

DNA methylation in white blood cells

Association with risk factors in epidemiologic studies

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Alterations in DNA methylation patterns, both at specific loci and overall in the genome, have been associated with many different health outcomes. In cancer and other diseases, most of these changes have been observed at the tissue level. Data on whether DNA methylation changes in white blood cells (WBC) can serve as a useful biomarker for different health outcomes are much more limited, but rapidly emerging. Epidemiologic studies have reported associations between global WBC methylation and several different cancers including cancers of the colon, bladder, stomach, breast, and head and neck, as well as schizophrenia and myelodysplastic syndrome. Evidence for WBC methylation at specific loci and disease risk is more limited, but increasing. Differences in WBC DNA methylation by selected risk factors including demographic (age, gender, race), environmental exposures (benzene, persistent organic pollutants, lead, arsenic and air pollution), and other risk factors (cigarette smoke, alcohol drinking, body size, physical activity and diet) have been observed in epidemiologic studies though the patterns are far from consistent. Challenges in inferences from the existing data are primarily due to the cross-sectional fashion and small size of most studies performed to date, as well as to the differences in results across assay type and source of DNA. Large, prospective studies will be needed to understand whether changes in risk factors are associated with changes in DNA methylation patterns, and if changes in DNA methylation patterns are associated with changes in disease endpoints.

DNA methylation may play an important role in causing disease by silencing genes through hypermethylation or activating genes through hypomethylation.¹⁻⁷ In addition to gene-specific DNA methylation, lower genome-wide aberrant DNA methylation (also referred to as global methylation) in regions that are normally methylated, such as repeats or transposable elements can lead to genomic instability and altered gene transcription impacting normal growth and development in target tissues. Although a large literature exists on DNA methylation changes at the tissue level, less is known about whether DNA methylation measured in WBC can be used as an intermediate biomarker for different health outcomes. As blood biomarkers

can be measured repeatedly over time in epidemiologic studies, they can be very useful to understand how disease risk changes over the life course. Several requirements need to be met when evaluating whether a potential biomarker is useful for over time monitoring. First, the intermediate marker needs to be associated with the endpoint of interest. Second, exposures that logically precede the measurement of the biomarker should be correlated with the marker. Third, changes in the exposure levels should result in changes in the intermediate biomarker. Fourth, changes in the intermediate biomarker should be associated with change in the endpoint. Here, we review whether WBC DNA methylation fulfills the requirements to be used as a potential biomarker in human epidemiology studies, specifically focusing on whether the studies to date suggest that risk factors are associated with WBC DNA methylation.

A number of methods are available for the analysis of CpG methylation levels.⁸ Overall, levels of 5-methyl-cytosine (5-mC) can be quantified by enzymatic hydrolysis of the DNA, chromatographic separation of the nucleosides, and analysis by HPLC or MS. A methyl acceptor assay that is dependent upon the ability of a bacterial enzyme to specifically methylate unmethylated Cs in CpG sites using [³H] S-adenosylmethionine has also been used extensively (referred to here as the [³H]-methyl acceptor assay). The Luminometric Methylation Assay (LUMA) targets all CCGG sequences in the genome by using methylation-sensitive restriction enzymes to discriminate methylated and unmethylated DNA followed by pyrosequencing. Repetitive element sequences, such as Alu, LINE-1 and Sat2, have also been analyzed in a number of studies, as a surrogate for genome-wide methylation levels; such analysis requires bisulfite conversion of cytosine to uracil followed by PCR. In the pyrosequencing analysis of repetitive elements, the PCR step amplifies a given region using primers outside the target sequence containing CpG sites so that both methylated and unmethylated sequences are amplified. This is followed by pyrosequencing of the region of interest. For methylation specific assays such as MethyLight, a Taqman quantitative real-time PCR assay is performed, using primers and probes that are methylation specific and require all CpG sites in the region to be methylated for amplification to occur. To assay gene specific methylation, MethyLight and pyrosequencing are the most commonly used methods in large human population studies. However, studies employing array methodologies are beginning to emerge as well.⁹⁻¹¹

