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Acquired epigenetic and chromosomal changes in women treated for breast cancer

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Table of contents **Page**

List of Tables

Table **Page**

List of Figures **Page**

Abstract

ACQUIRED EPIGENETIC AND CHROMOSOMAL CHANGES IN WOMEN TREATED FOR BREAST CANCER

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Improved survival for women receiving chemotherapy for breast cancer (BC) has been accompanied by the development/persistence of psychoneurological symptoms (PNS) that compromise their quality of life. The biological basis for these PNS is unknown, but could reflect the acquisition of soma-wide chromosomal/epigenetic alterations. An important first step in testing this hypothesis is to determine if somatic genetic/epigenetic changes arise and persist following treatment. To answer this question we longitudinally studied 71 women (ages 23-71) with early-stage BC and

collected measures before chemotherapy (baseline), and 4 weeks (mid-chemo); six months (during radiation therapy for a subset of women); and one year following the initiation of chemotherapy. Acquired lymphocyte chromosomal instability (scored by micronuclei frequencies [MNF]) showed a significant increase in post-treatment compared to baseline time-points ($p<0.0001$), with these increases persisting for at least one year following chemotherapy. Significant predictive associations were observed between MNF and **tumor characteristics** [luminal B (lower MNF; $p=0.0182$); triple negative (higher MNF; $p=0.0446$)], **radiotherapy** (higher MNF; $p=0.0004$), the **type of chemotherapy** received ($p=0.0463$), **race** (Caucasians > African Americans; $p=0.0037$), **perceived stress levels** (positive-association; $p=0.0123$), and **cognitive flexibility domain measures** (positive-association; $p=0.0238$). Genome-wide acquired methylation changes were also measured in peripheral blood cells, with 1265 sites showing significant differential methylation following chemotherapy. These sites were localized to open sea, shores, shelves, and CpG island sequences and included sites within genes involved in cell cycle, DNA repair, transcription regulation, signal transduction pathways, neuronal regeneration, and immunity. To determine if the genetic/epigenetic alterations acquired in peripheral blood cells correlated with those in tumor cells, BC tumors from 10 participants were analyzed using a genome-wide copy number/targeted mutations (CN/M) microarray. While no clear blood-tumor cell correlations were detected, genome-wide CN/M evaluations showed promise for stratifying tumors. Lastly, in an unrelated project studying a rare case of fetuses in fetu, methylation changes acquired in embryogenesis were shown to be influenced by both environmental and genetic cues. In summary, acquired chromosomal/epigenetic

alterations do arise following chemotherapy (and in embryogenesis). Further delineation of these acquired changes could increase our understanding of the biological basis for cancer-related side-effects and help to identify “at risk” individuals.

Chapter 1

Background and Significance

Breast cancer (BC) is the most common invasive cancer in females, accounting for about 29% of all women's cancers (Siegel et al 2014). Although BC is the 2nd most common cancer leading to death, advances in early detection and management have led to an increased survival rate (Jemal et al 2010). About 89% of diagnosed women are expected to survive at least 5 years after diagnosis (American cancer society 2013). However, the quality of survivors' life is inevitably affected by short and long-term side effects of BC and its treatment (Mehnert et al 2007, Mehnert et al 2009, Winnie et al 2009, Byar et al 2006). Anxiety (Badger et al 2007), depression (Badger et al 2007), cognitive dysfunction (Hurria et al 2007), sleep disturbances (Bender et al 2005), fatigue (Winnie et al 2009, Byar et al 2006), and pain (Winnie et al 2009) are the most common side effects observed in women who survive following BC treatments (Winnie et al 2009, National Institute of Health 2004, Kim et al 2008).

The clustering tendency of these psychoneurological symptoms (PNS) led researchers to question whether or not at least some of these symptoms might share a common biological etiology, and whether they could be collectively treated (Kim et al 2009, Misakowski 2006, Misakowski & Aouizerat 2007, Cleeland et al 2003). One of the most accepted theories for a shared biological mechanism is the cytokine-induced sickness behavior mechanism (Misakowski & Aouizerat 2007, Cleeland et al 2003, Dantzer 2001). Despite the animal studies and the clinical evidence that support this hypothesis (Konsman et 2002, Lee et al 2004, Cleeland et al 2003, Nozaki et al 1997, Capuron et al 2002, Raison

et al 2005), this mechanism has not been studied extensively in women treated for BC, nor has it been investigated with consideration of all the PN symptoms mentioned above. While some investigators have suggested mechanisms for a subset of these symptoms independently (Collado et al 2006, Ahles et al 2007), no effective prevention or treatment for the multiple symptoms associated with BC has yet been developed. Moreover, it remains unclear how long-term side effects persist even after the recovery from BC and its treatment, and from the inflammatory induction associated with BC and chemotherapy. These PN symptoms are observed in a major subgroup of BC survivors (Ahles et al 2007, Gatson-Johansson et al 1999, Janz et al 2007, Zabora et al 2001), but the reasons behind their occurrence in only a subgroup of survivors remains unknown.

Many factors could lead to the development of these PN symptoms in a major subset of BC survivors. Akin to the causes underlying most complex disorders, such as cancer and psychiatric problems, the factors influencing the development of PN symptoms, such as cognitive dysfunction and depression, are thought to include both environmental and genetic factors (Wichers et al 2009). Examples of environmental factors include the stress associated with the diagnosis and its treatment, nutrition, and BC therapeutic drugs. An individual's genetic background could affect their susceptibility to develop the PN symptoms. Moreover, these factors could interact with each other, and could also trigger cellular biological changes such as inflammation, which have been evidenced to associate with sickness behavior. These observations raise many questions. In particular, how do all these factors interact and trigger biological changes, such as inflammation, that could lead to the development of PN symptoms in a subset of BC survivors?

A. Feinberg, who is one of the pioneers and leaders in the field of epigenetics, proposed

that epigenetics is an epicenter of modern medicine, serving to explain the relationships between environment, genetic background and disease (Feinberg 2007, 2008). Epigenetics is referred to as mitotically heritable modifications in gene expression and phenotype that do not result from changes in genetic sequences. It plays an important role in the development and differentiation of cells. Mounting evidence shows an additional major role of epigenetics in inflammation and in developing cancers and psychiatric diseases (Wierda et al 2010, Kristensen et al 2009, Pidsley 2011, Mehler 2009). Although epigenetic changes are known by their stable heritability, they have also been characterized by their plasticity and reversibility (Reik 2007, Narayan& Dragunow 2010). The heritability phenomenon could explain how long-term PN symptoms persist even after the recovery from BC and chemotherapy. The plasticity and sensitivity of epigenetics to genetic background, environment influences, and their interactions could explain why PN symptoms happen only in a subset of survivors. The reversibility of epigenetic changes makes them an attractive target for manipulation and drug therapies (Narayan & Dragunow 2010).

Cytokine-induced sickness behavior: "A biological mechanism at the cellular level"

One of the cellular biological mechanisms that is thought to have an important biological basis for the development of at least some of the PNS associated with cancer treatment is the cytokine-sickness behavior mechanism. It is thought that inflammation resulting from BC, or its treatment, has a role in symptom production (Cleeland et al 2003). From animal models, preclinical, and clinical evidence it is believed that sickness behavior requires an immune-to-brain communication. A cascade of pro-inflammatory cytokines is initially released by the action of macrophages. This released cascade then activates other

immune cells, such as lymphocytes and neutrophils. Peripheral cytokines could induce PNS through multiple pathways where cytokines are required to communicate with the brain. Cytokines could be produced not only by macrophages, but also by other cell types, such as tumor cells in cancer patients and nervous system cells (Reviewed in Cleeland et al, 2003). However, this mechanism alone cannot explain the occurrence of all these symptoms and their persistence, even after the recovery from BC and its treatments and in the absence of inflammatory induction. Therefore, inflammation could have a basic role in initiating the development of at least a portion of these symptoms. However, there must be more than one mechanism, perhaps - a series of interactive events - that occurs before and after inflammation to lead to the final observed outcome. Therefore, our hypothesis is that while inflammation may be one of the early biological changes leading to the development of these PNS, it is just one of a cascade of events that interact and together lead to the development of these short and long term symptoms (Figure 1).

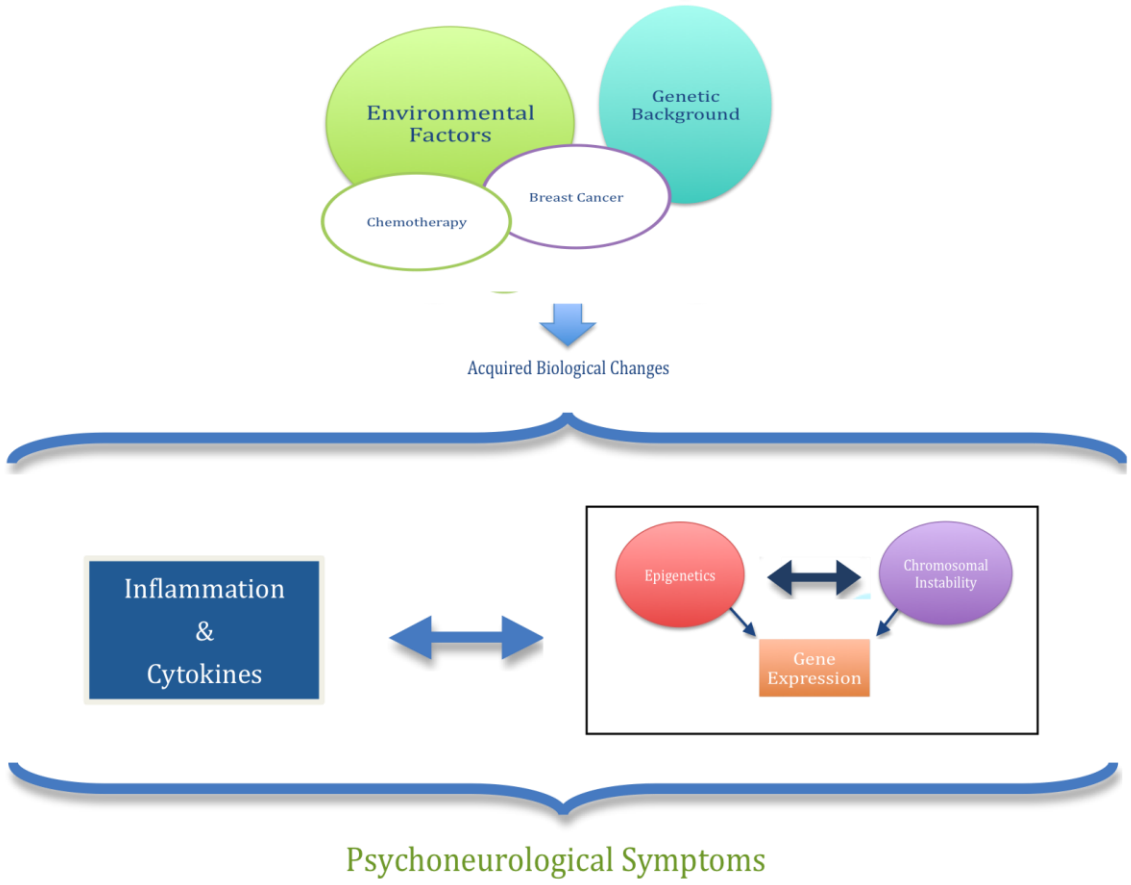


Figure 1. Schematic illustrating the hypothesized mechanism(s) leading to PN symptoms associated with BC. Both environmental factors and genetic susceptibility lead to the development of BC in women before receiving BC treatments. BC and its treatment, in addition to the stress associated with the diagnosis, induce acquired biological changes such as inflammation activation and genetic changes involved in the inflammation process. Our hypothesis is that inflammation is one of the initiators of a cascade of events that arise, interact, and together lead to the development of PN symptoms. These events include acquired changes in epigenetic profiles, gene expression and chromosomal instability. Epigenetic changes are heritable on a cellular level, but are characterized by their plasticity and potential for reversibility. Thus, these changes are persistent, yet sensitive to genetic environmental interactions (E X G), making them a viable therapeutic target.

Environmental factors and genetic susceptibility:

Environmental factors include, but are not limited to, nutritional factors, the stress associated with BC diagnosis and its treatment, and BC treatments such as chemotherapeutic drugs, radiation and hormonal therapies. One important nutritional factor could be folic acid. Results of many studies have shown the important role of folate in the DNA methylation process. Folate provides the one-carbon units required in converting homocysteine to methionine, which is then converted to the universal methyl donor S-adenosylmethionine (Jacob R, 2000). Folate deficiency has been associated with birth defects and tumorogenesis (Jacob R. 2000, Blount BC et al. 1997). Moreover, folic acid concentration correlates significantly and inversely with frequencies of micronuclei in human lymphocyte cultures, indicating an association with genomic instability (Fenech M. 2007). Most importantly, the results of some studies suggest the role of folate deficiency in developing depression (Coppen and Bolander-Gouaille. 2005). Increasing evidence also links folate levels with inflammation (Mangoni A. 2006). Due to the role of folate in affecting methylation, gene expression and genomic instability, and its link with inflammation and depression, it is important to consider it in the complete final analysis of this study (part of full study; results reported elsewhere).

Baseline "germline" or heritable genetic background influences could also affect a woman's susceptibility to develop PN symptoms following treatment for BC. Background genetic influences involve the genomic sequence with its gene mutations, including those that might influence DNA repair mechanisms. In addition to germline genetic influences, acquired genetic changes could contribute to differential outcomes for women with BC. For example, tumor mutations, as well as baseline epigenetic modifications and

chromosomal instability levels, could play a role in a patient's health outcome. Therefore, both environmental factors and genetic background can lead not only to the development of BC, but also to the susceptibility of a woman to develop inflammation and, consequently, PN symptoms. For example, one mechanism where both genetic and environmental factors could be integrated is through the regulation of a methylation site overlapping with a single nucleotide polymorphism (SNP) that is associated with the development of a phenotype (Taqi MM et al. 2011).

Breast Cancer:

BC is characterized by its heterogeneity and is highly challenging when it comes to its classification and treatments decisions (Polyak K. 2011). Genetic and epigenetic research has helped in improving the ability to understand the development of BC, its classification, treatment decision and its prognosis.

BC classification:

BC has traditionally been classified clinically and histopathologically (Westbrook and Stearns 2013, Viale G 2012). The tumor node metastasis (TNM) stage and tumor grade, along with the histopathological classification, remain included in the report and have been implicated in the prognosis of BC (Viale G 2012). BC has also been subdivided by immunohistochemistry and/or fluorescence in situ hybridization (FISH) characterization according to the presence or absence of the human epidermal growth factor receptor-2

(HER2), estrogen (ER), and progesterone (PR) receptors. The results of gene expression microarray analyses have assisted in subdividing BC tumors into the molecular intrinsic subgroups (Basal like, Luminal subtypes, HER-2 enriched, and normal like) (Perou et al 2000, Sorlie et al 2001, Sorlie et al 2003). The presence or absence of each of the three receptors helped in refining the molecular subtypes as; luminal A (ER+ and/or PR+; HER2-), Luminal B (ER+ and/or PR+; HER2+), basal-like (ER-, PR-, and HER2-) , and HER2-enriched (ER-, PR-, and HER2+)(Sinn P et al. 2013, Byler et al. 2014). The later classification has been extremely useful in directing the treatment and the prognosis of the cancer (Byler et al 2014). However, the hormone responsiveness doesn't always determine the gene expression molecular subtypes. Many other genetic factors have been evaluated, underscoring the complexity of the tumors and the difficulties in accurately identifying the subtypes. In addition to the receptors noted above, BC tumors have been evaluated for different gene mutations, frequencies of mutations and also different types of mutations (non-sense or frame shift)(Byler et al. 2014). The heterogeneity and complexity when classifying BC is due to the tremendous number of genetic changes that could be implicated in its development. These changes include somatic gene mutations, copy number variations, alterations in gene expression and epigenetic changes. In Table 1 a summary is presented of some of the genes that have been found to show mutations or copy number aberrations in BC.

The oncotype DX, and MammaTyper, are some of the currently available tools specific for gene expression markers that were developed to aid in diagnosis, prognosis and therapy decisions relating to BC (Sinn et al. 2013). However, due to the inherent instability of

mRNA, which makes its use challenging, there is a need for more stable and reproducible tools. DNA methylation is an alternative tool. The stability and accessibility of DNA makes it a more attractive target, especially if whole blood cells could be used rather than the tumor tissue itself. Methylation will be more extensively discussed in the DNA methylation section.

Treatment:

As more knowledge is being gained, there is a constant evolution of therapies given to patients with BC. Treatments include hormonal therapies, targeted biologic therapy, cytotoxic chemotherapy, and radiotherapy (Westbrook and Stearns 2013).

Hormonal Therapies:

Several hormonal therapies are available and approved for treating BC. The selective estrogen receptor modulators (SERMs) -such as tamoxifen and raloxifene, and the third generation aromatase inhibitors (AIs), including anastrazole, exemestane and letrozole, are two classes of drugs commonly given to patients with BC (Westbrook and Stearns 2013). Tamoxifen is the most widely prescribed SERM given to women with early stage hormone responsive BC (Westbrook and Stearns 2013). It is also effective in treating women with metastatic hormone receptor-positive BC and women having ductal carcinoma in situ (Rose & Mouridsen, 1984, Cuzick et al 2002, Westbrook and Stearns 2013).

Targeted biologic therapy:

Trastuzumab is one example of a targeted biologic therapy. This drug is a humanized monoclonal antibody (HMAB) given to suppress cell proliferation caused by the overexpression of the HER2 protein. It works by binding specifically to the HER2 receptor (Westbrook and Stearns 2013). It is given in combination with chemotherapy and has led to a significant improvement for treating HER2 overexpressing tumors and a significant reduction in recurrence and mortality for women having this type of tumor (Piccart-Gebhart et a 2005, Romond et al 2005, Slamon et al 2011, Westbrook and Stearns 2013).

Trastuzumab is not the only drug that targets HER2+ tumors. Pertuzumab also has been approved to be used in treating HER-2 + metastatic BCs by the European Medicine Agency in 2013 (Sabatier & Goncalves 2014). It could be used in combination with Trastuzumab and docetaxel. Moreover, lapatinib, which is a dual tyrosin kinase inhibitor could also be used in targeting both EGFR and HER2 (Zhu et al 2013).

Another example of a targeted therapy is Bevacizumab, which is HMAB that targets the vascular endothelial growth factor A (VEGF-A). (Westbrook and Stearns et al 2013).

Cytotoxic Chemotherapy:

The following are some of the cytotoxic drugs most often given to patients with BC.

Antimicrotubules Agents:

Taxanes, including paclitaxel (Taxol) and docetaxel, are very effective chemotherapeutic treatments given to patients with both adjuvant and metastatic BC. They cause cell death by disrupting microtubule depolymerization and spindle formation during cell replication (Westbrook and Stearns 2013).

Anthracyclines:

Both doxorubicin and epirubicin are anthracyclins that have been widely used in treating BC. Although doxorubicin is widely used, the mechanism(s) for its effectiveness in preventing/reducing cancer cell proliferation is/are not fully known, with some investigators proposing that it induces cell apoptosis by inhibiting topoisomerase II, while others cite roles for DNA adduct formation, oxidative stress, ceramide overproduction and/or torsion-induced nucleosome destabilization as the causal means for this drug's efficacy (Gewirtz 1999; Senchenkov , et al., 2001; Minotti, et al., 2004; Yang, et al., 2014, Westbrook and Stearns 2013).

Cyclophosphamide:

Cyclophosphamide is considered a backbone in BC treatment, which is incorporated into many adjuvant BC chemotherapeutic drugs. (Westbrook and Stearns 2013)

Carboplatin:

Is a platinium analog, which induce double stranded DNA breaks. It has shown more efficacy in treating BRCA1- mutated tumors and triple negative breast cancers (Sikov et al 2014)

Outcome:

Interestingly, patients treated with equivalent drugs show a significant variability in response and in survival outcomes (Polyak et al 2011, Westbrook and Stearns 2013). While the survival rate has improved significantly during the last few decades, it is time now to consider the quality of women's life after surviving BC, along with improving their survival chances. One of our goals in this study is to understand how different types of treatments are affecting patients' methylation patterns or causing chromosomal instability and how these latter changes relate to the acquired PNS affecting the patient's quality of life.

There is a need for a discovery of tools that can be used in biomonitoring a patient's response to a therapy (including its efficacy and toxicity) influenced by their genetic makeup. Advances in the genetic technology allowed the identification of candidate genes and SNPs that might associate with BC outcome. Some studies have been conducted with the goal of identifying drug targets. Table 2 summarizes some of the candidate genes that are thought to be associated with the outcome when a patient is treated with a specific drug. While results from some studies support the associations between these genes and a patient's response to treatment, others failed to confirm an association. Thus, insufficient

evidence is available to allow for unequivocal recognition of these associations on health outcomes in women treated for BC.

Epigenetic modifications:

Epigenetics refers to the mitotically heritable modifications in gene expression and phenotype that do not involve any changes in the underlying DNA sequence (Russo et al 1996). Epigenetic information is important for normal mammalian development and in cell differentiation (Reik 2007). It is not only involved in controlling gene expression, but also in the formation of heterochromatin, which can play an essential role in chromosome segregation and chromosomal stability (Herrera 2008). Feinberg et al, 2008, suggested epigenetics as a link between environment, genetic background, and disease. Rapidly growing evidence shows the major role of epigenetics in inflammation, developing cancers and psychiatric diseases (Wierda et al 2010, Kristensen et al 2009, Pidsley and Mill 2011). This process includes DNA methylation, histone modification, chromatin structure, and noncoding RNA-mediated regulation of gene expression (Kouzarides 2007)). Among these processes, DNA methylation and histone modifications are the most understood.

DNA methylation:

DNA methylation involves the addition of a methyl group to position 5 of the cytosine pyrimidine ring (C) of a cytosine guanine dinucleotide (CpG) (Klose and Bird 2006). This process is catalyzed by the DNA methyltransferase (DNMT) enzymes (Klose and Bird

2006). DNA methylation is one of the most stable and well-characterized epigenetic mechanisms, which makes it an attractive area to focus on in our study (Jirtle and Skinner 2007). Typically, in normal cells, promoter CpG islands are hypomethylated while repetitive DNA sequences in the genome are hypermethylated. Studies of genome-wide DNA methylation in cancer cells revealed that the methylation patterns can be reversed (Feinberg et al 2006). DNA methylation patterns in different tissues (normal and cancerous) including blood, are known to be sensitive to environmental insults such as age, alcohol intake and smoking (Table 3).

Genetic and epigenetic research has been very helpful in clarifying and understanding the development of cancer/BC (Tables 1, 4-6). Most of the genetic research has been performed using breast tumor tissues after the development of BC for purposes of classification, treatment decisions and prognosis prediction. Epigenomic research in cancer is a relatively new field. Studies of the role of epigenetics in cancer were initiated by searching for candidate genes that are known to be involved in breast and other tissues' carcinogenesis using tumor tissues from patients with cancer. The recently available whole-genome methylation techniques allowed investigators to look for more global changes and search for epigenetic signatures that might add more information to the existing classification, with a goal of finding a better assessment of prognosis. Some current findings from DNA methylation studies on breast tissues from patients with BC are summarized in Table 4.

Researching the tumor itself is necessary for classification and treatment decisions for

the management of BC. However, an alternative approach that might be more powerful for evaluating a patient's response to a treatment and to be able to more accurately predict systemic PN symptom outcomes could be through the study of peripheral blood specimens. Studies of methylation patterns in blood samples, especially circulating cell free DNA found in the serum or plasma, has been conducted with the purpose of finding biomarkers for breast cancer risk and diagnosis, or for prediction of treatment outcome and prognosis. DNA methylation patterns specific for cancer were found in the circulating cell-free DNA, which is released from dead cancer cells (Sidransky 2002, Van De Voorde et al 2012). DNA methylation biomarkers of BC found in the serum or plasma have been extensively reviewed by Van De Voorde et al. (2012). A summation of these findings is presented in Table 5. Panels composed of two, three or more biomarkers have been developed (Van De Voorde et al 2012). It is important to note that methylation of many of these genes identified as biomarkers are not specific for BC and could be found in other types of tumors (Table 5). Problems of studying cell free DNA found in serum/plasma include the limited amount of DNA present, requiring the use of very sensitive techniques; possible contamination with DNA from whole blood cells; and limitations in following up acquired changes in methylation patterns associated with the treatment after the surgical removal of the tumor, since this cell free DNA is released from dead cancer cells.

Few studies have been done to investigate the association between BC -or cancer in general- and DNA methylation patterns in peripheral blood cells (PBCs). The limited numbers of studies using PBCs reflects concerns regarding whether the methylation patterns in PBCs are suitable as a surrogate for changes arising in other tissues.

Investigators evaluated DNA methylation levels in PBCs of specific candidate genes as well as global genomic levels. Based on the limited number of available studies, global hypomethylation in PBCs has been associated with the presence of BC (Choi et al 2009). Also, methylation of specific loci have shown promise for use as markers for the presence of BC or an increased risk for developing BC (Iwamoto et al 2011, Wong et al 2010, Brennan et al 2012, Widschwendter et al 2008). Fewer studies have been performed to monitor chemotherapy effects on methylation of PBCs. Available data support the hypothesis that PBCs are a good representative cell for evaluation and could be used as a biomarkers indicating an increased risk for developing cancer, the presence of cancer, or changes acquired as a result of cancer (Table 6).

Cancer is a systemic disease involving many tissues (including peripheral tissues) rather than just the tumor by itself. It becomes even more systemic when patients are treated for cancer, since all their tissues are exposed to cyto/genotoxic regimens that are mainly toxic to the tumor, but also toxic to tissues within the human body as a whole. There are limited numbers of studies investigating the effect of treatments on methylation patterns in PBCs from patients with cancer. The importance for using blood samples comes from the accessibility of blood, the non-invasive means for collection, and the ability to detect risk and follow-up acquired changes in methylation patterns longitudinally, allowing for biomonitoring progression and changes associated with specific treatments for prediction of outcome. Tumor tissues are not obtainable until after the development of the cancer and it is not possible to follow up changes in the tumor tissue after its surgical removal.

