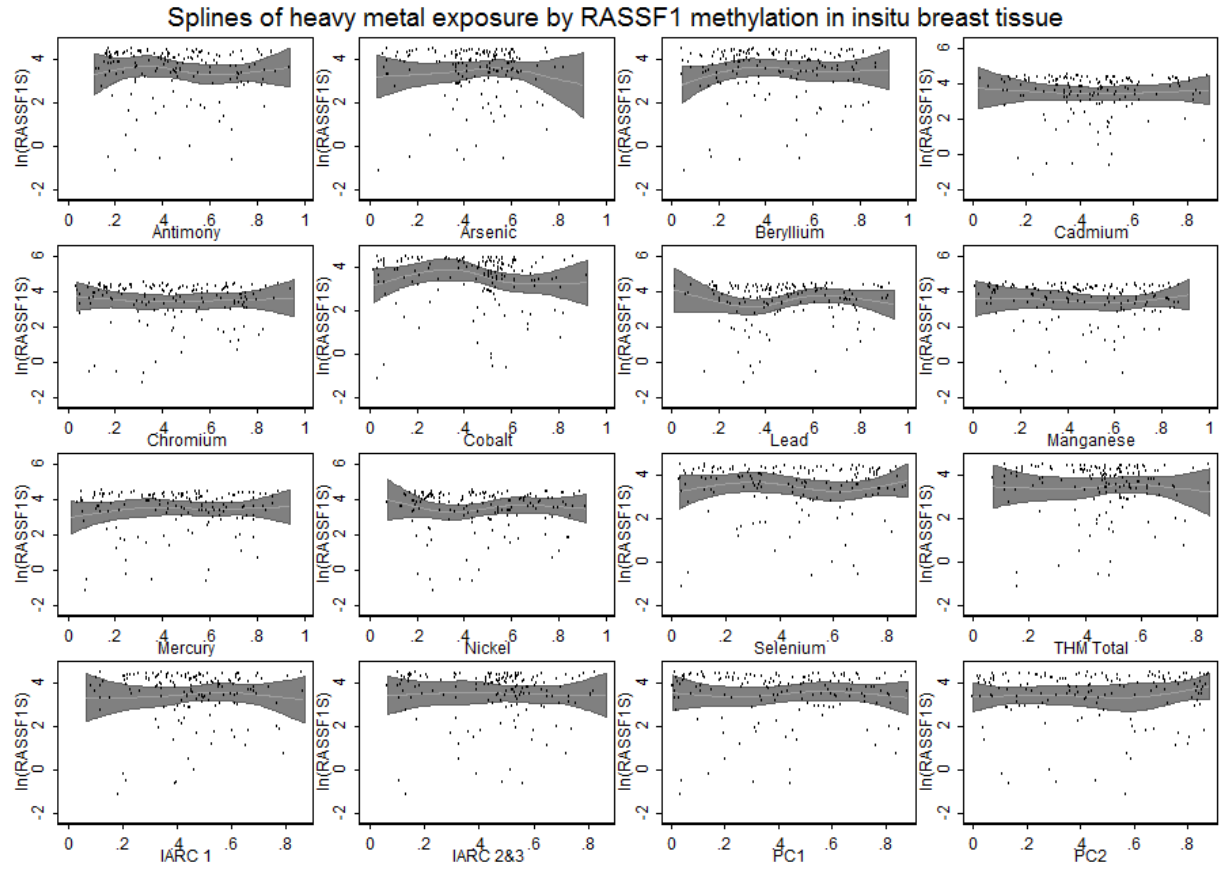

Figure 23. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *RASSF1* methylation in *in situ* samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

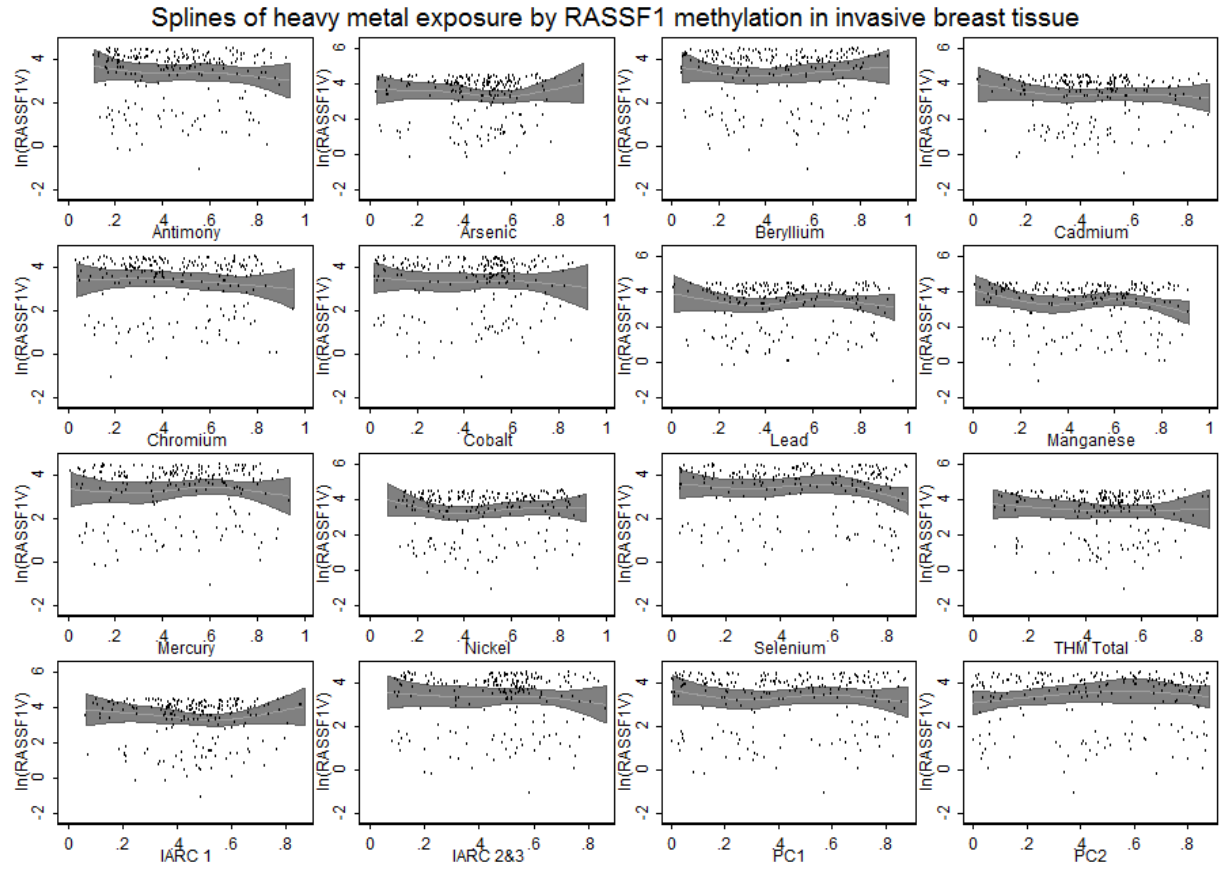

Figure 24. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *RASSF1* methylation in invasive samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

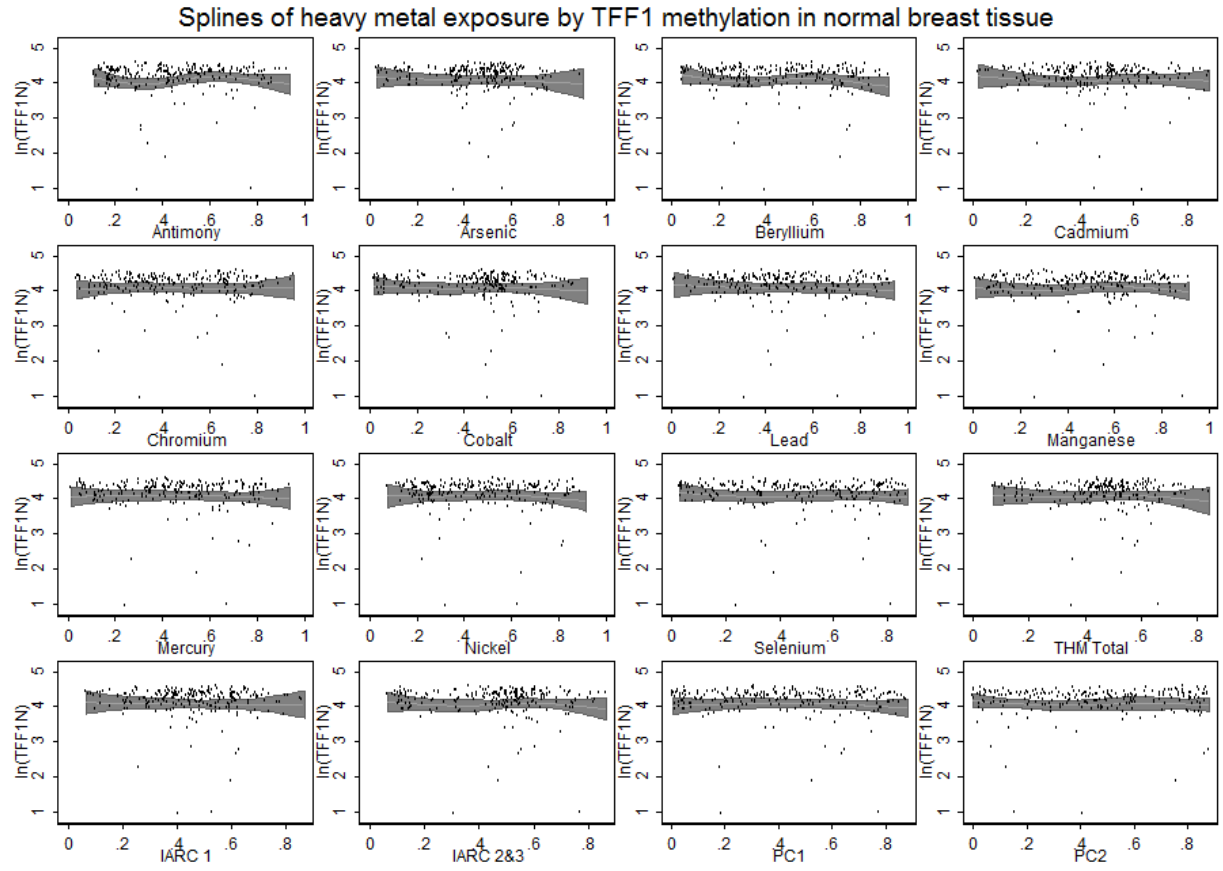

Figure 25. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *TFF1* methylation in adjacent normal samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

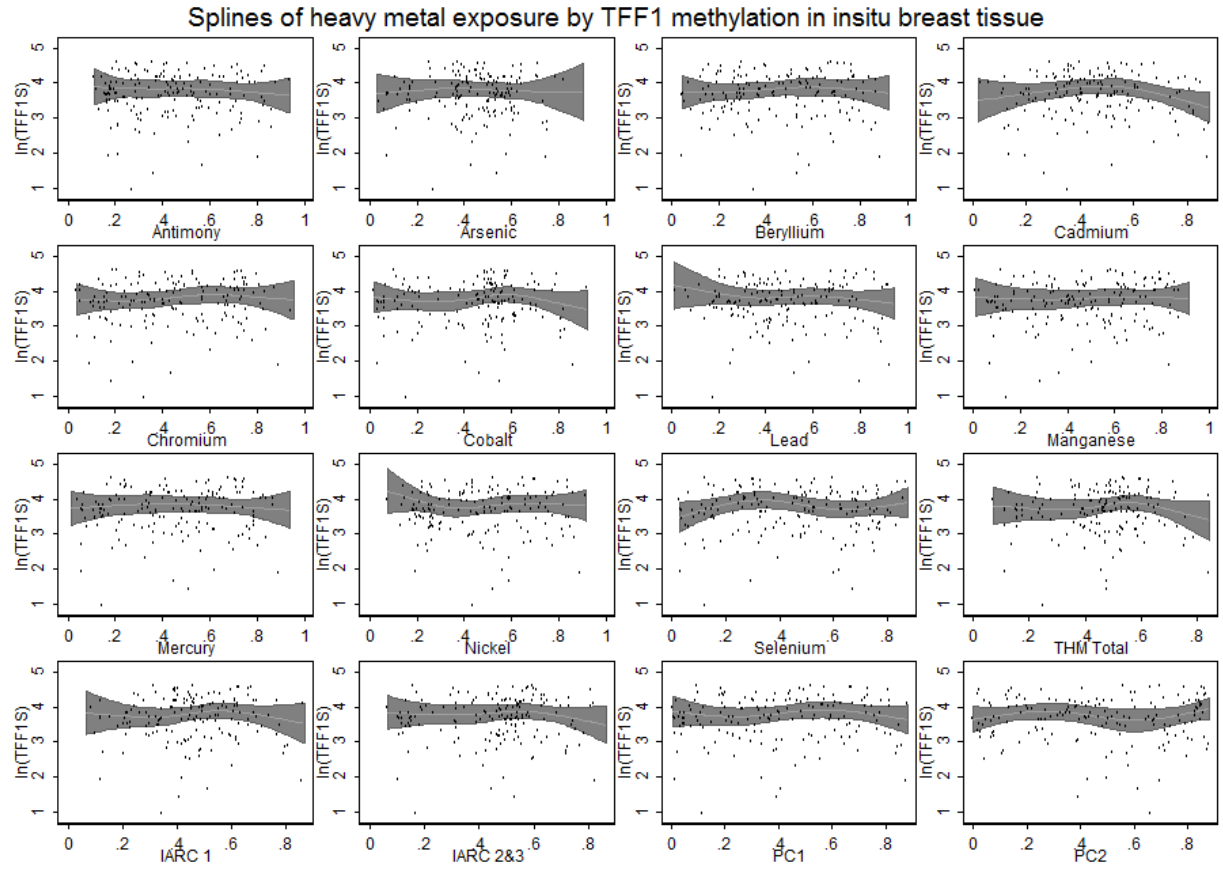

Figure 26. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *TFF1* methylation in *in situ* samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