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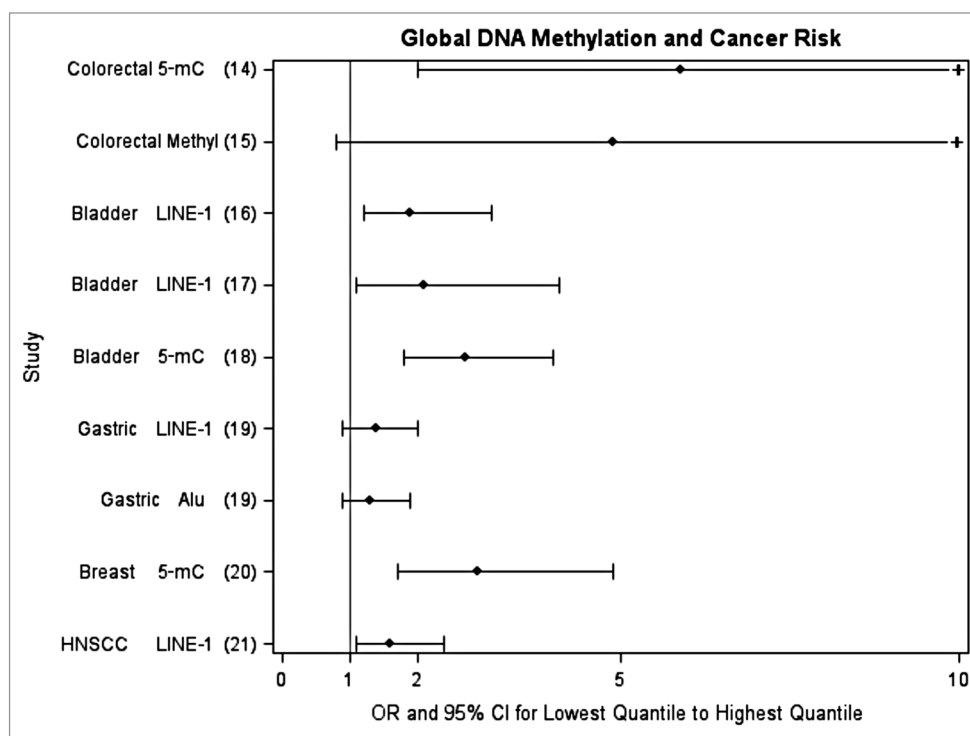


Figure 1. Epidemiologic studies investigating the association between WBC global DNA methylation and cancer risk (comparing lowest quantile to highest quantile). Tertiles used are from references 14–16 and 19–21. Quartiles used are from reference 18. Deciles used are from reference 17. +, upper 95% confidence limit exceeds 10.

WBC Methylation and Disease

Alterations in DNA methylation patterns, both at specific loci and overall genomic level, have been associated with many different cancers.¹² These alterations include hypermethylation of tumor suppressor genes, hypomethylation of oncogenes, and overall lower levels of global methylation, and have primarily been observed at the tissue level. More recently, DNA methylation changes have also been observed in plasma as a marker of circulating tumor DNA¹³. Less is known about changes in WBC DNA methylation levels and cancer risk, although studies are rapidly emerging. Specifically, at least eight studies measuring overall WBC global DNA methylation in different cancer types including colon,^{14,15} bladder,^{16–18} stomach,¹⁹ breast,²⁰ and head and neck cancer²¹ have found an elevated risk for cancer between those in the lowest quantile of global DNA methylation compared to those in the highest quantile of risk; the association was statistically significant in six of the studies (see Fig. 1 for summary). The few studies that exist investigating gene-specific methylation in WBC and cancer risk have mostly focused on selected genes for breast cancer and colon cancer but also support the potential for gene-specific methylation measured in WBC as a biomarker of risk^{22–27} (see Fig. 2 for summary). However, data are still very limited and systematic and unbiased approaches to the selection of genes have not been routinely employed. The first paper using Illumina Infinium

