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# Epigenetic Biomarkers of Breast Cancer Risk: Across the Breast Cancer Prevention Continuum

**Mary Beth Terry**,
Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, NY, USA. Herbert Irving Comprehensive Cancer Center, Columbia University, New York, NY, USA

**Jasmine A. McDonald**,
Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, NY, USA

**Hui Chen Wu**,
Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY, USA

**Sybil Eng**, and
Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, NY, USA

**Regina M. Santella**
Herbert Irving Comprehensive Cancer Center, Columbia University, New York, NY, USA. Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY, USA

## Abstract

Epigenetic biomarkers, such as DNA methylation, can increase cancer risk through altering gene expression. The Cancer Genome Atlas (TCGA) Network has demonstrated breast cancer-specific DNA methylation signatures. DNA methylation signatures measured at the time of diagnosis may prove important for treatment options and in predicting disease-free and overall survival (tertiary prevention). DNA methylation measurement in cell free DNA may also be useful in improving early detection by measuring tumor DNA released into the blood (secondary prevention). Most evidence evaluating the use of DNA methylation markers in tertiary and secondary prevention efforts for breast cancer comes from studies that are cross-sectional or retrospective with limited corresponding epidemiologic data, raising concerns about temporality. Few prospective studies exist that are large enough to address whether DNA methylation markers add to the prediction of tertiary and secondary outcomes over and beyond standard clinical measures. Determining the role of epigenetic biomarkers in primary prevention can help in identifying modifiable pathways for targeting interventions and reducing disease incidence. The potential is great for DNA methylation markers to improve cancer outcomes across the prevention continuum. Large, prospective epidemiological studies will provide essential evidence of the overall utility of adding these markers to primary prevention efforts, screening, and clinical care.

Correspondence to: Mary Beth Terry.

**Keywords**

Biomarker; Breast cancer; DNA methylation; Plasma; Prevention; Prognosis; Recurrence; Serum; Survival; Breast tissues

## Introduction

Breast cancer mortality rates have steadily decreased since 1990; however, breast cancer remains the second leading cause of cancer deaths in women in the United States [1]. Breast cancer is the most common cancer in women in the United States, and the incidence is increasing dramatically in very young women under age 40 years [2]. Women at higher risk of breast cancer due to family history and/or specific genetic alternations have an earlier age of onset than women at average risk and screening mammography is less sensitive in younger women [3]. Early detection of breast cancer increases treatment options, including surgical resection and therapeutic interventions [4]. Thus, finding markers that can help detect cancer early, particularly in younger women, that complement and/or improve existing methods will help in reducing incidence and mortality from breast cancer.

Biomarkers can be a useful tool for monitoring disease risk and prognosis. For example, in cardiovascular disease, blood pressure and blood markers such as lipid levels are measured routinely throughout adulthood. These markers prove particularly useful when combined with other cardiovascular disease risk factors in predicting risk through models that can readily be employed in the community and clinic. Breast cancer risk assessment models provide estimates of the absolute risk of breast cancer within a fixed time horizon (e.g., 5 or 10 years) or for the remaining lifetime of a woman. For example, women with a 5-year risk of 1.67 % or higher are classified as “high-risk” and are eligible for taking tamoxifen or raloxifene to reduce breast cancer risk based on the FDA guidelines. The Gail model is the most frequently used risk prediction tool in United States clinics; however, the model is not recommended for high-risk women such as those with a strong family history of breast cancer [5, 6]. Breast cancer risk assessment methods, just like cardiovascular disease models, may benefit from the addition of biomarker and intermediate marker information. However, at present, there are no existing validated plasma/serum biomarkers for breast cancer. Only a few biomarkers (such as estrogen receptor) have utility for diagnosis and prognosis (reviewed in [7]). Thus, there is a great need for sensitive biomarkers to detect early neoplastic changes and to facilitate the detection of breast cancer at an early treatable stage.

Epigenetic modifications (e.g., DNA methylation) refer to heritable and modifiable markers that regulate gene expression without changing the underlying DNA sequence. DNA methylation may play an important role in tumorigenesis by silencing tumor suppressor genes [8–12]. Emerging evidence suggests that aberrant DNA methylation can begin very early in breast tumor progression [13] and can be detected in body fluids [14]. Similarities between methylation patterns found in primary tumor specimens and in blood plasma indicate the potential utility of blood-based molecular detection of breast cancer [15–18]. Emerging evidence has shown that DNA methylation of select genes measured in plasma

results in sensitivities > 90 % for detecting breast cancer [15, 19]. These results suggest that DNA methylation has promise for screening. As we review, however, the evidence base is far from complete with many small studies and of a cross-sectional design that limit any inferences about temporality. Where there are gaps, we suggest study designs and the types of evidence that may prove useful in addressing these gaps.

Breast cancer is a heterogeneous disease with very different therapeutic responses and outcomes. Gene expression profiles have been used for breast cancer classification and have served as prognostic and therapeutic predictors. However, there are still major challenges in accurate early prediction of breast cancer incidence, detection and prognosis. Given that DNA methylation changes are plausibly critical components of the molecular mechanisms involved in breast cancer, distinct DNA methylation profiles may help improve the accuracy of prediction of incidence, detection and prognosis. The number of genes identified as being aberrantly methylated in breast cancer is rapidly growing (reviewed in [20]). These genes encompass multiple pathways leading to malignancy, including the six alterations proposed by Hanahan and Weinberg required to transform a healthy cell into a cancer cell: unlimited replication potential, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, sustained angiogenesis, and tissue invasion and metastasis [21].

In this chapter, we review the methods used to assay DNA methylation in human studies and the evidence to date from clinical and epidemiological studies on DNA methylation and breast cancer. We focus our review on describing the most common measurement techniques used to ascertain DNA methylation in human studies and then evaluate the evidence base for DNA methylation to enhance tertiary prevention (reduction of morbidity after diagnosis and improving overall survival), secondary prevention through early detection of disease, and primary prevention as a risk marker to reduce overall breast cancer incidence.

## DNA Methylation, Definitions and Measurement Methods

Epigenetics is defined as changes in gene expression in the absence of changes in DNA sequence. Levels of DNA methylation, histone modifications and microRNA expression are the three main epigenetic drivers of altered gene expression. As the evidence base is largest for DNA methylation biomarkers, here, we concentrate on studies of DNA methylation, specifically 5-methylcytosine (5mC), which results from the addition of a methyl group to the 5′ position of cytosine primarily in CpG sequences. DNA methylation is essential in development and cell differentiation, silencing of transposable elements, genomic imprinting and X-chromosome inactivation. In cancer, it is well established that tumors have lower levels of total 5mC than adjacent tissues (reviewed in [22]). This hypomethylation is primarily in repetitive elements which make up the majority of our DNA and leads to their reactivation, increased illegitimate recombination, and genomic instability. This loss of methylation is an early event in carcinogenesis. Gene-specific hypomethylation can also occur and results in the re-expression of affected genes. Gene-specific hypermethylation, particularly in CpG island promoters, is the more common and well-studied event and is associated with gene inactivation. Thus, we now know that inactivation of tumor suppressor genes is not only the result of mutation but also of DNA methylation. In breast cancer, as

discussed below, a large number of genes have been identified as having hypermethylated CpG island promoters and include those involved in DNA repair, cell-cycle regulation, apoptosis, chromatin remodeling, cell signaling, transcription and tumor cell invasion.

In addition to 5mC, which is present at levels of about 4 % of the cytosines, 5-hydroxymethylcytosine (5hmC) is present but at much lower levels. This is the result of Tet enzyme oxidation of 5mC [23]. This family of enzymes can further oxidize 5hmC to 5-formylcytosine and 5-carboxylcytosine, both of which are substrates for thymidine–DNA glycosylase, a DNA repair enzyme. This pathway of oxidation and base removal and repair is believed to be a mechanism for removal of the methyl group from cytosine.

