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Impact of Bodyweight on Tissue-Specific Folate Status, Genome Wide and Gene-Specific DNA Methylation in Normal Breast Tissues from Premenopausal Women

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IMPACT OF BODYWEIGHT ON TISSUE-SPECIFIC FOLATE STATUS, GENOME
WIDE AND GENE-SPECIFIC DNA METHYLATION IN NORMAL BREAST
TISSUES FROM PREMENOPAUSAL WOMEN

A Thesis Presented

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ABSTRACT

IMPACT OF BODYWEIGHT ON TISSUE-SPECIFIC FOLATE STATUS, GENOME WIDE AND GENE-SPECIFIC DNA METHYLATION IN NORMAL BREAST TISSUES FROM PREMENOPAUSAL WOMEN

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Obesity has reached an epidemic level in the United States. A number of epidemiological studies have established obesity as a critical risk factor for postmenopausal breast cancer (post-BC), whereas a reverse association holds prior to menopause. A significant scientific gap exists in understanding the mechanism(s) underpinning this epidemiological phenomenon, particularly the reverse association between obesity and premenopausal breast cancer (pre-BC). This study aimed to understand how folate metabolism and DNA methylation informs the association between obesity and pre-BC. Fifty normal breast tissue samples were collected from premenopausal women who underwent reduction mammoplasty. We developed and measured the breast tissue folate by a Lactobacillus Casei microbiological assay, and the DNA methylation of LINE-1, a biomarker of genome-wide methylation, and the promoter methylation and gene expression of SFRP1, a tumor suppressor, were measured by pyrosequencing and real-time PCR. We found a high BMI is associated with increased folate level in the mammary tissue, with an increase of 2.65 ng/g of folate per every 5-unit increase of BMI ($p < 0.05$). The LINE-1 DNA methylation was significantly associated with BMI ($p < 0.05$), and marginally associated with folate concentration ($p = 0.087$). For the 8 CpG

sites analyzed in the promoter region of the SFRP1 gene, no associations were observed for either BMI or tissue folate (p > 0.05), although a high expression of SFRP1 was observed in subjects with high BMI or high folate (p < 0.05). This study demonstrated that, in premenopausal women, obesity is associated with an increased mammary folate status, genome-wide DNA methylation and SFRP1 gene expression, indicating that the improved folate and epigenetic status is potentially responsible for the reverse association between obesity and pre-BC. More studies are warranted to further understand how obesity mediates pre-BC via altering folate metabolism and DNA methylation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed cancer in women and the third leading cause of cancer-related death. (CDC 2017) When compared to postmenopausal, premenopausal women with BC have poorer prognosis and respond less to chemotherapy. Within the same molecular subtypes, the signatures of genetic and epigenetic alterations are shown to be different in premenopausal versus postmenopausal BC. Tumors found in young women are biologically distinct from those found in women over 50 and may necessitate distinct preventive and therapeutic approaches. Obesity is an independent prognosis factor in BC. Unlike the positive association between obesity and postmenopausal BC risk, general adiposity exhibits an inverse effect with overall risk in premenopausal women. The mechanism for this protective effect is yet unknown.

Epigenetic alterations occur well in advance of observable changes in DNA integrity or dysregulated cellular proliferation patterns. DNA hyper- or hypomethylation can be gene-specific or genome-wide. Changes in the methylation levels of cytosine-guanine dinucleotide-rich areas (CpG sites), of tumor supressor or repair genes induce genomic instability; an early event in breast tumorigenesis. BMI and menopausal status have been shown to co-vary with gene-specific methylation patterns in BC. BMI is associated with hypermethylation levels of epiobesigenic genes governing critical cell functions across all molecular subtypes of BC.

Though BMI is associated with lower global methylation levels overall, results from studies on global methylation, body weight status and BC risk are varied. This

variance may be due to heterogeneity between measurement assays. Global methylation as measured by LINE-1, a transposable element that composes more than 45% of the human genome, was positively associated with BMI in a recent cross-sectional study with a healthy cohort. A 2014 study by DeRoo et al. reported an association between LINE-1 methylation from peripheral blood and BC risk in a dose-dependent manner. In a recent review, two out of the thirteen studies with significant results using LINE-1 found a reduced risk of BC with higher DNA methylation levels.

Folate availability, the substrate for DNA methylation, may contribute to the potentially reversible epigenetic alterations associated with BC. Regardless of menopausal or weight status, epigenetic alterations resulting from deficiencies in one-carbon metabolism (OCM) related compounds such as folate have been shown to drive BC tumorigenesis. A deficiency in dietary folate has been shown to lead to gene promoter region hypermethylation as well as lower global hypomethylation. Epidemiological studies on the association between folate status and the risk of BC have shown inconsistent results. Meta-analyses suggest a U- or J-shaped curve relationship between folate intake and BC risk. A non-linear dose-response relationship between 200-320μg/d of dietary folate and lower risk of BC was recently reported, verses a significant increase in risk with daily folate intakes above 400ug. In other prospective and case-control studies, dietary folate status as assessed between categories of highest and lowest intakes was not associated with a higher relative risk (RR) of BC for metastatic or invasive carcinoma. Conversely, when stratified into quartiles between lowest and highest intake as measured by a food frequency questionnaire (FFQ), folate was associated with an overall reduced RR of BC across all subtypes. A possible explanation to these mixed results could be timing or assessment

methods for measuring levels of folate intake. Folate's role in BC etiology may depend heavily on age of dietary exposure.

DNA methylation patterns differ between metabolic states, and fluctuations throughout the human lifespan of both folate status and BMI elicit modifiable changes in the epigenome. Epigenetic modifications are sensitive to folate availability and folate levels have been shown to change with BMI status. After adjustments for overall intake, overweight and obese women were reported to have lower serum folate and higher red blood cell (RBC) folate levels when compared to women of the same weight categories. In addition, BMI has been shown to affect the distribution of folate from circulation into tissue. This weight-dependent redistribution of folate may affect the availability of folate as a substrate for both global and gene-specific methylation.

Whether changes in DNA methylation in response to folate levels and bodyweight are deleterious or protective against BC risk may heavily depend on menopausal status. Though the mechanisms underlying these associations have yet to be discovered, DNA methylation patterns in premenopausal overweight or obese women with BC are often significantly different from postmenopausal overweight or obese women with BC. Results from the few investigations on BMI, folate, methylation, menopausal status and BC risk are currently inconclusive. A possible explanation for these mixed results is that the relationship between these factors may be contingent on age, area of fat disposition, and metabolic activity of adipose. Despite the current lack of overall consensus, DNA methylation is highly associated with BMI and BMI status is associated with folate concentrations, when measured in adipose tissue. Weight-related differences between folate status as well as epigenetic responses to folate interventions provide significant

evidence in support of the effect of overweight and obesity on epiobesigenic methylation

levels and BC risk.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast Cancer

Breast cancer chapter text. Aside from some skin cancers, breast cancer (BC) is the most frequently diagnosed cancer in women regardless of race or ethnicity. BC is the leading cause of cancer-related death in women aged 20-39 years. (CDC 2017) The American Cancer Society estimates that one in eight women in western countries will be diagnosed with BC during their lifetime. BC is projected to account for 30% of all new cancer cases, with about 255,180 new cases of invasive BC projected in 2018. It has been known since the mid-1980s that survival rates for all types of BC are significantly lower in premenopausal women versus postmenopausal. (Adami, Malker, Holmberg, Persson, & Stone, 1986) Younger age at onset is considered an independent risk factor for overall risk, tumor aggressiveness, recurrence and survival. (Bleyer et al., 2008; El Saghir et al., 2006)

Rather than a single disease, BC is a collection of various subtypes with distinct underlying genetic profiles, molecular signatures and phenotypes. Instances of the most lethal subtype of BCs, such as triple negative, are higher in premenopausal women than in postmenopausal. (Anders, Johnson, Litton, Phillips, & Bleyer, 2009) Though incidence rate has remained relatively stable over the last decade, BC mortality rate in premenopausal women is slowly increasing. (Siegel, Miller, & Jemal, 2017) Common screening measures such as mammography are encouraged, as early detection has been shown to improve overall prognosis, yet these early stage events are often masked in mammograms of premenopausal women due to overall breast density. (Boyd et al., 2007)

Women with extensive mammographic density have a 17 times greater risk of developing BC within the 12 months following a negative screening due to this masking effect

2.2 Obesity and Breast Cancer

Well-established risk factors for BC are numerous. These risk factors include age; race and ethnicity; socioeconomic status; hormonal status and parity; breast density; diet, physical activity and body weight status. Of these modifiable and non-modifiable risk factors, body weight status is of high significance with regard to incidence and prognosis. (Calle, Rodriguez, Walker-Thurmond, & Thun, 2003). Body weight status as measured by body mass index (BMI), is significantly correlated with overall BC risk. (Tamimi et al., 2016)