HumanMethylation27 arrays that interrogate 27,000 CpG sites to differentiate ovarian cancer cases from controls suggests the potential of this approach as a tool for detecting or predicting certain cancers.¹¹

While research in this field is at an early stage, increasing evidence is accumulating on the role of epigenetics, including DNA methylation (genome-wide hypomethylation and gene-specific hypo- and hypermethylation) as well as histone modifications, in numerous other diseases in addition to cancer, including autoimmunity, asthma and neurologic diseases. For example, alterations in gene expression impacted through epigenetic mechanisms can lead to the development of autoreactive lymphocytes in systemic lupus erythematosus, synoviocyte proliferation in rheumatoid arthritis or neural demyelination in multiple sclerosis (reviewed in ref. 28). Studies in asthma are also leading to a new understanding of the role of environmental exposures in altering DNA methylation as well as histone modifications (reviewed in ref. 29). Twin studies in Alzheimer disease support the notion that epigenetic mechanisms mediate risk (reviewed in ref. 30). However, since many studies of gene-specific methylation are performed on brain autopsy samples, it is not clear whether the observed epigenetic changes actually represent a cause or a consequence of the disease. Studies on WBC DNA methylation and other disease points are more limited but are starting to emerge with respect to Alzheimer's disease, schizophrenia and myelodysplastic syndrome.^{30–32}

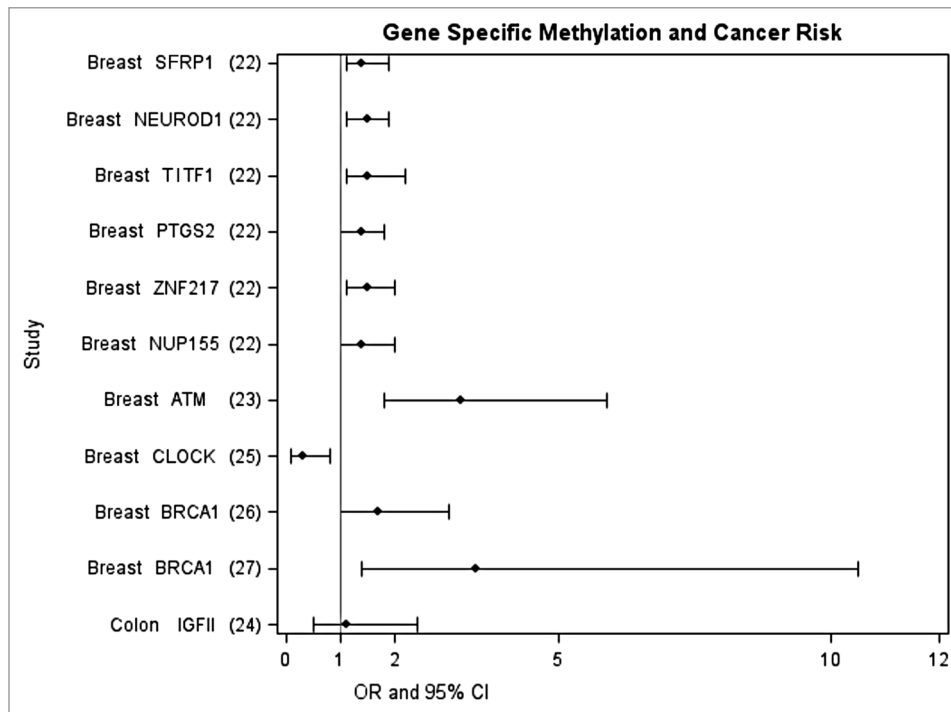


Figure 2. Epidemiologic studies investigating the association between WBC gene-specific DNA methylation and cancer risk. Methylation cutpoints specific to each study, for details see references 22–27.