A large number of methods have been developed for analysis of DNA methylation including evaluation of total 5mC; levels of methylation in repetitive elements that are a large fraction of the human genome as an indirect measure of global methylation; and levels in specific genes, primarily in CpG-rich promoter regions, but also in gene bodies and regions more distant from genes. While a large number of methods have been developed for the analysis of DNA methylation (reviewed in [24–28]), a much more limited range of assays has been applied to human health studies. These methods as well as their strengths and limitations are given in Table 1. Early studies digested DNA to nucleosides and analyzed levels of 5-methyldeoxycytidine (5mdC) by high performance liquid chromatography (HPLC) or used antibodies to bind to 5mC to obtain qualitative data. More recently, liquid chromatography-mass spectrometry (LC/MS) that allows the use of an internal standard for highly accurate and sensitive quantitation has been used [29]. This has also facilitated the quantitation of 5hmdC; however, this method as well as HPLC generally requires 1μg of DNA [30]. Another method takes advantage of the ability of the *Sss*I prokaryotic methylase enzyme to indiscriminately methylate all unmethylated CpG sequences using [$^{3}$H]*S*-adenosylmethionine as the methyl group donor [31]. Therefore, the ability of DNA to incorporate [$^{3}$H] methyl groups *in vitro* is inversely related to endogenous DNA methylation. Another method that looks at general levels of DNA methylation is the luminometric methylation assay (LUMA) which specifically analyzes 5mC in C$^{m}$CGG regions. It takes advantage of a pair of isoschizomer restriction enzymes that cut DNA differentially based on methylation status. Sequencing of the product allows determination of methylation but only in CCGG sequences [32].

A major advance in analysis of DNA methylation resulted from the demonstration that treatment of DNA with sodium bisulfite resulted in deamination (the removal of an amine group) of unmethylated cytosines converting them to uracil while leaving 5mC intact. Since uracil pairs with adenine, polymerase chain reaction (PCR) primers can be designed with either an A or a G opposite the position of the C in CpG sequences. Cs in non CpG sequences, since generally not methylated, will be converted to U and an A will be used in the PCR primer. Upon PCR, the U is amplified as a T. Thus, Cs in unmethylated CpG sites are converted to Ts while methylated CpG sites remain as Cs. In methylation specific PCR (MSP), two sets of primers are designed specifically for the modified DNA strand encompassing several CpG sites, one assumes a C and the other a T in Cs in CpG sites. PCR is then followed by gel analysis for qualitative determination of whether methylated and/or unmethylated DNA is present [33].

This basic bisulfite treatment methodology has also been applied to real time fluorescence PCR eliminating the need to run gels, as well as to microarray analysis, sequencing and other types of assays. There are a number of variations of the real time assays, some using a combination of methylated and unmethylated primers with cyber green for quantification of amplified DNA and others using a control gene [21–23]. A specific variation of real time PCR, the MethyLight assay uses Taq-Man probes for quantification [34]. The fluorescence-based PCR assays are much more sensitive than MSP, but also allow high throughput since they can be run on 96- or 384-well plates. All the PCR methods that use methylation specific primers/probes detect only those DNA strands that are fully methylated for the CpG sites that are interrogated by the primers or probe; they cannot discriminate between 5mdC and 5hmdC. While small quantities of DNA are required for each PCR reaction, bisulfite modification is generally carried out on a minimum of 250 ng of DNA. All bisulfite-based assays also are dependent on the complete conversion of C to T for accurate data. In addition, differential PCR efficiency with methylated and non-methylated primers can impact results.

Bisulfite sequencing has been used extensively in epidemiologic studies for analysis of methylation. For both analysis of specific genes as well as repetitive elements (e.g., LINE-1, Alu), pyrosequencing has been the method of choice due to its relatively low cost [35]. In contrast to real time PCR, primers do not contain CpGs so that both methylated and unmethylated DNA will be amplified. The sequencing probe that sits adjacent to the region of interest also does not contain CpG sites. Synthesis of the DNA strand from the 5′ to 3′ direction is carried out one base at a time by incubation with the appropriate triphosphate (dNTP) based on known DNA sequence. Each incorporation event is accompanied by the release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide. ATP sulfurylase coverts the PPi to ATP in the presence of adenosine 5′phosphosulfate and this ATP drives an enzymatic reaction that generates light. When sequencing through positions that might contain either a C or a T, both G and A dNTPs are sequentially added, which allows calculation of average level of methylation of each CpG site in the region sequenced, which is generally < 300 base pairs in length. Allele-specific methylation data, or methylation along a single strand of DNA, can only be obtained if PCR products are cloned prior to sequencing, but this is not feasible in epidemiologic studies. Pyrosequencing is also not accurate at very low or high levels of methylation. The sensitivity limitation for pyrosequencing is ~ 5 %. Next generation bisulfite sequencing is the most comprehensive method of analysis as it allows determination of methylation of multiple regions at the same time or even across the genome. Different platforms utilize different technologies, but all provide large amounts of data even with relatively small amounts of DNA. However, there are cost limits in the utilization of these platforms in epidemiological studies.

Bisulfite treated DNA has also been analyzed using Illumina Infinium Human-Methylation BeadChips that evaluate methylation of > 27,000 or > 450,000 CpGs. Two types of chemistries are used on the 450 K chips that lead to some differences in data, but both provide beta values or percent of methylation at each site. The low cost per data point and ease of data interpretation have made these arrays commonly used in epidemiologic studies. While results are frequently referred to as genome-wide analysis data, they are limited to the

specific CpG sites on the chip. However, the 450 K array covers 99 % of RefSeq genes, with an average of 17 CpG sites per gene region distributed across the promoter, 5′UTR, first exon, gene body, and the 3′UTR [36]. The 450 K array covers 96 % of CpG islands, with additional coverage in island shores and the regions flanking them. One challenge with methylation studies is knowing which region of the DNA to analyze [37]. Most gene-specific methylation studies evaluate promoter regions upstream and downstream of transcription start sites. While these regions are clearly important, we now know that that intragenic CpG sites as well as CpG shores may also be important (reviewed in [38, 39]). While levels of gene expression are often of primary interest, the relation between methylation levels and gene expression is rarely evaluated.

DNA methylation is dynamically changing over the lifecourse, but most studies only have samples from one time point. Here, we describe how DNA methylation markers may be useful in improving prognosis and overall survival (tertiary prevention), early detection (secondary prevention) and primary prevention. The importance of DNA methylation markers across all stages of the prevention continuum is strengthened by the recent data from The Cancer Genome Atlas (TCGA) on DNA methylation of over 800 breast tumors using Illumina Infinium HumanMethylation BeadChips. The data have dramatically expanded the number of genes identified as aberrantly methylated in breast cancer [40]. Knowing whether these aberrantly methylated genes in the tumor tissue are influenced by modifiable factors across the lifecourse, and/or affect early detection and tumor growth, and/or response to treatment and overall survival will have major implications for primary, secondary, and tertiary prevention efforts. In TCGA, unsupervised clustering analysis of the methylation array data identified five distinct DNA tumor groups. Group 3 showed a hypermethylation phenotype, was significantly enriched in the *luminal-B* mRNA subtype, and was under-represented for *PIK3CA, MAP3K1* and *MAP2K4* mutations. Group 5 showed the lowest levels of DNA methylation, overlapped with the basal-like mRNA subtype, and had a high frequency of *TP53* mutations. Other studies examining the associations between whole-genome DNA methylation and breast cancer classification found that there were distinct methylation patterns by hormone receptor status [41, 42] and by *BRCA* mutation state [43]. Methylation profiling was also shown to reflect the cell type composition of the tumor microenvironment, specifically T lymphocyte infiltration [44]. In addition, methylation patterns in selected genes were significantly associated with disease progression [41, 42] and survival [45]. Thus, DNA methylation markers by enhancing molecular characterization of breast tumors show potential utility in population health prevention and screening and clinical care. Here we review the evidence to evaluate its potential across the cancer prevention continuum starting with improving outcomes after diagnosis and ending with primary prevention.