Chromosomal instability:

Micronuclei (MN) frequencies are widely used as an indicator of chromosomal instability. Studies in our lab and other labs showed that high frequencies of MN associate with aging, environmental exposures, and cancer (Table 7) (Fenech and Morley 1986, Fenech and Morley 1985, Jones et al. 2011). MNs are thought to form as a result of chromosome lagging at anaphase. They contain either whole chromosomes or chromosomal fragments that are not incorporated in the daughter nuclei at the completion of mitosis (Leach and Jackson-Cook 2001). Increased chromosomal instability has been observed in tumor tissues, peripheral blood of patients with cancer, and following the exposure of cells to chemotherapy and ionizing radiation (Table 7). Investigators have shown that cells with increased levels of methylation alterations are more susceptible to chromosomal instability (Herrera et al 2008). It has also been suggested that global DNA hypomethylation could lead to chromosomal instability, aneuploidy, and contribute to alterations in chromatin structure and cancer progression (Ulrich et al 2012).

Table 1: Summary of a subset of genes implicated in the development of BC:

Type of genomic changes	Genes	References
Somatic gene mutations:		
	Well established genes in BC development:	
	Genes Involved in apoptosis, cell cycle regulation, and transcription regulation:	
	BRCA1 , RB1, TP53, PTEN, AKT1, CDH1, GATA3, PIK3CA	Stephens et al 2012 *
	Genes Involved in Signal transduction:	
	APC, ARID1A, ARID2, ASXL1, BAP1, KRAS, MAP2K4, MLL2, MLL3, NF1, SETD2, SF3B1, SMAD4, AND STK11	
	A recently identified group of genes:	
	ARIDIB, CASP8, MAP3K13, MAPK2K13, NCOR1, SMARCD1, and CDKN1B	
	Well established genes in BC:	
	RB1, TP53, PTEN, AKT1, CDH1, GATA3, PIK3CA, MLL3, MAP3K1, CDKN1B	
	Mutation in 3 genes occur in > 10% incidence across all BC:	The Cancer Genome Atlas 2012*
	TP53, PIK3CA, and GATA3	
	Novel genes:	
	TBX3, RUNX1, CBFB, AFF2, PIK3R1, PTPN22, PTPRD, NF1, SF3B1, and CCND3	
Copy number aberration:		
	Amplification of regions containing PIK3CA, EGFR, FOXA1, and HER2 correlated with different mRNA subtypes	The Cancer Genome Atlas 2012 *
	Deletion of regions containing MLL2, PTEN , RB1, and, MAP2K4 correlated with different mRNA subtypes	
	Used the MIP probe and identified 19 CNI that discriminate recurrence risk among early stage BC	Thompson et al 2011
	The data suggest that specific CNI might promote or limit tumor spread in some cases	

* Reviewed by Byler et al 201

Table 2: Summary of candidate genes potentially related to BC outcome in response to a treatment*

Agent	Proposed Outcome	Candidate Variant Genes
Hormonal Therapy:		
Tamoxifen	Poorer outcome	CYP2D6 poor metabolizers, SULT1A1, UGT2B15, ABCB1, ESR1
	Increased likelihood of tamoxifen discontinuation	CYP2D6 extensive metabolizers
	Decreased hot flashes	ESR2
Targeted biologic therapy:		
Trastuzumab	Better outcome	Fc gamma RIIIa-158V/V
Cytotoxic chemotherapy:		
Antimicrotubules: Docetaxel	Increased myelosuppression	SLCO1B3
Anthracyclines: Doxorubicin	Better outcome	ABCB1 C354T, MPO, NOS
	Greater reduction of tumor burden , increased neutropenia	CBR311A
Cyclophosphamide	Better outcome	CYP2B6
	Poorer outcome	ALDH1A1
	Poor pathological CR, reduced risk of hematologic toxicity	GSTP1

*Adapted from review by Westbrook et al., 2013

Table 3: Examples of environmental influences on DNA methylation in blood and other tissues in health and disease

Environmental influences	Tissue	Tool(s) used for assessment	Finding	References
Aging	WBC, Lung & other solid tissues (normal and cancer) Cervix, mesenchymal stem cells and ovarian cancer	Illumina Infinium 27k Goldengate assay	Found age-dependent DNA methylation signatures of genes in WBC and in other tissues and were independent of disease state, sex, tissue and cell type. The signature is present in preneoplastic conditions and might be associated with carcinogenesis	Teschendorff et al 2010
Aging	WBC, CD4+ T cells, CD14+ monocytes, buccal cells	Illumina Infinium 27k	Identified aging aDMRs signatures with a multitissue phenomenon.	Rakyan et al 2010
Alcohol intake, folate and tumor size	BC tumor	GoldenGate	BC DNA methylation profiles are associated with alcohol & folate intake	Christensen et al 2010
Alcohol intake	WBC	LC-ESI-MS/MS for 5-mdC Pyrosequencing for LINE-1	Among controls alone or combined with cases of BC, alcohol intake was inversely associated methylation levels	Choi et al 2009
Smoking	WBCs	Illumina Infinium 27k, Mass spectrometry & Sequenom EpiTYPER	Identified a single locus (F2RL3) differentially methylated between exposure groups The gene was fully replicable in an independent sample	Breitling et al 2011

Associated differentially methylated regions (aDMRs)

Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS)

Table 4: Methylation patterns in BC tissue tumor:

Tool(s)	Findings	References
Genome Atlas Network	BC can be classified into several groups that are related to the cellular origin of the tumor and are different than current gene expression classification, with some overlap with intrinsic subgroups	The cancer Genome Atlas 2012 (1)
MeDIP* (Familial BC) Validation by pyrosequencing and epityper	Studied familial BC and identified distinct profiles defined by mutation status Identified a distinct subtype of BRCA tumors based on their methylation profile	Flanagan et al 2010
Illumina 27 k	Methylation of 4 genes is affected by ER/PR status	Li et al 2010 (2)
Illumina 27 K	Identified a group of genes where their methylation is associated with relapse free survival	Hill et al 2011 (2)
Illumina 27 k	Identified a methylator phenotype that is associated with lower risk of metastasis and better survival rate	Fang et al. 2011 (2)
Illumina Golden Gate methylation Validation by Sequenom EpiTYPER	Tumor grade, size, estrogen and progesterone receptor status, and triple negative are associated with altered methylation Association with race, and tumor size.	Christensen et al 2010

* Methylaed DNA immuneprecipitation (MeDIP)

(1) Reviewed by Byler et al 2014

(2) Reviewed in Szyf , 2012

Table 5: Summary of biomarkers for BC based on studies of cell free DNA from serum/plasma*:

Type of Biomarkers	List of genes
Biomarkers related to BC:	
Markers involved in cell cycle regulation	BRCA1, GSTP1, RASSFA, APC,RAR-β , MGMT, P16 INK4A , P14ARF , 14-3-3-σ, TMS1, Cyclin D2
Markers related to hormone receptor	ESR1, ESR2, PR
Markers involved in cell adhesion	CDH1, TIMP-3, Slit2
Biomarkers associated with poor prognosis	RASSF1A, APC , GSTP1, BRCA1, 14-3-3-σ, PITX2, P16INK4A, ERB, RARβ2,
Biomarkers for response to treatment	
Surgery and Tamoxifin	Surgery significantly reduced the methylation of MGMT, PAX5, and RARB2, but not MDGI
	Tamoxifen had a more pronounced effect on MDGI
	Methylation of PR PROX was equally affected by surgery and tamoxifen
	Methylation of ESR1 and MSH1 was initially reduced by surgery, but increased during tamoxifen treatment
Neoadjuvant chemotherapy	Methylation levels of RASSF1A in serum may indicate tumor shrinkage
	could be used in monitoring response during neoadjuvant chemotherapy
Markers found in other types of tumor	Lung: BRCA1, APC, RAR-β, P16,
	Ovarian: BRCA1, RASSF1A, Slit2
	Liver:GSTP1, Hepatocellular cancer: Slit2
	Prostate: GSTP1, RASSF1A, Slit2, ER
	Bladder: RASSF1A,
	Stomach: APC, Gastric cancer: CDH1, Gastrointestinal tract: P16, 14-3-3-σ,
	Colorectal: APC
	Glioblastoma: RAR-β, P14ARF, TIMP3, Glioma:MGMT, P16, Neuroblastoma: TMS1
	Lymphoma: P16, Cyclin D2, Multiple myeloma: P16
	Cervix: P14ARF
	Cholangiocarcinoma: TMS1
	Pancreas: TMS1
	Melanoma: ER

Endometrial: PR

* Adapted from reviewed by Van De Voorde et al., 2012

Table 6: Methylation biomarkers in WBCs for different types of cancer

	Tissue/type of tumor	Tools	Finding	References
Gene:				
IGF2	PBL / (P&D CM)	Imprinting analysis	LOI at IGF2 in PBL could be a valuable predictive marker of risk for CRC	Cui et al 2003
IGFE	PBL	Quantitative hot-stop PCR for imprinting analysis	LOI at IGFE in PBL as a hereditary risk factor for colon neoplasia	Cruz-Correa et al 2004
49 genes *	WBCs/BC	MethylLight assay	DNA methylation in WBCs provides a good prediction of BC risk	Widschwendter et al 2008
BRCA1 promoter	WBCs/BC	Methylation specific qPCR	BRCA1 promoter methylation in WBCs is significantly associated with risk of BC tumors with BRCA1 promoter methylation	Iwamoto et al 2011
BRCA1	WBCs/BC	MethylLight and (MS-HRM)	BRCA1 methylation in WBCs identifies a small group of women in the population who have increased risk for an early-onset BC	Wong et al 2010
ATMmvp2a & ATMmvp2b / LINE-1	WBCs	Bisulfite pyrosequencing	DNA methylation at ATM could be a marker of BC No differences in LINE-1 between cases and controls	Brennan et al 2012
Global:				
	WBCs/ BLC	Illumina 27k	DNA methylation profiles associate with risk of BLC	Marsit et al 2011
	WBC/ BLC	HPCE & Hpa II D&D	Hypomethylation associate with increased risk of developing BLC	Moore et al 2008

	WBC/ (HNSCC)	Modified version of CBRA of LRE1 sequence	DNA hypomethylation is associated with HNSCC, Complex relationship with the known risk factors ex: smoking and folate intake	Hsiung et al 2007
	WBCs / PaC	GoldenGate (phase I) & ICVC (phase II)	DNA methylation signature that can detect (PaC)	Pedersen et al 2011
	WBCs/ OC	Illumina 27k	DNA methylation signature that can detect or predict risk of OC	Teschendorff et al 2009
	WBCs /BC	(LC-ESI-MS/MS) for(5-mdC) Pyrosequencing for(LINE-1)	Global DNA hypomethylation is associated with development of BC	Choi et al 2009
	WBCs/BC	MethylLight Pyrosequencing	DNA methylation levels in WBC from control girls are associated with family history of BC	Wu et al 2011
Sat2M1:				
	WBCS/BC tumor/ & adj. normal tissue	MethylLight	Sig. correlation in methylation of Sat2m1 between the 3 tissue types Sig. differences in methylation of Sat2M1 between cases and controls	Cho et al 2010
LINE-1:				
	WBCs / RCC	Pyrosequencing in triplicate, & averaged across 4 CpG sites	High LINE-1 methylation levels were positively associated with RCC The association was stronger with current smokers than with former / never smokers	Liao et al 2011
	Lymphocyte/BLC	Pyrosequencing	LINE-1 hypomethylation is associated with BLC risk among non-smokers	Cash et al 2012

			Reduced LINE-1 methylation in current smokers with high cytochrome index	
	WBCs/ BLC	Pyrosequencing	Line-1 hypomethylation is associated with BLC risk; especially among women	Wilhelm et al 2010
BC and Chemotherapy	Blood Mononuclear cells	Illumina 450k	8 CpG sites were hypomethylated in women treated with chemo as compared to control (not treated) women; After radiation only 4 CpG sites remained Methylation at these sites was associated with inflammation but not with fatigue	Smith et al 2014

* Lymphocyte (PBL)

Quantitative analysis of methylated alleles (QAMA)
loss of imprinting (LOI)
Colorectal cancer (CRC)
49 GENES included a) 19 ERT genes b) 4 genes ER-a target genes(diffrentially methylated based on hromone receptor status) c)20 PCGT genesmore likely have a promoter DNA hypermethylation in cancer . D) 6 genes that are known to be methylated in BC (MBC)

proximal and distal colonoic mucosal (P&D CM)

Head and neck squamous cell carcinoma (HNSCC)

Pancreatic adenocarcinoma (PaC)

Bladder Cancer (BLC)

Ovarian Cancer(OC)

Renal cell cancer(RCC)

Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS)

Methylation Sensitive-High Resolution Melting (MS-HRM)

high-performance capillary electropheresis(HPCE)

combined bisulfite restriction analysis (CBRA)

digestion & densitometry (D &D)

Illumina. custom VeraCodemethylation (ICVC)

Table 7: Summary of previous studies related to BC or its treatment evaluating MN frequencies in peripheral blood lymphocytes

Topic	References
Breast Cancer Effects	
Higher MN frequencies in untreated women with breast cancer irrespective of smoking and aging	Santos et al., 2010
Significant differences in MN frequencies at baseline between patients with breast cancer and controls	Cardinale, et al. 2012
Chemotherapy Effects:	
In vitro study showing variation in response to doxyrubicin-induced MN between lymphocyte cell lines; Polymorphism in the GSTP1 gene was associated with an increased MN frequency	Ramos et al., 2011
In vitro study showing a significant increase in MN frequencies in breast cancer cell lines following exposure to doxyrubicin; methoxyamine alone did not significantly increase MN frequencies; use of both methoxyamine and doxorubicin significantly increased levels of MN frequencies (combination higher than single exposure values)	Guerreiro et al., 2013
In vivo study showing MN frequencies in lymphocytes and buccal mucosa cells from children with malignant tumors (various types) increased significantly following chemotherapy	Minicucci et al ., 2008
In vivo longitudinal study (1 year) showing MN frequencies increased significantly after one cycle of chemotherapy and remained increased for 2 months after the completion of chemotherapy (Cisplatin); MN frequencies also correlated with the cumulative dose of Cisplatin and with Cisplatin-induced nephrotoxicity	Elsendoorn et al 2001
Radiation Effects:	
In vivo study showing MN frequencies increased significantly during radiation and further increased immediately following the completion of radiation; MN frequencies not influenced by age	Jagetia et al., 2001
Higher MN frequencies during radiotherapy; while decreases in frequency were observed 6 months and one year after radiotherapy, MN frequencies did not return to pre-therapy level in the majority of patients; older subjects (ages 75-80; also had more advanced tumors) showed higher MN frequencies than younger subjects (ages 57-70)	Gamulin et al., 2010
In vivo studies showing radiation-induced MN frequencies were correlated with dose and quality of occupational, medical, accidental, or therapy-related radiation	Vral et al., 2011
In vitro study showing significant increase in MN frequencies in cells exposed to ionizing radiation, with the difference in frequency responses of patients with breast cancer compared to controls exceeding the differences observed at baseline	Cardinale, et al., 2012
Chemotherapy and Radiation Effects:	
In vivo study showing an increased frequency following radiation alone and following radiation in combination with chemotherapy regardless of age or smoking	Milosevic-Djordjevic et al., 2011

In vivo longitudinal study (18 months) showing MN frequencies increased after radiation (20 patients received radiation) but not after chemotherapy (only 9 out of 20 patients received and were assessed after chemotherapy) MN frequencies were not significantly correlated with tumor size, receptor status, nodal status, family history of cancer, age, or smoking habits	Aristei et al., 2009

Hypothesis, goals, aims, study design and significance of the present study:

Hypothesis:

Treatment advances have resulted in improvements in survival for women diagnosed with breast cancer, but many of these survivors report acquiring persistent adverse side effects following treatment. The etiology of the observed PNS is not fully known. Chemotherapy, or the tumor itself, induces short-term effects and long-term PN effects through a series of events and interactions, which result in acute and chronic biologic changes, where some could be persistent for years after completing a successful treatment. We offer the hypothesis that inflammatory activation is an early step in a cascade of biological changes leading to genomic and/or epigenetic alterations that contribute to PNS. Alternatively, epigenetic alterations may occur first, leading to inflammatory activation. These epigenetic changes could also lead to chromosomal instability. Each of these genomic/epigenetic factors, acting either singly or in concert, could contribute to the development/persistence of PNS since they would provide a means for "remembering" the biological effects after chemotherapy and have plasticity to explain responsiveness to environmental exposures (as well as G x E interactions). They also have the potential for reversibility, with the latter attribute being important for the potential development of therapeutic interventions (Figures 1 & 2).

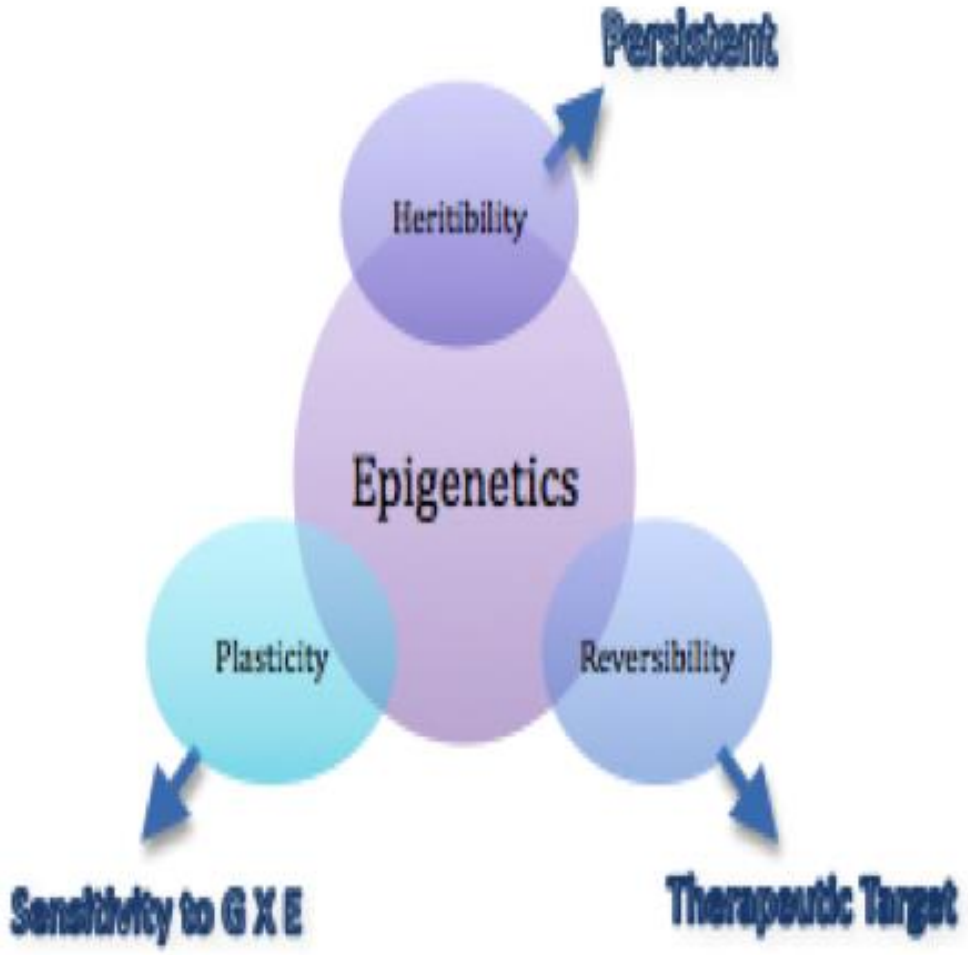


Figure 2: The Strength of investigating epigenetic mechanisms: Epigenetic mechanisms provide a means for "remembering" the biological effects after chemotherapy and have plasticity to explain responsiveness to environmental exposures (as well as G x E interactions). They also have the potential for reversibility, with the latter attribute being important for the potential development of therapeutic interventions.

Goal:

Investigate the biological basis for the development of the clustering PNS associated with chemotherapy, or the cancer itself, in women treated for BC.

Specific aims:

The primary aims of this study were to collect blood specimens from women having a diagnosis of BC and longitudinally determine:

1) The frequency of acquired chromosomal instability and genome wide methylation changes present at baseline and following treatments.
2) The relationship between acquired chromosomal alterations and the development of PNS.

Overall study design: (Note: This thesis is a part of a larger study design)

In order to understand the epigenetic and the chromosomal instability changes, and their role in developing PNS, which are associated with BC and its treatments, we designed a longitudinal study to follow a total of 76 women treated for BC at 5 time points within a two-year time period. For the facet of the study reported in this thesis, acquired changes in PBCs methylation patterns and frequencies of MN formation in binucleated lymphocytes were examined at 4 time points: before the initiation of chemotherapy, at the 4th chemotherapy, 6 months after the initiation of chemotherapy (at which time some women are receiving radiation therapy), and 12 months after

the initiation of chemotherapy. Because it is not completely clear whether the tumor itself or the treatment is initiating the development of these symptoms, we initiated copy number assessments in tumor tissues obtained from a subset of women who provided their consent to have their tumor specimens evaluated.

The tissue sample choice:

In order to identify acquired epigenetic changes/chromosomal instability associated with the development of PN symptoms observed in a subset of women treated for BC, one would think of the brain tissue as one of the most affected or permanently affected tissues. While acquired epigenetic changes in brain cells are likely to be associated with some of the observed symptoms, such as cognitive dysfunction, they would not be anticipated to be clearly associated with all of the observed symptoms, such as fatigue. Also, it is not possible to sample brain tissue from living individuals (especially since multiple brain areas would need to be evaluated to most accurately reflect the neural tissue changes that might arise).

Inflammation, which results from BC, and its treatment, has been suggested to have a role in PNS production (Konsman et al 2002, Lee et al 2004, Cleeland et al 2003). Because white blood cells (WBCs) are involved in the inflammatory process, blood samples are optimal. As mentioned earlier, animal models, preclinical and clinical evidence showed that sickness behavior requires an immune-to-brain communication, which involves cytokines and (WBCs) (Cleeland et al 2003). Therefore, we believe that blood samples are one of the best sample choices to be investigated in

our study. Moreover, we are investigating acquired changes associated with chemotherapy. Thus, blood cells are one of the first cells to be exposed and affected by the therapy. Indeed, leukocytes could reflect one of the earliest acquired epigenetic changes associated with BC chemotherapy and inflammatory induction, which happen before triggering other changes that cause the occurrence of the permanent changes in brain cells. Blood samples are easily obtained and all parts of our study could be easily performed on this type of tissue, including inflammatory biomarkers measurement, acquired epigenetic changes, gene expression and MN frequencies assessments. Furthermore, for future implications, if epigenetic signatures or MN assessments could be used to estimate an individual's risk or could be treated, this sample will be the easiest to obtain and follow up.

Another sample that would be interesting to look at is cells from the tumor cells themselves. The tumor may be the source that initiates a cascade of changes, including inflammation, and acquired epigenetic changes in other tissues such as WBCs. Cytokines could be produced not only by immune cells, but also by other cell types such as the tumor cells in cancer patients (Cleeland et al 2003). It remains unclear how chemotherapy affects women and associates with the development of PNS in a subgroup of women treated for BC. Thus, it is important to investigate not only blood samples, but also the tumor tissue itself. It is possible that the tumor cells define or are related to the susceptibility of women to develop PNS as a result of chemotherapy.

Significance:

The results from this study will enable us to gain a better understanding of the biological basis for the development and persistence of PN symptom in women with BC, with the hope of enhancing the quality of survivors' lives. With advances in the pharmacology of epigenetics, if the development of PN symptoms could potentially be detected at an earlier point, they might be prevented or alleviated through treatment. Alternatively, the results of this study could provide a basis for recognizing women who are most "at risk" for developing PNS, thereby allowing them to make more informed decisions regarding their therapy options. This study is the first of its kind where multiple biological mechanisms and symptom assessments are followed during the treatment course with a hope of helping BC survivors to live with a better quality of life.

Chapter 2

Acquired Chromosomal Changes in Women Treated for Breast Cancer and their Association with the Development of Psychoneurological Symptoms

Micronucleus frequencies in lymphocytes

Introduction

Breast cancer is the most common invasive cancer in females, accounting for approximately 29% of all women's cancers (Siegel et al, 2014). According to estimates from the American Cancer Society, about 12.3% of women in the US will develop breast cancer during their lifetime (American Cancer Society, 2013). Due to advances in early detection and management, approximately 89% of women diagnosed with breast cancer will survive for at least 5 years (American Cancer Society, 2013). Unfortunately, these advances in survivorship have been accompanied by the acquisition of treatment-related adverse side effects that can compromise the quality of life for many women who receive treatment for breast cancer. These side effects, which include anxiety (Badger et al. 2007), depression (Badger et al. 2007), fatigue (Berger et al. 2010), cognitive dysfunction (Wefel et al 2011) , sleep disturbance (Lee et al 2004), and pain (Utne et al 2010, Valeberg et al 2008), are collectively referred to as psychoneurological symptoms (PNS) and can persist for years after treatment.

The biological basis for the genesis of PNS in women treated for breast cancer is not known. The results of some cross sectional studies have shown an association between markers of inflammation, most notably proinflammatory cytokines, and PNS (Cleeland et al 2003, Fumaz et al 2012). However, investigators have not attained a clear consensus regarding the etiological role,

if any, of cytokines in chemotherapy-related PNS (Dantzer et al 2001, Cleeland et al 2003, Lee et al 2004). More recently, conjectures about the biological basis underlying the development and persistence of chemotherapy-related PNS have shifted to include mechanisms that would account for both the short-term presentation of symptoms, as well alterations that could be biologically "remembered" or retained for months/years. One potential means whereby chemotherapy could induce the development and persistence of PNS is if that therapy resulted in the acquisition of somatic chromosomal instability. This soma-wide instability could result in the development of a clonal population(s) of cells having a chromosomal imbalance, which, in turn, could lead to the development of PNS and/or other health conditions. Recent advances in methodology support the premise that somatic mosaicism and/or mutations are associated with a variety of health conditions (Grayson, et al., 2010; Forsberg, et al, 2013; Poduri, et al., 2013; Li, et al., 2014). In particular, spontaneous somatic chromosomal instability/mosaicism has been associated with the development of cancer (Santos et al . 2009, Cardinale et al , 2012, Chang et al. 2010); dyskeratosis congenital (Jongmans, et al., 2012); rheumatoid arthritis (Tascioglu et al., 2005); and osteoarthritis (Castellanos et al. 2004, Dahlen et al., 2001, Kinne et al., 2001, Mertens et al., 1996, Broberg et al., 1998). Somatic chromosomal mosaicism has also been associated with several conditions involving behavior and/or cognition, including but not limited to, Alzheimer's disease (Zekanowski and Wojda 2009; Yurov, et al., 2013), Parkinson disease (Migliore et al 2001), age-related cognitive decline (Faggioli, et al., 2011), schizophrenia (Yurov, et al., 2013; Ruderfer, et al., 2013), and stressful events (York, et al., 2013).