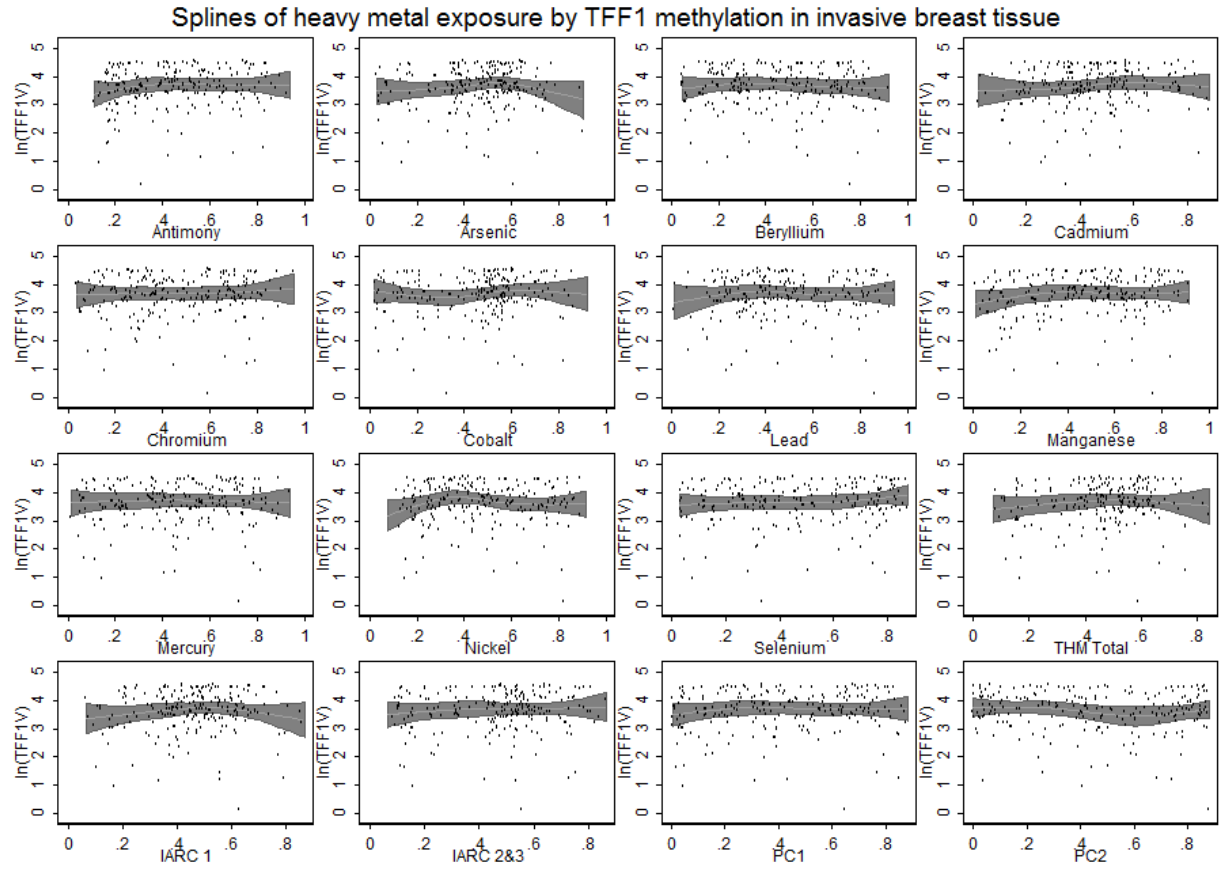

Figure 27. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with *TFF1* methylation in invasive samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

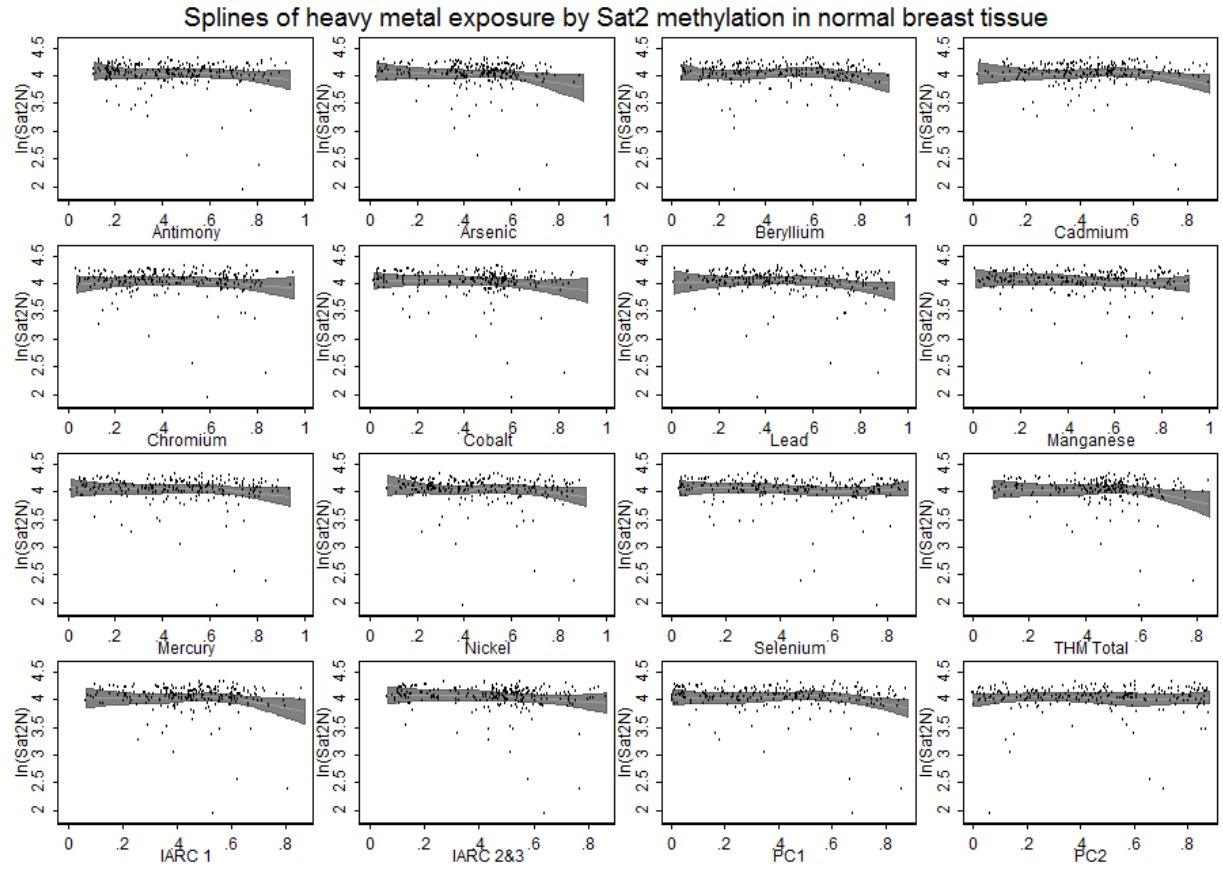

Figure 28. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with Sat2 methylation in adjacent normal samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

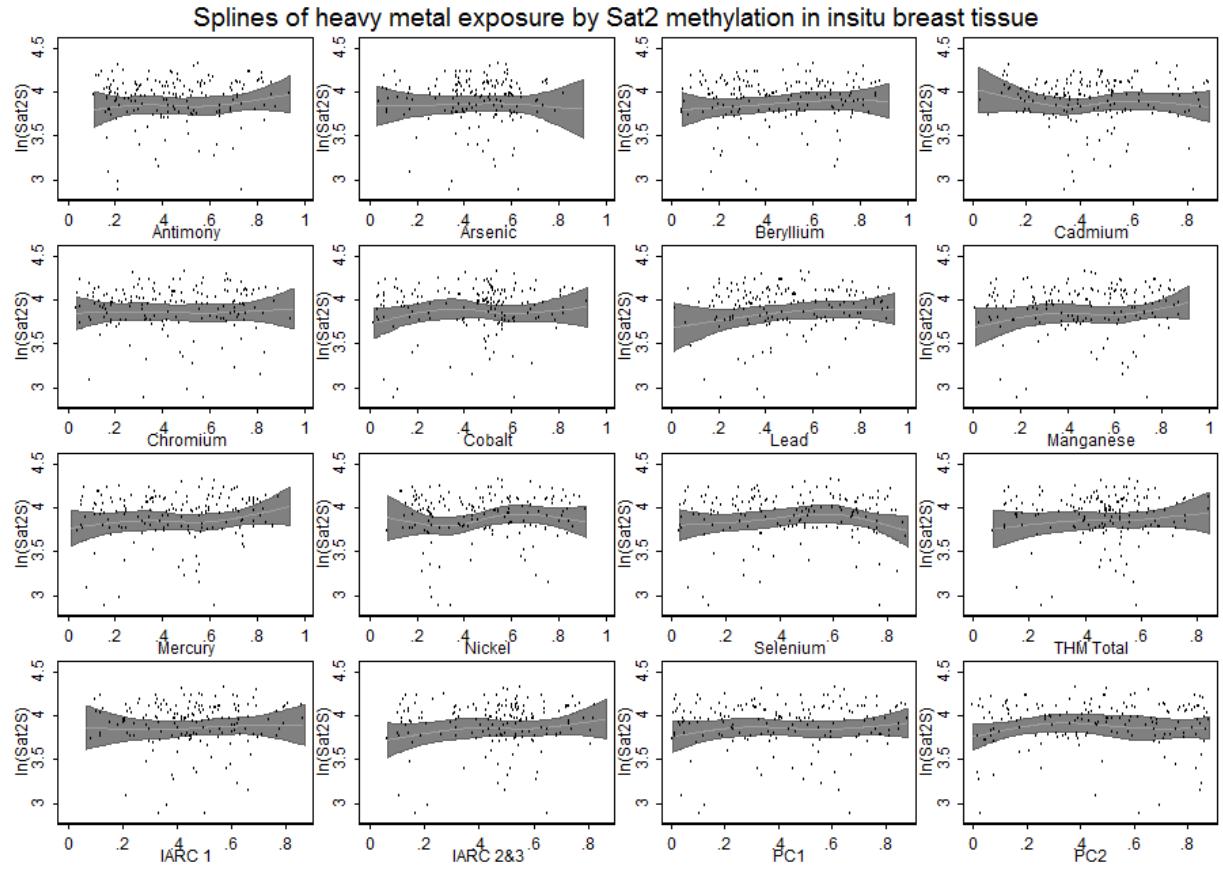

Figure 29. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with Sat2 methylation in *in situ* samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

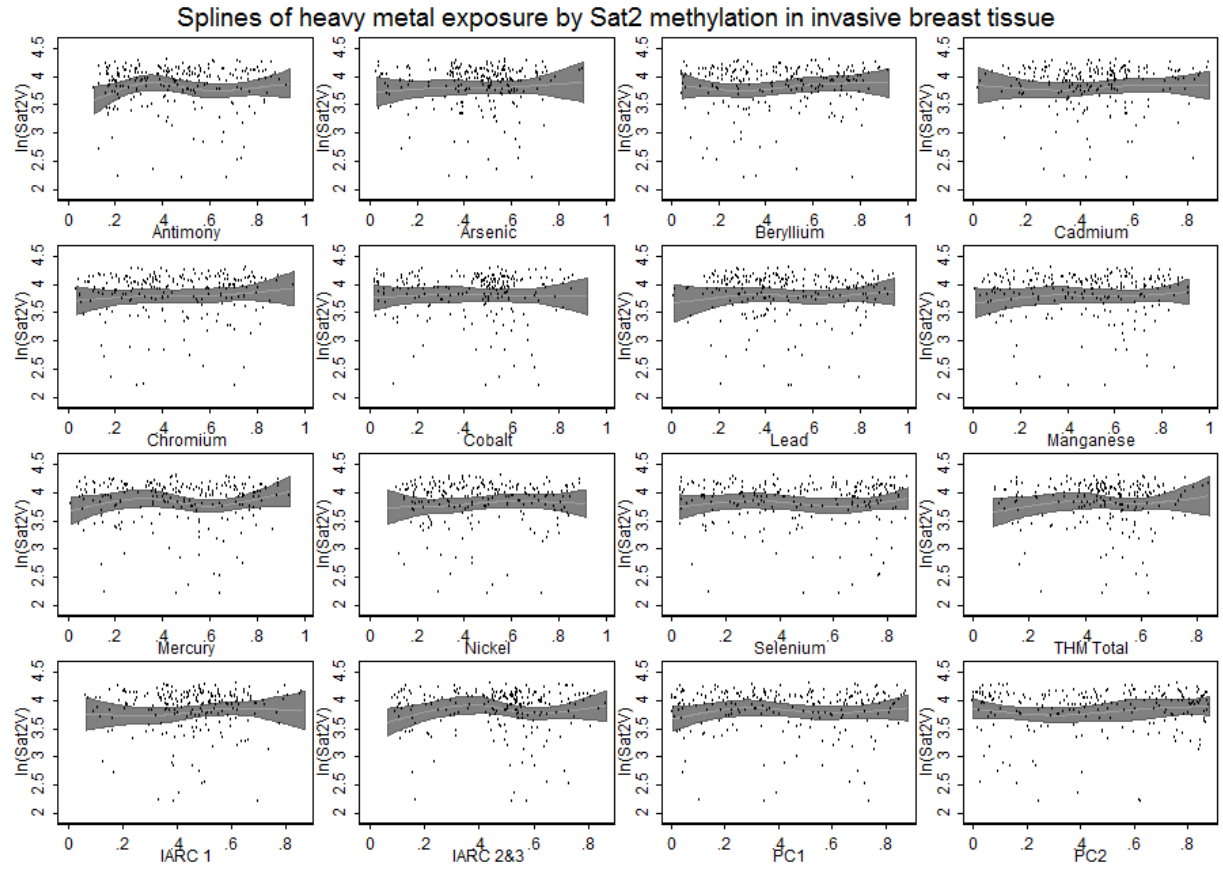

Figure 30. Splines depicting the shape of the association between the chronic single and combined heavy metal markers with Sat2 methylation in invasive samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