## DNA Methylation Markers and Tertiary Prevention and Role in Prognosis

Extensive data examining DNA methylation in tissue samples at the time of diagnosis exist, however, there are far fewer studies that have prospectively followed breast cancer cases to examine how DNA methylation patterns at the time of diagnosis relate to overall survival and prognosis after breast cancer diagnosis. For example, although there have been several thousand studies that report DNA methylation and breast cancer, when we used the

following search strategy in MEDLINE from the earliest available publication to September 2014 (the following search terms included forms of methylation + breast cancer + prognosis or recurrence or survival + serum or plasma in varied combinations) using two separate and independent reviewers, we only found 82 studies of DNA methylation in tissue or plasma at the time of diagnosis that examine DNA methylation and prognosis. Of these 82 studies, we reviewed the subset that specifically followed up patients longitudinally to evaluate whether DNA methylation markers are related to overall prognosis and mortality and that met the following additional criteria: (1) reported on either disease-free survival (DFS) or overall survival (OS) using survival regression methods and (2) had at least 30 events of either relapse or death (Table 2). We used these criteria because we specifically wanted to focus on whether DNA methylation markers predicted DFS or OS, over and beyond the standard clinical prognostic markers. As evidenced by TCGA, many DNA methylation markers map to subtypes of tumors [40]. For clinical utility, it is necessary to know whether new markers add to the prediction of DFS and OS after considering standard clinical metrics like stage, grade, tumor size, and nodal status. To do so, multivariable regression models are needed; such models require large sample sizes to yield precise estimates. For example, in one study that we did not include in Table 2 because it did not meet the criterion for the number of events, the overall unadjusted association of methylation in the *NEU-ROD1* gene with relapse free survival was 0.8 (relative risk (RR) = 0.8, 95 % confidence interval (CI) = 0.3–1.8) but the adjusted association was over six-fold (relative risk (RR) = 6.2, 95 %CI = 1.6–24) after adjusting for tumor size, grade, lymph node metastases, and menopausal status [46]. There were only 10 events in the group with low methylation in *NEUROD1* and 11 events in the group with high methylation [46]. Thus, with so few events, large associations in multivariable models may result from model over-fitting.

As reviewed in Table 2, only 17 studies of 82 studies on DNA methylation met the criteria that were large enough to adequately address the relation between tissue-based DNA methylation markers and DFS or OS have identified a number of markers that are independently related to outcomes after diagnosis. For example, the methylation patterns in selected genes including *RASSF1A* have been associated with disease progression [42, 47] and relapse-free survival [13, 42, 47–49]. Paired-like homeodomain transcription factor 2 (*PITX2*) DNA methylation has also been validated using a robust assay for paraffin-embedded tissue for clinically relevant outcome prediction in early breast cancer patients treated by chemotherapy [50], suggesting that DNA methylation signatures have important therapeutic implications in guiding the use of epigenetic drugs in anticancer therapy [51]. The Long Island Breast Cancer Study Project, a population-based case-control study that followed cases for prognosis and survival, evaluated ten genes in breast tumors of 670 invasive cancers and found that methylation of the tumor suppressor genes *GSTP1, Twist* and *RARβ* was significantly associated with higher breast cancer-specific ($n$ = 86) mortality with a mean follow-up time of 8 years [47, 49]. Compared to cases with an unmethylated promoter in tumor tissues, those with a methylated promoter had a 71, 67 and 78 % increased risk of dying from breast cancer at the end of follow up for methylated *GSTP1* (hazard ratio (HR) = 1.71, 95 % 1.10–2.65), *Twist* (HR = 1.67, 95 %CI 1.01–2.79), and *RARβ* (HR = 1.78, 95 %CI 1.15–2.76), respectively. Similar associations between methylation status and all-cause mortality ($n$ = 161) were observed [47].

In Table 2, with one exception [52], all studies have a follow-up of at least 5 years. Among the studies that do not report mean or median follow-up time, based on the Kaplan Meier curves, we assume follow-up time spanned at least 5 years. Few studies examined recurrence [50, 53–55] with only two observing significant associations with methylation [50, 54]. Among chemotherapy-treated patients, higher methylation of *PITX2*, a gene associated with tumor aggressiveness and tamoxifen resistance, was associated with time to distant metastasis [50]. Gene-specific methylation, the number of methylated genes, as well as global gene methylation were all significantly associated with poor DFS (HR 2–3 fold) [54, 56–58] and poor OS (HRs in the range of 1.2–3.0) [54, 57, 59–61]. One study exceeded these observed effect sizes. Among women treated with adjuvant chemotherapy, *BRCA1* methylation was associated with poor survival with effect sizes between 12–16 fold; however, measures were imprecise given the large confidence intervals [57]. While the majority of studies found methylation associated with poor prognostic outcomes, methylated *NT5E*, another gene linked to tumor aggressiveness, was associated with improved survival [62]. Table 2 also demonstrates that when examining methylation and tertiary prevention, the association between methylation and prognosis can vary in direction and magnitude across subpopulations which can be based on tumor (e.g. triple-negative) and/or sociodemographic characteristics (e.g. age). In addition to these studies, three large studies that do not report event rates, with median follow-up time exceeding 5 years, found higher methylation associated with worse OS in single gene analyses [63] or gene panels [64, 65]. As is evidenced in Table 2, a number of important genes for breast cancer are also methylated and the methylation status affects outcomes. In summary, although limited prospective evidence exists, studies reported to date suggest that promoter methylation, particularly for a panel of tumor suppressor genes, has the potential to be used as a biomarker for predicting breast cancer prognosis; however, the data so far are very limited and the predictive value of the small number of DNA methylation signatures that have been identified is unclear.

Although the bulk of the epidemiologic evidence is with breast-tissue specific methylation, associations of plasma and serum DNA methylation and prognosis have also been observed. For example, patients with methylated *RASSF1A* and *APC* had worse prognosis than those without [66]. Several studies have examined recurrence [67–69] and survival [52, 64, 66, 67, 70–73]; with some focusing on the prognostic value of serum/plasma DNA methylation post therapeutics [64,69,66]. The source of the blood sample for the sera or plasma can also vary and may lead to different prognostic results. Peripheral blood plasma and bone marrow plasma samples were collected from 428 breast cancer patients during primary surgery with a median observation time of 51 months (interquartile range 35–68 months). In Kaplan-Meier analyses, methylated *PITX2* and *RASSF1A* in peripheral blood plasma were significantly associated with DFS and OS while associations were weaker in bone marrow plasma. Moreover, there was stronger prognostic value for DFS and OS when combining methylated *PITX2* and *RASSF1A* [70] indicating that plasma source, as well as gene panels, are important factors when testing prognostic biomarkers. Studies have also shown that methylation patterns change over time [74–76]. In one study, using cell-free plasma DNA, methylation patterns changed after surgery and tamoxifen treatment suggesting that methylation may also be used to monitor treatment [77]. Serum markers may also have prognostic utility. Studies have shown that tumor methylation patterns are highly correlated

with serum methylation [15–17]. For example, the correlation coefficient of *GSTM1* methylation in breast tumor tissues and serum was 0.365 [15]. Therefore, DNA methylation in plasma or serum is an attractive prognostic tool as it can be measured repeatedly and may help monitor response to therapeutics, DFS, and OS over time. In addition, plasma or serum DNA is an easier sample to procure in comparison to tissue samples.