Chromosomal instability has been observed by several investigators in cells from breast cancer tumors (Yoon et al 2003, A'Hern et al 2013, Burell et al 2010 , Kabalar et al 2012, Niu et al 2013,

Ottesen et al 2003, Jiang et al 2014), as well as in cells from peripheral tissues (typically blood) of people diagnosed with cancer prior to their receipt of treatment (Santos et al 2010, Milosevic-Djordjevic et al 2010, Harsimran et al 2009). While few in number, studies have also been completed to evaluate the frequency of chromosomal instability in peripheral blood following exposure to various treatments for breast cancer, with these reports noting an increase in chromosomal instability values soon after radiation treatment (Jagetia et al 2001; Gamulin et al 2010; Vral et al 2011; Cardinale et al 2012) or radiation and chemotherapy (Aristei et al 2009; Milosevic-Djordjevic et al 2011).

Given that chromosomal instability appears likely to arise after treatment for breast cancer, it follows that these chromosomal aberrations might result in somatic mosaicism that could compromise the function of the cells containing these aberrations. Based on this premise, we hypothesized that an increase in micronuclei frequencies arises following chemotherapy and that this increase in somatic chromosomal instability is associated with the development and/or persistence of PNS in women receiving therapy for breast cancer. To test this hypothesis we designed a 2-year longitudinal study to determine the frequency and retention of acquired chromosomal changes in women diagnosed and treated for breast cancer. This current study reports the findings from one year of follow-up for this longitudinal study. The time points evaluated include: baseline (before the initiation of chemotherapy); mid-cycle (typically at the 4^{th} chemotherapy treatment); 6 months following the initiation of chemotherapy (at which time a subset of women have recently received radiation therapy); and 1 year following the initiation of chemotherapy.

Materials & Methods

Study Participant Ascertainment and Specimen Collection

A total of 76 women with early stage (I to IIIA) breast cancer, who ranged in age from 23 to 71 years old, were ascertained through 5 regional cancer centers in Central Virginia. To identify potential study participants, a member of the research team attended the weekly interdisciplinary Breast Health meetings that were held at the Virginia Commonwealth University Medical Center (VCUMC). Study eligibility criteria for participants were: (1) 21 years or older; (2) diagnosis of early stage breast cancer with a scheduled visit to receive chemotherapy; and (3) female (males were excluded since too few male participants were available for study). Exclusion criteria included: (1) a history of previous cancer, or chemotherapy; (2) a diagnosis of dementia; (3) active psychosis; or (4) a history of immune-related diagnoses (e. g. multiple sclerosis; systemic lupus erythematosus). After providing informed consent (VCU IRB #HM 13194), participants were enrolled and the first study visit was scheduled prior to the initiation of chemotherapy. Study visits took approximately one hour to complete. A peripheral blood sample was collected at each of the 4 time points by venipuncture or through an existing access device and transported (same day) to our (Jackson-Cook) cytogenetics laboratory. The specimens were coded prior to their delivery to the lab to ensure that the cytogeneticists were unaware of the clinical history and PNS status of each participant at the time of sample processing and evaluation.

Demographic, Lifestyle, Breast Tumor and Other Health Information

Demographic information for the study participants was collected by self-report using a questionnaire format. Demographic variables evaluated in this current report included age, race, and income. Similarly, lifestyle factors [nutritional practices (eating vegetables/fruit); smoking status, and ethanol usage] and health attributes not directly related to the diagnosis of breast cancer [menopausal status (pre- or post-)] that were evaluated in this 1 year follow-up report were obtained from self-report. Medical records were used to determine body mass index, breast tumor attributes, and chemotherapy regimens. Based on the tumor fluorescent in situ hybridization (FISH) and/or immunohistochemical testing results, which were obtained from the medical records, the tumors were categorized as luminal A (human epidermal growth factor receptor 2 [HER2] -; estrogen receptor and/or progesterone receptor +), luminal B (HER2+; estrogen receptor and/or progesterone receptor +), HER2 positive (HER2+; estrogen receptor -; progesterone receptor -), or triple negative (HER2-; estrogen receptor - ; progesterone receptor -) (Perou, et al., 2000; Hu, et al., 2006, Fan, et al., 2006; Voduc, et al., 2010; Carey, et al., 2013).

Chemotherapy and Radiation Treatments

The chemotherapy treatments received for study participants were determined by medical record review. Three primary types of chemotherapy administered included: (1) taxotere (docetaxel), adriamycin (doxorubicin), and cyclophsophamide [TAC]; (2) taxotere and cyclophosphamide [TC]; or (3) taxotere (docetaxel), carboplatin (paraplatin); and herceptin (transtuzumab) [TCH]. The recognition of each participants' receipt of radiation therapy (or lack thereof) was also derived from reviews of the medical records. All blood specimens were collected

within 1 month after treatment, with most chemotherapy specimens being collected within 2 weeks of exposure.

Chromosome Instability Methodology

Chromosomal instability levels were quantified for each specimen using the cytokinesis-block micronucleus (CBMN) and cytome assay (Fenech, 2006). A micronucleus (micronuclei) (MN) is (are) small chromatin containing structure(s) juxtaposed to a parent nucleus (Figure 3). The CBMN assay was selected for the chromosomal instability assessments because it allows for: (1) the quantification of both numerical and structural cytogenetic findings; (2) the analysis of a large number of cells, thereby enhancing one's ability to detect even low levels of instability; and (3) minimization of in vitro growth selection (only 1 round of cell division is completed in vitro) and minimization of technical artifacts due to processing (for example, the CBMN assay does not show chromosome "loss" due to cell breakage at harvesting or slide making, which can occur when assessing metaphase chromosomes).

Leukocytes, which were isolated using Histopaque-1077 (Sigma), were established in culture according to the protocol of Fenech (1993). Briefly, following their mitogenic stimulation using phytohemaglutinin (PHA), lymphocytes were arrested at cytokinesis by adding cytochalasin B to the cells 44 hr after their culture initiation. The cells were harvested 72 hours after culture initiation and slides prepared (2 per specimen) as described previously (Leach and Jackson-Cook, 2001) (Figure 4)

Micronuclei, buds, and/or bridges were visualized following giemsa staining (4% Harleco Giemsa solution) and identified according to the criteria established by Fenech (2006). The proportion of abnormalities was calculated by adding the values obtained from the two replicate scores (1000 binucleates were evaluated from each of two slides for a total of 2000 binucleates per study participant). The total number of binucleates with abnormalities was then divided by the total number of binucleates scored.

PNS Assessment

PNS were assessed based on study participant self-reporting or computer interfaced performance measures using well established biobehavioral tools. Briefly, cognitive dysfunction was evaluated using the CNS Vital Signs (CNSVS, https://www.cnsvs.com) computerized neurocognitive testing system, in which practice effects are minimized by randomly generated alternate test forms or random selections from item banks (ref). Cognitive measures evaluated in this study included the overall cognitive index, memory, complex attention, psychomotor speed and cognitive flexibility domains. Depressive symptoms and anxiety were identified using the hospital anxiety and depression scale (HADS), which is a brief (14-item) self-report questionnaire that has well-established reliability and validity for both depressive symptoms and anxiety in women with breast cancer. Fatigue symptoms were evaluated using the Brief Fatigue Inventory (BFI), which is a clinically validated tool used to assess cancer-related fatigue and its impact on daily functioning. Participant sleep disturbances were assessed through self-report using the 21-item General Sleep Disturbance Scale (GSDS), which evaluates various aspects of sleep disturbance (quality and quantity of sleep, sleep onset latency, number of awakenings, excessive

daytime sleepiness, and medication use) over the past week. Pain was evaluated using the Brief Pain Inventory (BPI), which assesses the severity and location of pain, pain medication use, amount of pain relief in the past 24 hours or the past week, and the impact of pain on daily functions. The arithmetic mean of the 4 severity items were used as a measure of pain severity, and the arithmetic mean of the 7 interference items were used as a measure of pain interference. Perceived stress was measured using the 10-item Perceived Stress Scale (PSS).

Statistical analysis

Descriptive statistics of MN/cytome frequencies were computed for the demographic, tumor characteristic, treatments, and PNS symptoms variables noted above. All data were examined graphically to determine their distributional properties. It was anticipated that the MN/cytome frequencies would follow a log-normal distribution as is typical with MN frequency data (Ceppi, et al., 2011). However, because of the relatively high number of MN in this population, the MN frequencies were approximately normally distributed.

Using the model building approach proposed by Hosmer and Lemeshow (2000), a mixed effects linear model (Brown and Prescott, 2006) was fit (by R. K. Elswick, Jr.) to determine the best subset of predictors of MN/cytome frequencies. In the first stage of the model building process, a base model was selected that represented the design of the data collection and of the timing of the treatments (chemotherapy and radiation). Fixed effects included time (baseline, mid-chemo, six months and 1 year post-chemo), chemotherapy (TAC, TC and TCH), time by chemotherapy interaction (chemotherapy was administered only during time point 2), radiation

(Yes/No); time by radiation interaction (radiation was only administered at time point 3), and a random effect for study participant. In the second stage, each potential predictor was fit individually with the base model and, if the *p*-value was 0.25 or less, that predictor was used in the next stage. Potential predictors included demographic variables (age, race, BMI, income, menopausal status, current smoking status & alcohol consumption, and lifestyle nutrition), tumor characteristic variables (grade, stage. luminal A, luminal B, triple negative & HER 2 positive status), surgery, PNS variables (HADS depression, HADS anxiety, BPI worst, BPI least, BPI average, BPI now, BPI relief, BPI interference, BFI total, BFI now, BFI usual, BFI worst, BFI interference, GSDS total, total PSS) and cognition variables (NCI, Memory, Complex attention, Psychomotor speed & Cognitive flexibility). In the third stage, all potential regressors ($p< 0.025$) were put into a multiple variable model. This initial model was further refined by sequentially removing variables from the model with the highest p-values (backward stepwise) until all remaining factors had a *p*-value of 0.05 or less. At this stage, all pairwise interactions were added. Again using a backward stepwise approach, all interaction terms with *p*-values less than 0.05 were removed. This model was considered the final prediction model. The SAS v9.2 and JMP v11.1 statistical packages were used for these analyses (SAS 9.3 and JMP 11.1: SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27513.). In order to limit type I errors, an alpha level of $p<0.05$ was used for all statistical tests.

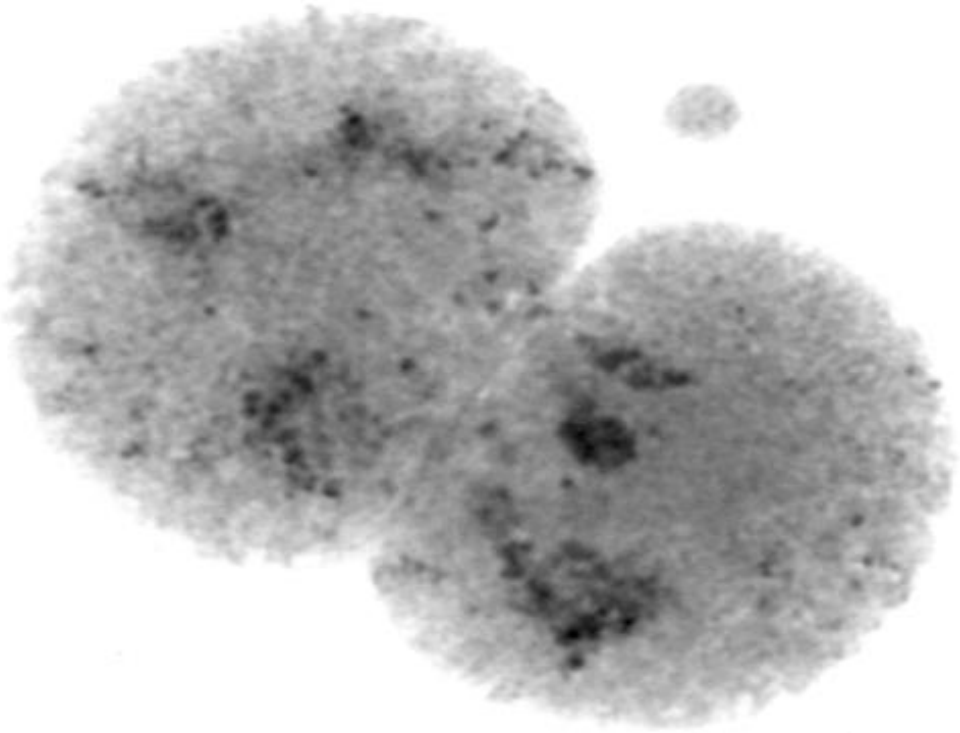


Figure 3: The appearance of a micronucleus (MN) juxtaposed to a binucleated lymphocyte stained by Giemsa.

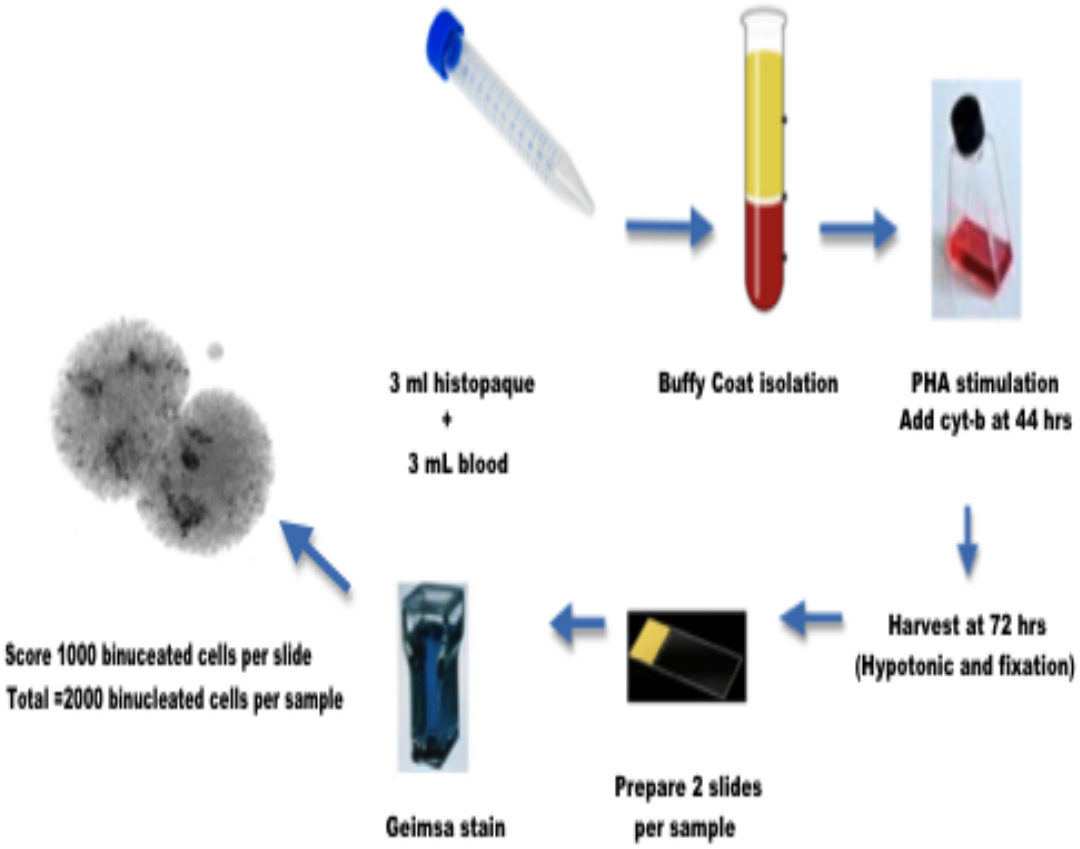


Figure 4: Description of the Cytokinesis Block MN (CBMN) assay. Briefly, the buffy caot consisting of leukocytes was isolated using Histopaque. Following their mitogenic stimulation using phytohemaglutinin (PHA), Lymphocytes were arrested at cytokinesis by adding cytochalasin B to the cells 44 hr after their culture initiation. The cells were harvested 72 hours after culture initiation and slides were prepared (2 per specimen), stained and scored.

Results

A total of 73 of the 76 women initially recruited for this longitudinal study elected to complete the first year of sample collections (96% retention rate), with 1 participant providing only a baseline specimen (withdrew due to lack of time/interest); and 2 participants failing to provide specimens for all 4 times (both women cited lack of time as their reason for not continuing). For times 3 and 4, assessments were completed for 67 and 50 of the study participants, respectively, with the smaller number of specimens evaluated for these times reflecting pending specimen collections in this ongoing, 2-year longitudinal study. The majority of the women in this study received a TAC chemotherapy regimen (n=39), followed by TC treatment (n=21), or TCH treatment (n=11). Two of the women received a cyclophosphamide, methotrexate and 5 fluorouracil treatment (CMF). Given the small number of women receiving this treatment, this group was excluded from the analysis. Thus, comparisons of factors influencing chromosomal instability frequencies were completed for a total of 71 women.

Demographic data, tumor characteristics, and treatment information is/are shown for the 71 women fully participating in this longitudinal study in Tables 8-10. These participants included 22 women of African American heritage and 49 women of Caucasian heritage. Only 2 women (1 in the African American cohort and 1 in the Caucasian cohort) self-reported that they were of Hispanic ethnicity. Thus, given the small number of women having a Hispanic heritage, this sub-group was not analyzed separately. Since none of the 14 women who reported smoking met the criteria of a heavy smoker, as suggested by Fenech et al. (2006) for assessments of the influences of smoking on micronuclei frequencies, smoking habits were not included in our statistical model.

The average age of the study participants was 51.59 years, with a significant difference in age being observed between the African American (mean= 47 years, s.e.=1.89 years) and Caucasian (mean= 53 years, s. e. =1.46 years) sub-groups (p=0.006) (Table 8). Annualized income levels also varied between the racial groups, with the Caucasian patients having significantly higher incomes than the African American women (p<0.0001). As expected, the majority of women (56%) were post-menopausal with 44% being either pre- or peri-menopausal (Table 8). No significant difference in menopausal status was detected between the African American and Caucasian study participants. However, significantly more Caucasian women reported consuming alcohol (69%) than African American women (27%) (p=0.0016) (Table 8).

Due to the inclusion criteria established for this study, all of the study participants had early stage breast cancer (stages I to IIIA). The participants' clinical pathological characteristics and their pre-chemotherapy treatments are summarized in Tables 9 and 10, respectively. While the proportion of African American women having grade 3 (Grade 3 - 40.91%) tumors tended to be lower than the proportion of Caucasian women (Grade 3 – 59.18%) these tumor distributions were not significantly different (p=0.153). Similarly, while no significant difference in the proportion of the 4 intrinsic subtypes of tumors was observed between the racial sub-groups (p=0.107), there was a trend toward a higher proportion of triple negative tumors and a lower proportion of luminal A tumors in the African American women (triple negative tumors-36.36%; luminal A tumors-36.36%) compared to the Caucasian women (triple negative tumors-24.49%; luminal A tumors-61.22%) (Table 9). As expected, due to the lack of a significant difference in the tumor characteristics in the African American compared to Caucasian women, no significant differences

were observed in the treatment regimens between the racial groups (chemotherapy p=0.2011; radiation p=0.213).

To determine if any of the treatment, health, demographic, or symptom variables were associated with MN/cytome abnormality frequencies, the data were fit to a mixed effects linear model (Table 11). The final optimized model identified 8 variables that had a significant predictive association with MN/cytome abnormality frequencies (Table 11). These variables included: (1) the time point at which the specimen was collected (p<0.0001); (2) the type of chemotherapy treatment (p=0.0463); (3) exposure to radiotherapy (p=0.0004); (4) race (p=0.0037); (5) having a luminal B tumor categorization (p=0.0182); (6) having a triple negative tumor categorization (p=0.0446); (7) total perceived stress (p=0.0123); and (8) cognitive flexibility domains (p=0.0238).

The strongest significant association between MN/cytome abnormality frequencies was the time at which the specimens were collected (p<0.0001) (Table 11 & Figure 5). Almost all women showed a significant increase in their chromosomal instability frequencies at time point 2 (the mid-chemo specimen). Those participants who received radiation showed an additional increase in their MN/cytome abnormality frequencies at time point 3 (figure 6). Interesting, while the chromosomal instability frequencies were highest at times 2 and 3, the time 4 values (1 year following the initiation of chemotherapy) tended to remain higher than baseline values (Table 11).

The frequency of MN/cytome abnormalities also differed based on the type of treatment the participants received. The greatest increase in MN/cytome abnormality frequencies in response to

the administration of chemotherapy (time point 2) was observed for the women who received TAC treatment (Figure 5). However, the women who received TCH treatment had the highest overall value of chromosomal instability (mean = .071, SE = .0042) when compared to women receiving TAC (mean=.063 SE =.0043) or TC (mean=058 ; SE = .0048) chemotherapy regimens (p=**0.0463)** (Table 4 & Figure 2). Moreover, the subset of women who received radiation had significantly higher MNF (mean=.073; SE=.0033) than women who didn't receive radiation (mean=.057; SE=.0042) (Figure 6 & Table 11).

Race was also observed to be predictive of chromosomal instability frequencies (*p*=0.0037), with values being significantly lower in the African American participants (mean=.059; SE= .0043) compared to the Caucasian participants (mean=.071; SE= .0031). One might conjecture that this association between acquired chromosomal instability and race is confounded by other influences (e. g. age differences between the racial sub-groups). However, the consistent recognition of an association between MN/cytome abnormality frequencies and race following the stepwise removal of other potential confounding variables (including age and tumor characteristics) suggests that there may be a separate, differential impact of race on chromosomal instability frequencies following treatment for breast cancer (Table 11).

Tumor characteristics were also observed to be predictive of MN/cytome abnormality frequencies with luminal B tumors being associated with lower overall chromosomal instability values (mean=.057; SE=.0060), as compared to other types (mean=.073; SE=.0024) (p=.0182). Conversely, triple negative tumors were associated with higher values (mean=.0693; SE=.0043) as compared to other tumors (mean=.0611; SE=.0031) (p= .0446) (Table 11). Given that the tumor

characteristics determine the type of therapy regimen received by the study subjects, it is feasible that this association is confounded by the impact of chemotherapy and/or radiation. However, as noted previously in regard to race, the retention of consistent significant association values between these tumor characteristic variables and MN/cytome abnormality frequencies following the stepwise removal of the potentially related therapy variables suggest the presence of a separate effect (above that related to therapies) for the tumor sub-types on peripheral blood MN/cytome abnormality frequencies.

The best fit model also showed that the participants' total perceived stress levels were predictive of chromosomal instability frequencies ($p=0.0123$), with women reporting higher stress levels also having higher values of instability. A second symptom associated with MN/cytome abnormality frequencies was cognitive flexibility, with a significant positive relationship being observed (higher instability values in women with higher cognitive flexibility domains)($p=.024$). Although complex attention measures were also initially suggestive of an association with MN/cytome abnormality frequencies (Table 11), this variable did not yield a significant difference in the reduced model. No other PNS evaluated showed significant associations with acquired lymphocyte chromosomal instability frequencies.

Other variables that were tested and found to show no significant association with micronuclei frequencies in the initial or final models were age, alcohol consumption, income, nutritional habits, body mass index, menopausal state, tumor grade, tumor stage, luminal A and HER2+ tumor sub-groups, types of surgery, depressive symptoms, anxiety, pain measures, fatigue measures, memory, psychomotor speed, and overall cognitive function.