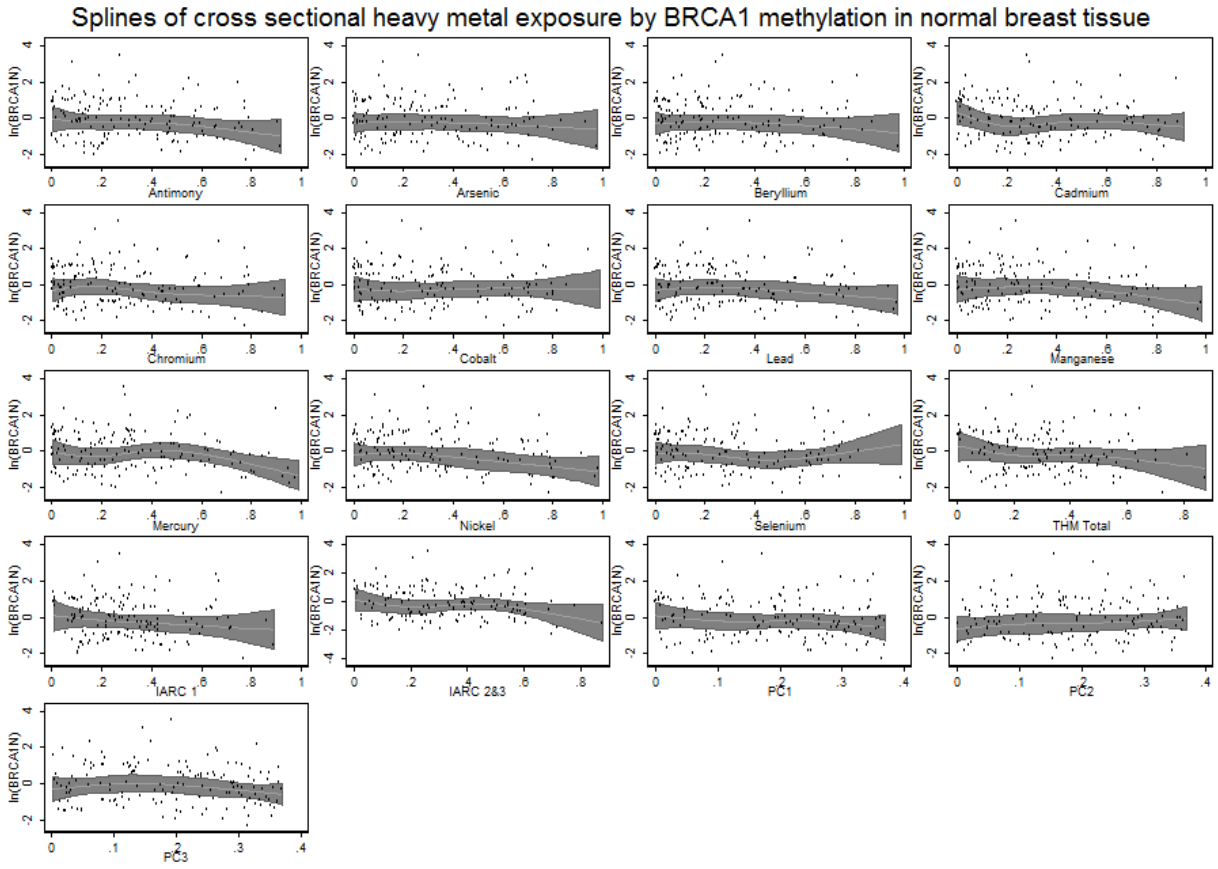

Figure 31. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *BRCA1* methylation in adjacent normal samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

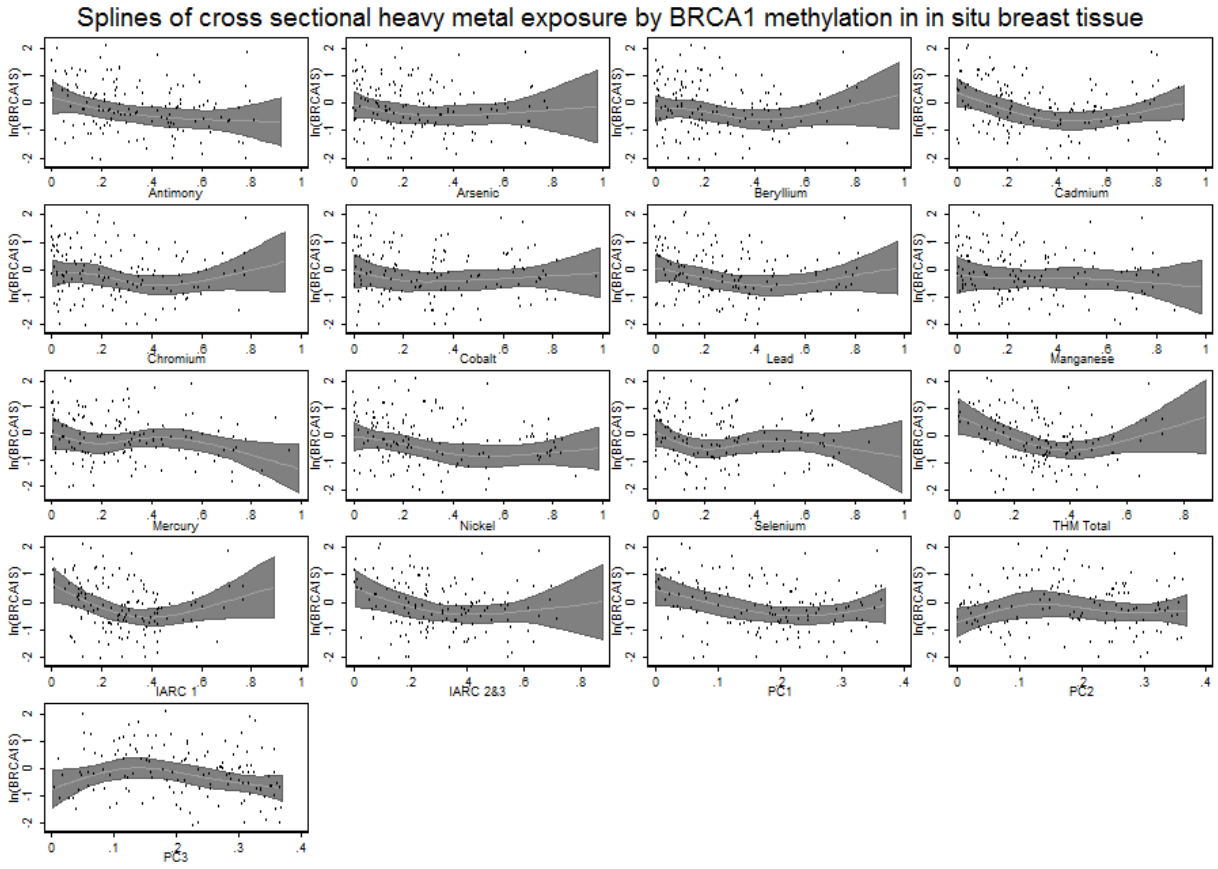

Figure 32. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *BRCA1* methylation in *in situ* samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

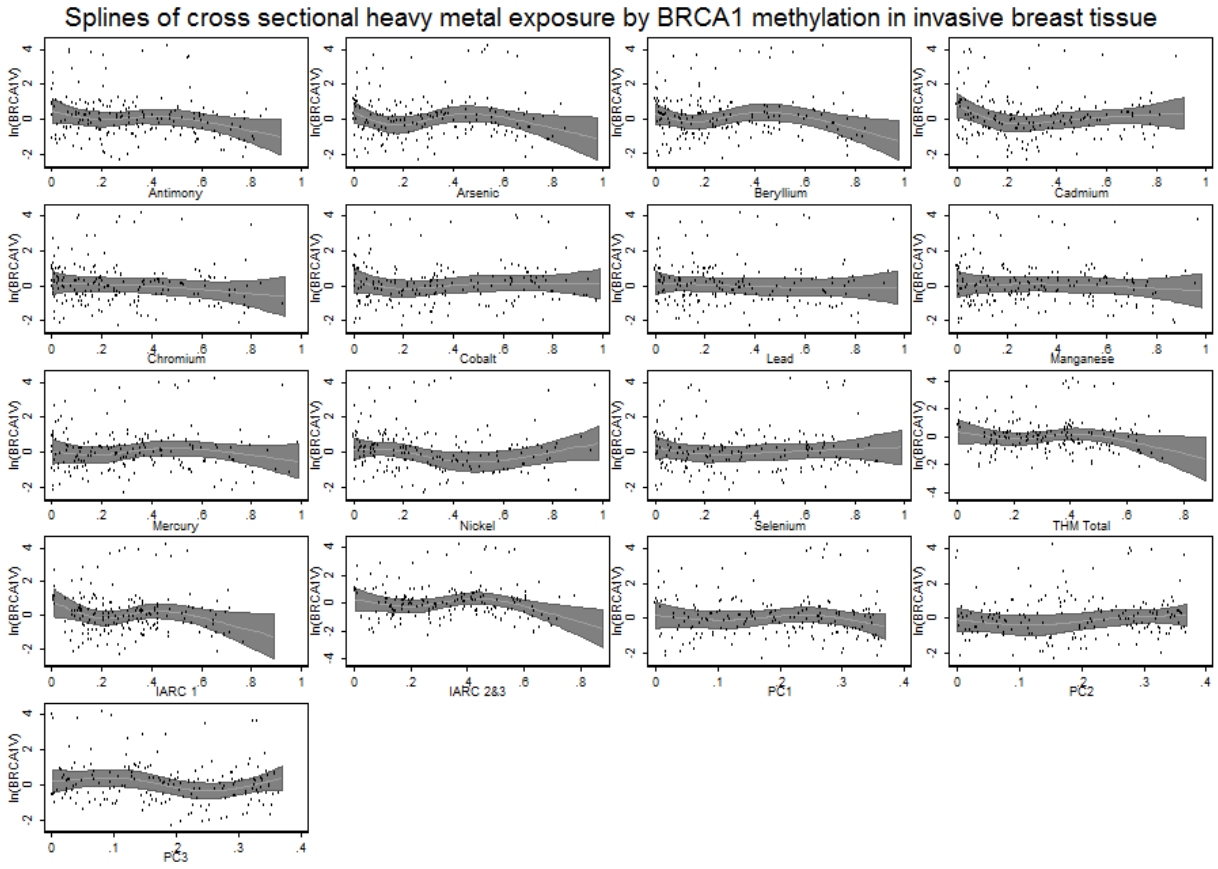

Figure 33. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *BRCA1* methylation in invasive samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