In summary, the evidence that markers of DNA methylation, both in breast tissue, plasma, and serum collected at diagnosis may be important prognostic markers is intriguing and growing. As the evidence to date has primarily been relatively weak with breast cancer specific outcomes (e.g., breast cancer specific mortality), and also with only a select sample of markers, larger prospective studies that address a panel of markers are needed. It will be critical to identify those studies that collect extensive data on other clinical markers so that the contribution these methylation biomarkers make over standard clinical markers such as stage, grade, tumor size and molecular subtype in predicting DFS and OS can be determined. In addition to general prospective observational cohorts, clinical trial data using stored breast tissue and plasma samples from diagnosis, where available, have the advantage of examining the impact of gene-methylation over and beyond detailed therapeutic information among a cohort of individuals who may be more homogenous with respect to stage and overall treatment than participants in an observational epidemiologic study. In addition to the markers measured at baseline, it will be very useful to evaluate if repeated plasma samples can be useful to complement screening protocols after diagnosis.

## DNA Methylation Markers for Secondary Prevention and Early Detection

Regular mammographic screening has greatly improved breast cancer mortality among women ages 40–74 [78, 79]; however, mammography has limited sensitivity and specificity particularly in women with dense breasts [80, 81] and in younger women [82, 83]. Moreover, breast cancer is a complex disease that is difficult to detect in early stages by a single-marker approach. A variety of different markers and risk factors combined and weighted using robust and validated statistical models are needed to improve disease screening sensitivity [84]. Thus, identification of other markers for improved early detection is critical. In addition, more accurate risk assessment and risk stratification will improve the population effectiveness of these screening modalities.

While plasma and serum biomarkers have been used as a prognostic tool to determine treatment and diagnosis, there has been limited use of blood biomarkers as a reliable secondary prevention screening tool. For a plasma/serum biomarker to have adequate screening ability, the marker must be able to identify as positive those with the disease (high sensitivity) and be able to identify as negative those without the disease (high specificity). The most well-known examples of plasma/serum cancer screening biomarkers are the prostate-specific antigen (PSA) test for the detection of prostate cancer and CA-125 for ovarian cancer. While both screening tools are widely used, the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial has demonstrated that neither PSA nor CA-125 screening meet the criteria of a good sensitive and specific screening tool [85, 86]. Cancer research continues to identify serum/plasma biomarkers as independent and synergistic

cancer screening tools for secondary prevention as the biological processes involving cancer detection in blood is well established.

That cell-free DNA is released from tumors and can be found circulating in the plasma was first discovered by analysis of mutations in *KRAS* and *P53* [87, 88]. Although they can vary widely, in general, levels of circulating DNA are higher in cancer cases than controls, ranging from 0 to > 1 μg/ml of plasma; healthy individuals generally have < 25 ng/ml (reviewed in [89, 90]). In individuals without cancer, an increase in DNA in the blood can be caused by exercise, inflammation, and tissue injury [91]. In cancer cases, tumor circulating DNA is thought to originate from necrotic and apoptotic tumor cells. While initial studies analyzed mutations in circulating DNA, it was also found to contain methylation patterns similar to those found in primary tumors, suggesting the potential utility of blood-based molecular detection of cancer including breast cancer (reviewed in [89, 92–94]).

DNA hypermethylation of selected biomarkers, such as *RASSF1A* and *RARβ2*, was found to occur early in breast cancer development, suggesting that plasma DNA methylation might be useful as an early marker of disease [13]. Multiple genes that are now more frequently evaluated include *CDH1, RASSF1A, APC, BRCA1, GSTP1, RARβ*, and others (reviewed in [19, 93, 94]), with many studies showing reasonably consistent results. However, methylation of a single gene often results in low sensitivity; using a panel of epigenetic markers seems to achieve a more reasonable sensitivity with high specificity in breast cancer detection [93]. Most studies of methylation in serum/plasma DNA used samples collected at or just after breast cancer diagnosis. This retrospective design can result in bias if methylation levels are affected by disease progression or treatment. In order to evaluate the usefulness of DNA methylation markers in plasma as potential screening tools, it is important to understand whether methylation markers can be detected in plasma years prior to diagnosis. In a pilot study, we measured methylated *RASSF1A* in plasma DNA collected before diagnosis from 28 women with breast cancer and 10 of their unaffected siblings as well as from 33 women with breast cancer and 29 age- and ethnicity-matched population-based controls [95]. We found 18 % of cases were positive for methylation of *RASSF1A* in their plasma DNA collected before diagnosis, while only 5 % of controls were positive. Tumor tissue was available for 12 cases and all were positive for *RASSF1A* methylation. Our results suggest that aberrant promoter hypermethylation in serum/plasma DNA may be common among high-risk women and may be present years before cancer diagnosis. However, another study measured methylation in *RASSF1A, GSTP1, APC* and *RARβ2* using a nested case-control ($n$ = 50 cases and 100 controls) study design within the prospective New York University Women's Health Study cohort [96]. While the frequency of methylation in each gene was lower than expected among cases and higher than expected among controls, the frequencies did not differ between cases and controls.

While many studies have used plasma for early detection [14, 59, 70, 97–99], studies of plasma DNA methylation must take into account the technical aspects of plasma collection. For example, the time between blood collection and processing may impact the amount of DNA obtained due to the potential for lysis of white blood cells (WBC). The ratio of tumor to normal DNA in plasma may also be low given that plasma DNA can come from all tissues. There are conflicting data on the percentage of circulating DNA that comes from the

tumor, with a range of 10–90 % reported (see review in [89]). Another limitation is that circulating DNA is highly fragmented, typically 160–180 bp in length [100]. So care must be taken in the design of PCR primers to ensure that most samples will give a PCR product. In our own studies of plasma DNA in liver cancer, we found that the success of PCR decreased from 80 to 100 % for reactions with products < 200 bp to 63 % for one reaction with a 248 bp product [101]. Finally, it is clear that plasma DNA is lost rapidly when the source is removed. This was first observed in studies of pregnancy where it was found that fetal DNA disappeared from the mother's blood within hours of delivery [102]. Much optimization remains to be done, both in terms of increasing the sensitivity of both assays and guarding against false positives.

The great potential of plasma markers for screening enhancement is that it could complement the existing protocol of Magnetic Resonance Imaging (MRI) and mammography in very young women if the plasma markers are sensitive and specific. Currently, according to National Cancer Institute (NCI) Guidelines [103] as well as the National Comprehensive Cancer Network (NCCN) [104], women who are high-risk should be screened with MRI and also mammography starting as early as 25. Criteria to define high-risk vary by guidelines but include (*BRCA1* or *BRCA2* carrier, lifetime risk of > 20 % as defined by models that are largely dependent on family history, or 5-year risk of > 1.7 %. This means that a woman with lower DNA repair capacity because of mutations in *BRCA1* or *BRCA2*, among other genes, typically may have 15–20 mammograms by their early 40s. Instead, if MRI could be coupled with sensitive plasma markers for monitoring, this would translate into a substantially lower radiation dose. In order to evaluate this, prospective observational cohorts, ideally utilizing a range of sample collection and/or repeated blood collection so that a panel of genes can be evaluated in the plasma would be essential for uncovering whether or not plasma markers can enhance the potential of screening with MRI. For average risk women who are already postmenopausal, mammography has already been shown to be highly effective at reducing mortality [1, 105] so the main question for subsequent research would be whether plasma markers can help make the findings from mammography more useful in terms of informing the screening interval (number of years between screens) as well as improve the overall specificity of mammography to reduce the false positives.