Table 8. Demographic, health and lifestyle findings in study participants receiving chemotherapy for breast cancer

	African American n=22 (100)			Caucasian n=49 (100)			
	TAC	**TC**	**TCH**	**TAC**	**TC**	**TCH**	**Total**
Demographic Variable	n=10 (45.45[1])	n=6 (27.27)	n=6 (27.27)	n=29 (59.18)	n=15 (30.61)	n=5 (10.20)	n=71 (100)
Income							
Less than 30,000	5 (22.73)	4 (18.18)	3 (13.64)	6 (12.24)	1 (2.04)	0	19 (26.76)
30,000-59,999	3 (13.64)	2 (9.09)	3 (13.64)	6 (12.24)	1 (2.04)	0	15 (21.13)
60,000-89,999	1 (4.55)	0	0	6 (12.24)	7 (14.29)	3 (6.12)	17 (23.94)
90,000+	1 (4.55)	0	0	11 (22.45)	6 (12.24)	2 (4.08)	20 (28.17)
Age	44.00, 3.40[2]	47.00, 2.52	50.50, 2.93	52.17, 1.73	57.13, 2.64	50.20, 6.35	
Menstrual Status							
Pre- or Peri-	7 (31.82)	3 (13.64)	2 (9.09)	11 (22.45)	5 (10.20)	3 (6.12)	31 (43.66)
Post-	3 (13.64)	3 (13.64)	4 (18.18)	18 (36.73)	10 (20.41)	2 (4.08)	40 (56.34)
BMI	31.99, 2.68[2]	29.84, 2.54	35.56, 6.26	30.09, 1.47	26.77, 0.88	37.28, 4.70	
Nutrition[3]	2.59, 0.18[2]	2.22, 0.21	2.35, 0.25	2.72, 0.11	2.92, 0.13	2.67, 0.30	
Current Smoking Status							
Yes	2 (9.09)	3 (13.64)	2 (9.09)	4 (8.16)	2 (4.08)	1 (2.04)	14 (19.72)
No	8 (36.36)	3 (13.64)	4 (18.18)	25 (51.02)	13 (26.53)	4 (8.16)	57 (80.28)
Current Ethanol Consumption							
Yes	3 (13.64)	1 (4.55)	2 (9.09)	19 (38.78)	11 (22.45)	4 (8.16)	40 (56.34)
No	7 (31.82)	5 (22.73)	4 (18.18)	10 (20.41)	4 (8.16)	1 (2.04)	31 (43.66)

[1]Number in parenthesis is the percentage of study participants for the category (%)
[2]Mean, standard error
[3]Nutritional assessments for the participants' intake of fruits and vegetables

Table 9. Study participants' breast tumor characteristics

Tumor Characteristic	African American N=22 (100*)			Caucasian N=51 (100*)			
	TAC	**TC**	**TCH**	**TAC**	**TC**	**TCH**	**Total**
	(n=10) (45.45*)	(n=6) (27.27)	(n=6) (27.27)	(n=29) (59.18)	(n=15) (30.61)	(n=5) (10.20)	(n=71) (100)
Luminal A							
Yes	6 (27.27)	2 (9.09)	0	21 (42.86)	9 (18.37)	0	38 (53.52)
No	4 (18.18)	4 (18.18)	6 (27.27)	8 (16.33)	6 (12.24)	5 (10.20)	33 (46.48)
Luminal B							
Yes	0	0	2 (9.09)	2 (4.08)	0	3 (6.12)	7 (9.86)
No	10 (45.45)	6 (27.27)	4 (18.18)	27 (55.10)	15 (30.61)	2 (4.08)	64 (90.14)
Triple negative							
Yes	3 (13.64)	4 (18.18)	1 (4.55)	6 (12.24)	6 (12.24)	0	20 (28.17)
No	7 (31.82)	2 (9.09)	5 (22.73)	23 (46.94)	9 (18.37)	5 (10.20)	51 (71.83)
HER2+,ER-,PR-							
Yes	1 (4.55)	0	3 (13.64)	0	0	2 (4.08)	6 (8.45)
No	9 (40.91)	6 (27.27)	3 (13.64)	29 (59.18)	15 (30.61)	3 (6.12)	65 (9.15)
Grade							
1	1 (4.55)	0	0	2 (4.08)	2 (4.08)	0	5 (7.04)
2	5 (22.73)	3 (13.64)	4 (18.18)	12 (24.49)	4 (8.16)	0	28 (39.44)
3	4 (18.18)	3 (13.64)	2 (9.09)	15 (30.61)	9 (18.37)	5 (10.20)	38 (53.52)
Stage							
I	1 (4.55)	4 (18.18)	0	5 (10.20)	8 (16.33)	2 (4.08)	20 (28.17)
IIA	6 (27.27)	2 (9.01)	3 (13.64)	10 (20.41)	6 (12.24)	1 (2.04)	28 (39.44)
IIB	3 (13.64)	0	3 (13.64)	6 (12.24)	1 (2.04)	2 (4.08)	15 (21.13)
IIIA	0	0	0	8 (16.33)	0	0	8 (11.27)

*** Number (%) of participants.**

Table 10. Study participant treatments

Treatment	African American N=22 (100*)			Caucasian N=51 (100*)			
	TAC	**TC**	**TCH**	**TAC**	**TC**	**TCH**	**Total**
	(n=10) (45.45*)	(n=6) (27.27)	(n=6) (27.27)	(n=29) (59.18)	(n=15) (30.61)	(n=5) (10.20)	(n=71) (100)
Surgery							
Biopsy	3 (13.64)	0	0	2 (4.08)	0	0	5 (7.04)
Lumpectomy	4 (18.18)	0	1 (4.55)	8 (16.33)	6 (12.24)	1 (2.04)	20 (28.17)
Segmental	1 (4.55)	5 (22.73)	3 (13.64)	1 (2.04)	6 (12.24)	0	16 (22.54)
Simple	2 (9.01)	1 (4.55)	2 (9.01)	18 (36.73)	3 (6.12)	4 (8.16)	30 (42.25)
Neoadjuvant							
Yes	3 (13.64)	0	1 (4.55)	2 (4.08)	1 (2.04)	0	7 (9.86)
No	7 (31.82)	6 (27.27)	5 (22.73)	27 (55.10)	14 (28.57)	5 (10.20)	64 (90.14)
Radiation							
Yes	9 (40.91)	5 (22.73)	5 (22.73)	23 (46.94)	12 (24.49)	1 (2.04)	55 (77.46)
No	1 (4.55)	1 (4.55)	1 (4.55)	6 (12.24)	3 (6.12)	4 (8.16)	16 (22.54)

* Number (%) of participants.

Table 11. Mixed Effects Linear Model Fitting Assessment of Predictive Associations of Variables with Micronuclei/Cytome Abnormality Frequencies.

	Base Model[1]	Final model[2]		
Variable	**p value**	**Least Squares Mean**	**Std. Error**	**p value**
Visit				**<0.0001**
1		**0.050**	**0.0044**	
2		**0.067**	**0.0042**	
3		**0.075**	**0.0043**	
4		**0.069**	**0.0045**	
Age	0.973			
Race				**0.0037**
African American		**0.059**	**0.0043**	
Caucasian		**0.071**	**0.0031**	
Chemotherapy				**0.0463**
TAC		**0.063**	**0.0043**	
TC		**0.058**	**0.0048**	
TCH		**0.071**	**0.0042**	
Radiation				**0.0004**
Yes		**0.073**	**0.0033**	
No		**0.057**	**0.0042**	
Radiation by visit				**0.0077**
BMI	0.437			
Income	0.373			
Menopausal status	0.507			
Alcohol consumption	0.078			
Tumor grade	0.805			
Tumor stage	0.234			
Luminal A tumors	0.926			
Luminal B tumors				**0.0182**
Yes		**0.057**	**0.0060**	
No		**0.073**	**0.0024**	
Triple negative tumors				**0.0446**
Yes		**0.0693**	**0.0043**	
No		**0.0611**	**0.0031**	
HER2 +	0.640			
Surgery	0.262			
HADS depressive symptoms	0.814			
HADS anxiety	0.104			
BPI Measures				
Worst pain	0.329			
Least pain	0.293			
Average pain	0.166			
Current pain	0.416			
Pain relief	0.598			
Pain interference	0.236			
BFI Measures				
Total fatigue	0.188			
Current fatigue	0.664			
Usual fatigue	0.100			

Worst fatigue	0.171			
Fatigue interference	0.1571			
Perceived Stress				**0.0123**
Nutrition (fruits and vegs)	0.323			
Cognitive Measures				
NCI	0.495			
Memory	0.991			
Complex attention	0.013			
Psychomotor speed	0.318			
Cognitive Flexibility				**0.0238**

[1]The base model, which was determined by the study design, was:

MN frequency=Visit + Chemotherapy (3 types) + Radiation therapy + Visit by Chemotherapy* + Visit by Radiation therapy* with the study subject being a random effect

*The visit by chemo and visit by radiation interaction variables allowed these values to differ across time points.

[2]The final model reflects the variables that remained significantly associated with MN frequencies after stepwise removals.

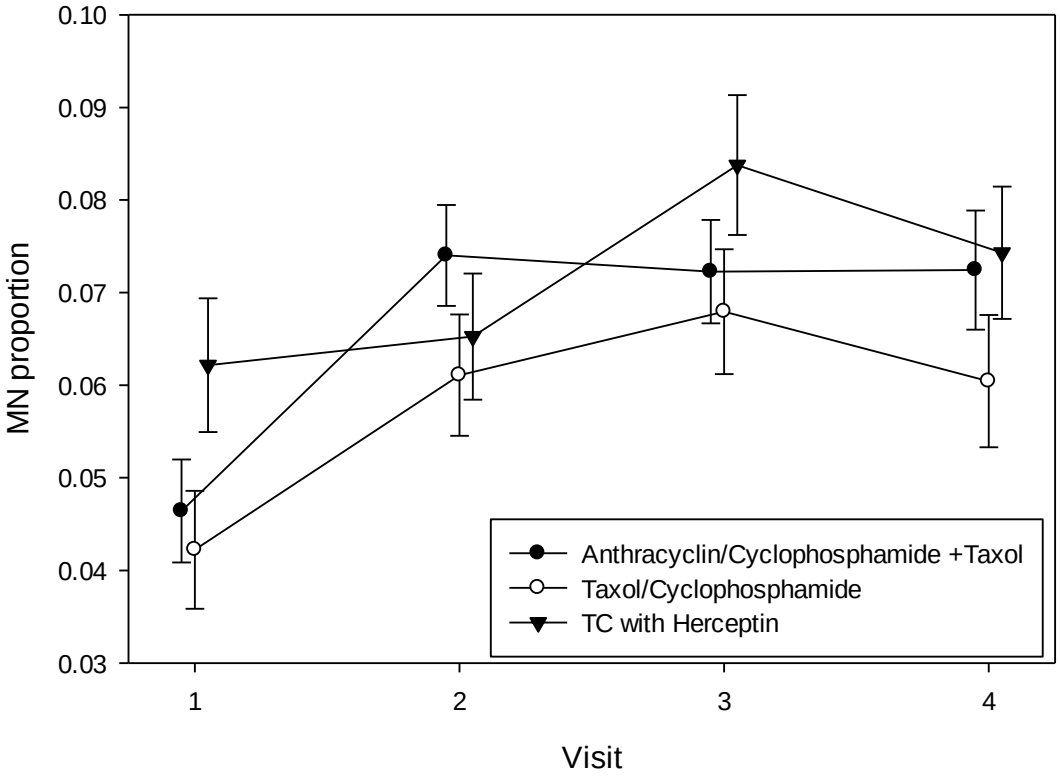


Figure 5: The effect of different types of chemotherapy on MN proportions.
Women treated with TCH (mean=**0.071)** showed the overall highest MNF followed by TAC (mean=0.063) then TC (mean=0.058) (p=0.04). However, at visit 2 (during the administration of chemotherapy), patients treated with TAC had the highest level of acquired changes in MNF followed by TC, then TCH.

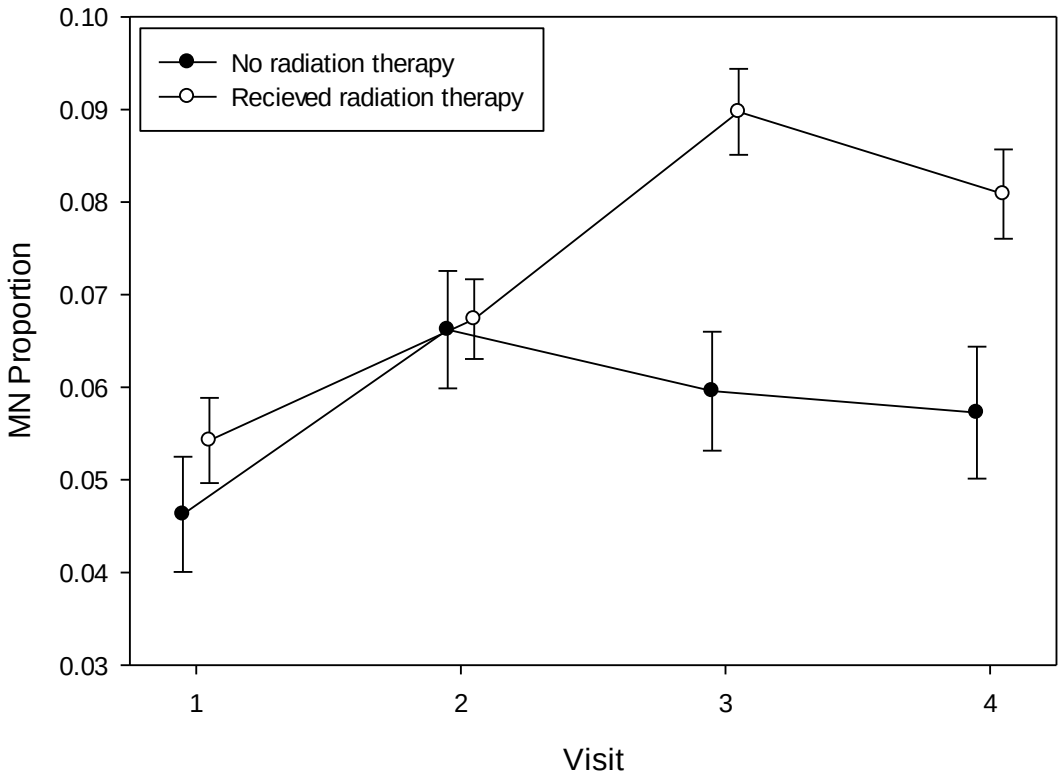


Figure 6: The effect of radiotherapy on MN proportions.

MNF in women treated with radiation were not significantly different from those of women who were not treated with radiation at visit 1 or 2 (before the administration of radiation). However, at visit 3, the subset of women treated with radiotherapy had significantly higher MNF. (Radiation by visit, p=0.0077). Women treated with radiation had overall higher frequencies (mean=0.073) than women who were not treated (mean=0.057) (p= 0.0004)

Discussion

In this longitudinal study we showed that MN/cytome abnormality frequencies in lymphocytes increase after treatment for breast cancer and remain at an increased level for at least a year following the initiation of treatment. In addition to the timing of the receipt of therapy, other factors significantly associated with acquired MN/cytome instability frequencies were therapy regimens (types of chemotherapy and/or radiation), race, tumor characteristics (luminal B or triple negative tumors), and a subset of PNS (total perceived stress and cognitive flexibility domains).

The largest influences on lymphocyte MN/cytome abnormality frequencies were the chemotherapy and radiation treatments given to the participants and the time points of assessment relative to the administration of these treatments. Since both of these types of treatment are genotoxic and can induce DNA damage, it is not surprising that they were associated with significant increases in MN/cytome abnormality frequencies. At visit 2, during the administration of chemotherapy, women treated with TAC acquired the greatest increase in chromosomal instability levels. The fact that the instability levels were higher in the TAC compared to TC treatment cohorts points to adriamycin as the primary factor contributing to this comparative increase. Adriamycin is a member of the anthracyclin class of compounds. Although this drug is widely used, the mechanism(s) for its effectiveness in preventing/reducing cancer cell proliferation is/are not fully known, with some investigators proposing that it induces cell apoptosis or senescence by inhibiting topoisomerase II, while others cite roles for DNA adduct formation, oxidative stress, ceramide overproduction and/or torsion-induced nucleosome destabilization as the causal means for this drug's efficacy (Gewirtz 1999; Senchenkov , et al., 2001; Minotti, et al.,

2004; Yang, et al., 2014). While the mechanism(s) of action for adriamycin (also known as doxyrubicin) remain(s) controversial, this drug has consistently been shown to induce chromosomal instability (Table 7), with telomere dysfunction also consistently being associated with this increase in acquired chromosomal abnormality frequencies (Elmore, et al., 2002; Khan, et al., 2009; Li, et al., 2012). Thus, the results of our study are in agreement with the *in vitro* findings of several other investigators and the *in vivo* results of the few other groups reporting frequencies following treatment in humans.

While the cohort of participants receiving TAC treatment had the highest levels of chromosomal instability at time point 2, the cohort receiving TCH showed the highest overall change in MN proportion levels Table 4. Explanations for this difference in MN/cytome abnormality frequencies between treatments based on the time of specimen collection may reflect the length of the chemotherapy regimen (e.g. the number of cycles for the treatment), the mechanism(s) of the drugs used (docetaxel causes microtubule depolymerization leading to apoptosis, the administration of Herceptin (a targeted monoclonal antibody that binds to the HER2 receptor), and/or the follow-up radiation treatments administered. Radiation therapy was also identified to have a significant predictive influence on the women's acquired lymphocyte chromosomal instability frequencies. As with Adriamycin, radiation exposure has been consistently associated with increased levels of chromosomal instability in human cells, primarily through *in vitro* study designs (Table 7).

An unexpected finding in this study was the significant predictive association of race on MN/cytome abnormality frequencies, with the Caucasian study participants having higher overall

instability frequencies than the African American participants. To our knowledge, associations between lymphocyte MN/cytome abnormality frequencies and race in women treated for breast cancer have not been previously reported. Also, there is a paucity of information available regarding MN frequencies in healthy subjects from different racial groups (Huen, et al., 2006). However, the results of population cytogenetics studies are controversial, with some investigators suggesting a difference in constitutional chromosomal abnormality frequencies between people from different racial backgrounds, while most have seen no clear or consistent differences (Sherman, et al, 2007; Hook, et al., 1999; Hook and Porter, 1977). It is interesting to note that African American women have been described to have significantly lower levels of dietary-related oxidative DNA damage than white women, suggesting that there may be race-related differences in the cellular processes involved in oxidative DNA damage recognition/repair (Watters et al 2007). While our step-wise statistical approach suggests this observation is an independent association, given the inter-relatedness of human epidemiology variables, one cannot fully rule out the possibility that it may be confounded by other factors, such as age or tumor characteristics. For example, the Caucasian women participating in this study were significantly older than the African American participants. This difference is consistent with data from the American Cancer Society, which shows that African American women develop more aggressive forms of breast cancer at younger ages and show a poorer prognosis than Caucasians. However, given that increasing age has consistently been shown to be positively correlated with MN frequencies in healthy women (reviewed by Fenech, et al., 2011; Volders-Kirsch, et al., 2011), it is possible that the age of the Causasian participants contributed to their observed increased rate of MN/cytome abnormalites. We also observed differences in annualized income levels and alcohol consumption rates between the racial groups in our study, with the Caucasian women having significantly higher incomes and

alcohol consumption practices than the African American women. Therefore, given the inter-related nature of the data, the basis for the association between race and MN/cytome abnormality frequencies in this study is not clear.

One aspect of breast cancer that has been associated with racial differences is the proportion of breast cancer tumor sub-groups in African American compared to Caucasian populations (Carey, et al. 2006). While not significantly different, a trend toward a higher proportion of HER2 positive (18.2%) and triple negative (36.4%) tumors was noted in the African American participants in our study compared to those noted in the Caucasian participants. This trend is consistent with the pattern reported by Carey, et al (2006) in their study of women from North Carolina. The proportions of tumor sub-types among the African American and Caucasian women participating in this investigation were not significantly different from those reported by Carey, et al. (2006) (Table 7). Thus, the breast tumors in our participants appear to be representative of those seen in larger populations. The treatment regimen for each study participant is determined, in large part, by her breast tumor characteristics. Therefore, if treatments are related to chromosomal instability levels, one might also anticipate detecting the presence of a relationship between tumor characteristics and acquired chromosomal changes. Indeed, our stepwise reduction model showed a predictive influence of tumor characteristics on MN/cytome abnormality frequencies for the subset of women having luminal B or triple negative tumors that appears to be a separate influence (beyond that of treatment). The interesting observation of a potential influence of tumors on acquired chromosomal instability levels in peripheral blood is in agreement with the previous reports by other investigators who have consistently detected higher pre-treatment MN frequencies

in women (and men) having a variety of different types of cancer when compared to age-matched controls without cancer (reviewed by Fenech, et al., and Kirsch-Volders, et al. 2011).

The basis for the influence of tissue-specific tumors on acquired chromosomal instability levels in peripheral blood (and possibly other somatic tissues) is not clearly known. However, one factor that could contribute to this increase in our population is stress, since the baseline values were collected after the participants had received their diagnosis of cancer (but before treatment). To our knowledge, this is the first study to identify a significant predictive association between stress and acquired lymphocyte chromosomal instability in women with breast cancer, with elevated stress levels being associated with higher frequencies of MN/cytome abnormalities. In the only other previous study in which a significant association was observed between stress and an increased rate of peripheral blood chromosomal instability, twins exposure to childhood sexual abuse were found to have significantly higher frequencies of MN than their unexposed identical co-twins (York et al. 2013). Furthermore, the data from the discordant identical twin study suggested that chromosomal instability accumulated throughout the lifespan of the twins exposed to childhood sexual abuse, leading the authors to speculate that the increased incidence of health problems observed in people experiencing early life trauma might be associated with acquired somatic chromosomal abnormalities (York, et al., 2013). While there is a paucity of studies investigating associations between stress and acquired chromosomal instability frequencies in women with breast cancer (or any type of cancer), several investigators have reported an association between stress and telomere shortening (Shalev et al 2013, Surtees et al. 2011); especially chronic stress (Quinlan et al 2014). Given that telomere shortening has also consistently

been observed to result in increased frequencies of acquired chromosomal abnormalities, it follows that stress would also be associated with chromosomal instability.

A second PNS identified to have a significant predictive association with MN/cytome abnormality frequencies was cognitive flexibility domain measures. Given that cognitive flexibility domains have been one of the aspects of cognition impacted by chemotherapy (along with working memory, processing speed, multitasking, and attention)(Wefel et al 2012; Kesler et al 2013) this association was of particular interest. However, the relationship observed between chromosomal instability and cognitive flexibility domain measures was positive, with women having higher cognitive flexibility values also having higher chromosomal instability frequencies. Our initial hypothesis was that increased levels of soma-wide chromosomal instability could lead to the presence of a clone(s) with a chromosomal imbalance and that this imbalance would lead to compromises in cognitive function. Therefore, the observation of higher cognitive flexibility domains being predictive of higher MN/cytome abnormality frequencies seemed contradictory of our initial conjecture. This impression is based on the concept that higher MN/cytome abnormality frequencies are considered to be a negative outcome indicating the presence of chromosomal instability. However, an alternative interpretation is that these cytological structures serve as indicators that the cell has successfully recognized the presence of chromosomal instability and has eliminated the abnormality from the cell, thereby reducing/preventing the accumulation of DNA/chromosomal damage. Thus, it is feasible that women having higher MN/cytome abnormality frequencies in response to genotoxic agent exposures may actually have more efficient repair systems than women with lower MN/cytome abnormality values. Interestingly, the results from *in vitro* time-lapse studies have shown that the frequency of apoptosis is higher in

cells having a MN than cells without a micronucleus, suggesting that these cytome measures are related to cellular fate (Utani et al 2010). While intriguing, this association between lymphocyte instability and cognitive flexibility warrants further study before clear conclusions can be derived.

In addition to the significant associations we observed, we also showed a lack of predictive associations for several factors. Of note, no significant association was observed between age and MN/cytome abnormality frequencies in this study. Other investigators have also failed to detect an age effect for MN frequencies in patients with cancer, or specifically with breast cancer, despite the clear association between age and MN frequencies that has consistently been observed in healthy adults (Table 7)(Fenech, et al., 2011; Kirsch-Volders, et al., 2011). This lack of a clear, independent influence of chronological age on lymphocyte MN frequencies in women with breast cancer could reflect the narrow age range of the participants sampled in this (and other studies) of women with breast cancer. Alternatively, the influence(s) of factors related to breast cancer development may over ride or "mask" the effects of age. For example, the large effects of the treatments may mask any impact attributable to age.

In summary, we determined that lymphocyte chromosomal instability frequencies increase in women following their treatment for breast cancer, with these increased frequencies persisting for at least one year following treatment. We identified 8 factors (time from treatment, the type of chemotherapy, radiotherapy, race, luminal B tumor sub-group, triple negative tumor sub-group, stress, and cognitive flexibility domains) that were predictive of these MN/cytome abnormality frequencies. Of particular interest was the recognition of stress as a contributor to increased levels of chromosomal instability since this factor has the potential to be modulated through intervention.

The recognition of biomarkers associated with the development/persistence of PNS, such as MN/cytome abnormality frequencies, could lead to a better understanding of the biological basis for the adverse side-effects associated with cancer treatment and could also provide a basis for the future development of an algorithm to identify individuals most “at risk” for acquiring PNS.

Chapter 3

Acquired Epigenetic Changes in Women Treated for Breast Cancer: Methylation patterns in the peripheral blood.

Introduction

Breast cancer (BC), which affects approximately 1 in 8 females in the US, is the most common invasive cancer in women (American Cancer Society, 2013). BC accounts for about 29% of women's cancer and is the second most common cause leading to mortality in females (American Cancer Society. 2013, Siegel et al. 2014). Due to advances in early detection and management of BC, the survival rates for women with BC have steadily increased during the last two decades, culminating in an estimation of about 2.5 million BC survivors. However, a major subset of BC survivors develop short and long term side effects as result of their treatments, or the cancer itself. These side effects include anxiety (Badger et al 2007), depression (Badger et al 2007), fatigue (Berger et al. 2010), sleep disturbances (Lee et al 2004), pain (Utne et al 2010, Valeberg et al 2008), and cognitive dysfunction (Wefel et al 2011) and can be collectively referred to as psychoneurological symptoms (PNS). While there is an essential need to retain and continue to enhance survivorship following treatment for BC, there is also a critical need to reduce the adverse side effects associated with BC and its treatment to allow for improvements in the quality of life for the survivors.

BC is a heterogeneous condition, making pathological diagnostic classifications, and, in turn, treatments decisions, challenging. Moreover, significant variability in drug response and

outcomes has been observed in individuals treated with identical therapy regimens (Polyak et al 2011, Westbrook & Stearns 2013). Gene expression analyses of tumor cells have resulted in improvements in recognizing optimal therapeutic approaches to be followed to prevent cancer cell proliferation and metastasis, but there is a paucity of information available about "personalized medicine" approaches to identify the prevalence or causes of the soma-wide adverse side effects that arise following treatments for BC (or the cancer itself) (Perou et al 2000, Sorlie et al 2001, Sorlie et al 2003; The Cancer Genome Atlas 2012, Byler et al. 2014). An optimal approach for treating each person diagnosed with BC would not be determined solely by the classification of their tumor's characteristics, but would also include recognition of the individual's genetic/exposure-related susceptibility to cellular responses from the therapeutic agents (chemotherapy and radiation) being administered, as well as the potentially long term systemic cellular biological changes that might result from the tumor and its treatment, such as potential acquired epigenetic alterations.