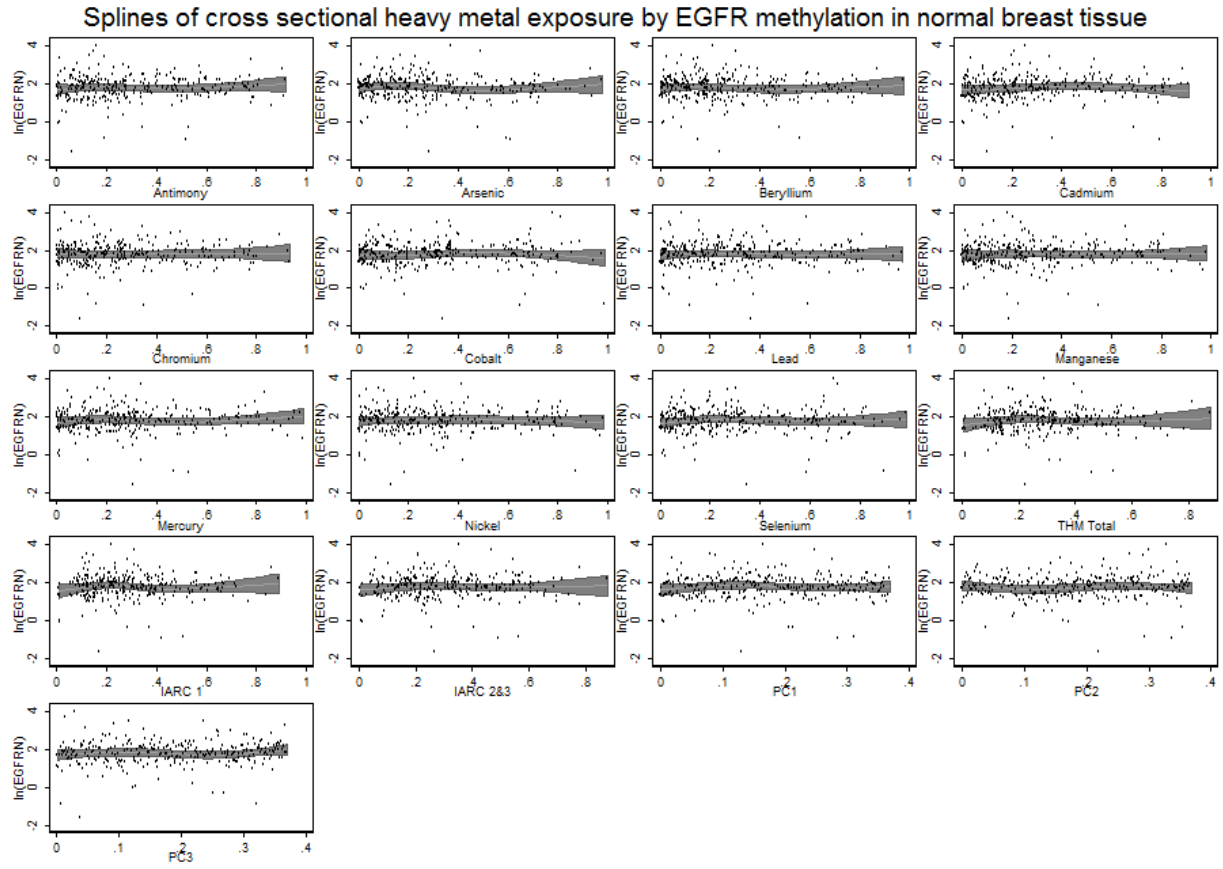

Figure 34. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *EGFR* methylation in adjacent normal samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

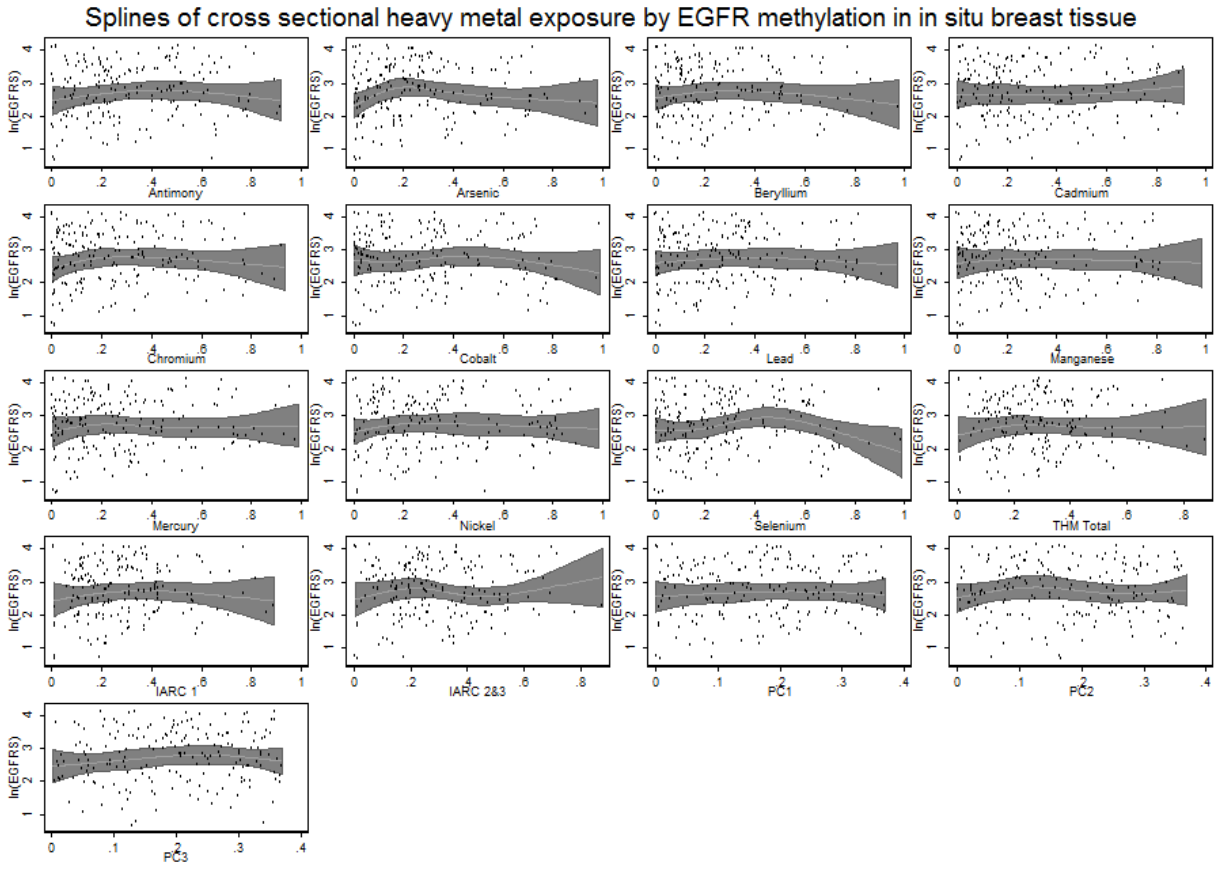

Figure 35. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *EGFR* methylation in *in situ* samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

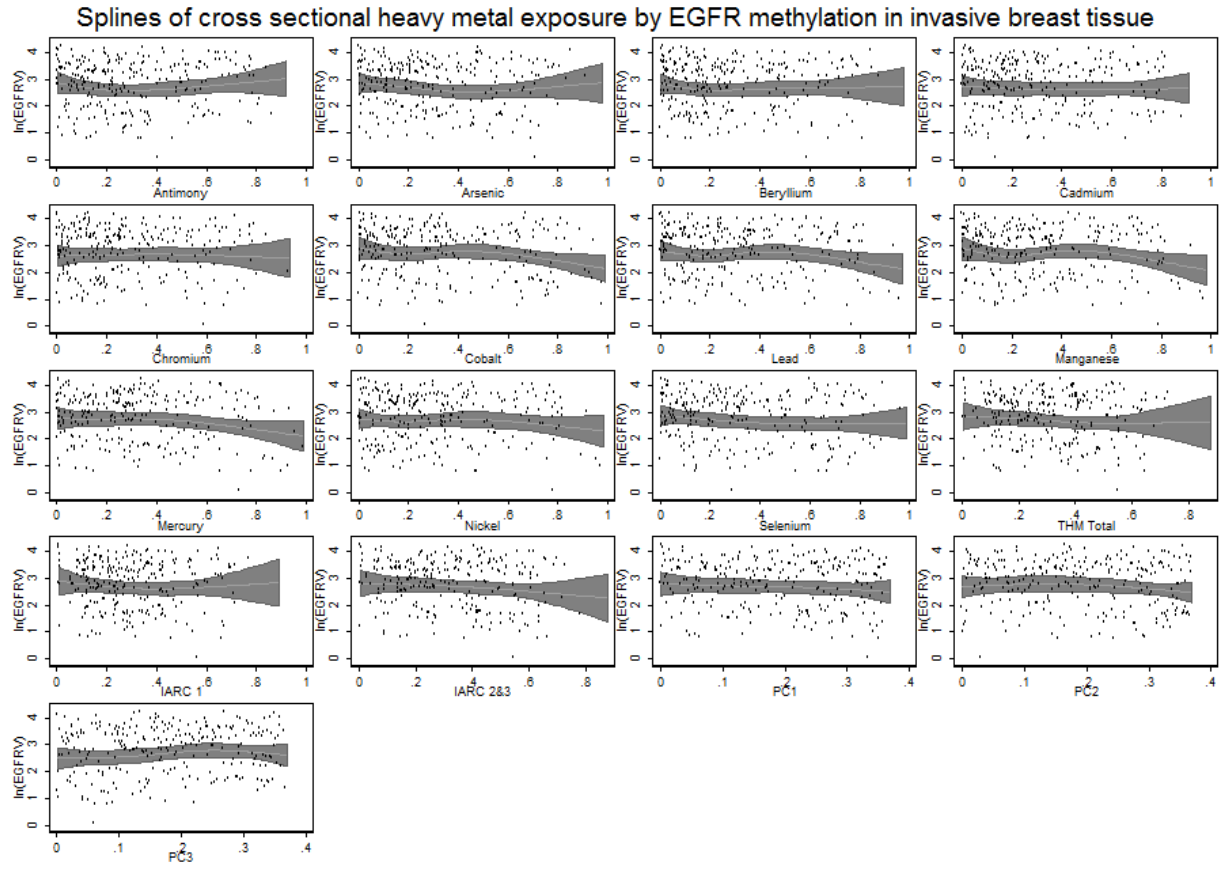

Figure 36. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *EGFR* methylation in invasive samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

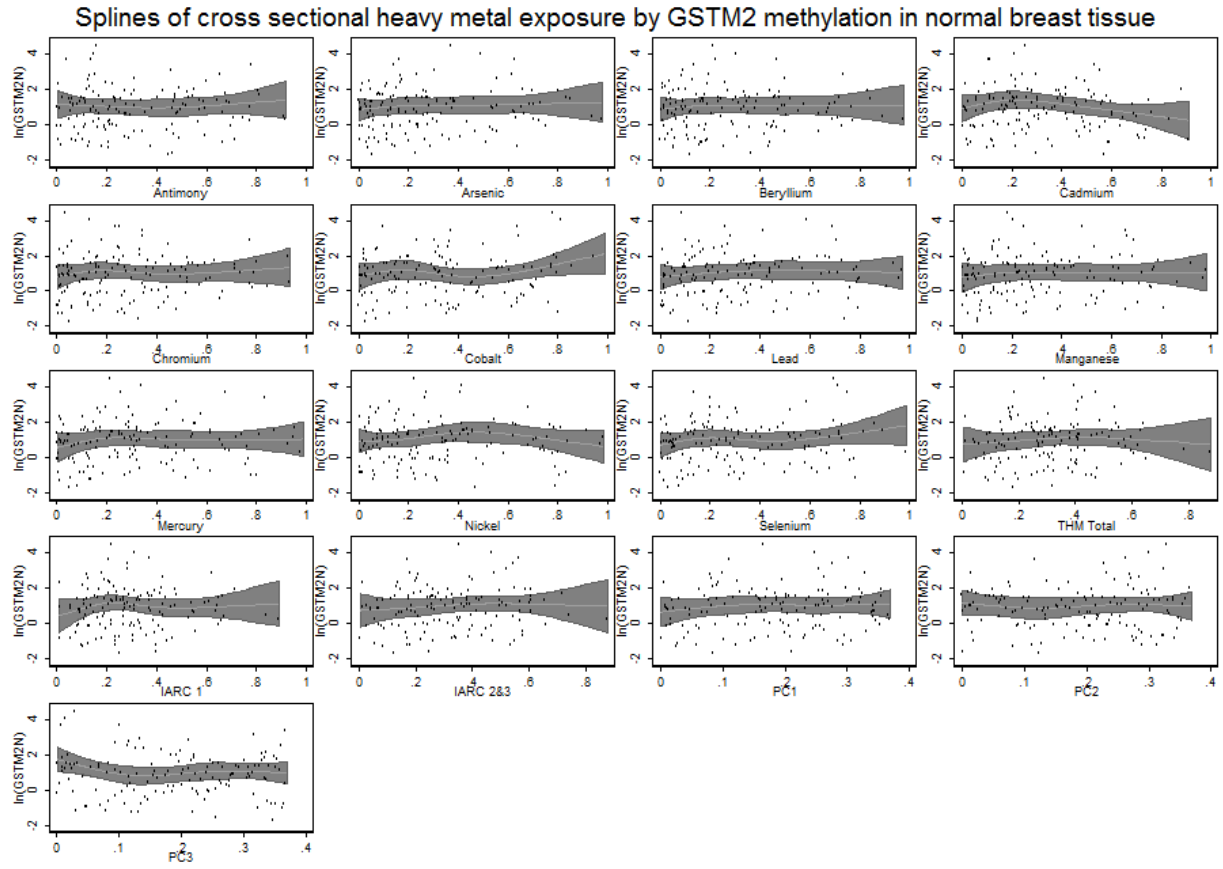

Figure 37. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *GSTM2* methylation in adjacent normal samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