Higher BMI, higher overall energy intake and lower physical activity levels have been associated with increased postmenopausal BC risk in prospective cohort studies. (Silvera, Jain, Howe, Miller, & Rohan, 2006) It was originally speculated that body weight status was also positively associated with the risk of premenopausal BC. (Silvera et al., 2006) However, in recent studies, though overweight or obese weight status (BMI of 25.0-29.9kg/m^2 or $\geq$30kg/m^2, respectively) has been continuously shown to be positively associated with postmenopausal BC risk, this correlation has been indicated to be reversed for women of childbearing age. (McCormack et al., 2006) In 2008, the Renehan laboratory reported their findings from a systematic review of meta-analysis of 141 articles. The authors found that each 5 kg/m^2 increase in BMI was associated with a 12% increased RR of postmenopausal BC, versus an 8% decreased RR of premenopausal BC. (Renehan, Tyson, Egger, Heller, & Zwahlen, 2008) Findings from recent large-scale

epidemiologic studies also support a reverse relationship between overall weight status

and premenopausal BC risk. (Munsell et al., 2014; Nelson et al., 2012) A meta-analysis

of sixty-six studies conducted by Nelson et al. found an inverse association between

premenopausal BC and metabolically healthy overweight/obese phenotype. In eighteen of

the studies included in the meta-analysis, reduced risks were found for women in the

overweight (RR, 0.86 [95% CI, 0.82, 0.90]) and obese (RR, 0.74 [CI, 0.68, 0.81]) BMI

categories when compared to the reference group (normal and underweight combined).

(Nelson et al., 2012) Another meta-analysis of 89 epidemiological studies conducted in

2014 by Munsell et al. found that premenopausal obese women showed a 20% reduced

risk of hormone receptor-positive BC when compared to underweight (summary RR,

normal vs. obese or overweight 0.78, 95% CI, 0.67, 0.92). (Munsell et al., 2014) Adipose

tissue is functionally a metabolically and hormonally active organ. Physiological

variations between normal weight and overweight or obese premenopausal women

related to BMI status may help to explain the weight-dependent variation in BC risk with

regard to hormonal status

2.3 Epigenetic Alterations in Breast Cancer

Though epidemiological studies have observed a significant negative correlation

between obesity or overweight and BC risk in premenopausal women, the mechanism for

this protective effect is yet unknown. One proposed mechanism for the reversible

association between weight and BC risk found prior to menopause is the epigenetic

modification of genomic DNA. BMI is known to elicit changes in the epigenome. (Meeks

2017) Further, BMI and menopausal status have been shown to co-vary with methylation patterns in BC. (Crujeiras et al., 2017)

2.3.1 Gene-Specific Methylation, Obesity and Breast Cancer

Changes in the methylation levels of the cytosines in cytosine-guanine dinucleotide-rich areas (CpG sites), of tumor supressor or DNA repair genes commonly induces genomic instability through gene transcription silencing. (Akhavan-Niaki & Samadani, 2013) These epigenetic alternations are early events in breast tumorigenesis. (Danforth, 2016) Abnormal hypermethylation of CpG sites in the promotor regions of tumor supressor genes is followed by loss of function, capable of increasing both somatic and germline mutations of genes in BC-related pathways. (Herman & Baylin, 2003)

Several studies have investigated the relationship between body weight status and BC risk with regard to gene-specific DNA methylation levels. BMI is associated with aberrant promotor methylation levels of epiobesigenic genes governing cell growth, immune response, DNA repair and adipogenesis across many molecular subtypes of BC. (Holm et al., 2010) With regard to specific genes, RASSF1A, CCND2, ER-alpha and PR are reported to be hypermethylated in BC, as well as PTEN, CDKN1A and ESR1. (Y. T. Huang et al., 2016; Campion, 2009) In addition, BC-specific mortality has been found higher in obese women with promoter methylation in the APC gene (HR = 2.47; 95 % CI, 1.43, 4.27). (McCullough et al., 2016) *Table 1* includes a more extensive list of commonly reported genes with differential methylation in BC

Table 1. Genes differentially methylated in breast cancer.

Function	Gene Name	Gene ID	Biological Role Implicated in Cancer
DNA Repair	BRCA1	672	Tumor supressor
	BRCA2	675	Tumor supressor
	MGMT	4255	Defense against mutagenesis and toxicity
	MSH2	4436	Mismatch repair gene, DNA damage signaling
	MLH1	4292	Tumor supressor, DNA mismatch repair gene
Cell-Cycle	SFRP1	6422	WNT inhibitor
Regulation	APC	324	Tumor supressor
	CCND2	894	Regulates G1-S (Cell growth to DNA replication phase)
	CDKN2A (P16)	1029	Tumor supressor
	CDKN1A	1026	Regulates cell cycle progression at G1
	DAPK1	1612	Apoptosis, autophagy
	SCGB3A1	92304	Cell proliferation and differentiation
ER+ vs ER-	RASSF1A	11186	Tumor supressor, blocks cell cycle progression
	CCND2	894	Regulates G1-S phase
	ESR1	2099	hormone binding, DNA binding, transcription activation
	PGR	5241	Cellular proliferation and differentiation
	CDH13	1012	Tumor supressor. cell adhesion
	HSD17B4	3295	Peroxisomal FA beta-oxidation pathway, differential methylation in HER2+ subtype
	BCL2	596	Inhibits apoptosis, Calcium signaling

With regard to methylation of genes in whole blood, the BC susceptibility genes BRCA1 and BRCA2 are two of the most frequently investigated. A recent meta-analysis of 40 studies supported the association between BRCA1 promotor hypermethylation in cancer versus non-cancer controls. (L. Zhang & Long, 2015) In contrast, in a healthy cohort of dominantly obese female nurses aged 40-60 years, obesity was not associated with BRCA1 or BRCA2 hypermethylation. (Peplonska et al., 2017) The relationship between methylation levels of BC susceptibility genes warrants further investigation.

2.3.2 Global Methylation, Obesity and Breast Cancer

In contrast to the hypermethylation of promotor regions of tumor suppressor genes, hypomethylation of non-coding genomic regions is characteristic of nearly all cancers, with BC as no exception. Global hypomethylation facilitates tumor promotion and progression through increased chromosomal instability. Results from studies on global methylation, body weight status and BC risk are varied, perhaps due in part to variations in inter-assay measurement validity. (Choi 2009, Cho 2010, Xu 2012) DNA methylation within specific regions of interest can be assessed with several different methods. Versions of High Performance Liquid Chromatography-based (HPLC) detection are considered the "gold standard" of global DNA methylation, but due to their considerable cost and need for special laboratory conditions, the use of HPLC for DNA methylation profiling is frequently less common. Two of the most popular substitutions for measuring whole-genome methylation profiles are long interspersed nuclear element-1 (LINE-1) and luminometric methylation assay (LUMA), as they exhibit higher specificity, sensitivity and lower assay-to-assay variability than other methods of approach. Both methods require very little

genomic DNA and so are often used in conjunction, with LUMA serving as an internal control accounting for variability in the amount of DNA input. Of these two methods, LUMA and LINE-1, only LINE-1 assay data correlates well with HPLC-derived measurements, and LINE-1 is often recommended over the use of LUMA alone to assess whole genome methylation patterns. (Kurdyukov & Bullock, 2016) As more than one-third of methylation in the human genome is found in the CpG-rich sequences of Long Interspersed Nuclear Elements (LINEs), these transposable elements are considered a valid surrogate marker for global methylation. (Beck et al., 2011; Carraro et al., 2016; Irahara, Nosho, Baba, & Shima, 2010; Park et al., 2014) The largest subclass of these transposable nuclear elements is LINE-1. Hypomethylation of LINE-1 parallels global DNA hypomethylation and is characteristic of cancer cells. (Tang et al., 2016)

Though evidence supporting blood-based DNA methylation as measured by LINE-1 as a biomarker for BC risk is currently limited, a dose-dependent association between LINE-1 methylation from peripheral blood and BC risk has been reported. (Deroo et al., 2014) The confounding effects of gender, age and lifestyle habits may explain the inconsistency in assessments between LINE-1 methylation and body weight status. Progressing age is associated with an increase in CpG island methylation but a decline in global methylation. (F. F. Zhang et al., 2011) LINE-1 methylation levels in white blood cells was negatively correlated with body fat mass but positively associated with a healthy lifestyle in a healthy, young cohort of men and women (mean age, 23 years; mean BMI = 22kg/m^2). (Marques-Rocha et al., 2016). Likewise, global methylation as measured LINE-1 was positively associated with BMI in a recent cross-sectional study with a healthy cohort of postmenopausal women. (Boyne et al., 2017)