Epigenetics is a relatively new, but very promising and rapidly growing area of research that has been suggested to be the epicenter of modern medicine since it provides explanations for the relationships between environment, genetic background and disease (Feinberg 2007, 2008). DNA methylation is one of the most stable and well-characterized epigenetic mechanisms (Jirtle and Skinner 2007). DNA methylation patterns in different tissues, including blood, are known to be sensitive to environmental insults, including (but not limited to) those associated with the aging process (Rakyan et al 2010, Teschendorff et al 2010) alcohol intake (Choi et al 2009, Christensen et al 2010) and smoking (Breitling et al 2011). It also plays an important role in inflammation (Wierda et al 2010), in developing cancers (Heyn and Esteller 2012) and in behavior/psychiatric

disorders (Mehler et al 2008, Narayan and Dragunow 2010). Typically, in normal cells, promoter CpG islands are hypomethylated, while repetitive DNA sequences in the genome are hypermethylated. Studies of genome-wide DNA methylation in cells from a wide range of different types of cancer have revealed that this typical scenario tends to be abnormally reversed (Feinberg et al 2006).

Investigations of DNA methylation patterns focused on BC tumors have been completed by several investigators, with their study results allowing for improvements in tumor classification, prognosis, and management (Szyf 2012, The Cancer Genome Atlas 2012, Byler et al. 2014, Van De Voorde et al 2012, Huang et al 2011). DNA methylation patterns thought to be specific to the tumor have also been found in cell-free DNA (which is released from dead cancer cells) that is present in serum/plasma (Sidransky 2002, Van De Voorde et al 2012), with some investigators studying the association between targeted methylation patterns and prognosis, while others have monitored changes in targeted methylation patterns that arise in response to a treatment. However, the utility of assessing cell free DNA in serum/plasma is limited due to: (1) the inability to adequately/accurately follow-up a patient's response to a treatment after the surgical removal of the tissue; and (2) the amount of presumed tumor-derived cell-free DNA in serum/plasma is very small and tends to lack sufficient quality (due to degradation) for use in the sensitive genetic techniques needed for genome-wide or targeted genetic/epigenetic evaluations. Thus, there is a need for an assay that can be used to evaluate potential associations between cellular responses that are acquired and persist following treatment for BC, with a goal of applying this technology to identify or stratify women who are most "at risk" for developing PNS or other adverse side effects following their treatment for BC.

Given that the effects of chemotherapy and radiation have been shown to have a soma-wide impact, one approach for monitoring genetic and/or epigenetic changes that arise in response to therapy would be to study the patterns of cells in peripheral tissues. Furthermore, based on the conjecture that inflammation plays a key initiating role in the cascade of biological changes resulting from chemotherapy (as well as its ease of access for evaluation), peripheral blood seems to be a strong specimen for evaluating biological changes related to the acquisition of adverse side effects related to BC treatment. Few investigators have examined the association between BC - or cancer in general- and DNA methylation patterns in peripheral blood. However, the limited number of investigations published have indicated that peripheral blood is effective for identifying biomarkers indicative of an increased risk for developing breast cancer, and/or the presence of cancer (Wu et al 2011, Choi et al 2009, Cho et al 2010 , Wong et al 2010, Iwamoto et al 2011, Widschwendter et al 2008).

An important first step in understanding factors contributing to the acquisition/persistence of PNS in a subset of BC survivors is to identify biological changes that arise in women's cells following their treatment for breast cancer and to determine if these changes vary with tumor characteristics and/or treatment regimens. We hypothesize that chemotherapy for BC leads to the acquisition of soma-wide epigenetic alterations and that these alterations can be detected in peripheral blood cells and will vary based on the type of chemotherapy administered. To test this hypothesis, we designed a longitudinal study to investigate acquired changes in methylation patterns in PBCs that were obtained from women diagnosed with BC; (1) before treatment (baseline) and (2) during the administration of different types of chemotherapy.

Materials and Methods

Study Participant Ascertainment and Specimen Collection:

A total of 68 women with early stage (I to IIIA) breast cancer, who ranged in age from 23 to 71, were ascertained through 5 regional cancer centers in Central Virginia, including the Massy Cancer Center (MCC) at Virginia Commonwealth University. To identify potential study participants, a member of the research team attended the weekly interdisciplinary Breast Health meetings at Virginia Commonwealth University Health System (VCUHS). Study eligibility criteria for participants were: (1) gender (only females were included since too few male participants were available for study); (2) an age of 21 years or older; and (3) a diagnosis of early stage breast cancer with a scheduled visit to receive chemotherapy. Exclusion criteria were: (1) a history of previous cancer, or chemotherapy; (2) a diagnosis of dementia; (3) active psychosis; or (4) a history of immune-related diagnoses (e.g. multiple sclerosis and systemic lupus erythematosus). After providing their informed consent (VCU IRB #HM 13194), participants were enrolled and their first study visit was scheduled prior to the initiation of chemotherapy. Study visits took approximately one hour to complete. A peripheral blood sample was collected from each participant prior to the receipt of treatment (baseline or visit 1) and at the midpoint of the chemotherapy treatments (visit 2). The blood specimens were collected by venipuncture or through an existing access device. The specimens were coded prior to their delivery (same day) to the cytogenetics lab to ensure that the geneticists completing this study were unaware of the clinical or treatment history of each participant at the time of sample processing and/or evaluation.

Chemotherapy Treatment Information

Medical records were used to determine chemotherapy treatments administered. The three primary types of chemotherapy regimens included: (1) Taxotere (docetaxel), Adriamycin (doxorubicin), and cyclophsophamide [TAC]; (2) Taxotere and Cyclophosphamide [TC]; or (3) Taxotere (docetaxel), Carboplatin (paraplatin); and Herceptin (transtuzumab) [TCH].

DNA preparation and methylation assessment:

Genomic DNA was isolated from whole blood according to standard methods using the Puregene DNA Isolation Kit (Qiagen). An aliquot (500 ng per study participant) of DNA was then sent to Hudson Alpha Institute for Biotechnology for bisulfite conversion, using standard methods (Zymo Research EZ Methylation Kit) and genome-wide methylation pattern assessment, using the 450K HumanMethylation Chip, according to the manufacturer's protocol (Illumina). The 450K HumanMethylation Chip interrogates 485,764 genome-wide targets. Both specimens (baseline and mid-chemo visits) from each study subject were localized to the same array to avoid any potential artifactual differences in methylation patterns that might arise due to "batch" effects. Also, to evaluate reproducibility of the assay and allow for the correction of any potential batch effects that might be present, replicate evaluations of a single specimen were completed (the same specimen was evaluated in every batch). Intensity data from the scanned arrays were imported into Illumina's GenomeStudio software and the *minfi* Bioconductor (Aryee et al 2014, Gentleman et al 2004) in the R programming environment (R core team 2014) to obtain β values for each probe.

Genome-Wide Methylation Data Analyses

Recognizing that high-throughput assays are subject to unwanted technical variation and that chemotherapy could elicit global DNA methylation changes, the functional normalization procedure was applied to the raw intensity values (Fortin et al 2014). Functional normalization builds upon quantile normalization by estimating the first 2 (default) principal components from inbuilt control probes as covariates to adjust for technical variation and is applied to the methylated and unmethylated intensities separately along with separate fits to the type I and type II probes; it has been shown to perform well even in the presence of batch effects. The primary motivation for this approach was to avoid placing unrealistic constraints across sample groups were likely to have very different marginal distributions (ie., pre- verus post-chemotherapy). In addition, since the performance of probes containing single nucleotide polymorphisms (SNPs) can be variable (Bibikova, et al., 2011), probes containing SNPs that overlapped the CpG site or at that were localized to the single base extension were excluded. Because the intensity value, β, reported for each CpG site represents "proportion methylated", which is constrained to an interval value of 0 to 1, where a β of 1 indicates complete methylation and 0 indicates no methylation, the normalized intensity values were transformed using the M-value procedure to promote normality (Du, et al., 2010). The M-value is calculated as a logit transformation of the methylated and unmethylated intensity ratio along with an added term to offset potentially small values.

Statistical analyses were then performed on the normalized M-values from the baseline compared to mid-chemotherapy samples. For each CpG site, differential methylation between baseline and treatment specimens was tested using a mixed effect linear model in the R programming environment (R Development Core Team, 2011), including a random effect term to

account for the paired status of observations. An additional term was included to account for the relative proportion of neutrophil levels (ie., the largest and most variable blood cell proportion) to account for changes in blood cell types that could differ between sample time points. This term controlled for a potential bias in results stemming from DNA methylation changes reflective of differences in white blood cell proportions following chemotherapy, but not the chemotherapy itself. To adjust for the multiple hypothesis tests, the p-values were used to estimate the false discovery rate (FDR) following Benjamini and Hochberg's (1995) method. (Normalization of the data, calculation of the M-Values and detection of the differentially methylated sites were performed by Dr. York).

The R program was then used to create pie charts and the DAVID gene functional classification tool (Huang, et al., 2007) was used to identify biological relationships among the differentially methylated sites.

Results

A total of 68 women were initially included in the study and methylation patterns were analyzed in each woman before (Baseline=T1) and during chemotherapy (Mid-chemo =T2). A single paired sample showed lower median intensity values for both the unmethylated and methylated set of probes as compared to the other samples. Therefore, this participant's specimens were excluded, resulting in a total of 67 paired samples that were analyzed. All women were diagnosed at early stage BC (I-IIIA). The women evaluated included 19 African Americans, and 48 Caucasians. The women were treated with either TAC (n=35), TC (n=22), or TCH (n=11). Using the normalization and SNP exclusion processes described in the methods section, a total of 18,131 probes were excluded from the analysis, resulting in a total of 467,633 probes that were evaluated for the paired specimens from each participant.

Acquired alterations in methylation patterns were observed in response to chemotherapy (Figure 7). When methylation patterns were compared between visit one (baseline) and visit 2 (mid-chemo), 1265 significant differentially methylated sites (DMSs) were detected (FDR<.01, <10% mean within pair difference) (Table 12). Acquired reduction in methylation (e.g. relative hypomethylaion) was seen more frequently than increases in methylation (relative hypermethylation) (Table 12 and Figure 8). A total of 788 of the 1265 significant differentially methylated CpG sites (62.3 %) showed relative hypomethylation following chemotherapy; while 477 of 1265 CpG sites (37.7%) showed relative hypermethylation. Observed changes involved multiple types of sequences, but were most prevalent in the "open sea" regions of the genome for

both hypo & hyper methylated sites (Table 12 and Figure 8). Of particular interest is the methylation status of the CpG island probes, since these sites are most clearly associated with gene function. Patterns differences showing increased methylation levels at the mid-chemo timepoint were observed in 8.0% of the CpG islands, with changes resulting in lower methylation values being seen in only 1.3% of the CpG islands. The significant DMSs showed altered patterns for genes involved in several biological processes or pathways including (but were not limited to) those involved in transcription regulation, signal transduction pathways, immune response, hemostasis, neuronal regeneration, cell cycle and cell death (Tables 13-15). In particular, 51% of the DMSs were associated with phosphorylation processes ($p=3.4x10^{-16}$). A subset of the significantly differentially methylated sites within interesting genes is listed in Table 16, along with a description of the genes' biological functions.

Table 12: Distribution of acquired methylation changes in relation to genomic sites.

Sites	Island* N (%)	N-Shelf* N (%)	N-Shore* N (%)	Open Sea* N (%)	S-Shelf* N (%)	S-shore* N (%)
Total Significant; 1265	48 (3.8)	82 (6.5)	106 (8.4)	851 (67.3)	98 (7.7)	80 (6.3)
Hypo; 788 (62.3 %)	10 (1.3)	55 (7.0)	61 (7.7)	545 (69.2)	69 (8.8)	48 (6.1)
Hyper; 477 (37.7%)	38 (8.0)	27 (5.7)	45 (9.4)	306 (64.2)	29 (6.1)	32 (6.7)

* CpG Island is defined as a DNA region > 500bp, GC >= 55% and observed/expected CpG ratio >.65 (Takai and Jones 2002).
* Shore is 2 kb from CpG island. The N and S indicate upstream and downstream of the CpG island, respectively.
* Shelf is 4-6 kb from CpG island. The N and S indicate upstream and downstream of the CpG island, respectively.
* Open sea > 6kb from CpG island

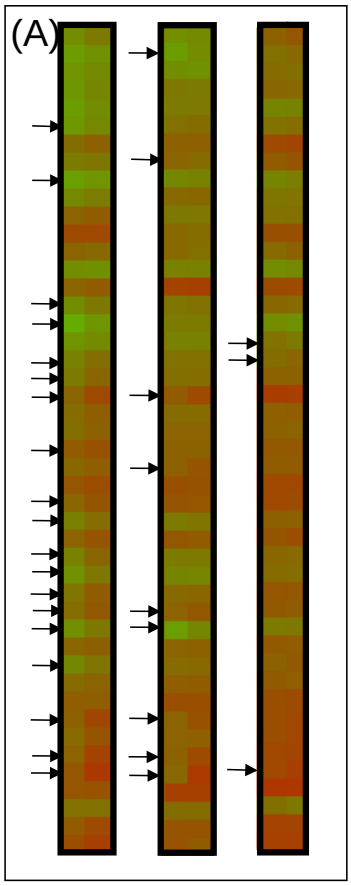
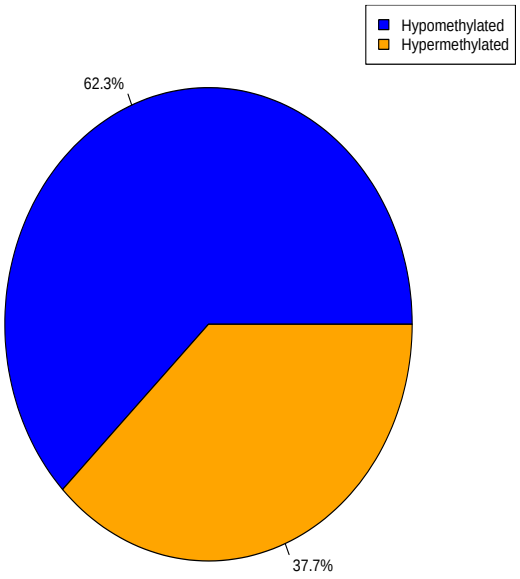
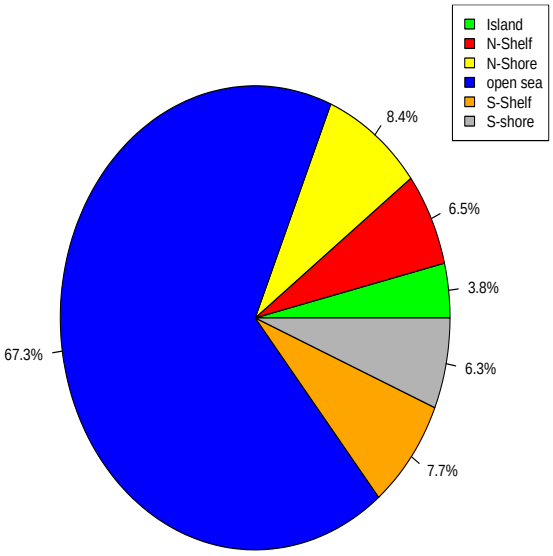


Figure 7: Variation in response to chemotherapy between women. Women with BC showed variability in acquired changes in DNA methylation patterns in response to chemotherapy, as indicated by the arrows.

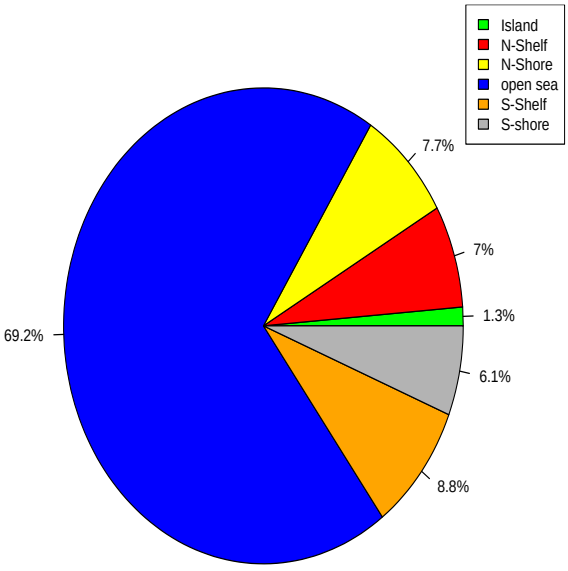
a) Sites with relative Hypo/Hyper methylation



b) Acquired alterations in DNA methylation



c) relatively hypomethylated sites



d) relatively hypermethylated sites

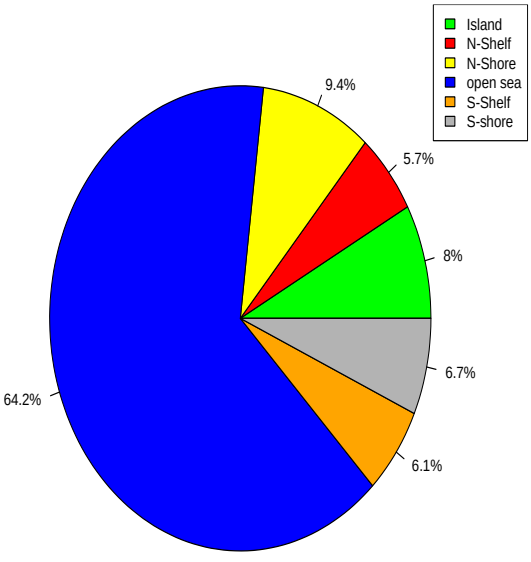


Figure 8: Significant acquired alterations in methylation patterns in peripheral blood cells following chemotherapy.

A total of 1265 significantly DMRs were detected at (FDR= 0.01 and >10% mean within pair difference) (a) sites with relative hypomethylation (62%) were acquired more frequently than hypermethylation (37.7%). (b) Acquired alterations in DNA methylation were distributed across different genomic sequences with the majority occurring in the open sea sequences (67.3%), followed by N-shore (8.4%), S-Shelf (7.7%), N-shelf (6.5%), S-shore (6.3%), and Islands (3.8%). (C) & (D) Distribution of acquired relative hypo & hyper methylation across the different sequences. Both were mainly involved in the open sea sequences (69.2% & 64.2%) respectively. More acquired hypermethylation alterations were acquired at CpG islands (8%) when compared to acquired hypomethylation (1.3%).

Table 13. Subset of Functional Roles Associated with the Significantly DMSs (based on Keywords)*

Term	Genes with DMSs		P-Value	Benjamini
	#	%		
Phosphoprotein	325	51.1	3.9E-16	1.5E-13
Cytoplasm	154	24.2	2.5E-7	5.5E-5
Activation of Protein Kinase Activity	16	2.5	6.9E-6	1.7E-2
Positive Regulation of Transferase Activity	24	3.8	7.3E-6	9.2E-3
Alternative Splicing	287	45.1	8.4E-6	1.3E-3
Negative Regulation of Gene Expression	38	6.0	8.6E-6	7.2E-3
Positive Regulation of Kinase Activity	23	3.6	1.3E-5	8.0E-3
Splice Variant	285	44.8	1.9E-5	3.3E-2
Regulation of Phosphate Metabolic Process	36	5.7	2.2E-5	1.1E-2
Regulation of Phosphorus Metabolic Process	36	5.7	2.2E-5	1.1E-2
Positive Regulation of Protein Kinase Activity	22	3.5	2.4E-5	9.9E-3
Sequence Variant	425	66.8	2.7E-5	2.3E-2
Polymorphism	409	64.3	4.1E-5	4.6E-3
Regulation of Phosphorylation	34	5.3	5.4R-5	1.9E-2
Mutagenesis Site	96	15.1	6.2E-5	3.6E-2
Intracellular Signaling Cascade	69	10.8	6.8E-5	2.1E-2
Regulation of Transferase Activity	29	4.6	7.0E-5	2.0E-2
Regulation of Protein Amino Acid Phosphorylation	18	2.8	8.3E-5	2.1E-2
Regulation of Kinase Activity	28	4.4	8.7E-5	2.0E-2
Negative Regulation of Transcription	33	5.2	9.4E-5	2.0E-2
Repressor	30	4.7	1.2E-4	1.0E-2
ATP-Binding	67	10.5	1.2E-4	8.9E-3
Regulation of Protein Kinase Activity	27	4.2	1.2E-4	2.4E-2
Coiled Coil	93	14.6	1.4E-4	8.8E-3
Transcription Activator Activity	30	4.7	1.8E-4	1.3E-1
Chromosomal Rearrangement	22	3.5	2.0E-4	1.1E-2
Zinc Finger, C3HC4 RING-Type	20	3.1	2.1E-4	2.0E-1
Transferase	68	10.7	2.9E-4	1.4E-2
Negative Regulation of Transcription from RNA Polymerase II Promotor	22	3.5	3.0E-4	5.2E-2
Calcium Transport	11	1.7	3.2E-4	1.4E-2
Type II Diabetes Mellitus	9	1.4	3.8E-4	5.3E-2
Phosphorus Metabolic Process	54	8.5	4.0E-4	6.5E-2
Negative Regulation of Transcription, DNA-Dependent	36	4.1	4.8E-4	7.3E-2
Nucleotide-Binding	78	12.3	4.8E-4	1.9E-2
Pleckstrin Homology-Type	23	3.6	4.9E-4	2.3E-1
Nucleoplasm	49	7.7	4.9E-4	1.8E-1
Phosphatidylinositol Signaling System	11	1.7	5.0E-4	3.6E-2
Binding Site: ATP	33	5.2	5.1E-4	2.0E-1
Limb Morphogenesis	12	1.9	5.2E-4	7.4E-2
Appendage Morphogenesis	12	1.9	5.2E-4	7.4E-2
Nucleotide Binding	105	16.5	5.4E-4	1.8E-1
Kinase	39	6.1	5.5E-4	2.0E-2
Phosphorylation	46	7.2	5.7E-4	7.7E-2
Calcium Channel	9	1.4	6.0E-4	2.0E-2
Phosphotransferase	17	2.7	6.1E-4	1.9E-2
Negative Regulation of RNA Metabolic Process	26	4.1	6.2E-4	7.9E-2

Positive Regulation of JUN Kinase Activity	7	1.1	6.3E-4	7.7E-2
Protein Amino Acid Phosporylation	40	6.3	6.3E-4	7.3E-2
Negative Regulation of Nucleobase, Nucleoside, Nucleotide and Nucleic Acid Metabolic Process	33	5.2	6.6E-4	7.3E-2
Embryonic Limb Morphogenesis	11	1.7	7.1E-4	7.5E-2
Embryonic Appendage Morphogenesis	11	1.7	7.1E-4	7.5E-2
Appendage Development	12	1.9	7.3E-4	7.4E-2
Limb Development	12	1.9	7.3E-4	7.4E-2
Positive Regulation of Macromolecule Metabolic Process	48	7.5	7.5E-4	7.3E-2
Adenyl Nucleotide Binding	78	12.3	7.7E-4	1.7E-1
Negative Regulation of Nitrogen Compound Metabolic Process	33	5.2	8.3E-4	7.7E-2
Zinc-Finger	78	12.3	8.3E-4	2.4E-2
Pleckstrin Homology	21	3.3	9.3E-4	2.8E-1
Nucleoside Binding	79	12.4	9.3E-4	1.6E-1
Regulation of Transcription from RNA Polymerase II Promotor	42	6.6	9.6E-4	8.6E-2
Apoptosis	25	3.9	9.7E-4	2.7E-2

*Subset selected to include only those associations having a P-value of E-4 or smaller

Table 14. Biological Pathways Associated with the significantly DMSs (Panther pathways search).

Term	Genes with DMSs		P-Value	Benjamini
	#	%		
PDGF Signaling Pathway	20	0.3	6.5E-4	5.7E-2
Endothelin Signaling Pathway	13	0.2	1.3E-3	5.7E-2
Inflammation Mediated by Chemokine and Cytokine Signaling Pathway	25	0.4	7.0E-3	1.9E-1
T Cell Activation	13	0.2	9.9E-3	2.0E-1
Angiotensin II-Stimulated Signaling Through G Proteins and Beta-Arrestin	8	0.1	1.0E-2	1.7E-1
VEGF Signaling Pathway	10	0.2	1.0E-2	1.4E-1
B Cell Activation	10	0.2	1.7E-2	2.0E-1
Integrin Signaling Pathway	18	0.3	2.6E-2	2.6E-1
Vitamin D Metabolism and Pathway	4	0.1	4.3E-2	3.6E-1
Purine Metabolism	3	0.0	5.1E-2	3.8E-1
Ras Pathway	9	0.1	6.0E-2	4.0E-1

Table 15. Biological Pathways Associated with the significantly DMSs (KEGG pathways search).