In addition to plasma markers, an intraductal approach to early breast cancer detection, which includes nipple aspiration, ductal endoscopy, and ductal lavage (DL), has also been explored within the context of DNA methylation (as reviewed in [94, 106, 107]). Epigenetic analysis of DNA methylation in DL fluid for early breast cancer detection has been evaluated [108–111] including in women at high genetic risk of breast cancer [112, 113]. Analysis of methylation of *Cyclin D2, RAR-β*, and *Twist* using cells from DL fluid found cancer-specific methylation in patients with ductal carcinoma *in situ* (DCIS); abnormal methylation in cells from some of the healthy women, who later developed breast cancer was also observed. These results provided the first direct evidence that DNA methylation can be used to detect cancer in asymptomatic individuals with non-suspicious mammograms and normal breast examinations [108]. However, the Breakthrough Breast Cancer Research group has provided evidence that, while DNA methylation detected in DL fluid may be a strong prognostic marker for cancer patients, such methylation lacks specificity. DNA

methylation was assessed in six tumor suppressor genes from tumor tissue, adjacent tissue and bilateral DL fluid of cases and from normal tissue and DL fluid of healthy controls. Between bilateral DL fluid and tumor tissue, the highest sensitivity for methylation markers was observed for *SCGB3A1* (90 %), *CDH13* (91 %), and *RAR-β* (83 %). There was poor discriminatory ability of DL biomarkers. The area under the curve (AUC) for the receiver operator characteristic curve for cancer DL DNA methylation ($n = 54$ samples) compared to healthy control DL DNA methylation ($n = 46$ samples) was 0.76 with a specificity, or error, of 22 % [109]. Biomarkers present promising utility for high-risk populations and limited studies have examined DL fluid and epigenetic analysis for women at high genetic risk [112, 113]. A prospective study of 34 *BRCA* mutation carriers (16 *BRCA1* and 18 *BRCA2)* measured hypermethylation of *CDKN2A, RASSF1A, Twist*, and *RAR-β* in DL fluid collected prior to breast cancer development in seven women. There was a significant association between *RASSF1A* methylation and the development of breast cancer and hypermethylation of *CDKN2A* was associated with *BRCA1* mutation status [112]. Larger prospective epidemiological studies are needed with larger gene panels to determine if DL fluid is a promising, non-invasive, screening tool for early breast cancer detection or diagnosis.

Studies examining the correlation of DNA methylation in breast tumor tissues and plasma show similarities between methylation patterns found in primary tumor specimens and those in plasma, indicating the potential utility of blood-based molecular detection of breast cancer. Overall, DNA methylation is a candidate bio-marker because of numerous characteristics: (1) in the process of carcinogenesis, promoter hypermethylation is a more frequent event than mutations [114], with estimates varying from 600 to 1000 aberrantly methylated genes per tumor [115], (2) methylation has been shown to be an early event in breast tumorigenesis [116–118], (3) not only the malignant cells but also the surrounding tissue shows methylation defects [15–18, 119], (4) DNA methylation is stable and can be amplified by PCR, which means that aberrations can be relatively easily analyzed in very small amounts of DNA [120] as opposed to other approaches such as gene expression profiling, (5) a hypermethylated sequence forms a positive signal against an unmethylated background, which makes it more easily detectable than genetic alterations such as loss of heterozygosity [121]. Emerging evidence has shown that DNA methylation of select genes measured in plasma results in sensitivities > 90 % for detecting breast cancer. These results suggest that DNA methylation has promise for screening. Yet, these small clinical studies were cross-sectional with no or limited corresponding epidemiologic data. Further, unlike more easily collected blood, tissue is not suitable for use as a screening method. Of those studies that used blood, samples were collected at diagnosis, raising concerns about temporality.

## DNA Methylation Markers and Primary Prevention

In addition to studies of tertiary and secondary prevention, DNA methylation markers have been evaluated to see if they are useful biomarkers for ascertaining risk. In these studies of individuals with and without breast cancer, the primary source for the DNA methylation markers are peripheral blood cells, as it is often difficult to get breast tissue in women

without breast cancer and plasma markers measure circulating tumor cells may not be useful to examine in samples collected many years prior to cancer diagnosis.

Studies examining the relationship between methylation of DNA from peripheral blood cells and breast cancer risk have largely been case-control investigations in which blood samples are collected from cases after the diagnosis of breast cancer. This study design feature makes it challenging to determine whether any observed differences in case vs. control DNA methylation levels are a consequence of the disease (or treatment), as opposed to a causative factor for breast cancer development. These studies have evaluated both gene specific and global methylation (reviewed in [122, 123]).

One early case-control study evaluated global methylation levels in WBC DNA using both the 5mdC and LINE-1 methodologies, initially in a subset of 19 breast cancer cases and 18 controls [124]. Blood from all cases in the study were collected prior to surgery or any chemotherapy. Levels of 5mdC were significantly lower in cases than controls, but there was no difference observed in LINE-1 methylation, nor did level of 5mdC correlate with LINE-1 methylation within this subset. The 5mdC assay was subsequently used to ascertain global methylation levels in a total of 179 cases and 173 controls, and lower methylation was significantly associated with breast cancer (odds ratio (OR) and 95 % CI comparing lowest tertile of methylation to highest tertile of methylation = 2.86 (1.65–4.94)). Xu and colleagues made use of data from the Long Island Breast Cancer Study Project, a population-based case-control study, utilizing both the LUMA assay and analysis of LINE-1 methylation to assess global DNA methylation in WBC DNA isolated from 1055 cases and 1101 controls [125]. Blood was collected from cases following diagnosis and it was possible to stratify the case population into those from whom blood samples were pre- vs. post-chemotherapy and also pre- vs. post-radiation therapy. No relationship with breast cancer was found for LINE-1, but for LUMA, higher levels of global methylation were associated with increased risk (OR 95 %CI) comparing quintile 5 of methylation to quintile 1 of methylation (OR = 2.41, 95 % CI 1.83–3.16). This is likely due to the fact that LUMA, which evaluates CCGG sites, is primarily measuring methylation in promoter regions. This over two-fold increase in breast cancer risk persisted when comparing prechemotherapy cases to controls and preradiation cases to controls, suggesting that the association was likely not a treatment effect. In our case-control studies, global methylation levels in Sat2 were correlated between 40 breast tumor tissues and matched WBC DNA isolated from blood samples collected from cases at the time of surgery and prior to chemotherapy [126]. Although intriguing, the literature is far from consistent and we did not observe an association with LUMA and breast cancer risk in a family registry of women at high-risk of breast cancer [127], and another case-control study found lower levels of methylation by the LUMA assay to be associated with breast cancer risk [128]. In addition to LUMA, other markers of global methylation have been examined, and we compared methylation of repetitive elements (Sat2, LINE-1, and Alu) in WBC and granulocyte DNA isolated from blood samples donated by sisters discordant for breast cancer development (282 cases and 347 sister controls; cases donated blood following breast cancer diagnosis) [129]. WBC DNA Sat2 hypomethylation was again associated with breast cancer risk, but no association was observed with granulocyte DNA Sat2 methylation, suggesting that differential global

DNA methylation of some repetitive elements may be associated with blood cell type counts.

In contrast to retrospective case-control studies, the nested-case control design has the same efficiency as in case-control studies but also ensures temporality, an important attribute for biomarker studies. A nested case-control study conducted within the prospective Breakthrough Generations Study and European Prospective Investigation into Cancer and Nutrition (EPIC) examined global DNA methylation in the LINE-1 repetitive element among the WBC DNA from 640 cases and 741 controls, but did not find any differences [130]. The Sister Study is another of the few prospective studies to examine global DNA methylation in WBC and breast cancer risk [131] and is methodologically strong because of its case-cohort design, with blood collected from cases prior to diagnosis. A total of 294 incident breast cancer cases and a sample of 646 non-cases in the study were selected for examination of global DNA methylation of LINE-1. Hypomethylation of WBC DNA LINE-1 was associated with subsequent development of breast cancer comparing quartile 1 of methylation to quartile 4 (HR = 1.75, 95% CI 1.19–2.59).