Associations Between WBC DNA Methylation and Epidemiologic Risk Factors

Epidemiologic studies have investigated the association between demographic, environmental and behavioral risk factors with both WBC global and gene-specific DNA methylation. These studies are summarized in **Tables 1 and 2**, respectively. In this review we searched for all articles published to date on each respective risk factor and WBC DNA methylation measured in human populations. We did not include studies that investigated DNA methylation in tissue.

Demographic factors. *Age.* Growing evidence supports that WBC global DNA methylation, particularly in blood, changes with age.^{9,33,34} In the two studies directly assessed the percentage of 5-mC content, a statistically significant decrease of 5-mC with older age was observed in one,³³ but not in the other.¹⁸ Age was also significantly associated with lower global methylation measured by the [³H]-methyl acceptor assay.³⁵ Studies have correlated age with two of the repetitive elements, LINE-1 and Alu. Although one study correlated lower levels of LINE-1 with increasing age,³⁶ most of the studies did not find an age-dependent effect on blood LINE-1 methylation.^{17,21,37-41} In contrast, all of the studies investigating age and Alu methylation supported a lower level of DNA methylation of this repetitive element with increasing age.^{34,36,37,41} Gene-specific methylation has been correlated with age in some studies (**Table 2**).^{34,42} However, studies looking across the genome have found infrequent age-dependent changes in CpG island methylation.⁴³⁻⁴⁵

The overall impact of age may account for a small proportion of inter-individual variation.^{33,36,37} Inter-individual variability in

methylation of repetitive elements has been reported to be in the range 5–25%.⁴⁶ Using information from monozygotic twin pairs, significant differences in total 5-mC content in WBC was found among older twins, suggesting environmental exposure throughout the life course might have a large impact on the decline in global DNA methylation over time.³⁴ Measurement of within-individual changes in WBC DNA methylation by LUMA on 111 individuals after 10 years found both increased and decreased methylation, with over half exhibiting >5% change.⁹ The CpG array data on a subset of the same subjects suggested that most sites lost methylation over time.⁹ This study also supported familial clustering in changes in DNA methylation, which indicates that both environmental and genetic factors are determinants of DNA methylation patterns.⁹ Evidence supporting a change in DNA methylation with time has led some to suggest that smoking, diet, and other epidemiologic risk factors may influence these epigenetic changes.⁴⁷⁻⁵⁰

Gender. Many studies have found that global DNA methylation was higher in males than in females.^{17,21,33,37,38,39,46} For example, total 5-mC content in WBC was higher in males than females in one study,³³ but this result was not replicated in a larger study measuring 5-mC.¹⁸ Of the repetitive elements, LINE-1 has been investigated more frequently and was associated with lower levels in females compared to males in most^{17,21,37-39,46} but not all studies.⁴¹ In contrast, there has been no consistent pattern associated with Alu methylation.^{37,41,46}

A genome-wide study of DNA methylation profile among twins and healthy singletons independently found that all CpG sites on the X chromosome were highly methylated in blood DNA from females, consistent with X-inactivation.^{44,51} A gender-dependent

Table 1. Risk factors investigated in studies of WBC DNA global methylation by assay type