Term	Genes with DMSs		P-Value	Benjamini
	#	%		
Type II Diabetes Mellitus	9	0.1	3.8E-4	5.3E-2
Phosphatidylinositol Signaling System	11	0.2	5.0E-4	3.6E-2
Gap Junction	11	0.2	2.1E-3	9.8E-2
Natural Killer Cell Mediated Cytotoxicity	13	0.2	5.1E-3	1.7E-1
Regulation of Actin Cytoskeleton	17	0.3	8.7E-3	2.2E-1
Chemokine Signaling Pathway	15	0.2	1.3E-2	2.7E-1
GnRH Signaling Pathway	10	0.2	1.3E-2	2.4E-1
mTOR Signaling Pathway	7	0.1	1.4E-2	2.3E-1
MAPK Signaling Pathway	19	0.3	1.5E-2	2.1E-1
Small Cell Lung Cancer	9	0.1	1.5E-2	2.0E-1
Non-Small Cell Lung Cancer	7	0.1	1.7E-2	2.0E-1
Renal Cell Carcinoma	8	0.1	1.8E-2	2.0E-1
Focal Adhesion	15	0.2	2.3E-2	2.3E-1
T Cell Receptor Signaling Pathway	10	0.2	2.3E-2	2.2E-1
VEGF Signaling Pathway	8	0.1	2.5E-2	2.2E-1
Oocyte Meiosis	10	0.2	2.6E-2	2.1E-1
Vascular Smooth Muscle Contraction	10	0.2	2.9E-2	2.2E-1
Fructose and Mannose Metabolism	5	0.1	4.1E-2	2.9E-1
Prion Diseases	5	0.1	4.5E-2	3.0E-1
Long-Term Potentiation	7	0.1	4.7E-2	2.9E-1
Neurotrophin Signaling Pathway	10	0.2	5.0E-2	3.0E-1
Apoptosis	8	0.1	5.1E-2	2.9E-1
Chondroitin Sulfate Biosynthesis	4	0.1	5.1E-2	2.8E-1
Nicotinate and Nicotinamide Metabolism	4	0.1	6.4E-2	3.3E-1
Dilated Cardiomyopathy	8	0.1	6.5E-2	3.2E-1
Chronic Myeloid Leukemia	7	0.1	6.9E-2	3.3E-1
Aldosterone-Regulated Sodium Reabsorption	5	0.1	7.3E-2	3.3E-1
Type I Diabetes Mellitus	5	0.1	7.8E-2	3.4E-1
Calcium Signaling Pathway	12	0.2	7.8E-2	3.4E-1
Fc Epsilon RI Signaling Pathway	7	0.1	8.1E-2	3.3E-1
Pathways in Cancer	19	0.3	8.3E-2	3.3E-1
Toll-Like Receptor Signaling Pathway	8	0.1	9.6E-2	3.7E-1

Table 16. Summary of a subset of genes differentially methylated in patients treated for breast cancer

GENE NAME (ABBREVIATION)	DESCRIPTION OF FUNCTION[1]	LOCATION[2]
RAD52 homolog (S. cerevisiae) RAD52	Cell cycle, Interact with DNA recombination protein RAD51	12: 1,021,255
RAD51-like 1 (S. cerevisiae) (RAD51L1)	Cell cycle, Nucleotide and ATP binding	14:68,286,496
phosphoinositide-3-kinase, catalytic, delta polypeptide (PIK3CD)	Apoptosis, Nucleotide and ATP binding, Intracellular signaling cascade, Transferase	1:9751525
MAD1 mitotic arrest deficient-like 1 (MAD1L1)	Regulate cell cycle	7:1937325
retinoic acid receptor, alpha (RARA)	Cytokine production, immune response, Intracellular signaling cascade, Zinc ion binding	17: 38497640
histone deacetylase 4 (HDAC4)	Regulator of cell proliferation, Cell death , Zinc ion binding	2:240016312
Janus kinase 1 (JAK1)	Nucleotide and ATP binding, Intracellular signaling cascade, Transferase	1:65298906
v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1)	Regulate cell cycle, Cell death, regulator of cell proliferation, immune response, Cell migration	11:128328656
Beta dystrobrevin (DTNB)	Zinc ion binding, ion binding	2:25600067
Vascular endothelial growth factor receptor 1 (FLT1)	Regulator of cell proliferation, Cell migration, Nucleotide and ATP binding, Intracellular signaling cascade, Transferase	13:28874483
deoxynucleotidyltransferase, terminal (DNTT)	Transferase, ion binding	10:98064085
tumor protein p63 (TP63)	Cell death, regulator of cell proliferation, cell-cell signaling, Neuron projection, Intracellular signaling cascade, Zinc ion binding	3:189349216
ubiquitin-conjugating enzyme E2, J2 (UBC6 homolog, yeast) (UBE2J2)	Nucleotide and ATP binding	1:1189292
DNA (cytosine-5-)-methyltransferase 3 alpha DNMT3A	Transferase, Zinc ion binding	2:25455830
SMAD family member 3 (SMAD3)	Transcription factor in the TGF-B pathway,positive regulator of cytokine production, cell death & proliferation regulator, regulate cell cycle, immune responce	15:67458493
tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10)	Induction of apoptosis, cell- cell signaling, immune response, Zinc ion binding	3:172223298
tumor necrosis factor (TNF superfamily, member 2) (TNF)	Regulate cell cycle , Induction of apoptosis, cytokine production, regulator of cell proliferation, immune response, Cell migration, Intracellular signaling cascade	6:31543344
Interleukin 1; alpha (IL1A)	Regulate cell cycle, cell death, Cytokine production, regulator of cell proliferation, immune response	2: 113.531492
Interleukin 18; interferon-gamma-inducing factor (IL18)	Cytokine production, regulator of cell proliferation, cell cell signaling, involved in behavior, immune response	11:112013974

[1]Functional descriptions obtained from DAVID clustering.

Interlaukin 1 family member 6 & epsilon (IL1F6)	Immune response	2:113763038
Ataxin 1 (ATXN1)	Cell death, cell cell signaling, involved in behavior, neurological system process	6:16299343
Huntingtin (HTT),	Cell death, cell cell signaling, involved in behavior, Neuron projection, neurological system process	4: 3076408
the myosin heavy chain 10 non muscle (MYH10)	Neuron projection, neurological system process, Cell cycle, Cell migration, Nucleotide and ATP binding	17:8377523
Myelin basic protein (MBP)	Cell-cell signaling, immune response, Neuron projection, neurological system process	18:74690789

[2]Chromosome starting nucleotide position

Discussion

The current longitudinal study shows that epigenetic alterations are present in peripheral blood obtained from women with BC following their receipt of chemotherapy. This data indicates the feasibility of using peripheral blood to assess an individual's soma-wide cellular response to different types of treatment regimens. To our knowledge, this is the first longitudinal study investigating the effect of chemotherapy on methylation patterns in peripheral blood obtained from women with BC before and after the administration of chemotherapy. We detected 1265 significant DMSs (both increases and decreases in methylation) associated with chemotherapy, with the majority of changes showing decreases that involved multiple types of sequences. The observed alterations associated with chemotherapy, included changes in genes involved in the immune response, transcription regulation, signal transduction pathways, neuronal regeneration, as well as genes involved in learning and behavior.

The observed acquired alterations in methylation could be directly caused by the chemotherapy. Alternatively, they could reflect a cascade of cellular reactions that occurred in response to chemotherapy, such as inflammation. Our use of a longitudinal study design enabled us to make direct comparisons within individuals to identify alterations associated with the administration of chemotherapy, without needing to adjust the data for potential confounding effects attributable to co-factors such as age, race, income, alcohol intake and smoking status, tumor characteristics, and other comorbidities. However, one biological factor that was adjusted for in the analysis was changes in the proportions of white blood cells that might arise following chemotherapy, the latter of which mainly affected the neutrophil cell counts.

We have identified only one other study in which investigators reported methylation changes in blood cells following exposure to chemotherapy. In that report, Smith et al. 2014, compared methylation patterns in peripheral blood mononucleated cells from 22 women treated with chemotherapy and compared their patterns to those of 39 women who were not treated with chemotherapy. These investigators detected only 8 DMSs that were significantly associated with chemotherapy, with each of these 8 sites showing lower methylation values in response to chemotherapy. Methylation on these sites was also associated with levels of inflammation. Given that many other factors differed between the treated and non-treated women evaluated in their study (including but not limited to different genetic/epigenetic backgrounds; environmental histories; lifestyle histories), their ability to detect differences attributable to chemotherapy was compromised. Nonetheless, it may not be fortuitous that we also observed decreased methylation values for 6 of the 8 DMSs identified by the Smith, et al research team [(TMEM49; cg12054453), (TMEM49; cg18942579), (TMEM49; cg16936953) (DTNB; cg25446789), (SMAD3; cg05438378) and (cg13518625)]. The increased number of significant DMSs associated with chemotherapy that was observed in our study as compared to theirs could be due to many reasons. First, one would expect more than 8 DMSs to be significantly associated with chemotherapy exposure including sites that are involved in mitosis, cell death, gene regulation, immunity and inflammation. However, probably due to the small sample size in their study, as well as the difficulty inherent with detecting differences between women having different genetic/epigenetic backgrounds; environmental histories; lifestyle histories, as noted above, they were limited in their ability to detect significant DMSs. Second, they compared treated to non-treated patients, which is a very big limitation of their study that prevented them from establishing a direct cause

and effect relationship between the observed differences and the treatment. Lastly, they used the strict Bonferroni criterion for significance ($p<1.03x10^{-7}$), while we used the FDR recommended by Benjamini and Hochberg's significant at both an FDR of 0.01 and >10% mean within pair difference). When a more strict significance level was used in our study (FDR of 0.01, >20% mean within pair difference), only 145 significant DMSs were detected.

The significantly DMSs identified in our study included sites that were localized to genes that have functions that are congruent with anticipated responses to chemotherapy. These included clusters of genes involved in the cell cycle, cell death, signal transduction, hemostasis, transcription regulation, immune response and neurogenesis. Interestingly, we observed methylation changes involving at least 25 genes having functions related to the immune system, such as increases in the methylation levels of the tumor necrosis factor (TNF) gene, as well as decreases in the methylation values for the tumor necrosis factor (ligand) superfamily member 10 (TNFSF10); Interleukin 1; alpha (IL1A); Interleukin 18; interferon-gamma-inducing factor (IL18); Interleukin 1 family member 6; and epsilon (IL1F6). The DMSs also included sites within at least 20 genes involved in neurogenesis, learning, memory and behavior, including decreased levels of methylation for sites localized in genes for Ataxin 1 (ATXN1) & Huntingtin (HTT), as well as increased methylation values for sites localized to the myosin heavy chain 10 non muscle (MYH10) and Myelin basic protein (MBP) genes.

Following chemotherapy in our cohort, the distribution of the significant DMSs across the genome revealed that both increases and decreases in methylation values occurred mainly in sites localized to "open seas" portions of the genome, but also occurred, albeit in lower percentages, in

the islands, shores, and shelves. Overall, more decreases in methylation were observed than increases in methylation.

While there is a paucity of reports of methylation changes in peripheral blood, methylation patterns present in tumor cells have been more widely studied. Overall, hypermethylation has often been observed at CpG islands localized to promoter regions of important tumor suppressors, while hypomethylation has often been observed globally and has been associated with genomic instability. The distribution of altered DNA methylation that contributes to tumorigenesis has been found to frequently happen in CpG islands, shores and shelves (Doi et al 2009, Irizarry et al 2009, Ogoshi et al 2011, Shen et al 2013). Less is known about methylation patterns in peripheral blood cells obtained from women with breast cancer at baseline. However, based on the limited number of available studies, global hypomethylation in peripheral blood cells has been observed to be associated with the presence of BC (Choi et al 2009). Also, methylation of specific loci have shown promise for use as markers for the presence of BC or an increased risk for developing BC (Iwamoto et al 2011, Wong et al 2010, Brennan et al 2012, Widschwendter et al 2008).

In summary, chemotherapy for BC, appears to impact the methylation patterns of peripheral blood cells, with alterations being observed that involve different biological pathways, including those involved in the immune system, cell death, transcription regulation, neurogenesis, cell cycle and signal transduction. These study results highlight the importance of further investigation to assess the role of acquired epigenetic changes in the cellular response to chemotherapy and its relation with inflammation, and also with the development of PNS.

These results support the premise that chemotherapy for BC leads to soma-wide alterations in epigenetic patterns. Further studies confirming these findings and comparing the acquired epigenetic alterations with acquired PNS following chemotherapy could provide insight about the biological changes underlying a person's propensity to develop adverse side effects following chemotherapy. Ultimately, these types of studies could lead to the development of tests to identify women most at risk for acquiring cancer or cancer treatment related side effects and could lead to "personalized medicine" approaches that might improve the quality of their life for BC survivors.

Chapter 4

Chromosomal Aberrations in Women with Breast Cancer

Copy number aberrations in breast tumor tissues.

Introduction

Breast cancer (BC) is a diagnosis that is shared by many women, since it is one of the most common cancers diagnosed in females (Siegel, et al. 2014). However, the clinical course associated with a diagnosis of BC varies markedly among these women. These differences in outcome have prompted pathologists and other scientists to study the characteristics of BC tumors in order to improve prognostication and therapy-decision making strategies. In particular, genetic research has revealed significant differences in the molecular "signatures" of tumors, the latter of which have served as the basis for improvements in BC classification and management (Polyak, 2011).

The types of genetic abnormalities observed in BC tumors include mutations, copy number alterations (CNAs), changes in gene expression, and epigenetic alterations. Of the genetic markers, gene expression profiles in tumors have been most heavily utilized in directing decisions regarding treatment regimens and prognosis assessments (Byler, et al. 2014; Sinn et al. 2013). The most well studied BC tumor subtypes are the gene expression intrinsic subtypes, which mostly overlap with the status of the progesterone (PR), estrogen (ER), and human epidermal growth factor 2 (HER2) receptors. These subtypes include (but are not limited to); Luminal A (Lum A), Luminal B (Lum B), HER2+, and triple negative [TNBC]).

In addition to gene expression changes, copy number alterations, loss of heterozygosity (LOH) and allelic imbalances are common characteristics of solid tumors, including BC. Studies utilizing comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) arrays have allowed for enhancements in our ability to identify copy number alterations (CNAs) and allelic imbalances associated with the molecular intrinsic subtypes (The Cancer Genome Atlas 2012). CNAs in tumors have also been associated with their gene expression patterns (Chin et al. 2006, Pollack et al. 2002, Han et al. 2008).

One challenge investigators have encountered when assessing CNAs (or other genetic changes) in BC tumors is that these cells do not always proliferate well *in vitro*, and the tissue culture process can be associated with the acquisition of genetic/epigenetic patterns that were not present in the tumor *in vivo*. An alternative approach is to study formalin fixed, paraffin embedded (FFPE) tissue that is directly obtained from the tumor. However, FFPE tissue typically has degraded DNA (as a result of fixation/embedding processes). Also, there is a limited quantity of DNA available for evaluation from FFPE specimens. These technological hurdles have thwarted progress in identifying CNAs associated with clinical outcomes. Nonetheless, a limited numbers of investigators have successfully reported highly promising results showing relationships between CNAs in BC tumors and clinical course (Bergamaschi et al 2006, Chin et al 2006, Van et al 2006). Recently, the utilization of molecular inversion probe (MIP) technology has facilitated the assessment of CNAs in FFPE tissue (Thompson et al 2011). Using the MIP technology, researchers studying BC were able to associate tumor CNAs with the patient's outcome, and have identified 19 CNAs that discriminate recurrence risk in early stage BC (Thompson et al 2011).

Investigations of the peripheral blood (PB) of patients with cancer, has revealed the presence of an increase in chromosomal instability and epigenetic alterations that are not only present in the tumor tissue, but are also observed in the patient's non-cancerous tissues. Many investigators have shown the presence of chromosomal anomalies (Barrios et al 1991, Trivedi et al 1998, Gebhart et al 1993, Pathak et al 1991, Udayakumar et al 1994, Rossner et al, 2005), and high levels of MN (Santos et al., 2010, Cardinale, et al. 2012) in the PB of patients with solid tumors. However, few investigators have examined the association between BC - or cancer in general- and DNA methylation patterns in PB. Also, there is a limited number of investigations published that compared chromosomal instabilities levels in tumors to those in blood. Therefore, it is of value to know if PB could be used as an easily accessible surrogate model for detecting genetic aberrations present in patients with solid tumors or to see if PB values could aid in identifying a person who has a high risk for developing cancer or the existence of a tumor.

We hypothesis that chromosomal CNAs present in the tumor tissue associate with acquired soma wide chromosomal changes in other tissues such as the PB. Thus, utilizing the recently available MIP technology (Oncoscan), chromosomal alterations in the tumor tissue were investigated, in order to test their associations with soma wide chromosomal alterations in the PB.

Materials and Methods

Study Participant Ascertainment and Specimen Collection:

For this pilot study a total of 17 women with early stage (I to IIB) breast cancer, who ranged in age from 41 to 63, were ascertained through the Massy Cancer Center (MCC) at Virginia Commonwealth University. These women were participants in a larger study that is being completed by our research team to determine potential associations between chemotherapy treatment for breast cancer, acquired chromosomal and/or epigenetic alterations, and the womens' development and persistence of psychoneurological side effects. The inclusion criteria for the larger study was : (1) 21 years or older; (2) diagnosis of early stage breast cancer with a scheduled visit to receive chemotherapy; and (3) female gender (males were excluded since too few male participants were available for study). Exclusion criteria included: (1) a history of previous cancer, or chemotherapy; (2) a diagnosis of dementia; (3) active psychosis; or (4) a history of immune-related diagnoses (e. g. multiple sclerosis; systemic lupus erythematosus). Women who provided their informed consent (VCU IRB #HM 13194), to participate in the larger, parental study were recontacted to determine if they were interested in having tissue from their tumors evaluated in this pilot study. Following their informed consent, the tumor specimens studied were obtained from the pathology lab via the Tissue & Data Acquision & Analysis Core (TDAAC) within the Department of Pathology at VCUHS. Peripheral blood specimens were collected by venipuncture or an existing access device prior to the initiation of chemotherapy. All specimens were coded prior to ensure that the cytogeneticists were unaware of the clinical history of each participant at the time of sample processing and evaluation.

Macrodissection of tumor tissue and tumor DNA extraction:

Tissue sections were prepared and reviewed by a pathologist, who identified the areas of the tissue that contained tumor to guide the macrodissection of the specimens. In addition to an H&E slide denoting the location of tumor, the laboratory received 5 unstained slides from the adjacent tissue. Deparaffinization was performed using the Protocol SafeClear (Xylene substitute from Fisher). Each of the deparaffinized, unstained slides was then aligned on top of the marked matching H&E stained slide. The tumor area was then marked from the back of the slide. A disposable blade was used to remove the non-tumor tissue. The remaining marked tumor tissue was scrapped off and transferred into a labeled 2.0 µl Sarstedt tube. FFPE Genomic DNA extraction (including tissue lysis, DNA purification and elution) was performed using the QIAamp DNA FFPE tissue kit, as recommended for the Oncoscan Assay following the manufacturer's protocol. Quantification of the eluted FFPE DNA was done using the Qubit ds DNA HS Assay Kit according to the manufacturer's instructions.

Tumor tissue copy number and somatic mutation analyses:

A total of 6 µl of DNA, at a concentration of 12ng/ul, was prepared for the processing of the OncoScan FFPE Assay (Affymetrix). This array allows the sample(s) to be interrogated for genome-wide copy number alterations, genome-wide LOH, and targeted mutations. This assay, which utilizes molecular inversion probe (MIP) technology, provides CNA assessments of approximately 900 cancer-related genes, with a resolution that ranges between 50kb-125kb. In addition, it provides genome-wide coverage with a resolution that ranges between 300-380 kb. The array also allows for the detection of 74 actionable somatic mutations from 9 different genes (BRAF, KRAS, EGFR, IDH1, IDH2, PTEN, PIK3CA, NRAS, TP53). Genome-wide LOH can

also be evaluated usng this platform (with a resolution of 3MB-10MB). However, we elected to exclude the assessment of LOH in this pilot study since we did not have data for paired normal and tumor specimens. All aspects of the OncoScan assay were performed following the manufacturer's instructions.

Peripheral Blood Chromosome Instability Methodology:

Baseline levels (prior to the administration of therapy) of chromosomal instability present in peripheral blood were quantified for each pilot study participant using the cytokinesis-block micronucleus (CBMN) and cytome assay (Fenech, 2006). A micronucleus (micronuclei) (MN) is(are) a small chromatin containing structure(s) juxtaposed to a parent nucleus. The CBMN assay was selected for the chromosomal instability assessments because it allows for: (1) the quantification of both numerical and structural cytogenetic findings; (2) the analysis of a large number of cells, thereby enhancing one's ability to detect even low levels of instability; and (3) minimization of in vitro growth selection (only 1 round of cell division is completed in vitro) and minimization of technical artifacts due to processing (for example, the CBMN assay does not show chromosome "loss" due to cell breakage at harvesting or slide making, which can occur when assessing metaphase chromosomes).

Leukocytes, which were isolated using Histopaque-1077 (Sigma), were established in culture according to the protocol of Fenech (1993). Briefly, following their mitogenic stimulation using phytohemaglutinin (PHA), lymphocytes were arrested at cytokinesis by adding cytochalasin B to the cells 44 hr after their culture initiation. The cells were harvested 72 hours after culture initiation and slides prepared (2 per specimen) as described previously (Leach and Jackson-Cook, 2001).

Micronuclei, buds, and/or bridges were visualized following giemsa staining (4% Harleco Giemsa solution) and identified according to the criteria established by Fenech (2006)(Figure 3). The proportion of abnormalities was calculated by adding the values obtained from the two replicate scores (1000 binucleates were evaluated from each of two slides for a total of 2000 binucleates per study participant). The total number of binucleates with abnormalities was then divided by the total number of binucleates scored.

Data analysis:

The OncoScan Console 1.0 software was used to determine that the experimental processes met or exceeded vendor established quality control standards. The OncoScan Nexus express software was used to identify CNAs, LOH, and somatic mutations in the tumor samples. Statistical Pearson's correlation comparisons between tumor CNAs and peripheral blood chromosomal instability were completed using the R programming environment (R Development Core Team, 2011).

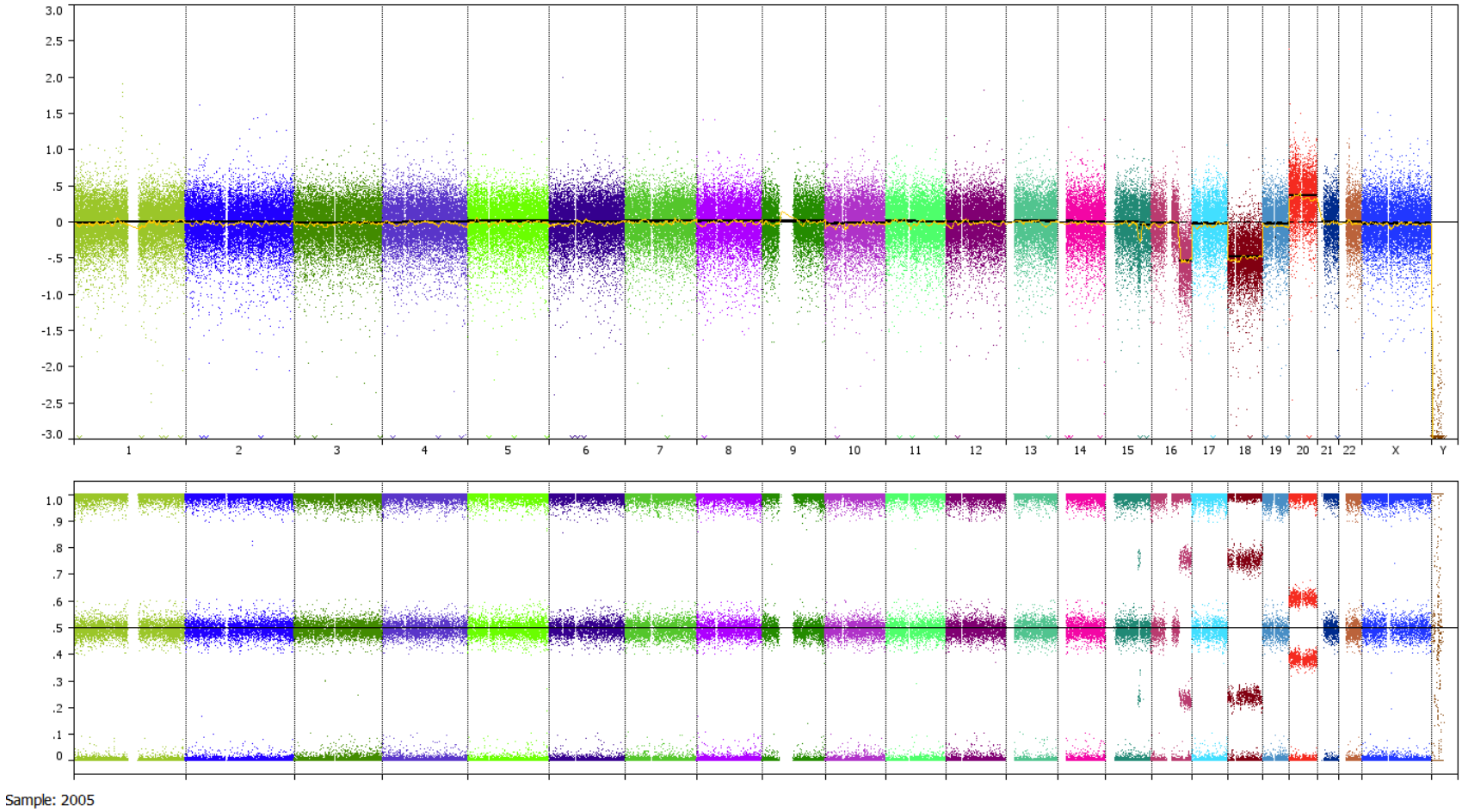


Figure 9: Log 2 ratio and B allele frequency plots for sample 2005.
CNAs were detected for chromosomes 15 (a loss at 15q); 16 (loss); 18 (a loss [monosomy]) and 20 (a gain [trisomy]). The tumors were estimated to be present in approximately 70% of the cells evaluated.

Results

Tissue amenable for use in the OncoScan array was available for 14 of the 17 participants who provided consent to have analyses completed for their tumor specimens, with three cases having an insufficient amount of residual tissue containing tumor that was available for assessment in this pilot study. The amount of DNA required for the OncoScan assay was successfully obtained from 11 of these 14 tumor tissues, with the cases having an insufficient amount of tumor all being derived from fine needle aspirates. Of the 11 cases for which DNA was successfully obtained, a total of 10 specimens yielded results that could be interpreted, with one sample having an array result that could not be unequivocally evaluated (possibly due to tumor heterogeneity and/or DNA degradation). One of these 10 tumor specimens was from a woman who died within 2 years following the initiation of chemotherapy (case 2001).

The specimens that were evaluated were all obtained from participants who were diagnosed at early stage BC (I-IIB). The mean age of the participants was 50 years old. The age, race, and tumor characteristics of the participants from whom the 10 specimens were evaluated using OncoScan are summarized in table 17.

Summaries of the CNAs, including amplifications, and the somatic mutations occurring in each patient are shown in tables 2-4. CNAs were detected in all of the 10 breast tumor tissues evaluated, with the number of events varying from tumors to tumors (Table 18). Genome-wide CNAs for each patient, and collectively for the pilot study cohort, are shown

in Figure 10. The most frequently observed CNAs were: Gains at 1q (70%), and 8q (70%), and losses at: 8p (70%), 17p (70%), 17q (60%), 18q (60 %), 13q (50%), and 22 (50%). As expected, since all the tumors were evaluated from females, loss of Y chromatin was observed for all tumors (far right of Figure 10) The most commonly observed pair of aberrations was loss of 8p and gain of 8q. High copy gain amplifications and homozygous losses are summarized in Table 19. Amplifications of regions including well-known oncogenes were observed, such as the ERBB2, MYC, and ERBB3. Amplifications (high copy [at least 4 copies] gains tended to be more frequent in HER2+ tumors (patients: 2001, 2011, 2028). Moreover, homozygous deletions of well-known tumor suppressors such as the RB1 were also detected.