With respect to gene-specific WBC DNA methylation, one of the earliest epigenotyping case-control studies made use of data on cases and age-matched controls from the ESTHER study [132]. The investigation utilized a multistep method consisting of selection of a broad array of 49 genes of interest based on those known to be methylated in breast cancer; methylation analysis of these genes in a small group ($n = 83$) of healthy, postmenopausal women to narrow down the genes investigated to 25, based on observed methylation patterns as related to *a priori* hypotheses; and examination of methylation of this smaller group of 25 genes among peripheral blood cell DNA isolated from individuals in the larger ($n = 353$ cases and 730 controls) case-control study. Adjusting for age and family history of breast cancer, the authors found differences in methylation of five out of 25 genes between cases and controls, with cases in each of these situations exhibiting lower levels of methylation. The genes with observed methylation differences were estrogen receptor-2 (ER-2) target genes (*NUP155, ZNF217*) and polycomb group target genes (PCTG) that play a role in stem cell biology (*TITF1, NEUROD1, SFRP1*). Lack of DNA methylation at these gene loci conferred a statistically significant 1.4- to 1.5-fold increased risk of breast cancer. In addition, invasive ductal and invasive lobular breast cancer was characterized by methylation of different sets of genes and methylation of ER-α target genes predicted estrogen receptor-positive breast cancer. Elevated *BRCA1* methylation has been observed in cases compared to controls in several case-control studies [17, 133]. In one study that enrolled 255 cases diagnosed with breast cancer prior to age 40 years and compared them to 169 controls, the prevalence of detectable WBC *BRCA1* methylation tended to increase as the tumors from cases contained more *BRCA1* mutation-associated morphologic features and methylation of *BRCA1* in WBC DNA was associated with a 3.5-fold (95 % CI 1.4–10.5) increased risk of breast cancer [17]. However, the largest case-control study carried out to date of *BRCA1* methylation did not observe a significant difference between cases and controls, although there was a trend of *BRCA1* promoter hypermethylation in cases vs. controls [134]. Other studies have evaluated the relationship between methylation of *ATM* in WBC DNA and breast cancer [130, 135], with the single prospective nested case-control examination reporting an increased risk of breast cancer associated with higher levels of methylation at

the *ATMmvp2a* locus comparing quintile 5 of methylation to quintile 1 of methylation (OR = 1.89, 95 % CI 1.36–2.64) [130].

The prospective Sister Study also used an efficient approach of a case-cohort design to study WBC methylation and breast cancer risk [136]. Specifically, they applied the Illumina Infinium 27k CpG HumanMethylation BeadChips arrays and identified 250 differentially methylated CpG sites between cases and controls in WBC DNA [136]. The AUC for the receiver operator characteristic curve estimated for five of these methylation markers (66 %) was larger than for the Gail model (56 %) or nine highly ranked single nucleotide polymorphisms from genome-wide association studies of breast cancer (56 %). The mean time from baseline blood draw to diagnosis among the breast cancer cases was only 1.3 years in the Sister Study [136]. Thus, the data cannot tell us whether epigenetic changes can predict risk years into the future or are, instead, a response to underlying disease. In addition, the differences in mean percent methylation for the identified sites, while statistically significant, were very small and almost entirely within two percentage points. Cohort studies with longer follow-up time and serial blood collections are needed to estimate lead times, clarify biology, and apply appropriate methods for evaluating predictive value of DNA methylation for diagnosis [137]. It is also necessary to validate data from Illumina arrays, which can be problematic at very high or low methylation levels. For example, in trying to validate some of the top candidates in the Sister Study in our subjects, we found that several sites with low methylation (~ 10 %) in the original Illumina data showed nondetectable methylation by pyrosequencing. In addition, there are also a number of potential problems with Infinium data such as cross-reactive probes, probes containing single nucleotide polymorphism (SNP) sites and probes giving high intensity data, which further underscores the importance of array data validation by other methodologies such as pyrosequencing [138].

A limitation of WBC DNA analysis is the concern that results may be related to differences in cell populations. This is particularly problematic for bloods collected at the time of diagnosis, since it is known that cancer patients have altered proportions of specific cell types compared to healthy controls [139]. We have demonstrated that global methylation profiles vary by different blood cell types [140]. A method has been reported that allows the use of DNA methylation array data to estimate WBC populations [141, 142]. A second limitation is that DNA methylation is known to be impacted by age, genetics, and environmental as well as lifestyle factors (reviewed in [122, 143]). Smoking, air pollution, heavy metals, micronutrients, and even stress have all been shown to alter DNA methylation in WBC. As mentioned above, differences in DNA methylation between cases and controls are small. The biological significance of these small differences is terms of gene expression and function is not clear. Finally, there have been objections to the use of WBC for DNA methylation analysis, given the lack of information on relationship to target tissue, in this case breast tissue. One paper has evaluated the use of WBC DNA as a surrogate for evaluating imprinted loci methylation in mammary tissue [144]. Of the six loci studied, after correction for multiple comparisons, for only one was there a correlation between tissue and blood and only for invasive tumor tissues not benign breast disease.

In addition to primary prevention studies of WBC DNA methylation, limited studies examined DNA methylation in breast tissue from mammoplasty patients. For example, one study of DNA methylation enrolled 141 healthy European and African American women (mean age (standard deviation (SD)) = 35 years (11) and mean body mass index (BMI) (SD = 32 kg/m$^2$ (7)) undergoing non-surgical breast reduction [145]. The focus of the study was to detect the likelihood of promoter methylation of *CDKN2A/p16INK4, BRCA1, ER-α, and RAR-β* across a spectrum of breast cancer risk factors. In age-adjusted models, family history of cancer was associated with a two to seven fold greater odds of *p16INK4, BRCA1* or *ER-α* hypermethylation compared to those without a family history of cancer. In race-stratified analyses of European-American women, hypermethylated *p16INK4* was independently associated with having a family history of any cancer and ever consuming alcohol. In African-American women, a family history of cancer was associated with *BRCA1* hypermethylation. While the large number of mammoplasty samples is a strength in this study, there was limited power, especially in the race-stratified analyses. Some of the cell counts had as few as two events, thus the results need to be interpreted with caution. In addition, though the women represent a high breast cancer risk population given their elevated average BMI, results of this study may not be generalizable. Confirmation in a larger population is required, but, mammoplasty samples are difficult to procure, as underscored by the recruitment timeline for this study [145].

In summary, the evidence base for DNA methylation markers and breast cancer incidence is accumulating but major gaps remain. First, most data come from retrospective studies where disease itself or cancer treatment may affect blood, saliva, and tissue methylation, limiting a determination of temporality and with methylation changes being a consequence rather than a cause. Second, the prospective studies that do exist, although growing, generally lack repeated measures making it difficult to establish whether environmental exposures change the levels of DNA methylation markers and, in turn, whether these changes alter breast cancer risk. Third, the evidence base until recently has focused on selected gene targets and markers of global methylation. As techniques improve to scan for a larger set of genes, using prospective studies with extensive environmental data, the evidence base for using DNA methylation markers as biomarkers will build.

## Summary and Next Steps

Although the scientific literature on DNA methylation and breast cancer is extensive, for specific questions about primary, secondary and tertiary prevention, as described above, the literature is in its infancy. In particular, methodological considerations for the type of prevention study need to be carefully considered. Table 3 summarizes some of the key methodological considerations that we have observed to be lacking from many of the existing studies. A significant consideration is whether or not the study design is prospective or retrospective, with the former needed to determine temporality. Another important consideration is whether the study design is large enough to have sufficient number of events for multivariable modeling to allow for assessment of the independence of the DNA marker over and beyond standard clinical markers.