Due to its complexity and mutability, the quantification of CpG islands in repeating elements LINE-1 is not a perfect biomarker for global DNA methylation. Aside from the DeRoo study, most associations between global methylation and BC risk find significance only when assessing the data with quantile analysis. Of the thirteen studies assessed in a review by Tang et al. in 2016, the four study results with significant odds ratios (ORs) had used different methods of measuring DNA methylation. The two studies which used the LUMA assay found that women in the highest quantile of global methylation versus the lowest had higher risks of BC. In contrast, the other two studies with significant ORs had used LINE-1 and/or 5-mdC levels to measure methylation. 5-methyldeoxycytosine methylation is measured through liquid chromatography mass spectrometry. These studies both found a reduced risk of BC with higher DNA methylation levels, though the results from the two measuring methods did not correlate with each other. (Choi et al., 2009) Importantly, changes in genome-wide or gene-specific methylation patterns may be confined to the microenvironment of the breast tumor site and specificity has been shown to depend on the originating tissue. (Knothe et al., 2016) Previous studies on obesity, BC risk and survival rates and global methylation as measured by LINE-1 almost exclusively use DNA extracted from whole blood. (Tang et al., 2016) Samples for testing methylation levels extracted from the localized area of the breast as opposed to whole blood could reduce potential bias. As an example, in one of the few studies that show a positive relationship between BC risk, genomic hypomethylation and promoter hypermethylation, tumor and adjacent-tissue specific methylation levels of LINE-1 in invasive ductal carcinoma of the breast were found to be lower than in healthy tissue. In contrast, higher levels of promotor hypermethylation were exhibited in tumor and adjacent tissue, when

compared to methylation levels of whole blood cell DNA from the same subjects. (Cho et al., 2012) Studies assessing weight status, methylation patterns and BC risk using local tissue samples may provide a more accurate picture of early epigenetic changes in the obesity phenotype.

In conclusion, though studies from the past five years may not show consistent associations between BMI and global DNA methylation (see review by Bell 2017), differing methods of measurement as well as originating tissue may be the significant contributors to this lack of consensus. Overall, gene-specific as well as sight-specific changes in methylation have been consistently observed in both case-control and larger intervention trials assessing BMI and BC risk. More studies addressing methylation, weight status and BC risk in premenopausal women specifically would need to be conducted to reach a consensus on these relationships.

2.4 Folate Status and Breast Cancer

Many of the critical cellular pathways disrupted in carcinogenesis are dependent on adequate folate status. Folate serves as the source of methyl groups for de novo nucleotide synthesis, DNA repair and the maintenance of the epigenetic landscape. Methyl donor depletion, as in a state of folate deficiency from inappropriate nutrition, significantly disrupts all areas of one-carbon metabolism. (Friso, Udali, De Santis, & Choi, 2017)

The anti-cancer effects of foods high in folate have been studied for many decades. Dietary folate intake is essential to proper nucleotide synthesis, DNA repair and methylation, all of which are disrupted in carcinogenesis, and adequate folate status exhibits a protective effect against risk of many cancers. (Kim 1999) Diets low in fruits

and vegetables often lead to diminished folate status. Overall vegetable intake has been associated with lowered premenopausal BC risk, though it is speculated that many compounds within the food (folate, fiber, phytochemicals, etc.) have a synergistic effect on risk reduction. (Freudenheim et al., 1996) More recently, cruciferous vegetable intake, a class of vegetables that are high in folate, has been associated with a decreased risk of premenopausal BC in a Japanese cohort. [multivariable RR for intake, highest vs. lowest = 0.64 (95 % CI, 0.38-1.10; p for trend = .046)]. (Suzuki et al., 2013) Furthermore, some evidence suggests that an adolescent diet low in green leafy and cruciferous vegetables may influence premenopausal BC incidence. (Harris, Willett, Vaidya, & Michels, 2017)

Several studies, including meta-analyses, have been conducted assessing the association between BC risk or BC-specific mortality and folate. Akin to the effect of certain food groups on premenopausal BC, epidemiological studies have shown mixed results as to folate's explicit role in associated BC risk. **(**See Table 2.)

Dietary folate (DF) status and total folate (TF, both dietary and from all sources) status, as measured through food frequency questionnaires (FFQs), are the most widely used exposures. Dietary folate status, when compared by lowest and highest intake, has not been associated with a higher RR of BC for both metastatic and invasive carcinoma in most meta-analyses. (Rohan et al., 2000, Cho 2003, Kabat et al., 2008, Bassett et al., 2013) This finding has remained true for larger-scale prospective cohorts, such as in the Canadian National Breast Screening Study (CNBSS) and Melbourne Collaborative Cohort Study (MCCS).

Table 2. Prospective studies and results on the association between dietary or total folate intake and breast cancer risk by premenopausal status.

Authors	Study Period	N	BC†	TF or DF‡	Age	Study Cohort	P for trend	Conclusions
Zhang et al., 1999	1980-1996	88,818	3483	TF	30-55	Nurses' Health Study	0.98	No association.
Rohan et al., 2000	1980-1993	5,382	1,336	DF	40-59	Canadian National Breast Screening Study	0.32	No association for DF. Suggestion of increased risk at highest quintile of DF in premenopausal women; 95% CI included unity.
Cho et al., 2003	1991-1999	90,655	714	both	26-46	Nurses' Health Study 2	TF 0.96 DF 0.94	No association.
Zhang et al., 2005	1980-2000	88,744	4,422	both	30-55	Nurses' Health Study	0.03	Higher TF intake associated with reduced RR of ER- but not ER+. ER+ associated with increased risk.
Cho et al., 2007	1991-2003	90,663	1,032	both	36	14 States (US)	TF 0.31 DF 0.77	No association (BC overall or ER- breast cancer).
Larsson et al., 2008	1990-2001	61,433	2,952	TF	53.3	Swedish Mammography Cohort	TF 0.84 ER+/PR- 0.01	No association for BC overall. Suggestion of inverse association with ER+/PR- tumors.
Kabat et al., 2008	1980-2000	49,654	2,491	DF	40-59	Canadian National Breast Screening Study	0.79	No association.

Shrubsole et al., 2011	1997-2008	72,861	718	DF	40-70	Shanghai, China	0.032	Higher dietary folate intake associated with a 40% decreased BC risk for high vs. low intake.
Bassett et al., 2013	2010	20,756	936	DF	41-86	Melbourne Collaborative Cohort Study	>0.07	No association.

† Breast cancer cases; ‡ total folate or dietary folate; N, number of study participants; BC, breast cancer; age, age at baseline; RR, relative risk; 95% CI ,95% confidence interval; ER+, ER-, Estrogen receptor positive, estrogen receptor negative; PR+, PR- Progesterone receptor positive, progesterone receptor negative.

In contrast, a meta-analysis of five studies assessing folate intake before diagnosis of BC by Li et al. reported a significant inverse relationship between DF intake and all-cause mortality (highest vs. lowest; pooled HR from all five studies 0.74, 95% CI, 0.60-0.92). (Li, Lu, Wang, & Zhang, 2015) All-cause mortality in BC patients was significantly lower in the highest levels of folate intake, showing a 14% risk reduction compared to lowest. Using two of the studies included in this meta-analysis, Sellers 2002 and Harris 2012, the authors also found a dose-response relationship between dietary folate intake at increments of 200µg/day and all-cause mortality (summary HR 0.86; 95% CI, 0.76-0.97). (Li et al., 2015) However, no association was found between total folate intake and BC risk.

In a meta-analysis of 14 cohorts from 19 studies (n=677,858), a non-linear relationship was observed between folate intake as measured by FFQ and BC risk (p-value for non-linearity = 0.0007). (F. Zhang et al., 2014) Zhang et al found that 200-320µg/d of dietary folate was associated with a lowered risk of BC, verses a significant increase in risk with daily folate intakes above 400µg. Intake at these levels (200-320µg/d) though below the RDA of 400µg/day, are often the median of intake in prospective studies on folate intake and BC risk. Similarly, a meta-analysis by Chen et al. in 2014 investigated the effects of folate on BC risk. After correcting for publication bias, statistical methods using models that assume linearity yielded different results than models that assumed non-linearity. In the 16 prospective studies, no linear association was found between DF and BC risk [pooled RR=1.04 (95% CI, 0.94,1.15)]. In contrast, women with DF intake between 153-400µg exhibited a reduced risk of BC compared to those whom consumed <153µg of folate daily, while women with average DF intakes above 400µg did not see this reduction in

risk. In the 25 case-control studies, a significant negative correlation was reported between BC risk and DF intake, though this result was not linear [pooled OR under linear model = 0.99 (95% CI, 0.84 -1.16)]. BC risk reduction was significant for women with higher DF intake compared with those with DF intake <130.5 μg per day, where a 100 μg per day increase of DF was associated with a 9% reduction in BC risk (OR = 0.91; 95% CI, 0.86–0.97)

Of consideration, results from the Chen et al. paper suggest higher folate intake is linearly associated with BC risk reduction in non-US populations, but not in American cohorts. This may be due to difference in folic acid fortification levels of the food supply; populations with relatively high folate status may confer a weaker protective effect. When stratified by menopausal status, DF was not associated with BC risk [highest vs. lowest category of intake, fixed- or random-effects model and 95% CIs, respectively: 1.05 (0.86–1.28), 1.02 (0.62–1.67)], though only 20 of the 42 included studies reported data on menopausal status.