In addition to evaluating CNAs, an assessment of targeted somatic mutations was also completed for the tumors (Table 20). Somatic mutations were detected for 6 of the 9 genes targeted in this array. A total of 7 of the 10 tumors had mutations within the TP53 gene; 6 had mutations in KRAS gene; 6 had mutations in the EGFR gene, 5 had mutations in the NRAS gene, 2 in the PTEN gene and 1 in the PIK3CA gene. Mutations involving the KRAS and at TP53 were found in 100% of ER+ patients, but in only in 25% of ER- patients (p=.03). Also, the total number of somatic mutations detected was higher in HER2+ tumors (HER2+ and luminal B) than HER2 negative tumors (luminal A and triple negative) (p=0.04).

When CNAs were compared on the basis of the tumors' intrinsic subtypes, significant differences were detected (Table 21). Gains involving chromosomes: 3 (q26.31 - q27.1);

10 (p11.22 - p11.21) and (q22.1); 16 (p13.3); and 22 (q13.1 - q13.2) were found in 100% of HER2+ tumors, and 0% of the other three subtypes (TNBC, Lum A, and Lum B)(p=0.02). Also, gains at 20q13.31 were seen in 100% of the TNBC tumors, but 0% of the other three subtypes (p=.02). Losses at 17p13.2 were present in the two HER2+ tumors, the 3 Lum B tumors , and 1 out of the 2 TNBC tumors, but were not present in any of the three Lum A tumors (p=0.03).

As expected, ERBB2 was amplified only in HER2+/Luminal B tumors. However, the amplification was not detected in 2 out of the 5 HER2+/Lum B patients (Patients 2001 & 2021). Tumors were also subdivided based on their estrogen receptor (ER) status. Losses involving chromosomes 12 (q13.13); 15 (q24.1 - q24.2) & (q24.3 - q25.1); and 17 (q21.32 - q21.33) & (q24.2 - q24.3) were present in 75% of patients with ER- status, but in none of the ER+ tumors (p= .03).

The tumors included in this study were determined to be either grade 2 or grade 3. Significant differences in the CNAs present in the grade 2 compared to grade 3 tumors were detected, with losses involving the proximal short arm of chromosome 4 (4p14; 4p13; and/or 4p12), the proximal short arm of chromosome 7 (7p11.2), and the proximal long arm of chromosme 17 (17q12) being present in 75% of the grade 3 tumors, but in none (0%) of the grade 2 tumors (p=0.03). Moreover, when groups were compared based on the stage of the tumor, gains at 20q13.31 were found in 100% of stage I tumors, but in none (0%) of the stage II (A or B) tumors (p=0.02). In addition, high copy gains at 8p11.22-

p11.21 were seen in 100% of stage IIB tumors, but in none (0%) of the lower stage tumors (I & IIA)(p =0.02), suggesting that this finding is associated with (or acquired in) more advanced tumors.

Comparisons were also completed to determine if baseline peripheral blood chromosomal instability levels were correlated with the CNAs or somatic mutations present in the women's tumors. While a trend of an association between MNF in PBCs and tumor mutations, the percent of the tumor genome changed, and tumor cells with amplification of MYC was observed, none of these associations were significant. A significant association was between MN and homozygous loss (p =.04) . (Table 22)

Table 17: Participants ' demographic and tumor characteristics.

Subject ID	Age	Race	Grade	Stage	ER+	PR+	HER2+	Subtypes
2001	56	AA	2	IIB	N	N	Y	HER2 +
2005	51	AA	2	I	N	N	N	Triple negative
2010	41	AA	2	IIA	Y	Y	N	Luminal A
2011	41	AA	2	IIB	Y	Y	Y	Luminal B
2012	63	C	3	IIA	Y	N	N	Luminal A
2018	52	AA	2	IIA	Y	Y	N	Luminal A
2021	58	AA	3	IIA	Y	Y	Y	Luminal B
2026	47	AA	2	IIA	N	N	Y	HER2 +
2028	43	C	3	IIA	Y	N	Y	Luminal B
2031	48	C	3	I	N	N	N	Triple negative

Table 18. Number of CNAs in tumors

Patient	Gains	High copy gains (4 or more)	1 copy Loss	Homozygous Loss	Total CNA	% Genome Changed	MNF
2001	29	38	19	2	88	31.73	0.034
2005	1	0	7	0	8	6.42	0.039
2010	2	1	5	1	9	10.24	0.031
2011	11	10	17	0	38	13.68	0.049
2012*	16	0	25	0	41	44.63	0.044
2018	6	13	16	0	35	25.16	0.061
2021*	3	1	10	2	15	22.4	0.039
2026*	18	3	14	0	35	26.97	0.063
2028*	4	14	11	0	29	10.47	0.067
2031*	18	5	19	0	42	45.96	0.064

* Tumors demonstrated patterns consistent with tumor heterogeneity.

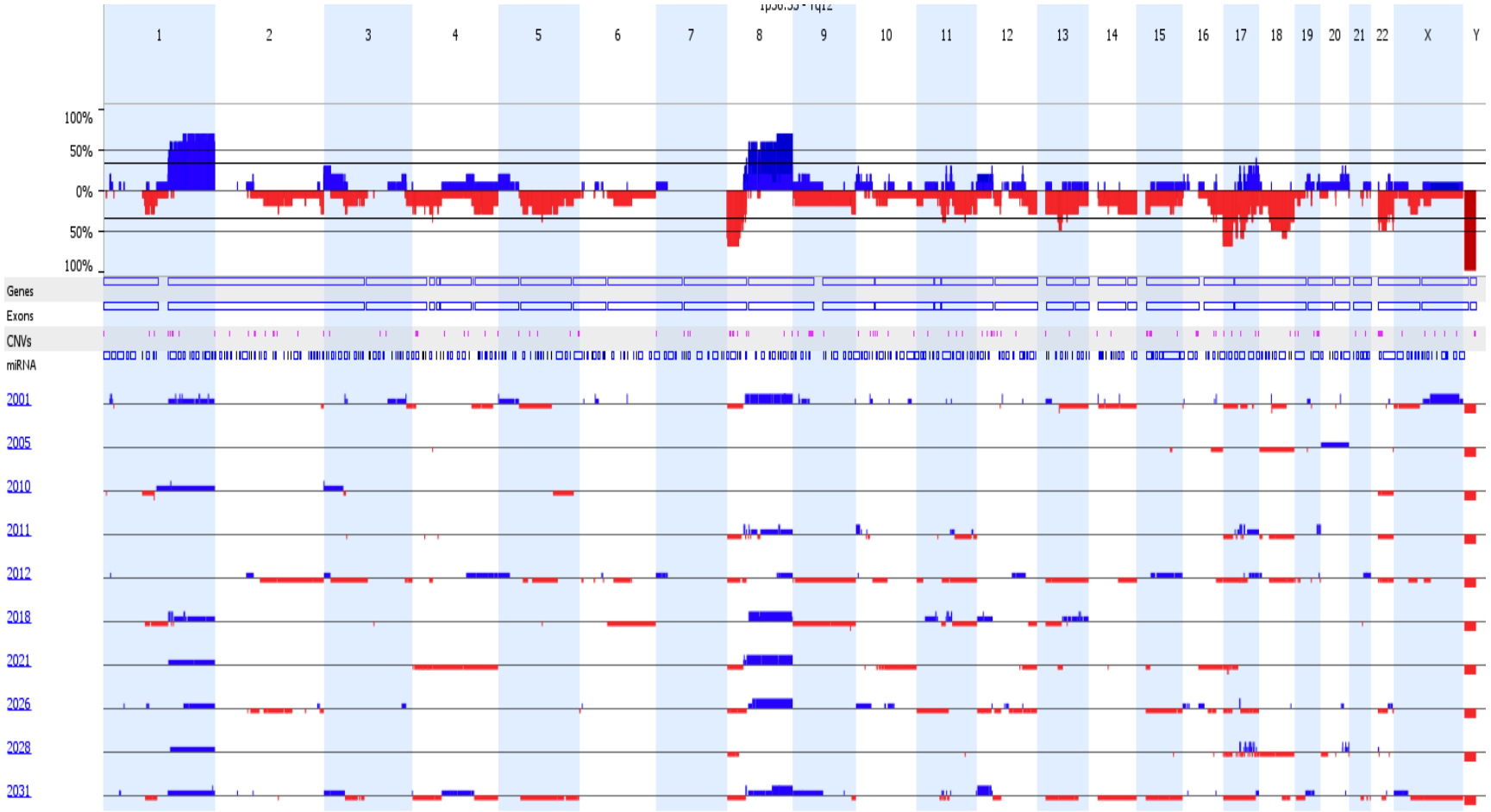


Figure 10: Copy number alterations (CNAs) in the 10 tumors evalauted.

(Red) indicates loss & (Blue) indicates gain. The uppermost plot shows the composite of findings observed in all participants. The lower plots show the findings for each tumor (identified by study participant number).

Table 19: Amplifications and homozygous losses occurring in tumors

ID	HCG*	Range of HCG	Important Oncogenes included	Homozygous loss	chr/gene
2001	38	(4-9 copies)	6 copies of MYC, 4 copies of ERBB3, 5 copies of RAD5IB, 4 copies of MAPK13 &14	2	RB1
2005	0			0	
2010	2	4 copies	4 copies of HORMAD1	1	1p13.2
2011	10	(4-8 copies)	6 copies of ERBB2	0	
2012	0			0	
2018	13	(4-23 copies)	4 copies MYC , 13 copies CCND1	0	
2021	1		4 copies MYC	2	17p12, 17p13.1
2026	3	(4-10 copies)	10 copies of ERBB2, 4 copies MYC	0	
2028	14	(4-11 copies)	11 copies of ERBB2	0	
2031	5	(4-7 copies)	4 copies MYC	0	

* HCG= High copy number gain

Table 20: Mutations detected in tumors

Patient	Type	High confidence
2001	Missense	KRAS
2005	None	None
2010	Missense	NRAS, EGFR, KRAS, TP53
2011	Missense Nonsense In-frame Deletion in Frame	NRAS, EGFR , KRAS, TP53 PTEN EGFR EGFR
2012	Missense	TP53
2018	Missensse	KRAS, TP53
2021	Missensse	NRAS, TP53
2026	Missensse Frame-shift	NRAS, PIK3CA, EGFR, KRAS, TP53 PTEN
2028	Missensse	NRAS, EGFR, KRAS, TP53
2031	Missensse	EGFR, TP53

Table 21 : Significant CNAs detected in tumors based on subgroup comparisons.

Chromosome	Event	Frequency of occurrence in the prominent group	Frequency in other groups	p-value
3 (q26.31 - q27.1)	Gain	100 % of HER2+	0% in (TRNG, Lum A, & Lum B)	0.02
10(p11.22 - p11.21)	Gain	100 % of HER2+	0% in (TRNG, Lum A, & Lum B)	0.02
10 q22.1	Gain	100 % of HER2+	0% in (TRNG, Lum A, & Lum B)	0.02
22(q13.1 - q13.2)	Gain	100 % of HER2+	0% in (TRNG, Lum A, & Lum B)	0.02
16 p13.3	Gain	100 % of HER2+	0% in (TRNG, Lum A, & Lum B)	0.02
20q13.31	Gain	100% of TRNG	0 % in (HER2+, Lum A, & Lum B)	0.02
17p13.2	Loss	85% of(TRNG,HER2+,Lum B)	0% of Lum A	0.03
17p13.2	Loss	100% of (HER2+ & Lum B)	20% of (TRNG and Lum A)	0.05
12 q13.13	Loss	75% of ER-	0% of ER+	0.03
15 (q24.1 - q24.2)	Loss	75% of ER-	0% of ER+	0.03
15 (q24.3 - q25.1)	Loss	75% of ER-	0% of ER+	0.03
17(q21.32 - q21.33)	Loss	75% of ER-	0% of ER+	0.03
17 (q24.2 - q24.3)	Loss	75% of ER-	0% of ER+	0.03
4 p14	Loss	75% of grade 3	0% of grade 2	0.03
4 p13	Loss	75% of grade 3	0% of grade 2	0.03
4 p12	Loss	75% of grade 3	0% of grade 2	0.03
7p11.2	Loss	75% of grade 3	0% of grade 2	0.03
17q12	Loss	75% of grade 3	0% of grade 2	0.03
20q13.31	Gain	100% of stage I	0% of stage IIA &IIB	0.02
8p11.22-p11.21	HCG	100% of stage IIB	0% of stage I & IIA	0.02
17 q12	Loss	100% of Caucasians	0% of AA	0.008
17q25.1	Gain	100% of Caucasians	14% AA	0.03

Table 22: Correlations between MNF in the peripheral blood and CNAs and mutations in the tumor tissue

Type of CAN	Correlation with MNF	p-value
Gain	0.066	0.86
>2 copy gain	-0.054	0.88
one copy loss	0.255	0.48
Homozygous loss	-0.651	**0.04**
Total (CNA)	0.047	0.90
% of genome changed	0.221	0.53
Mutations	0.4	0.34
Amplification of MYC	0.25	0.49

Table 23: Most frequently observed CNAs in patients with BC

Most frequently observed CNAs	References
Gains at 1q (35%), 8q (35%) , 11q (26%), and 16p (14%) Losses at 4q (58%), 5q(54%), 6q(43%), 8p(48%), and 14 q(48%).	Bergamaschi et al 2006
Gains at 1q (55%), 8q (41%), 16p (40%), 17q (28%), 20q (19%), and 11q (16%). Losses at 13q (27%), 16q (22%), 8p (18%), most frequent pairs: -8p/+8q, +17q/+20q, and -4q/-13q	Rennstam et al 2003
Gain at 1q (64.8%), 8q (61.4%), 17q (50.0%), 20q (33.0%), 3q (20.5%), 1p (17.0%), 5p (17.0%) and 15q (17%) Loss at 8p (19.3 %), 11q (11.4%), 16q (11.4%), 17p (11.4%) and 18q (10.2%).	Weber-Mangal et al 2003

Discussion

The CNAs detected in the small number of tumors evaluated for this pilot study were consistent with previously reported findings using chromosomal banding, chromosomal array CGH, and/or microarray methodologies. For example, the most frequently observed CNAs seen in the tumors assessed in this pilot study included: Gains at 1q, 8q, and 8p, and losses in 8p, 17p, 17q, 18q, 13q, 22q, 4p, 5q, 11q, and 16q (Figure 10). Most of these regions have also been recognized as areas of imbalance seen most often in BC tumors evaluated by other investigators (Thompson et al 2011, Bergamaschi et al 2006, Weber-Mangal et al 2003, Rennstam et al 2003)(Table 23). In agreement with our observations, losses at 8p and gains at 8q are one of the most commonly observed pairs of aberrations seen in BC tumors (Rennstam et al 2003), and are likely to reflect the presence of an isochromosome/isodicentric chromosome for the long arm of chromosome 8.

Our assessments of CNAs with tumor subtype also showed areas of agreement with the results of previous investigators. For example, we observed losses at 17p13.2 (which is located near the TP53 gene but did not include it) in 6 of the 10 tumors analyzed, but none of the 3 luminal A tumors showed this loss. Other investigators have also reported a higher frequency of 17p13.1 loss in luminal B and HER2+ tumors compared to luminal A and triple negative tumors. (Thompson et al 2011).

Interestingly, the tumor that was obtained from patient 2001, who died within 2 years following the initiation of her chemotherapy, is the only tumor evaluated that showed multiple

regions of high copy gains, including amplification of well-known oncogenes such as MYC, ERBB3, RAD5IB, MAPK13, MAPK14. This tumor also contained a homozygous deletion of the RB1 gene, losses involving 8p22 and Xp21, and a gain at 10p11.2. Many of these aberrations have been reported to be associated with a poor outcome.

As expected, ERBB2 was amplified only in the HER2+ & Lum B tumors. However, amplification for HER2 that was detected in diagnostic testing using FISH methodology was not detected in 2 of the 5 tumors evaluated with the OncoScan array platform (Patients #2001 & 2021). Tumors for patient 2021 showed high levels of heterogeneity in the microarray test. Thus, it is possible that the proportion of cells having amplification in the section of residual tissue available for evaluation was not large enough to allow for its detection above the "noise" present in this specimen. Indeed, the results of the Oncoscan assessment for this case showed at least two cell populations, with one of them being present in a low proportion of cells. An additional factor that might have contributed to the observed discrepancy between the FISH and microarray test results was the loss of chromatin from the short arm and centromeric region of chromosome 17, which is a finding that was noted in both discrepant cases. The OncoScan platform has been reported to allow for the detection of false positive HER2 FISH results, the latter of which have been observed for HER2 to control probe (localized to the centromeric region of chromosome 17) ratios that are greater than or equal to 2 due to a relative decrease in centromeric probe signals rather than in increase in HER2 signals, which is a finding that can be undetectable in a tumor having a near-tetraploid complement. Clearly, this observation requires further investigation through carefully designed clinical validation studies before the OncoScan platform is used for diagnostic testing.

While the number of tumors evaluated in this study was small, the tumors analyzed included representatives from each of the 4 intrinsic tumor subtypes. Although preliminary, the results of this study suggest that the evaluation of CNAs could provide additional information to facilitate the recognition of tumor subtypes. For example, gains at 3q26.31 to 3q27.1; 10q22.1; 16p13.3; and 22q13.1 to 22q13.2 were found only in two patients, both of whom were both HER2+. To our knowledge, none of these CNAs have been previously reported to be specifically associated with HER2+ tumors. Our observation of gains of 20q13.31 has also been detected in BC tumors by other investigators (Weber-Mangal, et al, 2003; Thompson, et al.,2011). However, the association of gains of 20q with tumor type is not consistent, with our data showing this finding only in patients diagnosed with TNBC, while other investigators have reported associations between gains for 20q in HER2 + and Lum B tumors (Thompson et al. 2011). Thus, the diagnostic and prognostic relevance of this finding appears to be limited.

No clear trend for an association between MNF in the peripheral blood and copy number alterations or mutations in the tumor was detected. The only significant correlation observed was between MN in PB and homozygous loss in the tumor (r= -0.6 , P=0.04) Table (6). However, this observation is based on only three homozygous deletions. Thus, this result should be interpreted with caution and the potential associations confirmed using a larger sample size.

In conclusion, the results of this "proof of principle" study support the use of the OncoScan genome-wide array for assisting with the recognition of differences between tumors. However, our data are too preliminary to determine if the CNAs recognized will lead to improvements in categorization or prognostication of BC tumors. Also, our study results showed no clearly significant associations between MNF in PB and any of the CNAs changes in the tumor, with the exception of homozygous deletions.

Through our future evaluation of a larger number of tumor specimens we hope to be able to answer the following questions: Could CNAs in the tumor tissue associate with CNAs and methylation patterns in WBCs? Do CNAs in the tumor tissue associate with the acquisition and/or persistence of adverse side effects related to chemotherapy? Could alterations observed in peripheral blood cells be used as early detectors of carcinogenesis in the tumor tissue? The use of WBCs as early detectors of cancer or as early predictors of an outcome is a concept that is worthy of more investigation.

Chapter 5

Conclusions

The primary goal of the current study was to investigate the biological basis for the development of psychoneurological symptoms (PNS) associated with chemotherapy, or the cancer itself, in women treated for breast cancer (BC). A longitudinal study was designed to investigate chromosomal/epigenetic changes in peripheral blood cells collected from women with BC at 5 time points and to examine the role of chromosomal alterations present in the tumor tissue at baseline.

The following conclusions were attained for the study aims that were examined.

Aim A) Determine the frequency of acquired chromosomal instability present before and following treatments and investigate its relationship to the development/persistence of PNS.

To date, micronuclei frequencies (MNF) have been scored for a total of 73 women at 4 times points, with 43 of these women also having data available for a fifth time point (2 year follow-up). The data presented in this dissertation included only the 4 time points that have been statistically analyzed. The final optimized statistical model identified 8 variables that had a significant predictive association with MN/cytome abnormality frequencies. These variables included:

(1) The time point at which the specimen was collected ($p<0.0001$)

(2) The type of chemotherapy treatment (p=0.0463)

(3) Exposure to radiotherapy (p=0.0004)

(4) Race (p=0.0037)

(5) Having a luminal B tumor categorization (p=0.0182)

(6) Having a triple negative tumor categorization (p=0.0446)

(7) Total perceived stress levels (p=0.0123)

(8) Cognitive flexibility domains (p=0.0238)

Decreases in MNF were observed 1 year following treatment, but did not return to baseline levels. At 2 years following chemotherapy, the preliminary results including a subset of women at time point 5 shows that MNF declined following the completion of all treatments. However, women showed variability, with levels of MN returning to baseline in a subset of women but remaining high in other women.

Aim B) Determine genome-wide epigenetic changes present at baseline and following chemotherapy to determine if methylation alterations are induced in peripheral blood cells following treatment.

To date, DNA methylation data has been collected for 69 women (at time points 1 & 2), and for 57, 45, and 10 women at time points 3, 4 and 5 respectively. The present dissertation includes results that have been statistically analyzed for 67 women before and during chemotherapy (T1 & T2). The following results have been concluded:

1) The results indicate that epigenetic changes associated with chemotherapy are acquired in peripheral blood.

2) A total of 1265 sites showing significant differential methylation after chemotherapy were detected at (FDR= 0.01 and >10% mean within pair difference). These sites included CpGs located within genes that are involved in the cell cycle, transcription regulation, cell death, signal transduction, neurogenesis, and in immune response.

3) Acquired decreases in methylation were more frequent than acquired increases in methylation.

4) Acquired alterations in DNA methylation were distributed across different genomic sequences, with the majority of changes occurring in the open sea sequences.

Aim C) Detect CNV present in BC tumor tissue and determine its relationship to peripheral blood MNF and methylation patterns at baseline.

Only 10 samples were included in this preliminary investigation. Not surprisingly, due to the small sample size, no clear associations were detected between MNF in blood and CNVs in tumors. However, the CNVs detected in the small number of tumors analyzed using the new OncoScan microarray platform were consistent with those previously reported in breast cancer tumors, thereby confirming that the genetic make-up of our study participants' tumors are representative of those observed in larger groups who were evaluated using alternative methods (classical chromosomal banding, chromosomal array CGH, and/or other microarray platforms).

Overall, our results support the primary hypothesis of this study, which states that in response to chemotherapy, biological changes arise in somatic tissue (PBCs) leading to genomic and/or epigenetic alterations that potentially contribute to PNS. Each of these genomic/epigenetic factors, acting either singly or in concert, could contribute to the development/ persistence of PNS since they would provide a means for "remembering" the biological effects after chemotherapy and have plasticity to explain responsiveness to environmental exposures (as well as G x E interactions). They also have the potential for reversibility, with the latter attribute being important for the potential development of therapeutic interventions.

This data also indicates the feasibility of using peripheral blood to assess and monitor an individual's soma-wide cellular response to different types of treatment regimens. Further investigation and continuation of this study, including all patients at all time points, could provide insight about the biological changes underlying a person's propensity to develop adverse side effects following chemotherapy, including but not limited to PNS. Ultimately, these types of studies could lead to the development of tests to identify women most at risk for acquiring cancer or cancer treatment-related side effects and could lead to "personalized medicine" approaches that might improve the quality of life for BC survivors.

Chapter 6

Methylation patterns in fetuses in fetu having disorganized development compared to those in their identical liveborn triplet

Fetuses in fetu (FIF) is a rare condition in which the growth of a twin/triplet erroneously occurs within the body of a co-twin/triplet. The incidence is estimated to be 1/500,000 births (Hoeffel et al., 2000). While the term fetus in fetu has been reported by Meckel in 1800, and the first case was been cited in 1809 by Young, a specific diagnostic criteria to distinguish it from teratoma was not developed until 1935 when Willis (Willis, 1935) defined the diagnosis of FIF by the presence of a vertebral column, which is often surrounded by other organs. In 1956, Lord suggested that in addition to the presence of the vertebral axis, an appropriate arrangement of other organs and limbs is required. The majority of cases are found to occur as an abdominal mass (Hoeffel et al., 2000). However, reports of FIF present in the sacrum, pelvis, liver, lungs, spleen, lymphnodes, pancreas, adrenal gland, scrotum and in the cerebrum have also been described (Gangopadhyay et al 2010, Heuer et al 2008, Escobar et al, 2008).

A mechanism for FIF is that they arise from the inclusion of a diamniotic monochorionic monozygotic twin in its co-twin due to the unequal division of the inner cell mass during embryogenesis (Lord, 1956, Beaudoin et al 2004). An alternative theory, termed the teratoma theory, states that the mass found in the fetus is a highly-organized, well differentiated teratoma (Magnus et al, 1999; Gilbert-Barness et al., 2003).

To gain insight about the embryonic origin of FIF Miura et al (2006) completed genotyping studies for two different cases of FIF and also looked at the methylation status at the human IGF2-H19 locus. They concluded that the genotypes of both cases of FIF were identical to the genotypes of the host infant, providing support for the monozygotic twin theory of development. They further conjectured that the implantation of the FIF occurred during the process of methylation establishment in the first case (based on differing patterns of methylation in the multiple clones evaluated), while it happened after the establishment of methylation in the 2nd case.

In 2012, Huddle et al published a case report on rare intraventricular fetuses in fetu. The case was diagnosed following both Willis' and Lords' criteria. Two fetiform structures were connected to the host's circulation by a vascular pedicle. Both masses were anencephalic and had a vertebral column with well-formed limbs. The two fetuses had other organs including CNS, gastrointestinal, and genitourinary tissues along with other less commonly observed organs such as the thymus, salivary glands, tooth buds, and adnexal structures. The organs were in a relatively correct anatomic arrangement to one another. Karyotyping and genotyping (using a SNP array analysis) on tissues obtained from the two fetuses and the host confirmed that the three structures were genetically identical females with a normal chromosomal complement. Thus, this case supports the monozygotic twin theory. These FIF also provide a unique opportunity to recognize genes that undergo methylation alterations during embryogenesis since they share identical genotypes, albeit in very different development environments. Therefore, the primary aim of this study was to determine the genome-wide DNA methylation patterns in the two fetuses in fetu and to compare those patterns to the

patterns seen in their genetically identical host triplet, the latter of whom showed normal development other than the brain malformations that resulted from the management of the FIF (Huddle, 2012).