Common to all types of studies is a consideration of the source of DNA. Using data from multiple DNA sources from the same individuals, we have observed variation in genomic DNA methylation within specific WBC types [140]. Given the variation in function and gene expression levels of specific WBC types, it is not surprising the both gene-specific and global methylation levels vary by type. This complicates investigation of the association of DNA methylation with disease. It is well known that there are differences in total WBC counts in healthy individuals, with a range of 5000–10,000/μl, but there are also differences in cell populations. Cancer patients also demonstrate alterations in specific cell types. For example, neutrophil and lymphocyte counts were elevated and reduced, respectively, in advanced stage uterine cervical cancer [139]. It is also clear that in metastatic cancer, there is a dynamic range of circulating tumor DNA [146]. Martin [147] suggested that definitive analyses require specific methodologies to account for shifts in cellular population heterogeneity. Moreover, a significant proportion of DNA variation might be due to the disparity in protocols for sample processing [148], and in time of storage of blood samples [149]. In addition, as future studies will need to address repeated measures of DNA methylation markers, issues surrounding the temporal stability of DNA methylation need to be understood. For example, comparing DNA methylation levels in blood collected at two visits, we have observed that changes in DNA methylation over time are highly associated with baseline values of the assay and vary by assay type [76]. These findings suggest that assays that change more over time may warrant consideration for studies that use DNA methylation as bio-markers. An additional challenge is that different assays measure varying aspects of DNA methylation. For example, MSP measures the relative amounts of fully methylated regions, while pyrosequencing measures average methylation levels at several CpG sites in a pool of DNA. It is important to verify and validate differences in DNA methylation using various assays.

These methodological considerations can be overcome by more research utilizing large, prospective studies that have careful biospecimen collection. The great potential of DNA methylation markers across the prevention continuum and in improving risk assessment may soon be realized. Absolute risk prediction models provide useful information for health care providers and patients and aid in the design and recruitment phase of studies of preventive interventions [150–152]. Clinical prediction modes such as Gail [153], and Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm (BOADICEA) [154] have been developed to estimate absolute age-specific breast cancer risk. The most frequently used risk prediction tool in United States clinics is the Gail model, which takes only first-degree family history into account and focuses on nongenetic risk factors [153]. Although the Gail model has been found to be well calibrated for women at average risk, its discriminatory ability is moderate and limits its clinical applicability, particularly for screening [155]. Moreover, the Gail model has not been recommended for high-risk women such as those with a strong family history of breast cancer [4,6]. Using information from the New York site of breast cancer family registry (BCFR), we previously reported that models developed using extended family and genetic data, such as the IBIS model, showed better discrimination (AUC = 69.5 %) than did the Gail model [6]. As mentioned above, Xu et al. [136], using prospectively collected blood samples, identified five methylation markers

(AUC = 66 %). Extending such models to include additional genetic or epigenetic information may improve performance in women across the breast cancer risk continuum.

In summary, DNA methylation markers are compelling candidate biomarkers because of numerous characteristics: (1) in the process of carcinogenesis, promoter hypermethylation is a more frequent event than mutations [114], with estimates varying from 600 to 1000 aberrantly methylated genes per tumor [115], (2) methylation has been shown to be an early event in breast tumorigenesis [116–118], (3) not only the malignant cells but also the surrounding tissue shows methylation defects [15–18,119], (4) DNA methylation is stable and can be detected by PCR methods, which means that aberrations can be relatively easily analyzed within very little material [120] as opposed to other approaches such as gene expression profiling, and (5) a hypermethylated sequence forms a positive signal against an unmethylated background, which makes it more easily detectable than genetic alterations such as loss of heterozygosity [121]. It will be essential as we move forward to fully evaluate and quantify the potential gains that can be made in primary, secondary, and tertiary prevention efforts by measuring through large, prospective studies the impact DNA methylation markers have in predicting incidence and OS after diagnosis.

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**Table 1**

Methods commonly used for analysis of DNA methylation in epidemiologic studies

| Assay | Method | Advantages | Disadvantages |
|---|---|---|---|
| 5methylC by HPLC or LC/MS | Enzymatic digestion of DNA followed by analysis | Absolute, specific quantification of total levels of 5mdC; can also measure 5hmdC | Expensive equipment especially for LC/MS; MS need internal standard |
| [$^3$H] Methyl acceptance assay | Enzymatic radioactive labeling of non-methylated CpG sites | Global measure of methylation | Uses radioactivity, variable with batch of enzyme and *S*-adenosylmethionine; requires highly accurate DNA quantitation |
| Luminometric methylation assay (LUMA) | Methylation sensitive restriction digestion followed by pyrosequences | Global measure of methylation at CCGG sites | Limited CpG sites evaluated; requires high quality DNA |
| *Bisulfite treatment-based assays* | | | |
| Combined bisulfite restriction analysis (COBRA) | Restriction digestion of PCR amplified regions | Provides semi-quantitiative data at specific regions | Analysis limited to specific restriction target sites; gel analysis limits high throughput |
| Methylation-specific PCR (MSP) | Separate primers for methylated and non-methylated DNA followed by gel analysis | Requires limited equipment; sensitive to 0.1 % methylated alleles | Gel analysis limits high throughput; not quantitative; only one region analyzed per assay |
| Fluorescence-based real time methylation-specific PCR | MSP but with fluorescence detection | Suitable for high throughput; highly quantitative; sensitive to 1/10$^5$ | Requires more expensive instrumentation than PCR; only measure DNA fully methylated on sites covered by primers; only one region analyzed per assay |
| MethyLight | Adds Taqman probe to real time PCR | Suitable for high throughput; highly quantitative | Requires more expensive instrumentation than PCR; only measure DNA fully methylated on sites covered by primers; sites covered by probe must also be fully methylated; only one region analyzed per assay |
| Pyrosequencing | Sequencing by synthesis after amplification with non-methylation-specific primers; sequencing probe also does not contain CpG sites | Relative level of methylation at each CpG analyzed; control for efficiency of bisulfite conversion | Requires expensive instrumentation; only one region analyzed per assay; sometimes impossible to design appropriate primers and probe for specific region |
| Illumina Infinium 27k and 450k CpG HumanMethylation BeadChip arrays | Two types of assays; type 1 uses two probes per CpG (methylated and unmethylated); type 2 uses degenerate probes and two colors | Information from across the genome; easily interpretable beta values for methylation level | Expensive instrumentation and arrays; only interrogates sites on the array; two chemistries, specific probes and SNPs require careful data analysis |
| Next generation sequencing | Varies by platform | High resolution analysis of each cytosine in the genome; also obtain genetic information | Expensive; large fraction of C converted to T (reduced sequence complexity) complicates sequence alignment |