A seminal prospective cohort study conducted in 1999 of more than 88,000 women (3483 cases of invasive BC) found total folate was not associated with an overall reduced risk of BC. Despite this, the excess risk of BC associated with 15g of alcohol intake per day was reduced by total folate intake. Folate intake above 600μg/d had a 7% decrease in risk compared to participants with intake between 150-299μg/d [multivariate relative risk (RR) of 0.55 (95% CI, 0.39-0.76; p for trend = .001)] This association was only slightly attenuated after additional adjustment for intake of beta carotene, lutein/zeaxanthin, preformed vitamin A, and total vitamins C and E. (S. Zhang et al., 1999)

There is limited evidence on hormone receptor-specific premenopausal BC risk and dietary folate intake. In a prospective study on a subset of the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (n=367,993 women), researchers found that higher DF intake may be associated with reduced risk of hormone-receptor-negative BC in premenopausal women. A statistically significant trend towards lower risk in estrogen receptor-negative BC (HR, highest vs. lowest = 0.66; 95% CI, 0.45-0.96, p for trend = .042) and progesterone receptor-negative BC (HR, highest vs. lowest = 0.70; 95% CI, 0.51-0.97, p for trend = .021) was exhibited. (Batlle et al., 2014)

In another cohort study with 3,016 overweight and obese women, a marginal inverse association between dietary folate intake and premenopausal status was found in African American women who consumed more than 230mcg/d of natural food folate but did not reach significance (highest vs. lowest quartile: OR=0.57, 95% CI, 0.33–1.00; *p* for trend = .06). After stratification by hormone receptor status, this association between premenopausal BC risk and folate status was further diminished. In their conclusion, the authors of this study suggested other B vitamins may modify the relationship between folate and premenopausal BC risk, as the lowest ORs were observed in women who consumed the highest levels of natural food folate as well as either B12 or methionine (OR = 0.60, 95% CI, 0.37–0.96 and OR = 0.42, 95% CI, 0.25–0.72, respectively). (Gong 2014)

Previous epidemiological studies investigating folate intake and their non-consensus among results may be due to differences in methods of folate status assessment (self-reported versus validated food frequency questionnaire) or due to the timing of measurement (pre- or post-BC diagnosis). Reported folate intake and relative status may differ; misclassification due to self-reported low intake may offer a causal explanation as

to why folate intakes at levels below the RDA are not associated with risk reduction. It is known that dietary assessments using self-reported intakes can exhibit systematic bias, especially with regard to energy underreporting in a female cohort. Therefore, it is suggested that self-reported intakes are used in conjunction with biomarkers for folate status such as serum or red blood cell (RBC) folate, especially when considering intakes at levels indicative of folate deficiency or excess. (Bailey et al., 2017)

It is important to note that higher folate levels, such as those reached with intakes of supplements or fortified foods, are more closely correlated with folate levels as assessed with self-reporting and validated with biomarkers. Systematic underreporting is prominent in epidemiological studies, which assess folate status by food frequency questionnaire or through 24-hour recall. This is of special concern with regard to studies investigating all-female cohorts, as women tend to systematically underreport energy intake more often than men. Caution is recommended when interpreting the relationship between health outcomes and lower folate intakes when measured by self-reported data. (Bailey et al., 2017) A further consideration: though self-reported dietary data in large epidemiological studies can be paired with biomarkers to increase the strength of observed folate status and health outcome associations, both serum and RBC folate are not true recovery biomarkers. Folate levels as assessed by these measures still cannot correct for the systematic bias of energy underreporting that is common to dietary assessments using FFQ.

Folate status and its role in BC etiology may also depend as heavily on timing of intake as well as relative intake levels, especially with regard to risk in premenopausal versus postmenopausal BC instance. Dietary intake pattern changes between pre- and post-diagnosis have been reported in up to 30-40% of participants in some epidemiological

studies on BC risk. (Salminen et al., 2000 and Maunsell et al., 2002) The authors suggested their findings support adequate folate intake pre-diagnosis, as opposed to supplementation with high doses post-diagnosis.

At present, study findings have suggested a U-shaped curve relationship between individual vitamin intakes and health risks, including folate. (Chen et al., 2014) Following a sharp observable increase in colorectal cancer after food fortification with B vitamins, early studies were held to investigate the relationship between increased folate intakes due to food fortification and various health outcomes. (Selhub & Rosenberg, 2016) The American Institute of Cancer Research (AICR) and World Cancer Research Foundation (WCRF) both advise meeting vitamin and mineral needs through dietary rather than supplemental sources, in order to keep intake below levels that may pose risk (WCRF and AICR, 2007).

Like studies using FFQs, results compared with circulating plasma or RBC folate levels as indicators of status are also disagreement with regard to folate's impact on BC. Serum and RBC folate are the two commonly used biomarkers to assess folate status. Where RBC folate levels correlate with tissue folate levels and are a more reliable measure of long term folate status, serum folate is subject to transient changes in dietary folate intake, and is therefore a poorer indicator of long term status. This lack of consensus with biomarker data may be due to fewer numbers of studies overall or due to differences in both lifestyle-related exposures (such as alcohol intake, a known folate disruptor, or smoking) and genetic predispositions in their cohorts. Further, few analyses on serum or RBC folate and BC have reported results with regard to both BMI and premenopausal status, specifically.

In a meta-analysis of twelve studies on BC risk and serum or RBC folate status by Lin et al. in 2008, no associations between folate biomarkers (serum or RBC) and BC risk reached statistical significance for ten of the studies, and only one study reported their findings with regard to menopausal status. (Lin et al., 2008).

In one of the studies that reached statistical significance in the Lin et al. meta-analysis, serum folate levels were assessed for carriers of the methyltetrahydrofolate reductase gene (MTHFR>C677T, wildtype or single nucleotide polymorphism) and compared to risk of BC instance. In the 141 BC patients and 109 age-matched controls, increased serum folate levels were significantly associated with a reduced risk of BC (quartiles, μg/L; p for trend = .001), but these results were not adjusted for menopausal status. (Beilby et al., 2004) In a later publication from this lab, using a sub-cohort within a larger prospective study in Caucasian West Australians, RBC folate but not serum folate was inversely associated with BC risk [n=569 women without cancer at first interview; adjusted HR per decrease of 100μg/L 1.96 (95% CI, 1.22, 3.12); p for trend < .01]. (Rossi et al., 2006). The trend for serum folate was similar but did not reach significance (p = 0.17). Participants in the lowest folate category (<200μg/L) had an adjusted HR of 6.46 (95% CI, 1.19, 35.07) for a subsequent cancer event. With a total of only 19 BC events in this sub-cohort, statistical power to assess the relation between folate levels and site-specific cancer instance was greatly diminished, compared to the original cohort, and caution should be exercised when interpreting the results of this study.

In the remaining study which reached significance within the Li et al. meta-analysis, a nested case control using the Women's Health Study cohort, plasma folate levels were positively associated with premenopausal BC but not postmenopausal BC [RR 1.42

(95% CI, 1.00, 2.02) for plasma folate, p for trend = .04 (adj. for BMI)]. (Lin et al., 2008) As described by authors of this study, additional research is needed for to explore these unexpected results.

In conclusion, though studies from the past five years may not show consistent associations between folate status and the risk of BC, different methods of folate status assessment, menopausal status, and obese state may be the significant contributors to this lack of consensus. In FFQ results, most studies do not describe a significant pattern. Results using biomarkers of folate status are mixed, as well. Going further, studies must account for menopausal status as well as bodyweight in order to delineate how these factors interact to influence the development of BC or BC-related mortality.

2.5 Folate and Methylation

Folate serves as both donor and acceptor of methyl groups in many one-carbon metabolism reactions, including DNA methylation. As a one-carbon donor deficiency will alter the rate of DNA methylation, fluctuations in folate levels disturb the maintenance of DNA methylation following replication. The deleterious effects of low folate on DNA methylation have been observed in numerous cancer cell models, though the effect and rage of impact of this depletion varies among cancer cell type. (Jin et al., 2009)

Indeed, *in vitro* studies have shown folic acid elicits changes in both the transcriptome and methylome of BC lines, though findings supporting the latter are varied. In an *in vitro* study by Price et al. using 1mg/d of folic acid supplementation, levels of folic acid in excess of normal recommended dietary intake levels (1mg/d versus the RDA of 400μg/d and 800μg/d for women of child-bearing age), on both normal and BC cell lines showed no variation between methylation of CpG sites on two breast-cancer related genes, BRCA1

and BRCA2. (Price, Lillycrop, & Burdge, 2015) In a more recent cell culture study by the same authors on both non-transformed and cancerous BC models, physiological concentrations of folic acid (100nm/l of folic acid) were shown to affect regulation of genes associated with apoptosis, senescence and cell proliferation. (Price 2016). Again, no affect was found on BRCA1 or BRCA2 DNA methylation by breast cell line in this study.