Materials and Methods

Subjects and sample collection:

The patient was a female child of Vietnamese ancestry who was initially ascertained following an ultrasound study at 37 weeks' gestation, the latter of which showed dilated lateral ventricles and a possible aqueductal stenosis. At birth, a head CT showed significant ventriculomegaly with a midline intraventricular sof-tissue mass having ossified stuctures suggestive of a fetiform mass. At 3 months of age, the child presented with neurological symptoms that necessitated the need for a craniotomy to remove the FIF masses. More details about the clinical findings in the patient and the fetus in fetu are provided in the case report by Huddle et al, (2012). At the time of surgery, a blood sample was collected from the child (host), and biopsies were collected from the FIF tissues. Tissues from the FIF were immediately established in culture, using standard procedures, to allow for the completion of the requested chromosomal studies. Genomic DNA for the FIF was extracted from the cells established in monolayer cultures using the Puregene DNA Isolation Kit (Qiagen) according to the manufacturer's methodology. Genomic DNA was isolated directly from the whole blood sample collected from the host child using this same procedure (Puregene DNA Isolation Kit by Qiagen). Requests to collect a skin biopsy from the host child (to allow for a more direct comparison of tissue types between the FIF and their liveborn triplet) were unsuccessful. Therefore, to assist with recognizing methylated regions that varied due to tissue-type differences, DNA was also isolated from the fibroblast-like cells from an amniotic fluid specimen. Also, to allow for comparisons of the methylation patterns seen in the FIF to those

present in multipotent cells, DNA was isolated from 3 unrelated breast adipose-derived mesenchymal stem cells (bASC1, bASC2, bASC3)(Sachs, et al., 2012)

Genome-wide DNA methylation pattern:

The 450K HumanMethylation Chip (Illumina) was used for determining genome-wide DNA methylation patterns in: (1) the fetuses in fetu; (2) the host child (peripheral blood); (3) 3 multipotent breast adipose-derived stromal cell lines; and (4) cultured cells from an amniotic fluid specimen. Following bisulfite conversion, the genomic DNA was hybridized to the 450K HumanMethylation Chip according to the manufacturer's protocol (Illumina).

Data visualization and statistical analysis:

The Genome Studio Methylation Module (Illumina) and R (R 2.15.1) were used for data visualization and statistical analysis. The minfi package from R was used for data normalization, Pearson's correlations estimation, and for Bean and density plot creations. To determine the specific CpG sites that were differentially methylated between the child and the two FIF the following strategy was followed: for each sample, bead level data were read into the R programming environment. After excluding beads having a negative or zero intensity in either the red or green channel, raw signal intensities for the Methylated and Unmethylated Infinium Type I designed beads were retained and for Infinium Type II designed beads, proportion methylated was calculated as β=(Red intensity)/(Red intensity + Green intensity) β=[0,1]. Prior to applying the logit transformation, any β values equal to 0 were imputed to be 0.0001 while β values equal to 1 were imputed to be 0.9999. Thereafter, a logit transformation was applied to the β values to promote normality.

For Infinium design type I beads, the logarithm of the signal intensity was modeled using an analysis of variance (ANOVA) where probe type (Methylated or Unmethylated), subject (Child, FiF), and their interaction was included as fixed effects in the model. The p-values from the two independent ANOVA models were then combined using Fisher's method for combining p-values. The false discovery rate was estimated using the combined p-values based on the Benjamini and Hochberg method (1995). CpG sites having both an FDR<0.01 and a $|\Delta\beta| \geq 0.20$ were considered significant. For Infinium design type II beads, the statistical analysis was restricted to bead types having at least two observations in all three samples. For each FiF and bead type, a two-sample t-test was performed to compare the logit-transformed β values between group (Child versus FiF). Again, Fisher's method was used to combine p-values resulting from the two independent t-tests and then Benjamini and Hochberg's method was used to estimate the false discovery rate.

Results

Bean plots for the beta values are shown in Figure 11 for each of the 7 specimens. The greatest level of variation was observed for hypermethylation states. While the methylation patterns of the 2 genetically identical FIF closely resembled one another, their patterns (especially hypermethylation) varied from those observed for the other specimens, showing patterns that more closely resembled those of the multi-potent stem cells, than their genetically identical "host" triplet, or the tissue comparable amniotic fluid specimen. Interestingly, the multi-potent adipose-derived stromal cells that were derived from 3 unrelated individuals showed similar patterns, suggesting that the methylation status of these cells was more closely associated with their multi-potent characteristic than their genotype.

The density of the beta values is shown in Figure 12 for each specimen. The samples tend to cluster into 3 groups: (1) the child and amnio case (highest density of hypermethylated loci); (2) the fetuses in fetu (mid-group); and (3) the multi-potent adipose-derived stromal cells (lowest densities for hypermethylated sites)

Genome-wide methylation states were highly correlated between the fetuses in fetu (Figure 13 A). However, the methylation states of the fetuses in fetu more highly correlated to the patterns of the multi-potent adipose-derived stromal cell lines than to those of the host child or the amniotic fluid case (Figure 13).

A genome-wide sample and locus cluster analysis confirmed the overall similarity in methylation patterns between the genetically identical (FIF), and the similarity between the

stem cells (dendograms on right based on complete data set). The observation that the host child was more closely related to the amniotic fluid case than the fetuses in fetu, suggests that the observed patterns reflect factors other than just genotype or tissue type. (Figure 14)

Over the 485,764 sites interrogated, clusters of similar patterns were observed that were: (A) consistent for the FIF and host child, but not the other specimens (likely to be genotype driven); (B) limited to the FIF; (C) common for the FIF and the multi-potent cells but not the child/amniotic fluid cells (influenced by environmental/developmental cues); (D) distinct for the multi-potent cells; (E) distinct to peripheral blood (shown) or amniotic fluid cells (not shown); or (F) similar across all specimens. The results of a cluster analysis that was completed for the entire data set 9 (Figure 14) confirmed the similarity in patterns between the FIF, with the greatest variation in patterns being observed between the genetically identical host child blood specimen and the FIF samples.

An analysis to identify specific CpG sites that were differentially methylated in the FIFs' and the child's genomes, based on the Infinium design type I beads, revealed 12,187 significantly differentially methylated CpG sites (using a false discovery rate [FDR] <0.01 and a $|\Delta\beta| \geq 0.20$). For the Infinium design type II beads, a total of 56,090 CpG sites showed significantly different methylation values (FDR<0.01 and a $|\Delta\beta| \geq 0.20$).

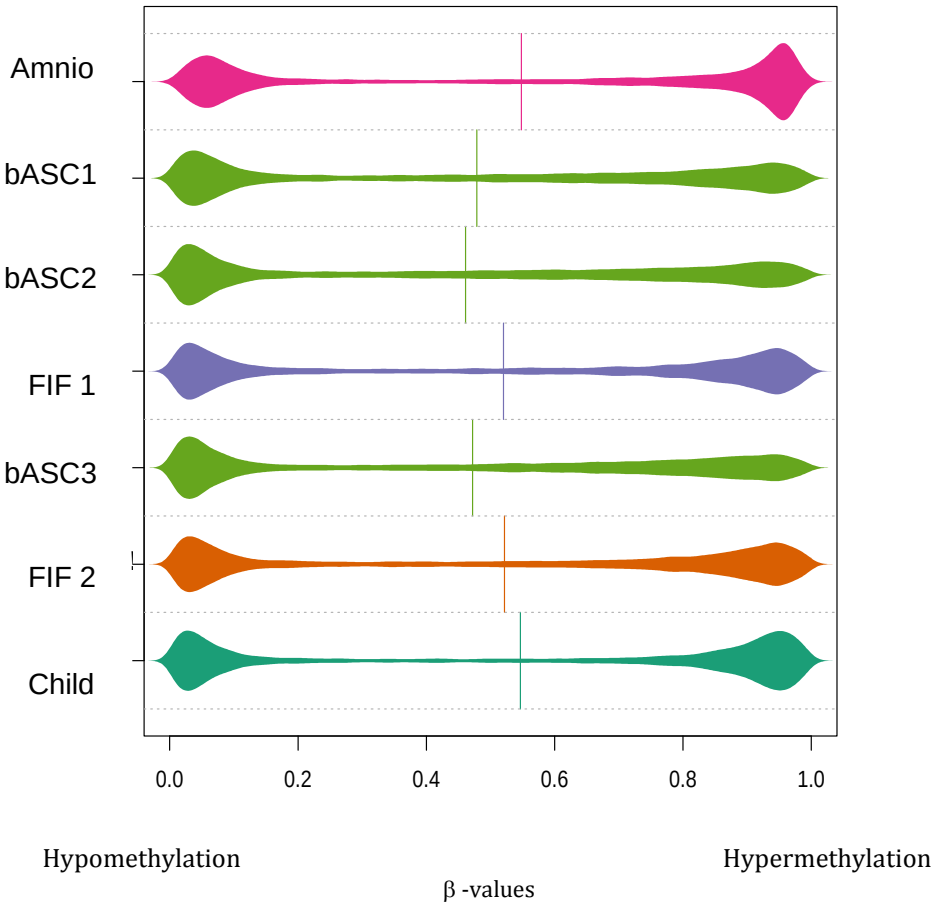


Figure 11: Bean plots for FIF: Bean plots are shown for the beta values for each of the 7 specimens, with the mean beta values being represented as a vertical line for each case. The greatest level of variation was observed for hypermethylation states, with the multipotent adipose-derived mesenchymal stem cells (bASC1, bASC2, bASC3 shown in green) having similar patterns between specimens derived from unrelated individuals. Also, the monozygotic fetus in fetu specimens (FIF 1 [purple]; FIF 2 [orange]) had similar patterns. However, despite being genetically identical, the fetuses in fetu had distinct patterns from those observed in the host child (aqua)

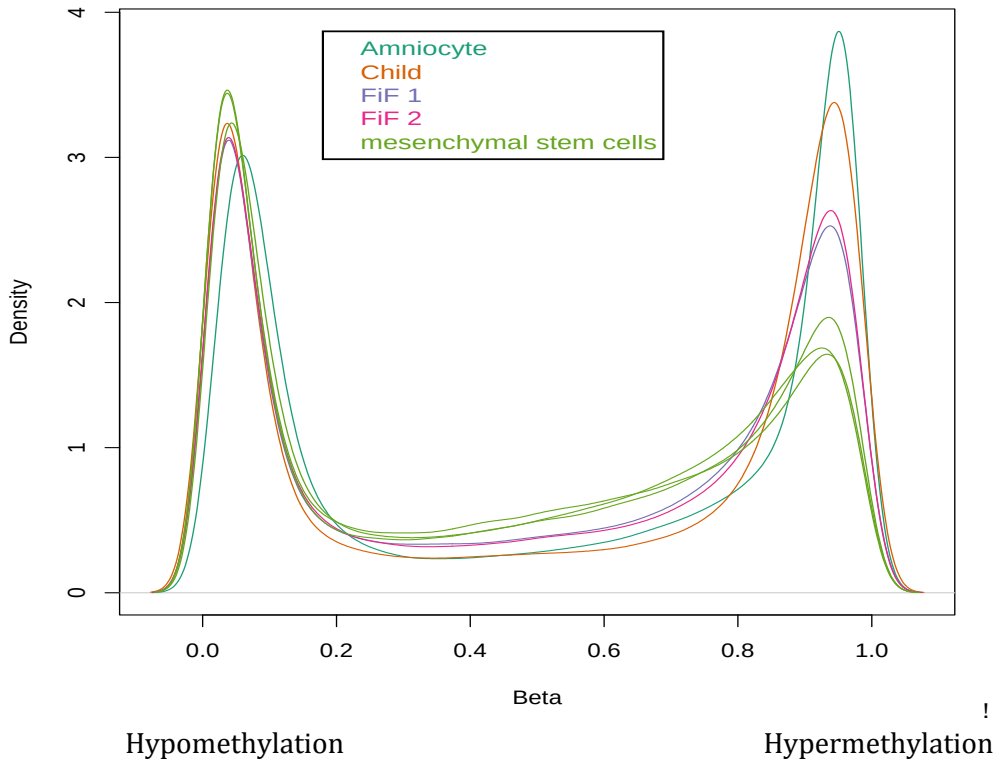
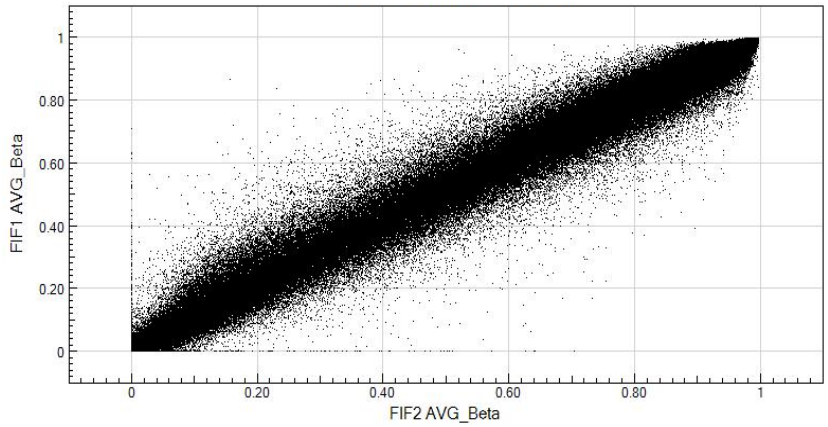
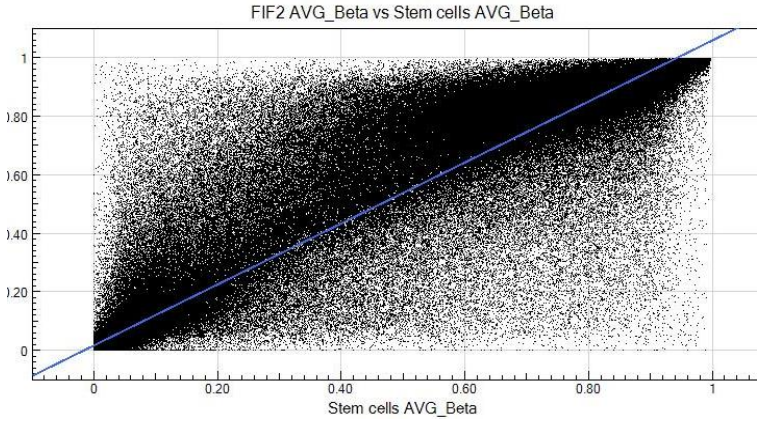


Figure 12: Density plots for FIF: The density of the beta values is shown for each specimen. For hypermethylation densities, the samples tend to cluster into 3 groups: (1) the child and amnio case (highest density of hypermethylated loci); (2) the fetuses in fetu (mid group); and (3) the stem cells (lowest densities for hypermethylated sites)

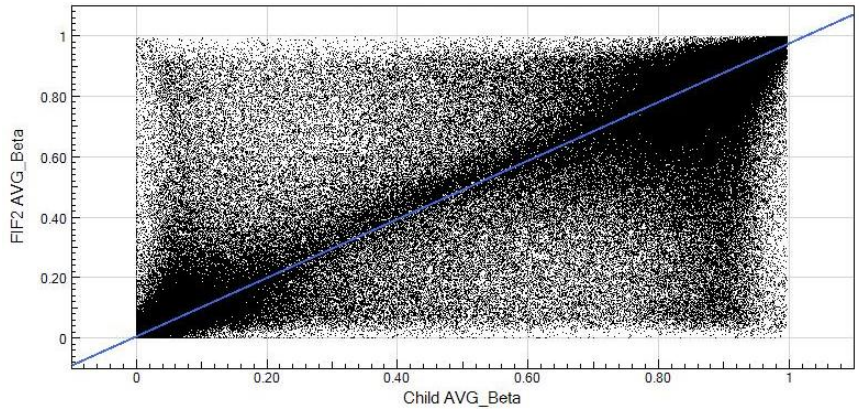
A)


FIF1 AVG_Beta
FIF2 AVG_Beta
0
0.20
0.40
0.60
0.80
1

B)


FIF2 AVG_Beta vs Stem cells AVG_Beta
Stem cells AVG_Beta
0
0.20
0.40
0.60
0.80
1

C)


FIF2 AVG_Beta
Child AVG_Beta
0
0.20
0.40
0.60
0.80
1

D)

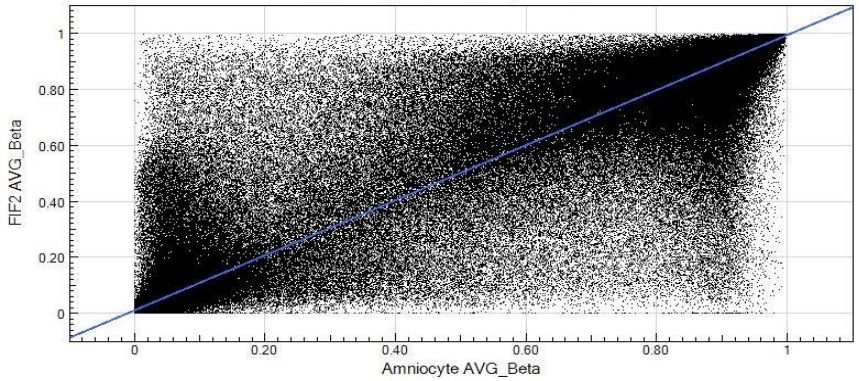


Figure 13: Correlations for FIF. Genome-wide methylation states were highly correlated between the fetuses in fetu (A). When comparing the methylation states of the fetuses in fetu to those of the other specimens, the following trend toward correlations of locus-specific sites was observed: Stem cells (B) correlation > Host child (C) or Amnio case

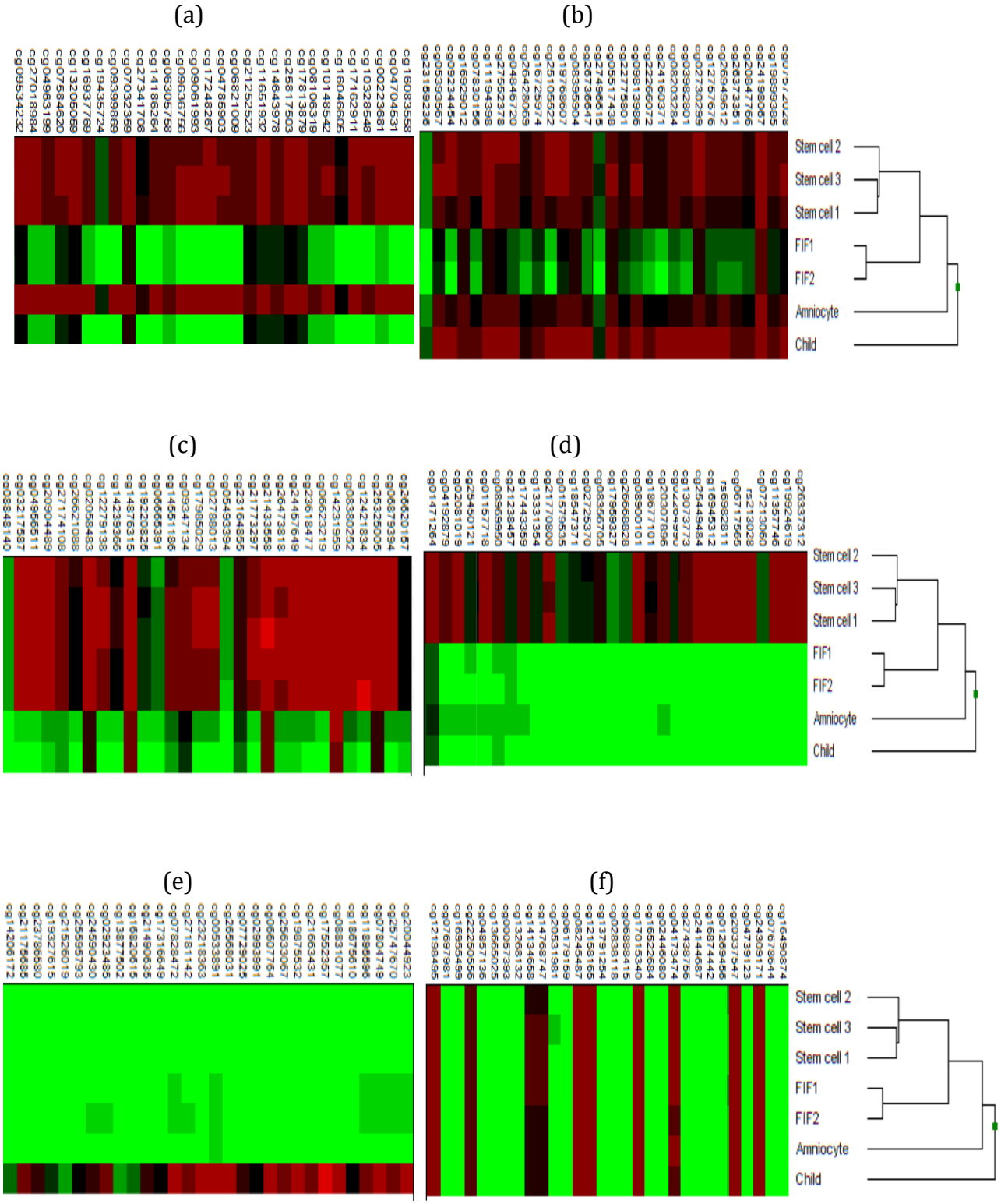


Figure 14: Heat maps for FIF

Heat maps showing examples of methylation patterns for subsets of 30 loci (columns) for each of the specimens (rows). Each square in the heat map shows the relative level of methylation for a specific site evaluated (values range from 0 to 1, with bright green corresponding to sites that are hypomethylated, while bright red corresponds to sites that are hypermethylated). Over the 485,764 sites interrogated, clusters of similar patterns were observed that were: (A) consistent for the FIF and host child but not the other specimens (likely to be genotype driven); (B) limited to the FIF; (C) common for the FIF and the stem cells but not the child/amnio (influenced by environmental/developmental cues); (D) distinct for the stem cells; (E) distinct to peripheral blood (shown) or amniocytes (not shown) or (F) similar across all specimens. The dendograms on the right show the cluster analysis results for the specimens based on the entire data set [not the subset of examples shown to illustrate the types of variation observed].

Discussion

Here, we present the first case of fetus/fetuses in fetu for which a genome-wide analysis of methylation patterns has been determined. The only other group to report methylation patters in FIF is Miura et al. (2006), who analyzed methylation patterns between the host/FIF duos for 2 kindreds using a targeted assessment of a single gene that is known to be differentially methylated (imprinted). Based on their results, they concluded that the embryonic timing of the implantation of the FIF could impact the methylation pattern of genes in the FIF.

Epigenetic reprogramming is a critical process during the normal development of an embryo. Upon fertilization, global demethylation takes place in whole genomic sequences except for imprinted loci, which are maintained. This erasure is followed by a whole genome de novo methylation. (Reik et al., 2001, Li, 2002, Owen and Segars, 2009) Since there is a lack of studies investigating human embryos, most of the findings in embryogenesis come from studies of mouse embryos. In mice, it has been shown that the paternal genome undergoes demethylation rapidly and reaches the lowest levels of methylation before the two-cell embryo stage (Haaf. T, 2006). However, the maternal genome imprints are maintained up to the two-cell embryo stage, then the methylation marks start to gradually drop until after the eight-cell embryo stage, when both paternal and maternal become equally demethylated (Haaf. T, 2006). Genome-wide de novo methylation happens preferentially in the inner cell mass o the blastocyst-stage embryos, thereby allowing for the establishment of the somatic

methylation patterns in the embryo's cells, which then differentiate to various embryonic lineages (Haff , 2006, Mayer et al 2000a, Reik et al 2001, Dean et al. 2001, Li, 2002).

Given that FIF is thought to originate in the blastocyst-stage of embryogenesis as a result of the unequal division of the inner cell mass, which is thought to be a stage that is important for epigenetic reprogramming (Lord, 1956, Beaudoin et al 2004; Haaf, 2006), studies of methylation patterns in FIF cases could provide insight regarding embryonic methylation and development. In this study, we showed that the greatest level of variation in methylation patterns between the FIFs and the host trio involved hypermethylation states. The three multi-potent adipose-derived stromal cells showed the lowest density of methylation at hypermethylated sites, followed by the two FIF, then the amniotic fluid cells and then the genetically identical FIF host child.

A shortcoming of this study was our inability to study a similar tissue type (skin) from the host child for comparison to the patterns seen in the fibroblasts from the FIF. However, the methylation patterns observed suggest that the differences reflect changes beyond those related to tissue-specific or genotype specific findings since the amniotic fluid cell patterns were more closely related to the host child then the FIFs (similar phenotype [normal], but different genotype and cell type). The observation of a higher density of hypermethylated sites in the host child compared to the FIF is consistent with a lack of the FIF specimens to complete the normal re-establishment of methylation patterns during embryogenesis. The data also suggest that epigenetic changes acquired during development are more heavily influenced by embryonic environmental cues than the developing embryo's genotype since the genetically

identical FIF and host child showed the greatest patterns of divergence. This is not surprising since establishing epigenetic imprints in the fetus is very critical during the formation of the blastocyst and it is known that the first trimester of pregnancy is very sensitive to environmental influences. Nonetheless, there were subsets of genes that did appear to be closely related to genotype, with other subsets being identified that had consistent methylation patterns across all specimens.

The observation that the methylation patterns for the multi-potent adipose-derived stem cells were more closely related to the FIF specimens than the other specimens is also interesting. One could speculate that the FIF cells retain their multi-potent capacity due to a failure to "silence" genes that would typically be methylated. The "cues" necessary for initiating these methylation alterations may involve microenvironments from the surrounding cells, rather than being strictly encoded through the organism's genome. A full assessment of the individual sites demonstrating differential methylation may provide clues as to genes/gene networks that are most closely related to methylation status to allow for normal embryogenesis to proceed.

In summary, further whole genome methylation studies of rare cases of fetus in fetu could provide valuable insight about epigenetic patterns that are acquired during human embryogenesis, as well as the influences those patterns play in directing tissue/morphological development.

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