**Table 2**

Summary of studies evaluating the tertiary prevention potential of DNA methylation markers using breast tissue or plasma

| | Sample size (N) | Follow-up time | Genes | Outcomes (HR (95 % CI)) | | |
|---|---|---|---|---|---|---|
| | | | | Disease free survival | Recurrence | Overall survival/mortality |
| *Tissue studies* | | | | | | |
| Huang 2013 [52] | 175 primary breast cancer samples | Mean 45.81 months (15–124 months) | *PTPRO* | – | – | Reference group: Unmethylated 3.27 (0.75–14.21) |
| Xu 2013 [56] | 167 triple-negative breast cancer treated with adjuvant chemotherapy | Median 9 years (0.41–15.1 years) | *BRCA1* | Reference group unmethylated | – | Reference group unmethylated |
| | 675 non-triple-negative breast cancer treated with adjuvant chemotherapy | | | 0.45 (0.24–0.84) for triple-negative | | 0.43 (0.19–0.95) for triple-negative |
| | | | | 1.56 (1.16–2.12) for non-triple negative | | 1.53 (1.05–2.21) for non-triple negative |
| Hsu 2013 [57] | 139 early stage breast cancer samples including 21 triple-negative | Up to 120 months[a] | *BRCA1* | Reference group unmethylated: 12.19 (2.29–64.75) | – | Reference group unmethylated: 16.38 (1.37–195.45) |
| Lu 2012 [53] | 348 primary breast cancer samples | Median 86 months (8–108 months) | *HOTAIR* | – | Reference group low methylated: 0.95 (0.52–1.73) | Reference group low methylated: 1.15 (0.58–2.31) |
| Lo Nigro 2012 [62] | 157 primary breast cancer samples including 119 ER-positive, including 11 HER2+ only, and including 26 triple negative | Up to 12.5 years[a] | *NT5E* | Reference group methylated: HR 2.7, $p = 0.001$ (95 % CI not listed) | – | Reference group methylated: HR 3.0, $p = 0.001$ (95 % CI not listed) |
| Van Hoesel 2012 [55] | 222 early stage breast cancer samples | Median 19.9 years (0.25–23.0 years) | *MINT17, MINT31, RARβ2* | Reference group meth-Neutral: meth-High: 1.51 (0.99–2.32) | Reference group meth-Neutral: meth-High: 1.70 (0.99–2.92) | Reference group meth-Neutral: meth-High: 1.36 (0.89–2.08) |
| | | | | meth-Low: 1.35 (0.91–2.02) | meth-Low 1.44 (0.85–2.92) | Meth-Low: 1.14 (0.75–1.72) |
| Li 2014 [59] | 98 | Median 60 months (43–70 months) | *PTPRO* | – | – | Reference group unmethylated: HR 2.7 (1.1–6.2) |
| Zeng 2012 [156] | 302 | Median 86.3 months (8.2–107.8 months) | *L3MBTL1* | Reference group<br>Low Meth:<br>Med Meth: 1.26 (0.73–2.18)<br>High Meth: 0.97 (0.55–1.71)<br>$P_{trend} = 0.97$ | – | Reference group<br>Low meth<br>Med Meth: 1.3 (0.66–2.56)<br>High Meth: 1.45 (0.75–2.77)<br>$P_{trend} = 0.27$ |

| | Sample size (N) | Follow-up time | Genes | Outcomes (HR (95 % CI)) | | |
|---|---|---|---|---|---|---|
| | | | | Disease free survival | Recurrence | Overall survival/mortality |
| Krasteva 2012 [157] | 135 sporadic invasive primary breast cancer samples | 5 years | *BRCA1* | – | – | Reference group unmethylated: 0.91 (0.24–3.41) |
| Cho 2012 [60] | 670 invasive breast cancer samples | Mean 8 years (0.3–9.4 years) | *HIN1, RASSF1A, DAPK1, GSTP1, CyclinD2, TWIST, CDH1* and *RARβ* | – | – | Breast cancer-specific mortality: Reference group unmethylated:<br>*HIN1* 1.17 (0.75–1.83)<br>*RASSF1A* 1.77 (0.86–3.67)<br>*DAPK1* 1.27 (0.72–2.22)<br>*GSTP1* 1.71 (1.10–2.65)<br>*CyclinD2* 1.18 (0.71–1.99)<br>*TWIST* 1.67 (1.01–2.79)<br>*RARβ* 1.78 (1.15–2.76)<br>All-cause mortality: Reference group unmethylated:<br>*HIN1* 1.07 (0.77–1.47)<br>*RASSF1A* 1.21 (0.76–1.93)<br>*DAPK1* 1.10 (0.72–1.66)<br>*GSTP1* 1.49 (1.08–2.07)<br>*CyclinD2* 1.27 (0.89–1.81)<br>*TWIST* 1.37 (0.93–2.01)<br>*RARβ* 1.45 (1.05–2.02) |
| Noetzel 2010 [58] | 195 primary, unilateral, invasive breast cancer Patients did not undergo neoadjuvant chemotherapy | Up to 140 months based on longest median recurrence free survival reported | *SYNM* | Reference group unmethylated: 2.94 (1.12, 7.71) | – | – |
| Akhoondi 2010 [48] | 68 (C1)b and 93 (C2)b breast cancer samples | Up to 5 years following diagnosis[a] | *FBXW7/hCDC4-β* | – | – | Reference group unmethylated:<br>C1: 0.53 (0.23–1.23)<br>C2: 0.50 (0.23–1.08) |
| Chen 2009 [158] | 536 breast cancer samples | Median 8 years (0.4–11.6 years) | *BRCA1* | Reference group unmethylated: Disease-free survival: 1.23 (0.84–1.80) Disease-specific survival: 1.27 (0.81–1.99) | – | – |
| Xu 2009 [159] | 851 breast cancer samples | Mean 5.6 years (0.2–7.4 years) | *BRCA1* | – | – | Reference group unmethylated:<br>Breast cancer-specific mortality: 1.67 (0.99–2.81)<br>All-cause mortality: 1.40 (0.94–2.08) |
| Hartmann 2009 [50] | 241 breast tumor samples All patients treated with adjuvant | Median 81.5 months | *PITX2* and 60 additional candidate genes | – | Reference group Q1 methylated: *PITX2P2* Q4 | – |

| | Sample size (N) | Follow-up time | Genes | Outcomes (HR (95 % CI)) | | |
|---|---|---|---|---|---|---|
| | | | | Disease free survival | Recurrence | Overall survival/mortality |
| | anthra-cycline-based chemotherapy | | | | methylated 1.28 (1.03–3.83) | |
| Nimmrich 2008 [61] | 412 (all LNN/HR+)c | Median 98 months (1–233 months) | *PITX2* | – | – | Reference group low methylation: 1.53 (1.21–1.92) |
| Van Hoesel 2012 [54] | 379 primary ductal breast cancer patients | Median 19 years (0–23 years) | *LINE-1* | Reference group methylated: 2.05 (1.14–3.67) for younger cases<br>0.83 (0.57–1.20) for older cases | Reference group methylated: 2.83 (1.53–5.21) for younger cases<br>0.67 (0.40–1.10) for older cases | Reference group methylated: 2.19 (1.17–4.09) for younger cases<br>0.73 (0.50–1.07) for older cases |
| *Plasma* | | | | | | |
| Gobel 2011 [70] | 428 primary breast cancer samples | Median 51 months (IQR[e] 35–68) | *PITX2 RASSF1A* | Reference group unmethylated: *RASSF1A* 3.4 (1.6–7.3) | – | Reference group unmethylated: *PITX2* 3.4 (1.2–9.8)<br>*RASSF1A* 5.6 (2.1–14.5) |

[a] Follow-up time not provided and estimated from Kaplan Meier curve

[b] Cohort 1 (C1) and Cohort 2 (C2)

[c] Lymph node-negative, steroid hormone receptor positive (LNN/HR+)

[d] Odds ratios (OR) were provided

[e] Interquartile range (IQR)

**Table 3**

Methodological considerations when conducting DNA methylation study across the breast cancer prevention continuum

| | **Primary prevention** | **Secondary prevention** | **Tertiary prevention** |
|---|---|---|---|
| Types of questions to address | Are DNA methylation markers related to breast cancer incidence? | Can DNA methylation markers augment standard screening? | Can DNA methylation markers predict prognosis (disease-free and overall survival)? |
| Study design considerations | Prospective studies needed to assure temporality | Repeated measures over time, prospective | Prospective studies with enough events to adjust for standard clinical markers |
| Source of DNA | WBC DNA | DNA methylation markers measured in plasma | DNA methylation markers in breast tissue at diagnosis |
| | Some markers in saliva have very different levels than blood | | |
| | Breast tissue will mean sample may be less generalizable | | DNA methylation markers in plasma repeated over time for recurrence |