2.6 Obesity, Folate Status, Methylation and Cancer

Folate serves as a methyl donor for adequate DNA methylation and folate levels are sensitive to weight status. Epigenetic responses to folate status have been shown to be bodyweight dependent. DNA methylation patterns differ between metabolic states, and fluctuations throughout the lifespan of both folate status and BMI elicit modifiable changes in the epigenome. (Meeks et al., 2017)

Both folate levels and distribution can vary by bodyweight. In a study by Tinker et. al., BMI was inversely associated with serum folate concentrations. (Tinker et al., 2012) Using NHANES data, Bird et al. found that young overweight and obese women had lower serum folate and higher RBC folate levels when compared to either men or older women of the same weight categories. (Bird et al., 2015) Even after adjustment for intake, BMI has been shown to affect the distribution of folate from circulation into tissue. (da Silva et al., 2013) This redistribution may affect the availability of folate as a substrate for global and gene-specific methylation.

Though many of the exact mechanisms underlying these associations have yet to be discovered, DNA methylation patterns in premenopausal overweight or obese women with BC are often significantly different from postmenopausal women with BC. Whether

changes in DNA methylation in response to folate levels and bodyweight are deleterious or protective against risk may heavily depend on menopausal status or age.

Differences between serum and RBC folate concentrations as well as epigenetic responses to folate interventions provide evidence in support of the significant effect of menopausal status and cancer risk. Many of the existing studies on folate status, bodyweight and premenopausal women have focused on the effects of folic acid supplementation against baseline serum or RBC folate levels. Within women of child-bearing age, folic acid supplementation has been shown to elicit different effects on methylation levels of folate metabolism-associated genes, dependent on weight status. Response to folic acid supplementation has been shown to be lower in healthy obese compared to healthy normal weight premenopausal women. (Da Silva et al., 2013) In obese women both before and after intervention with supplementation, open seas CpG sites of cancer-related genes in obese women were more likely to have higher degrees of change in response to folic acid supplementation, despite overall lower levels of serum folate. (Yong-Moon et al., 2017)

Results from the few investigations on BMI, folate, methylation, menopausal status and differences in BC risk have been mixed. A possible explanation for these mixed results is that the relationship between these factors may be contingent on age, area of fat disposition, and metabolic activity of adipose. Localized inflammation often precedes the development of BC, and obesity-related low-grade inflammation increases with age. (Frasca, 2017) A state of subclinical inflammation has been documented in the breast adipose of healthy, postmenopausal women with normal BMI as opposed to premenopausal women. It is speculated that obesity-associated metabolic or inflammatory

effects, such as increased Cytochrome P450 expression, may alter serum folate oxidation products and degradation rates, leading to changes in DNA methylation. (De la Rocha et al., 2016) Thus, age may play an inflammation-associated role in weight and methylation-related BC risk

Changes in epigenomic patterns are tissue-specific. Consequently, the method of folate assessment may also play a role in the varying results from the few investigations on BMI, folate, methylation, menopausal status and differences in BC risk. DNA methylation is highly associated with BMI when measured in adipose tissue, as opposed to blood, though the methylation patterns of women with a high BMI are more similar to patterns measured in peripheral tissues such as blood. (T. Huang et al., 2016) Results from studies assessing folate status and DNA methylation by bodyweight may be affected by measurement assessments; serum, RBC or whole blood DNA methylation profiles may show lower correlation with bodyweight status versus tissue-specific measurements of folate and DNA methylation patterns.

CHAPTER 3

HYPOTHESIS AND SPECIFIC AIMS

Obesity has increased with an alarming rate in the United States, and a further increase, from the current 1/3 of the US population to ~50% by 2030, is projected. Mounting epidemiological evidence suggests that overweight and obesity is a robust risk factor of many types of cancers. However, the connection between overweight and obesity and breast cancer is complex – a well-established positive association exists between obesity and postmenopausal BC, whereas a reverse association holds prior to menopause. A significant challenge for understanding the cellular and molecular mechanisms of action by body fatness is the apparent enhancement of risk for postmenopausal BC but a protective impact for premenopausal BC. A great number of studies have been conducted to understand the positive association between obesity and postmenopausal BC, and several mechanisms governing this association have been proposed and defined, including adipose tissue-driving circulating hormones (*e.g.* IGF-1, estrogens) and obesity-associated chronic low-grade inflammatory state. On the contrast, mechanistic studies to understand the negative association between obesity and premenopausal BC is extremely limited, and the pathways that inform this inverse relationship remain entirely un-delineated.

A recent publication from our laboratory using the National Health and Nutrition Examination Survey (NHANES) dataset demonstrated that despite lower dietary intake, overweight and obesity is associated with increased levels of RBC folate. This association particularly holds to be significant for premenopausal women. Based on this surprising

observation, we conceived our **hypothesis**: obesity exerts its protective impact on premenopausal BC via altering folate metabolism and DNA methylation. To elucidate this potential mechanism, we propose the following specific aims:

Specific Aim #1: to determine to what extent obesity affects folate status in mammary tissue from cancer-free premenopausal women.

Specific Aim #2: to determine how obesity, via altering folate metabolism, influences mammary genome-wide and gene-specific methylation.

CHAPTER 4

METHODS

4.1 Participants

Histologically normal breast biopsy samples were collected from 51 premenopausal women who underwent elective mastectomies at Baystate Medical Center in Springfield, Massachusetts. Data on parity, age and BMI status was included. Participants signed an authorization for release of medical information upon donation of tissue samples. Samples were stored in -80°C until use.

4.2 Assays

4.2.1 *Lactobacillus casei* Microbiological Folate Assay

The *Lactobacillus casei* microbiological assay is considered the gold standard of folate assessment today due to its affordability, sensitivity and ability to detect various conjugate forms of folate that are representative of folates naturally occurring in biological samples. Folate is essential for growth in *L. casei (formally known as L. rhamnosus.* so as such, relative concentrations of folate from tissue or serum samples available for microbial uptake can be estimated by comparison to the amount uptake of a known concentration of folic acid. The limit of detection (LOD) for folate forms using the *L. casei* assay is very low (>50 picomoles), making it suitable to detect relatively small concentrations of total folate, such as concentrations from breast tissue biopsy samples.

4.2.2 Generation of the Folic Acid Standard and Standard Curve

A known concentration of folic acid (10 mg/mL; Sigma F-7876) is dissolved into doubly-distilled H_2O and titrated with 0.1M NaOH until visibly clear. Folic assay standard stock is stored in a dilution of 1:20 of methanol, aliquoted in microcentrifuge tubes and wrapped in foil to prevent light degradation. Standard stock is prepared once, stored in -80°C and used within one month.

L. *casei* suspension consists of 10 μL of cryoprotected L. *casei* (Difco Lactobacilli Broth AOCA, Cat. 290100) and 150 μL of growth medium. 150 μL of L. *casei* suspension and 150 μL of potassium phosphate buffer (KPO4) are added to all wells of a 96-plate. A concentration of 6 ng/mL folic acid is added to the bottommost row and serially diluted up the columns, leaving the topmost row blank (concentrations of folic acid: 6 ng/mL to 0 ng/mL). Folic acid standard suspensions are run in multiple replicates (octuplet) on a 96-well plate to control for intra-assay variation with three wells of 300 μL KPO4 buffer serving as internal control for inter-assay variation. All plates for the folic acid standard will be run on the same day.

The relative turbidity of each well is caused by the extent of microbial growth, and consequently, indicative of available folates. Turbidity of samples can be measured by optical density (absorbance) on a spectrophotometer at the wavelength of 630 nanometers (nm). Concentration (g/l) of folic acid is then calculated with the following formula: molarity determined by optical density divided by molecular extinction coefficient for folic acid (ε=9260). Molarity is then multiplied by molecular weight for folate (441.4 g/mol) to determine concentration (g/l). As L. *casei* growth exhibits saturation kinetics and is repressed by excess folate, the standard curve is generated by log-transforming the growth

response rates of folic acid to L. *casei.* The generated standard folate growth curve must be log-transformed to accurately compare the unknown relative folate concentrations in the breast tissue samples to the known concentrations.

4.2.3 Breast Tissue Extraction and Folate Assay

Breast tissue samples are quickly weighed while still frozen, added to extraction buffer and homogenized. Samples are then boiled for 20 minutes, treated with rat plasma conjugase and incubated for 2 hours. After folate extraction, breast tissue samples are stored at -80°C until L. *casei* microbial assay is performed.

All microbiological folic assay reagents are prepared fresh on the day of assessment with ascorbic acid added to further prevent oxidation and degradation due to storage and light exposure. On the day of microbial folate assay with breast tissue samples, one aliquot of folic acid standard stock is diluted for use in KPO4. A serial dilution of the folic acid standard at a concentration is then performed in the first 2 columns of a 96-well plate. 10 µL of cryoprotected L. *casei* (Difco Lactobacilli Broth AOCA, Cat. 290100) is suspended in freshly prepared L. *casei* media and added to each remaining well of a 96-well plate (L. *casei* suspension total volume, 150 µL). Each breast tissue folate extraction sample is run in triplicate to reduce intra-assay variation. All plates are ran within one week, with 3 wells of L. *casei* suspension, human plasma and KPO4 buffer serving as the internal control. The prepared 96-well plates are mylar sealed and incubated overnight in a dry incubator at 37°C (18h) to be read on the spectrometer on the following day.