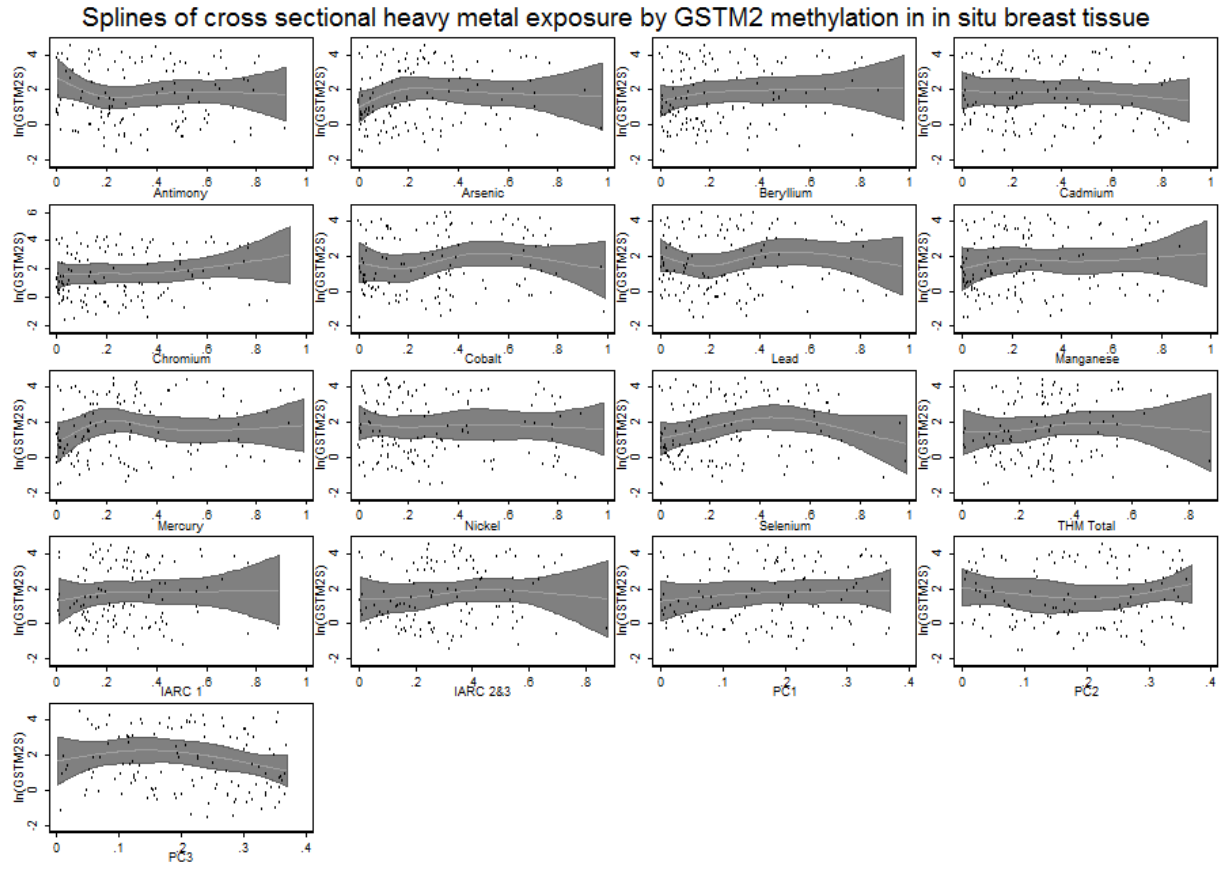

Figure 38. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *GSTM2* methylation in *in situ* samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

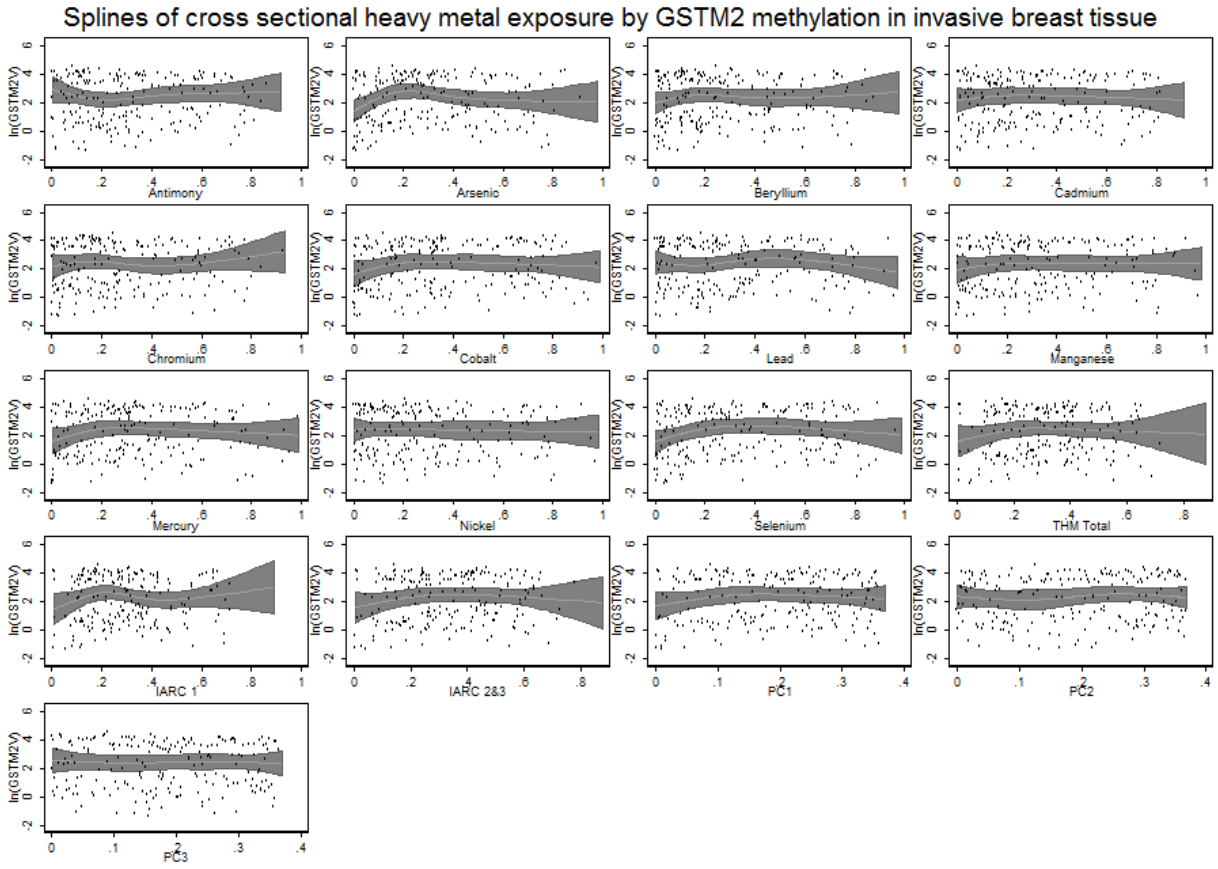

Figure 39. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *GSTM2* methylation in invasive samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

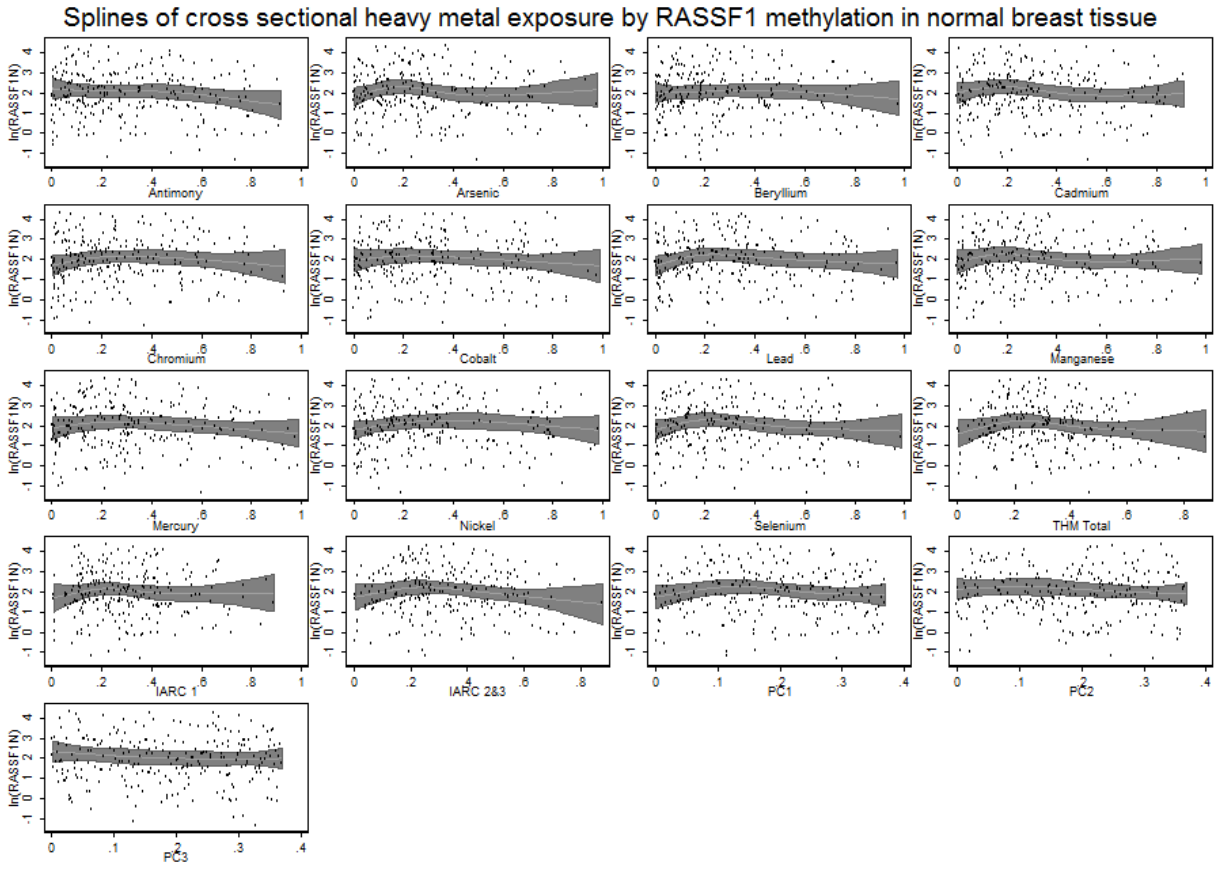

Figure 40. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *RASSF1* methylation in adjacent normal samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

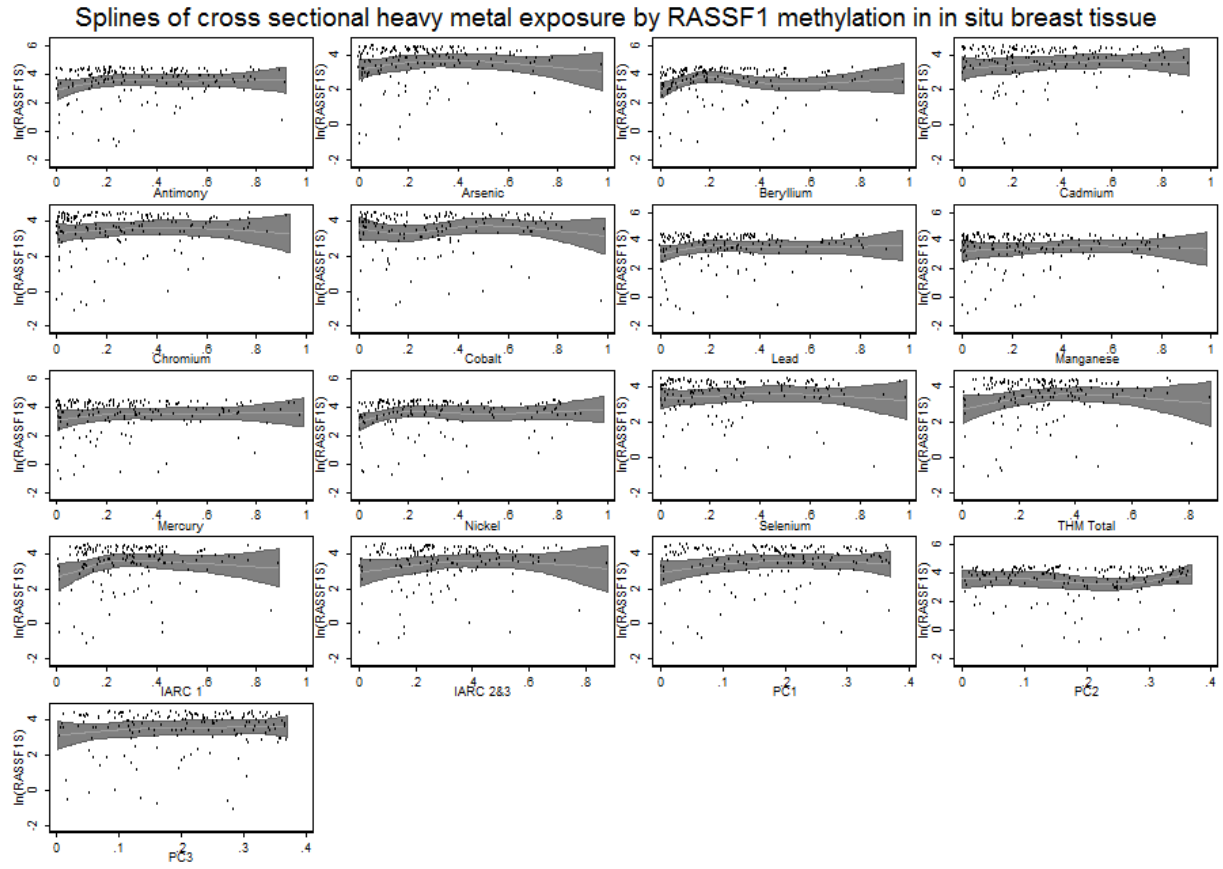

Figure 41. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *RASSF1* methylation in *in situ* samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