Total folate concentration is determined for each sample by photospectometry at 630 nm. Mean tissue folate concentrations for each sample will be calculated as the

weighted average of measurements ran in triplicate using the coefficient of variation between triplicates. Mean tissue folate concentration is determined by comparison to the known concentration of serially diluted folic acid standard.

4.3 DNA Extraction and Gene Expression

DNA is extracted from DNEasy kit (Qiagen). DNA concentrations and absorbance (260/280nm ratio) is quantified by Nanodrop spectrophotometer. Nineteen BC-related genes with aberrant epigenetic modifications were identified in existing literature. RT-PCR amplification will be performed to compare relative gene expression between BMI groups. Validated PCR primers for each gene were selected by a scan of the existing literature. RT-PCR will be performed on 96-well plates with the ViiA 7^{TM} Real-Time PCR (Life Technologies) and analyzed with accompanying software (Expression Suite).

4.4 Pyrosequencing for DNA Methylation

The average amount of both loci-specific and global DNA methylation analysis is conducted via targeted Next Generation Bisulfite Sequencing (pyrosequencing) by EpigenDX (Hopkinton, MA); where LINE-1 and Alu elements serve as the surrogates for global DNA methylation. EpigenDX's pyrosequencing methods include multiple quality control checkpoints, including bias testing for PCR conditions such as annealing temperature (ensuring nonbiased selection between methylated vs. unmethylated CpG sites) and sensitivity analysis. After Pyrosequencing analysis and data compilation, delivered results include both quantitative Pyrosequencing values (percentage methylation

values of each CpG site, provided in Excel) and raw Pyrogram data (provided in Microsoft Word).

4.5 Statistical analysis

Data are expressed as means ± SEM, and statistical analysis was performed using SAS (Version 9.4, SAS Institute, Cary, NC). Because the population of women undergoing reduction mammoplasty typically had high BMIs, we combined the normal (BMIs between 18-24.9 kg/m^2) and the overweight (BMIs between 25-29.9 kg/m^2) women together and grouped subjects into two categories; non-obese group with a BMI < 30 kg/m^2 and obese group with BMI ≥ 30 kg/m^2. To compare differences between these 2 groups, a T-test was used, and also when a multiple comparison was performed, significance was accepted with p < 0.05 and a FDR cutoff of q < 0.25. For the association analysis, Pearson correlation analysis was performed for the association between BMI and mammary tissue folate, BC related gene expression and genome-wide DNA methylation and gene-specific methylation.

For gene expression, each gene was normalized to the housekeeping gene *GAPDH* (ΔCt = Ct$_{target\ gene}$-Ct$_{GAPDH}$). Statistical analyses were performed based on differences in ΔCt between non-obese individuals with BMI < 30kg/m^2 and obese individuals with BMI ≥ 30 kg/m^2 and relative expression is reported as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta$Ct=ΔCt$_{BMI≥30}$-ΔCt$_{BMI<30}$.

CHAPTER 5

RESULTS

5.1 Participants

Anthropometric characteristics of study participants are shown in Table 3. Participants were characterized into BMI classes, Normal Weight, Overweight and Obesity based on the principal cut-off points established by the World Health Organization standards for BMI categorization (WHO). Mean BMI for the <30 kg/m^2 group was 26 kg/m^2 (n=27). Mean BMI for the >30 kg/m^2 group was 35 kg/m2 (n=23). There were no significant differences among the different BMI categories with regard to parity or age (p > 0.05). When compared to the Normal Weight group, age was marginally higher (p = 0.092). No others significant differences were measured.

### Table 3. Anthropometric Characteristics of subjects.

Categories	Parous		BMI (kg/m^2)*		Age (year)*	
	n		Mean	Range	Mean	Range
Normal Weight	8	4/8[a]	23 ± 0.61[a]	19 - 24	27 ± 3.01[a]	19 - 36
Overweight	19	11/19[a]	28 ± 0.32[b]	25 - 29	29 ± 1.90[a]	19 - 44
Obesity	23	14/23[a]	35 ± 0.75[c]	30 - 44	32 ± 1.30[a]	21 - 41
<30	27	15/27[a]	26 ± 0.50[a]	19 - 29	28 ± 1.59[a]	19 - 44
≥30	23	14/23[a]	35 ± 0.75[b]	30 - 44	32 ± 1.30[a]	21 - 41

*Different letters denote significant differences among the BMI categories (p < 0.001). There are no significant differences of numbers of parous women and age among the different BMI categories (p > 0.05), with a marginal higher age for the Obesity group when compared to the Normal Weight group (p = 0.092).

5.2 Obesity and Mammary Tissue Folate Status

The folic acid concentration standard curve was generated using a known concentration of folic acid. An example of plate layout is included in Figure 1. Tissue folate samples were

run in triplicate, KmNO4 buffer was used as an internal control and human plasma as a control for inter-assay variability.
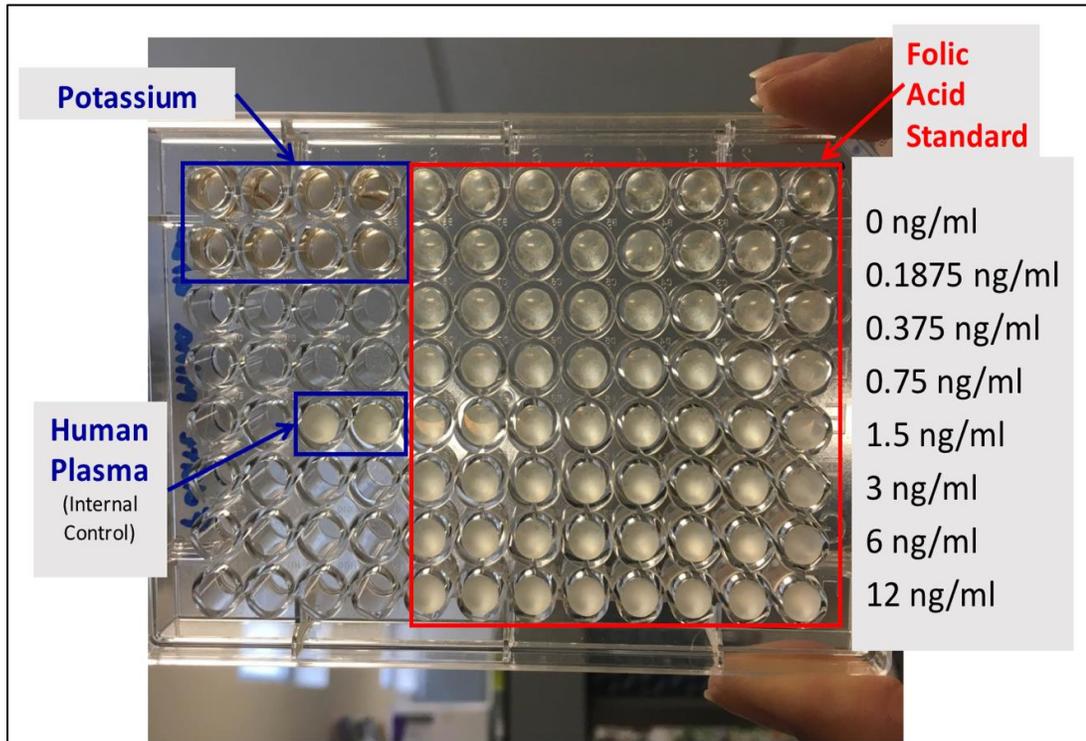
Figure 1. Lactobacillus casei folic acid assay standard plate layout

Mean tissue-specific folate for all groups was 17.042 kg/m^2. Mean tissue-specific folate level is significantly higher in obese versus non-obese women (<30 BMI 15.31, >30 BMI 19.59, p = 0.0439). (Figure 2) Breast tissue folate level in obese women (19.59 ng/g) is 28% higher (p < 0.05) when compared to folate status in non-obese women (15.31 ng/g). No statistically significant difference was found between normal and overweight BMI and tissue folate.
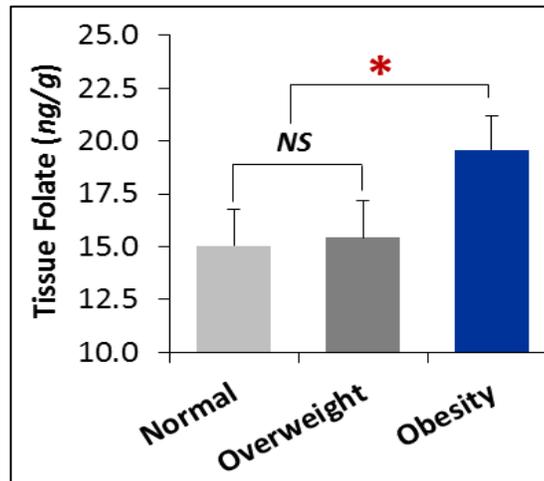
Figure 2. Association between tissue folate and BMI.
* denotes statistical significance (p <0.05).

A significant positive correlation (p < 0.01) was observed between breast tissue folate status and BMI (Figure 3). A high BMI is associated with increased folate levels in mammary tissue, with an increase of 2.65 ng/g of folate per every 5-unit increase of BMI (p-value for slope = 0.0064*).*
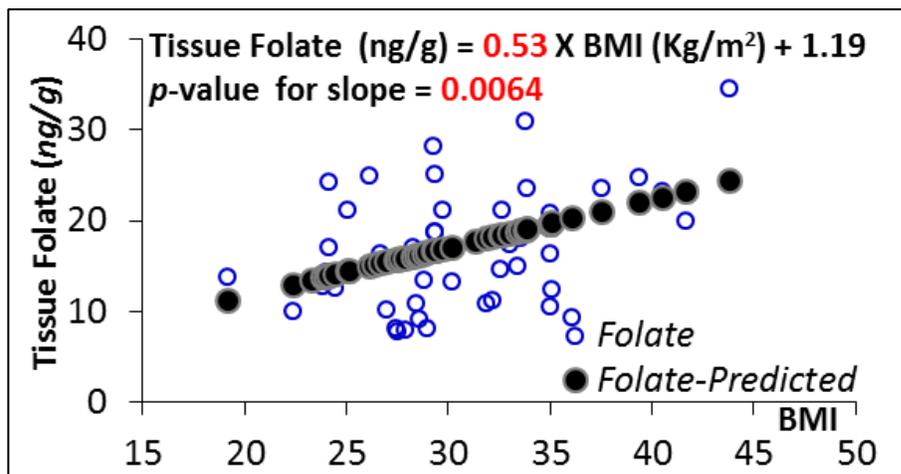
Figure 3. Linear association between BMI and tissue folate.

5.3 Global DNA Methylation

LINE-1 methylation was measured as a surrogate of global DNA methylation. LINE-1 methylation is significantly associated with BMI ($p < 0.05$). (Figure 5) The normal weight group was not significantly different from overweight or obese, and overweight and obese were not statistically different from each other. For global methylation as measured by 4 CpG sites along LINE-1 elements, BMI both overweight and obese groups showed higher percent LINE-1 methylation than the obese group ($p < 0.05$). The difference between overweight and obese was not significant. (Figure 4) LINE-1 methylation is also marginally associated with mammary tissue folate levels, but this association did not reach significance ($p = 0.087$, data not included). LINE-1 Methylation and BMI share a positive linear association. A 0.014% increase in LINE-1 methylation per unit of BMI was observed (p for trend $= .034$). (Figure 5)
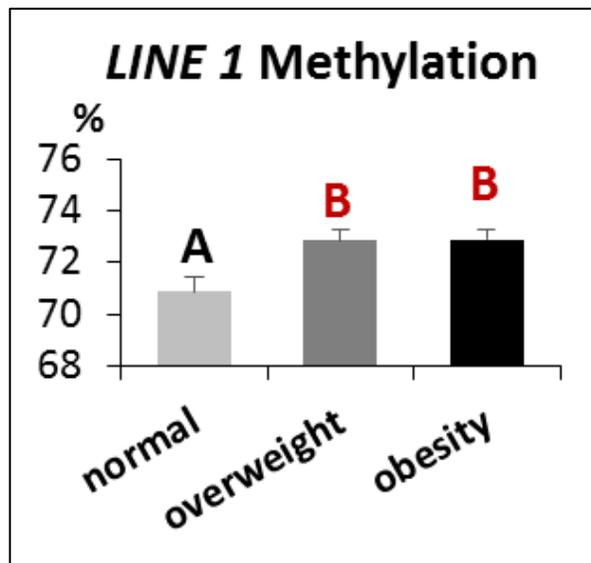
Figure 4. BMI status and percent LINE-1 methylation. Letters denote statistical significance between BMI categories ($p < 0.05$).
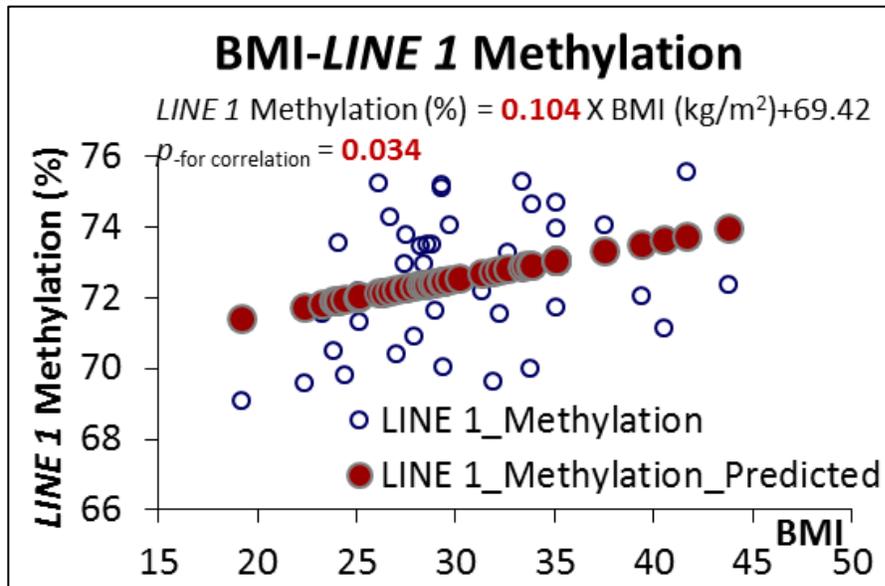
Figure 5. Linear correlation between BMI and percent LINE-1 methylation.

5.4 Gene Expression

Out of the eighteen genes selected from the literature for analysis via RT-PCR, only two provided significant results. Relative expression levels of SFRP1 correlated with BMI status. The SFRP1 gene codes for WNT pathway antagonist protein with tumor suppressor function. SFRP1 is downregulated in 46% of breast cancers. SFRP1 gene expression levels positively correlated with both BMI (p = 0.0105) and tissue folate (p = 0.0313). (Figure 6) BMI and SFRP1 gene expression also shared a positive linear correlation, with an increase of 0.112 relative fold for each unit of BMI (p for trend = 0.023, Figure 7).
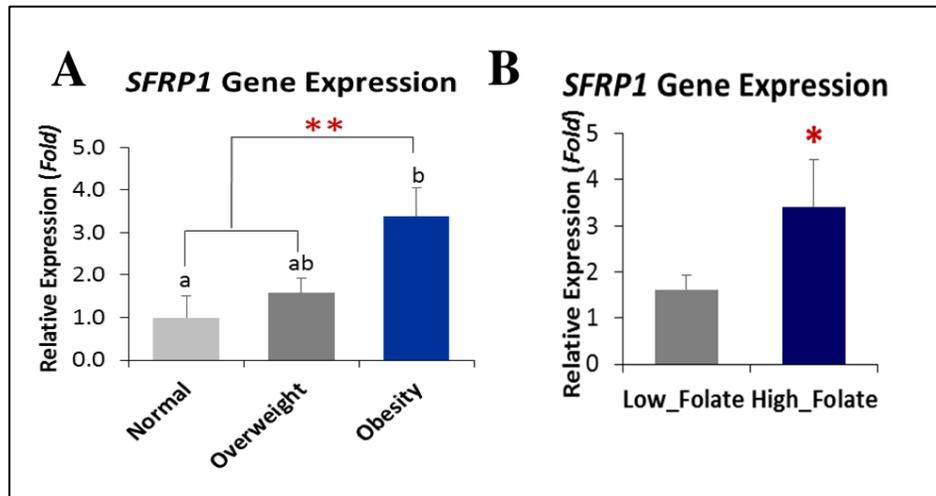
Figure 6. Relative gene expression of SFRP1. * denotes statistical significance (p < 0.01), ** denotes statistical significance (p < 0.001).
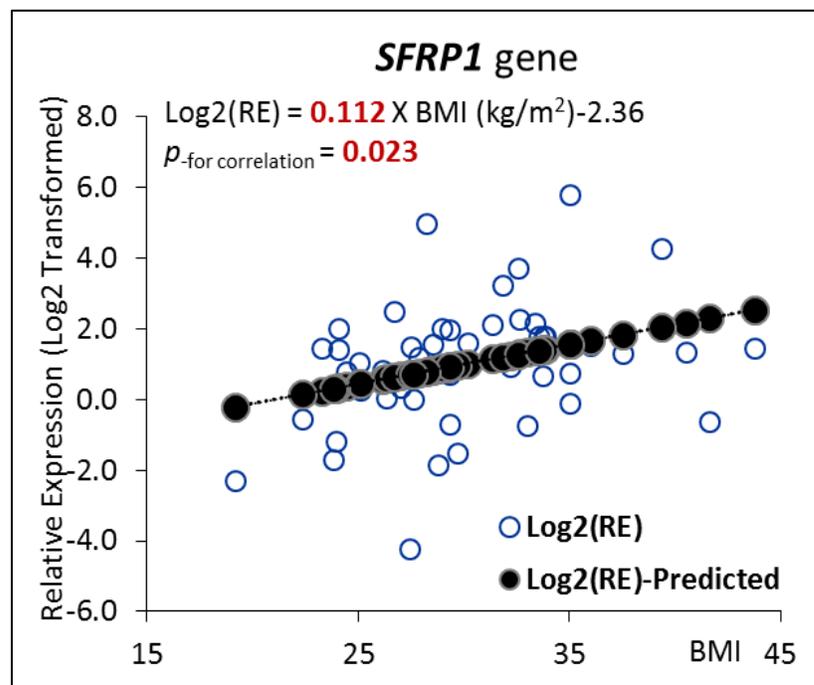
Figure 7. BMI and relative gene expression of SFRP1. SFRP1 gene expression levels positively correlated with BMI (P for correlation = 0.023).