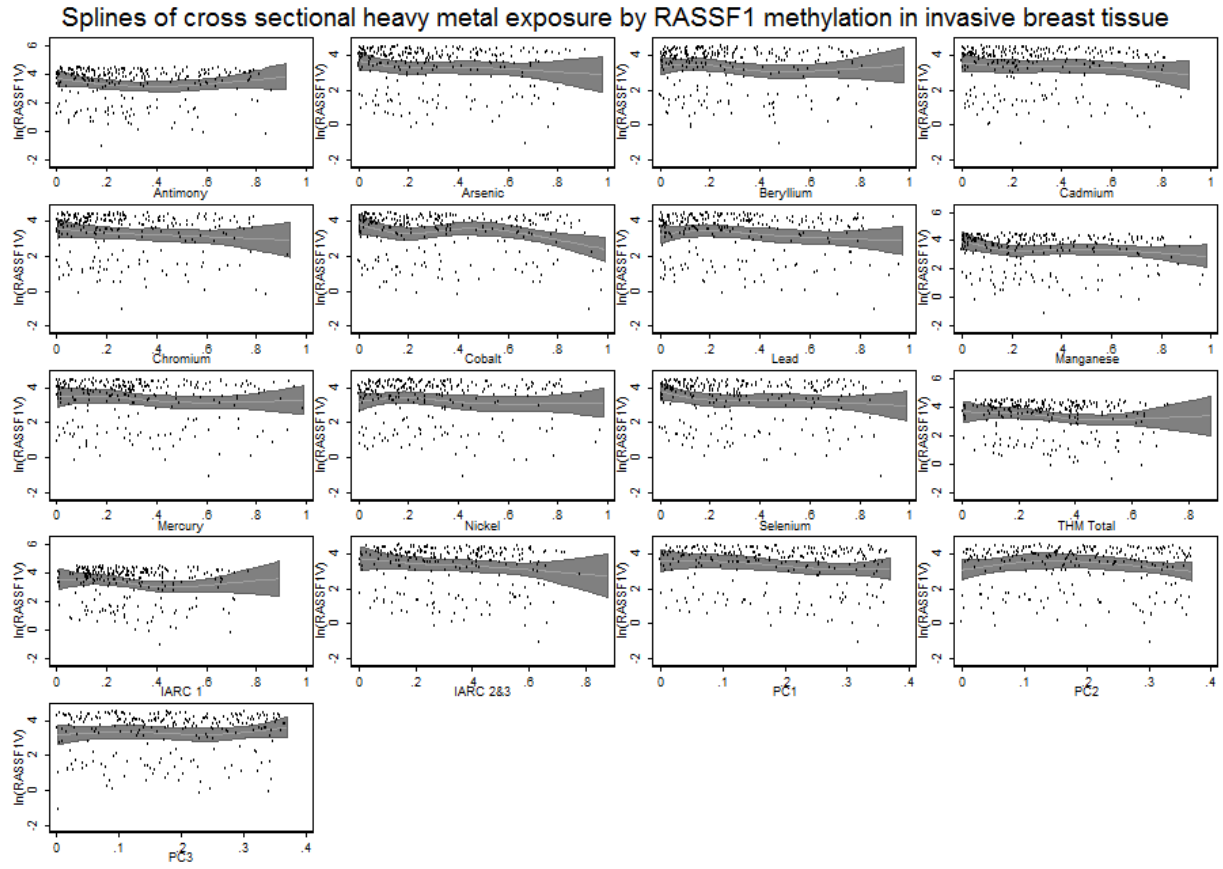

Figure 42. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *RASSF1* methylation in invasive samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

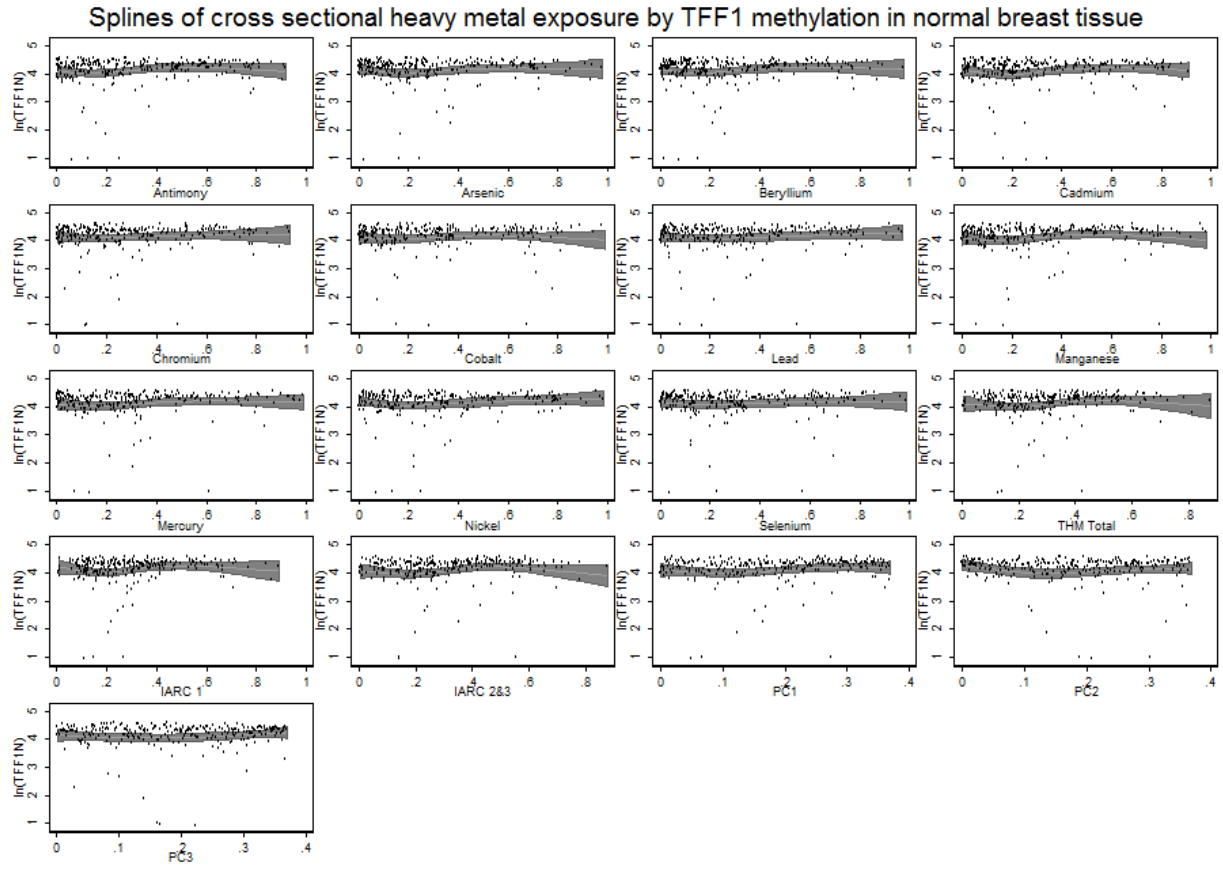

Figure 43. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *TFF1* methylation in adjacent normal samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

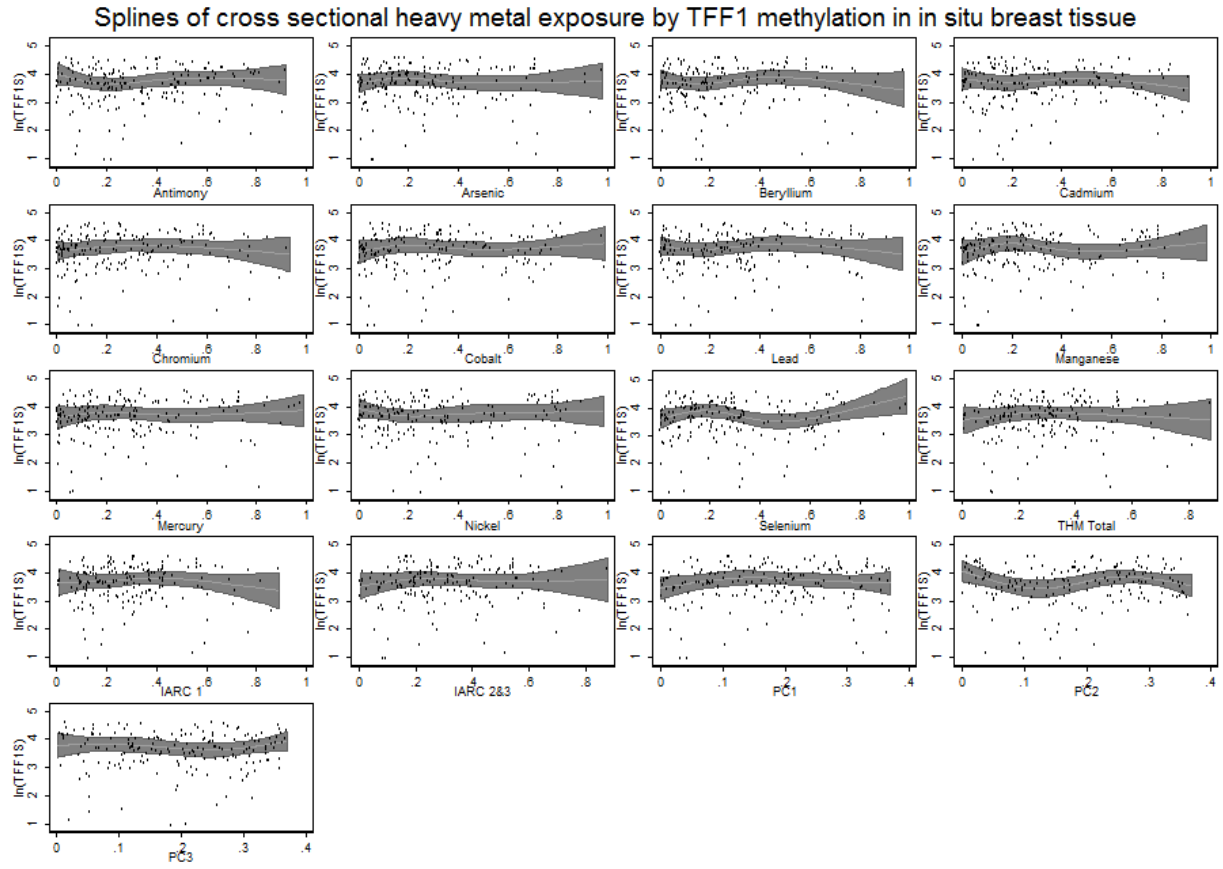

Figure 44. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *TFF1* methylation in *in situ* samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

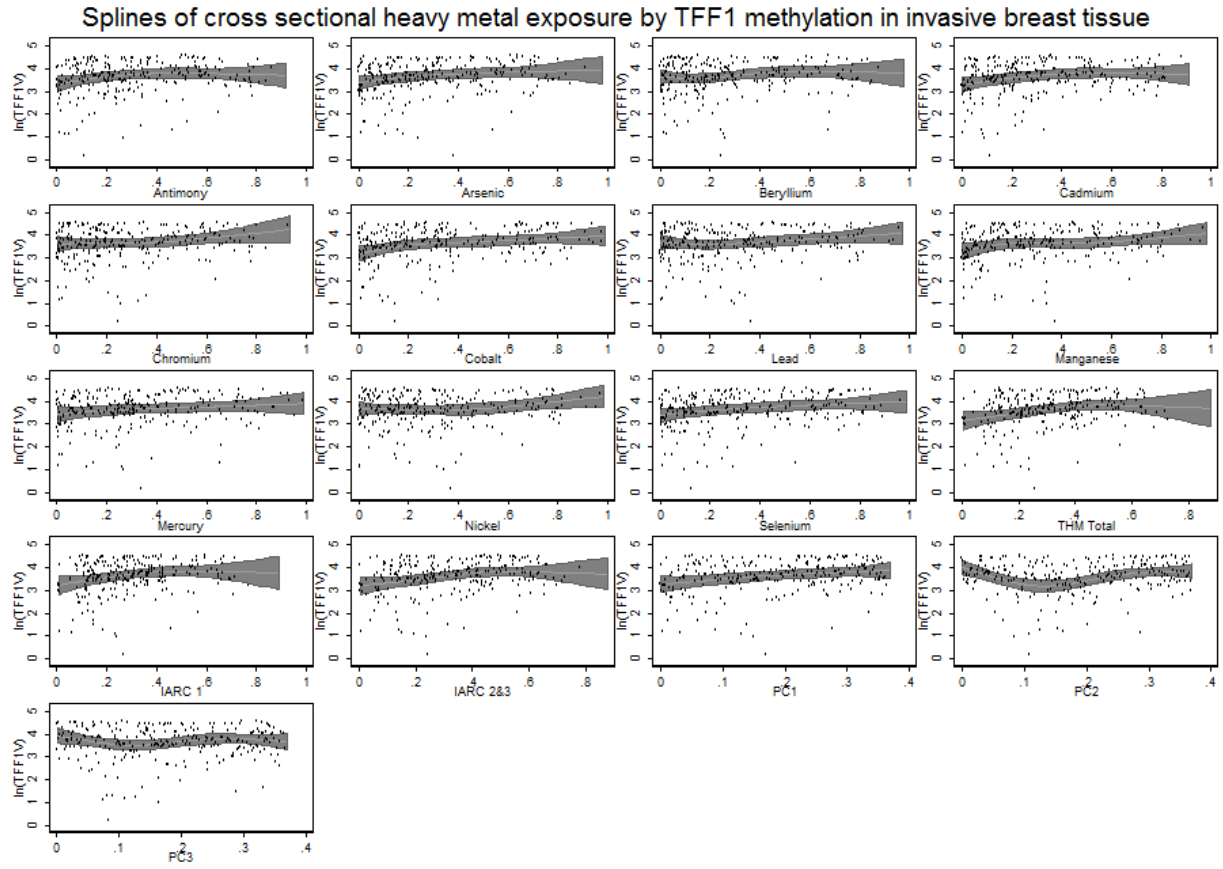

Figure 45. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with *TFF1* methylation in invasive samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