5.5 Gene-Specific DNA Methylation

SFRP1 was selected for promotor CpG site methylation analysis based on its significant correlations with regard to gene expression, BMI, and folate status. For the 8 CpG sites analyzed in the promoter region of the *SFRP1* gene, no associations were observed for both BMI and tissue folate ($p > 0.05$), although a high expression was observed in the subjects with high BMI or high tissue folate ($p < 0.05$).

CHAPTER 6

DISCUSSION

6.1 Strengths and Limitations

One strength of our study is the reliability of our results, due to the laboratory assays we elected to employ. We assessed global and gene-specific methylation levels using tissue samples from the breast, rather than whole blood. As our study was concerned with the localized environment of the healthy breast, and DNA methylation levels are known to vary by originating tissue, assessing the degree of methylation in mammary as opposed to peripheral tissue may better reflect the status of the healthy breast microenvironment. Further, our samples contained a heterogeneous selection of cell types, in that they contained adipose, epithelial and structural tissue. DNA methylation of adipose tissue is highly correlated with BMI, especially in individuals with a high BMI. (Huang 2016) With regard to folate status, biologically relevant tissue folate levels typically fall well above the limit of detection (LoD) for the *L. casei* assay. Biological concentrations of folate are generally in the nanograms per liter range, as opposed to nanomoles per liter (the assay's LoD). As such, our findings may accurately denote biological relevance despite the f fact that the quantification of tissue folate levels were relative to our folic acid standard rather than absolute values. The lowest measured point on our folic assay standard calibration curve was greater than 6 nmol/L, well above the limit of detection for the *L. casei* assay.

Additionally, both our standard curve for folate growth and calculated results for sample levels agreed with values previously reported by other laboratories. As the assay is very sensitive, measures were taken to reduce variability. Two internal controls were used,

human plasma and KPO4 buffer, and all reagents for the assay plates were generated on the same day by the same researcher (or used within less than one week). All plates were generated and analyzed by the same researcher, which reduced the likelihood of variability due to inconsistencies between technicians. In conclusion, not only are our *L. casei* assay results in agreement with typical concentrations in the previous literature, our study design may more accurately reflect the molecular and epigenetic heterogeneity that is present within the human breast

Another strength is the potentiality for method agreement between this investigation and large epidemiological studies. Despite the invention of protein-based assays to detect folate concentrations, the *L. casei* microbiological assay is still the most highly recommended assay to determine folate concentrations in biological samples. Thus, it is widely used in prospective cohorts. For example, NHANES is one of the largest and most detailed cohort studies in the United States. NHANES collects data on a wide range of variables beyond biospecimens related to micronutrient status, including lifestyle habits, environmental exposures, occupation, reproductive health. Many of such variables have been previously shown to be associated with BC risk. Though NHANES methodology briefly switched to protein-based assays for folate status assessment in the 2000s, it reverted back to the *L. casei* assay in subsequent years. Since we employed the same method of folate assessment, our results may be more readily compared against their outcomes.

Our study has several limitations. Tissue samples were donated from local women who underwent elective mammoplasty, and the accompanying data concerning the subjects' characteristics was limited to BMI category, menopausal status, age and parity.

Beyond these parameters and the classification as "healthy" women, we were unable to distinguish whether there were any pre-existing conditions that would influence their decisions to receive a breast reduction or speak to their underlying metabolic health. There are many factors beyond aesthetics that may influence a woman's decision to have breast reduction surgery, and these factors are not limited to; relieving lower back pain or stasis ulcers; improving sports performance or ability to breathe; augmentation as part of gender reassignment surgery, or many other various psychological or social reasons. Information as to the women's' personal motivations for the elective procedure may not be directly related to breast cancer risk, but data on these determinants was not provided. As our study is concerned with premenopausal breast cancer risk, any additional factors that would directly influence breast cancer risk such as breast density cannot be controlled for, and therefore may contribute to bias. Hence, there may be significant differences between our cohort of cancer-free women and the general population. Due to these concerns, we cannot eliminate the possibility that these tissue samples may have been from women who are at an increased risk of breast cancer, which would skew our results.

In addition, BMI is not an accurate indicator of fat deposition or metabolic activity. Some overweight and obese individuals exhibit "metabolically healthy" profiles, despite presenting with higher general adiposity. Without the accompaniment of biomarkers for metabolic function, no assumptions could be made as to the participants' overall health status. The lack of data pertaining to body fat distribution further limits our ability to conjecture on the relationship between the more nuanced aspects of bodyweight, folate, epigenetic status and premenopausal BC risk.

Another limitation of our study is related to our gene selection process. Gene selection for our assay was based on a scan of the existing literature. Though the field of epigenetics is nearing its hundredth birthday, investigations into the methylation patterns of specific cancer types are still quite young. Consequently, few systematic reviews on the epigenetic profiles of premenopausal breast cancer exist. The frequency and strength of support for evidence on the differential methylation of a particular epiobesigenic gene, outside of the most classically investigated such as BRCA1 and BRCA2 or RASSF1A, is limited. Many types of genes are strongly implicated with differential methylation levels by bodyweight, or folate status, but not for both factors as well as menopausal-specific BC. Going further, a more robust and objective screening method for gene inclusion should be utilized, strengthening the likelihood of finding a meaningful association between epigenetic signature and status.

CHAPTER 7

CONCLUSIONS

7.1 Implications

Because of the associated risks with increased bodyweight, such as cardiovascular

disease, stroke, type 2 diabetes mellitus and other metabolic perturbations, our current

level of understanding cannot support the recommendation of overweight or obesity for

premenopausal breast cancer risk reduction. Though our study is the first to describe a

linear relationship between bodyweight as measured by BMI, mammary tissue folate

status and increased global methylation, investigations into other candidate markers and

cellular processes are first needed to delineate this causal pathway. For example,

aberrations in reactive oxidative species production, dysfunctional glucose or lipid

metabolism, and immune cell function are common in the pro-inflammatory state of

obesity. Many critical components of normal one carbon metabolism and DNA

epigenetic modification are inexorably connected to normal cell functioning, and are

thus, affected by the overweight or obese state. As such, several lines of inquiry remain

open for investigation with regard to premenopausal breast cancer risk and its

relationship to bodyweight and tissue folate status.

7.2 Future Research Recommendations

The body of peer-reviewed literature on this subject is, at present, insufficient.

Though a fully powered, randomized control trial on the effects of these factors would

provide adequate evidence to support new recommendations for premenopausal BC risk

reduction related to increased bodyweight, further epidemiological studies are first

warranted. As stated above, our study uses some of the most widely accepted laboratory techniques in biomedical nutrition research, but our sample size is quite limited. Conducting a similar line of inquiry on a larger, more representative cohort would significantly improve generalizability. Equally important, the inclusion of data on other factors such as personal reasoning for the elective mammoplasty, would improve statistical power by reducing the potential for confounding. The addition of the descriptive data that is included in larger cohort studies, such as information on lifestyle factors (physical activity levels, dietary patterns, socioeconomic status) as well as more detailed analyses of body fat disposition would shed additional light onto this relationship. Controlling for several other variables would be necessary to provide the basis for a clinical trial, and in turn, adequate evidence to support tailored recommendations with regard to bodyweight status.

7.3 Conclusions

In conclusion, this study demonstrated that, in the normal mammary tissue from premenopausal women, obesity is associated with increased folate status, genome-wide DNA methylation and SFRP1 gene expression. These findings support our initial hypothesis and provide evidence toward weight-related folate metabolism and epigenetic changes as one of the driving factors behind the protective effect of obesity on premenopausal breast cancer. Whether there are optimal circumstances or periods for potential dietary or lifestyle interventions to promote the protective effects of overweight and obesity on premenopausal breast cancer remain to be illuminated.

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