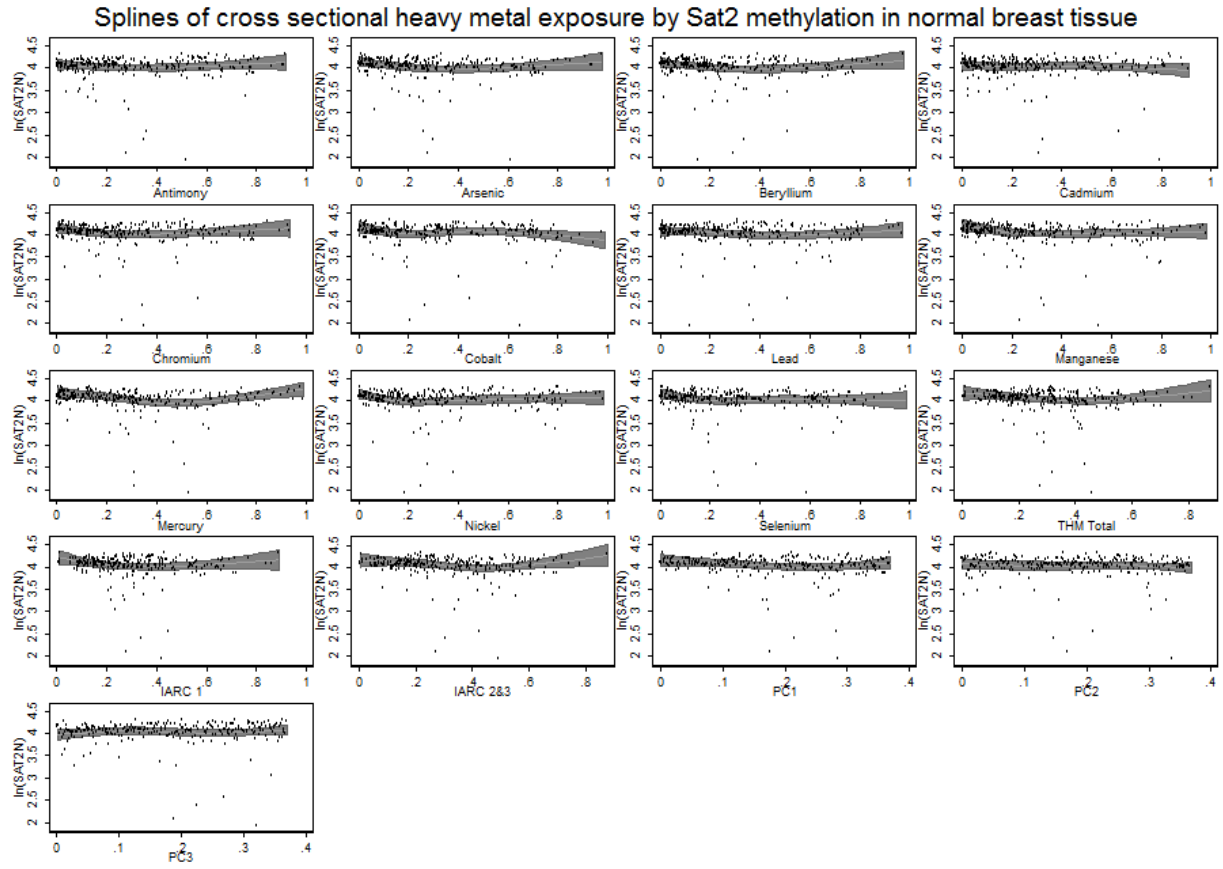

Figure 46. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with Sat2 methylation in adjacent normal samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

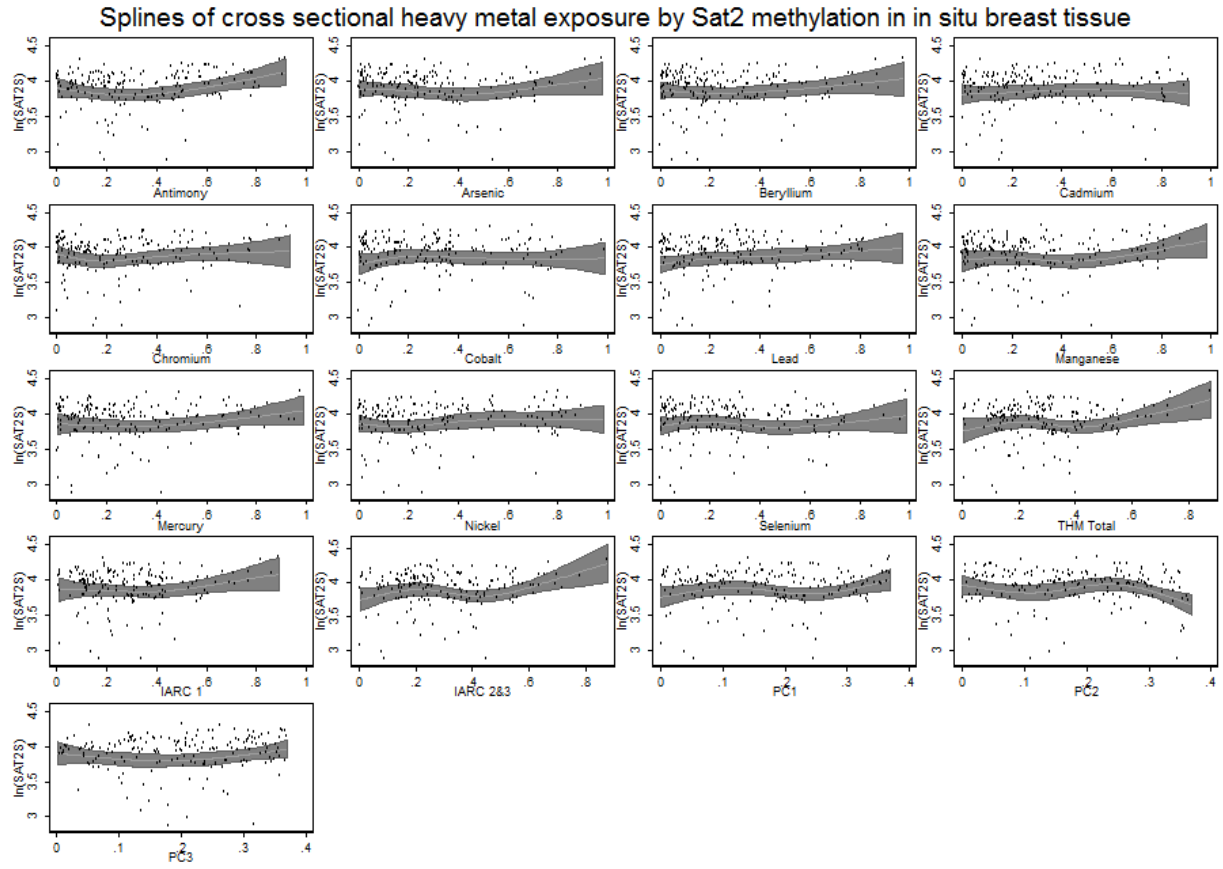

Figure 47. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with Sat2 methylation in *in situ* samples.

APPENDIX E (continued). SPLINES OF METAL EXPOSURE ASSOCIATIONS WITH METHYLATION BY GENE AND TISSUE COMPONENT

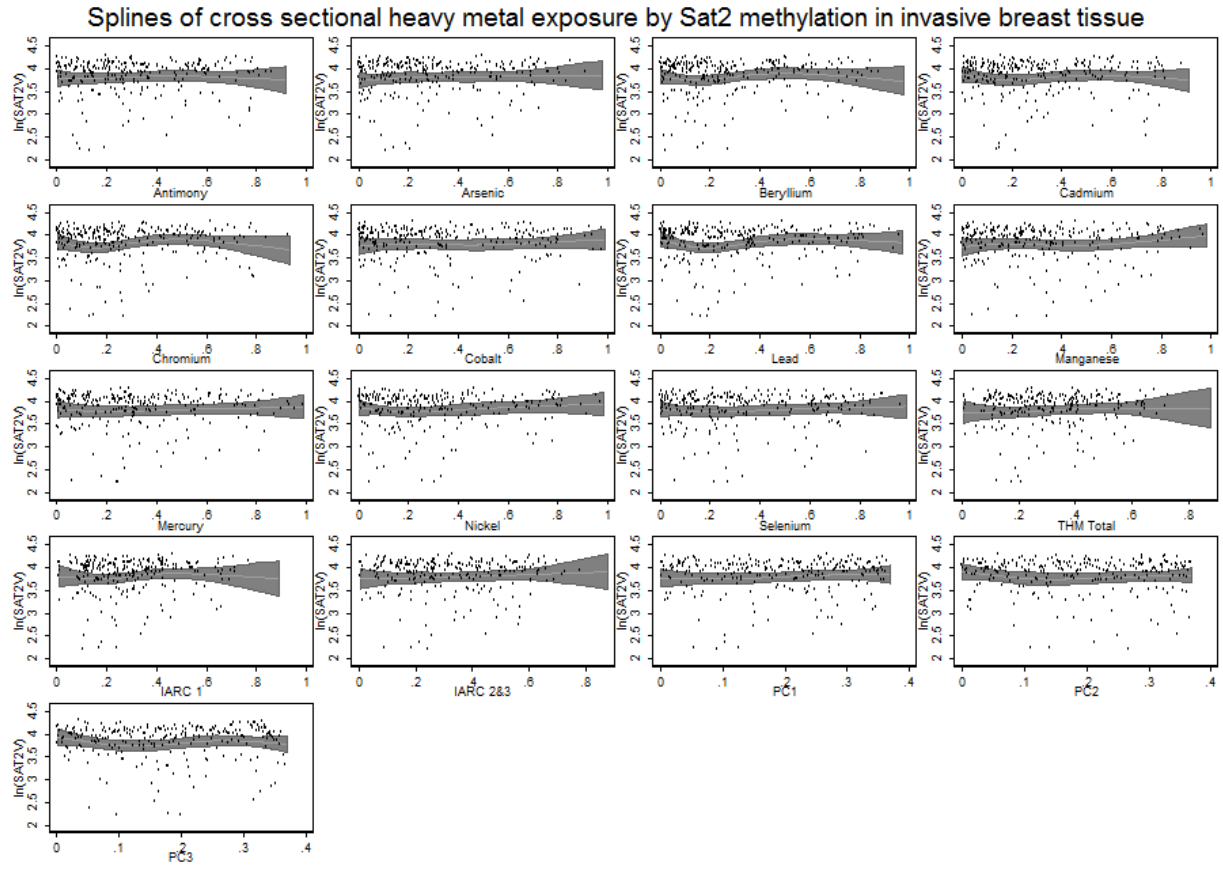

Figure 48. Splines depicting the shape of the association between the short-term single and combined heavy metal markers with Sat2 methylation in invasive samples.

APPENDIX F. IRB APPROVAL FOR STUDY

UNIVERSITY OF ILLINOIS
AT CHICAGO

Office for the Protection of Research Subjects (OPRS)
Office of the Vice Chancellor for Research (MC 672)
203 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Approval Notice

Continuing Review

October 13, 2016

Richard B. Warnecke, PhD

Institute for Health Research and Policy

1747 W Roosevelt

Room 472, M/C 275

Chicago, IL 60612

Phone: (312) 996-3109 / Fax: (312) 996-0065

RE: Protocol # 2004-0647

"Breast Cancer Care in Chicago"

Dear Dr. Warnecke:

Your Continuing Review was reviewed and approved by the Expedited review process on October 13, 2016. You may now continue your research.

Please note the following information about your approved research protocol:

Protocol Approval Period:	October 13, 2016 - October 13, 2017
Approved Subject Enrollment #:	8400

APPENDIX F (continued). IRB APPROVAL FOR STUDY

Additional Determinations for Research Involving Minors: These determinations have not been made for this study since it has not been approved for enrollment of minors.

Performance Sites: UIC, Illinois Department of Public Health, Rush University Medical Center

Sponsor: National Cancer Institute

PAF#: 00043776

Grant/Contract No: 1P50CA106743-01

Grant/Contract Title: UIC Centers for Population Health and Health Disparities

Research Protocol:

a) Revised Lay Summary and Research Protocol;10/14/2015

Recruitment Material(s):

a) Not applicable. Closed to subject enrollment.

Informed Consent(s):

a) Not applicable. Closed to subject enrollment.

Your research meets the criteria for expedited review as defined in 45 CFR 46.110(b)(1) under the following specific category:

(9) Continuing review of research, not conducted under an investigational new drug application or investigational device exemption where categories two (2) through eight (8) do not apply but IRB has determined and documented at a convened meeting that the research involves no greater than minimal risk and no additional risks have been identified.

Please note the Review History of this submission:

Receipt Date	Submission Type	Review Process	Review Date	Review Action
09/30/2016	Continuing Review	Expedited	10/13/2016	Approved

Please remember to:

→ Use your **research protocol number** (2004-0647) on any documents or correspondence with the IRB concerning your research protocol.

→ Review and comply with all requirements on the enclosure,

APPENDIX F (continued). IRB APPROVAL FOR STUDY

"**UIC Investigator Responsibilities, Protection of Human Research Subjects**"
(http://tigger.uic.edu/depts/ovcr/research/protocolreview/irb/policies/0924.pdf)

Please note that the UIC IRB has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

Please be aware that if the scope of work in the grant/project changes, the protocol must be amended and approved by the UIC IRB before the initiation of the change.

We wish you the best as you conduct your research. If you have any questions or need further help, please contact OPRS at (312) 996-1711 or me at (312) 413-3788. Please send any correspondence about this protocol to OPRS at 203 AOB, M/C 672.

Sincerely,

Rachel Olech, B.A., CIP

Assistant Director, IRB # 3

Office for the Protection of Research Subjects

Enclosure(s): None

cc: Robert Winn, Cancer Center, 432 MCA, MC 973
Robin J. Mermelstein, Institute for Health Research and Policy, M/C 275
OVCR Administration, M/C 672

JACOB K. KRESOVICH

3335 W. DIVERSEY AVE. APT. 225 • CHICAGO, IL 60647

PHONE (607) 227-2731 • E-MAIL JKRESO2@UIC.EDU

EDUCATION

PhD	2017 (expected)	Epidemiology University of Illinois at Chicago (Chicago, IL) Dissertation Title: Assessment of Breast Cancer Development and Aggression with Heavy Metal Exposures in Illinois Committee Chair: Garth H. Rauscher, PhD
MPH	2012	Epidemiology Columbia University (New York, NY) Thesis Title: Epilepsy and Paternal Age: A Population-based Study Thesis Chairs: Ruth Ottman, PhD Ezra Susser, MD, DrPH
BS	2008	Human Development Cornell University (Ithaca, NY)

RESEARCH EXPERIENCE

Research Interests: Genetics and Epigenetics, Environmental Epidemiology, Heavy Metals, Biomarkers, Cancer Etiology, Cardiovascular Disease Etiology

2015 - present	Pre-Doctoral Research Fellow Institute for Health Research and Policy University of Illinois at Chicago (Chicago, IL)
2016	Graduate Research Assistant Department of Ophthalmology University of Illinois at Chicago (Chicago, IL)
2012 - 2015	Graduate Research Assistant Division of Epidemiology and Biostatistics University of Illinois at Chicago (Chicago, IL)
2011 - 2012	Data Analyst Department of Epidemiology Columbia University (New York, NY)

2010 - 2011	Research Support Specialist Genome Resource Center Weill Cornell Medical College (New York, NY)
2008 - 2010	Lab Technician Microarray Core Facility Cornell University (Ithaca, NY)

TEACHING EXPERIENCE

2013 - present — Graduate Teaching Assistant
Division of Epidemiology and Biostatistics
University of Illinois at Chicago (Chicago, IL)

Courses Taught:
Introduction to Epidemiology (Graduate; for majors (3) and non-majors (1))
Epidemiologic Computing (Graduate (1))
Advanced Quantitative Methods for Epidemiology (Graduate (3))

PUBLICATIONS

Zheng Y, Sanchez-Guerra M, Zhang Z, Joyce BT, Zhong J, **Kresovich JK**, Liu L, Zhang W, Gao T, Dou C, Osorio-Yanez C, Carmona J, Wang S, McCracken JP, Zhang X, Chervona Y, Díaz A, Bertazzi P, Koutrakis P, Kang CM, Schwartz J, Baccarelli AA, Hou L. *Traffic-derived particulate matter exposure and histone H3 modification: A repeated measures study*. Environmental Research, 2016. In Press.

Rauscher GH, **Kresovich JK**, Poulin M, Yan L, Macias V, Mahmoud A, Al-Alem U, Balla AA, Wiley EL, Tonetti D, Ehrlich M. *Exploring DNA methylation changes as early events in breast cancer formation.* BMC Cancer. 2015. 15(816).

Kresovich JK, Argos M, Turyk ME. *Sex hormones and heavy metal exposures in males.* Environmental Research. 2015. 17(142): 25-33.

Susser E, Kirkbride JB, Heijmans BT, **Kresovich JK**, Lumey LH, Stein AD. *Maternal prenatal nutrition and health in grandchildren and subsequent generations.* Annual Review of Anthropology. 2012. 41:577–610

Kirkbride JB, Susser E, Kundakovic M, **Kresovich JK**, Davey Smith G, Relton CL. 2012. *Prenatal nutrition, epigenetics and schizophrenia risk: can we test causal effects?* Epigenomics. 2012. 4(3): 303-315.

PUBLICATIONS (Under Review)

Kresovich JK, Kibriya M, Islam T, Jasmine F, Yunus M, Parvez F, Ahsan H, Argos M. Tissue *DNA methylation as a cumulative biomarker of exposure to tobacco smoke.* Under Review, 2017. Epigenomics

Kresovich JK, Zhang Z, Fang F, Zheng Y, Sanchez-Guerra M, Joyce BT, Zhong J, Chervona Y, Wang S, Dou C, McCracken JP, Díaz A, Bertazzi PA, Koutrakis P, Kang CM, Bian S, Gao T, Byun HM, Schwartz J, Baccarelli AA, Hou L. *Histone 3 Modifications and Blood Pressure in the Beijing Truck Driver Air Pollution Study*. Under Review, 2017. Scientific Reports

Kresovich JK, Joyce BT, Bulka CM, Hibler EA, Schwartz J, Baccarelli AA, Hou L. *Dietary Manganese Effects on Systemic Inflammation in the Normative Aging Study*. Under Review, 2017. British Journal of Nurtition.

Kresovich JK, Zheng Y, Cardenas A, Joyce BT, Rifas-Shiman S, Oken E, Hivert MF, Gillman M, Hou L, Baccarelli AA. *Assessment of cord blood DNA methylation with adiposity phenotypes in early and mid-childhood.* Under Review, 2017. Clinical Epigenetics

Zhang Z, Joyce BT, **Kresovich JK**, Zheng Y, Zhong J, Patel R, Zhang W, Liu L, Dou C, McCracken JP, Díaz A, Motta V, Sanchez-Guerra M, Bian S, Bertazzi PA, Schwartz J, Baccarelli AA, Wang S, Hou L. *Blood pressure and expression of microRNAs in blood*. Under Review, 2017. PLoS One

PUBLICATIONS (In Preparation)

Khansari MM, Wanek J, Tan M, Joslin CE, **Kresovich JK**, Camardo N, Blair NP, Shahidi M. *Assessment of Conjunctival Microvascular Hemodynamics in Stages of Diabetic Retinopathy*. Target Journal: Diabetes

Blair NP, Wanek J, Felder AE, Joslin CE, **Kresovich JK**, Lim JI, Chau FY, Leiderman Y, Shahidi M. *Retinal Oximetry and Vessel Diameter Measurements with a Commercially Available Scanning Laser Ophthalmoscope in Diabetic Retinopathy*. Target Journal: American Journal of Ophthalmology

FUNDING

Current

2015 – present

National Cancer Institute (R25CA057699)

PI: Fitzgibbon M
Role: Pre-Doctoral Research Fellow

Cancer Education and Career Development Program

To prepare promising scholars to conduct research in the critical area of cancer disparities across the cancer prevention, control, and survivorship continuum.

2015 - present

Chancellor's Graduate Research Fellowship

PI: Kresovich JK
Role: PI
Amount: $8,000.00

Assessment of Breast Cancer Development and Aggression with Heavy Metal Exposures in Illinois

This project investigates the association between exposures to toxic heavy metals with development of aggressive breast cancer characteristics in the Breast Cancer Care in Chicago study.

Completed

2016

National Institute of Diabetes and Digestive and Kidney Diseases (1DP3DK104393)

PI: Shahidi M
Role: Graduate Research Assistant

Ocular Biomarkers of Microvascular, Neural and Metabolic Function in Diabetes

This project investigates the association of potential ocular hemodynamic and blood saturation biomarkers in patients with varying stages of diabetic retinopathy.

2015 - 2016

National Institute for Occupational Safety and Health (T42OH008672-10)

PI: Kresovich JK
Role: PI
Amount: $19,986.90

Assessment of Toxic Heavy Metal and Smoking Exposures on Breast Cancer Characteristics in Cook County, IL

This project investigated assessed smoking status of participants from the Breast Cancer Care in Chicago study using four pre-determined DNA methylation loci.

2012 - 2015

National Institute of Health (P50CA106743-06)
PI: Warnecke RB/Calhoun EA
Role: Graduate Research Assistant

Centers for Population Health and Health Disparities, Project 3: DNA Methylation and Differential Breast Cancer Aggressiveness by Race/Ethnicity (PD Rauscher)

Among other goals, Project 3 proposed to study differences in DNA methylation between Caucasian, Latin American and African American patients with breast cancer in Chicago.

PRESENTATIONS

Kresovich JK, Erdal S, HY Chen, Gann PH, Argos M, Rauscher GH. *Residential airborne heavy metal concentrations and breast cancer characteristics.* Poster presentation. October 18, 2016. University of Illinois Cancer Center Research Forum, University of Illinois at Chicago, Chicago.

Kresovich JK, Kibriya M, Islam T, Jasmine F, Yunus M, Parvez F, Ahsan H, Argos M. Tissue *DNA methylation as a biomarker of exposure to tobacco smoke.* Poster presentation. June 24, 2016. Annual meeting of the Society for Epidemiologic Research, Congress of the Americas, Miami.

Kresovich JK, Macias V, Mahmoud A, Poulin M, Erdal S, Argos M, Rauscher GH. *Assessment of airborne heavy metal exposures with breast cancer characteristics in Chicago, IL.* Poster presentation. April 5, 2016. School of Public Health Research Forum, University of Illinois at Chicago, Chicago.
****Winner of Poster Competition for Doctoral-level work**

Kresovich JK, Argos M. Tissue *DNA methylation as a biomarker of exposure to tobacco smoke.* Poster presentation. October 10, 2015. University of Illinois Cancer Center Research Forum, University of Illinois at Chicago, Chicago.

Kresovich JK, Al Alem U, Poulin M, Yang L, Ehrlich M, Rauscher GH. *Exploring DNA methylation changes as early and late events in breast cancer formation.* Poster presentation. April 15, 2015. Annual meeting of the Centers for Population Health and Health Disparities, Chapel Hill.

Kresovich JK, Argos M, Turyk ME. *Sex hormones and heavy metal exposure in males.* Poster presentation. May 7, 2015. School of Public Health Research Forum, University of Illinois at Chicago, Chicago.

Kresovich JK, Poulin M, Yan L, Macias V, Mahmoud A, Al-Alem U, Balla AA, Wiley EL, Tonetti D, Ehrlich M, Rauscher GH. *Exploring DNA methylation changes as early events in breast cancer formation.* Poster presentation. April 9, 2014. Annual meeting of the Centers for Population Health and Health Disparities, Los Angeles.

Kresovich JK, Al Alem U, Poulin M, Yang L, Ehrlich M, Rauscher GH. *DNA methylation and breast cancer disparities: A candidate gene approach.* Poster presentation. April 10, 2013. Annual meeting of the Centers for Population Health and Health Disparities, Boston.

HONORS AND AWARDS

2016	Chancellor's Student Service Award
2016	Poster Prize Winner, UIC School of Public Health Research Day
2015	Chancellor's Graduate Research Fellowship
2013	Golden Key International Honor Society

SERVICE & LEADERSHIP

2016	Graduate Editor, Interdisciplinary Undergraduate Research Journal
2015 - 2016	Student Director, Division of Epidemiology and Biostatistics Journal Club
2015	Graduate Reviewer, Interdisciplinary Under Graduate Research Journal

MEMBERSHIPS

2016	Student Member, American Association for the Advancement of Science
2015	Student Member, Society for Epidemiologic Research
2015	Associate Member, American Association for Cancer Research
2015	Student Member, Illinois Public Health Association

CERTIFICATIONS

2012 - 2016	Certification in Public Health (NBPHE)

NATIONAL WORKSHOPS

2016	*Epidemiological Approaches to Assessing Health Effects of Environmental Mixtures*. Society for Epidemiologic Research, June 21. Miami.
2016	*Epigenetic Epidemiology*. Society for Epidemiologic Research, June 21. Miami.
2015	*Integrative Molecular Epidemiology Workshop*. American Association for Cancer Research, August 10-14, Boston.

REVIEWER

Biological Trace Elemental Research
Biomedical and Environmental Research
Environmental Research
Environmental Science and Pollution Research
Scientific Reports