	Some support for lower global methylation	Little to no support of an association with global methylation	Some support for higher global methylation
Demographics			
Age	%5-mC ³³ [³ H]-methyl ³⁵ LINE-1 ³⁶ Alu ^{34,36,37,41}	%5-mC ¹⁸ LINE-1 ^{17,21,37-41}	
Gender (Females versus Males)	%5-mC ³³ LINE-1 ^{17,21,37-39,46} Alu ⁴⁶	%5-mC ¹⁸ LINE-1 ⁴¹ Alu ⁴¹	LUMA ⁴⁶ Alu ³⁷
Race (Black versus White)	[³ H]-methyl ⁵³ LINE-1 ³⁹	CCGG ⁵⁴	LINE-1 ²¹
Environmental factors			
Benzene	LINE-1 ⁵⁵ Alu ⁵⁵		
Persistent Organic Pollutants	LINE-1 ⁵⁷ Alu ^{41,57}	LINE-1 ⁴¹	
Lead	LINE-1 ^{58,59} Alu ⁵⁸	Alu ⁵⁹	
Arsenic			[³ H]-methyl ³⁵
Air Pollution	LINE-1 ⁶²⁻⁶⁴ Alu ^{62,64}	Alu ⁶³	
Behavioral Risk Factors			
Adult Active Cigarette Smoking		%5-mC ^{18,20} CCGG ⁶⁸ LINE-1 ^{17,21,37,41,57,69} Alu ^{37,41,57}	
Prenatal Cigarette Smoking	LINE-1 ⁷³ Sat2 ⁷³	Alu ⁷³	[³ H]-methyl ⁵³
Alcohol Drinking	Alu ³⁷	LINE-1 ^{21,37,41,79} Alu ⁴¹	CCGG ^{77,78}
Higher Body size	LINE-1 ⁸⁰	LINE-1 ^{37,41,79} Alu ^{37,41}	
Higher Physical Activity			LINE-1 ⁷⁹
Low Dietary Folate		%5-mC ^{18,81} LINE-1 ^{21,79} Alu ⁷⁹	

difference in gene-specific methylation in blood has also been reported for *CALCA*, *MGMT*, *MTHFR*, *MAOA*, *DRD4*, *SERT* and *F8*.^{42,46,52} Folate is essential for erythrocyte formation and development and, because menstruation regularly depletes their supply of erythrocytes, females may tend to have lower levels of circulating folate,²¹ potentially explaining some differences in gender levels of DNA methylation.

Race and ethnicity. Only a few studies have examined the association between race/ethnicity and DNA methylation in WBC.^{21,39,53,54} Among the repetitive elements, only LINE-1 has been investigated, with whites having higher levels of LINE-1

methylation compared with Hispanic and non-Hispanic black groups in one study,³⁹ and the opposite finding in another study.²¹

Environmental factors. Several studies in humans have established an association between DNA methylation and environmental factors, including benzene, persistent organic pollutants, lead, arsenic and air contaminants.

Benzene. Low-dose benzene exposure was associated with decreased methylation of LINE-1 and Alu sequences, and with hypermethylation of *p15* and hypomethylation of *MAGE-1*.⁵⁵ In 11 patients with benzene poisoning, the average methylation level of *p16*, but not of *p15*, was higher than in a control group.⁵⁶

Table 2. Risk factors investigated in studies of gene-specific DNA methylation

Some support for gene-specific methylation		Little to no support of an association with gene-specific methylation
Demographics		
Age	<i>PKDIP2</i> ³⁴ <i>GUK1</i> ³⁴ <i>CALCA</i> ⁴² <i>MGMT</i> ⁴²	<i>H19</i> ⁸² <i>IGF2</i> ⁸²
Gender (Females versus Males)	<i>MAOA</i> ⁵² <i>CALCA</i> ⁴² <i>MTHFR</i> ⁴² <i>MGMT</i> ⁴² <i>DRD4</i> ⁵² <i>SERT</i> ⁵² <i>F8</i> ⁴⁶	<i>H19</i> ⁸² <i>IGF2</i> ⁸²
Environmental factors		
Benzene	<i>p16</i> ⁵⁶ <i>p15</i> ⁵⁵ <i>MAGE-1</i> ⁵⁵	<i>p15</i> ⁵⁶
Air pollution	<i>NOS2</i> ⁶²	
Arsenic	<i>p16</i> ^{60,61} <i>p53</i> ⁶⁰	
Behavioral Risk Factors		
Cigarette Smoking	<i>F2RL3</i> ¹⁰	<i>COMT</i> ⁷² <i>MAOA</i> ⁷¹
Prenatal Cigarette Smoking	<i>IGF2</i> ⁷⁵ <i>BDNF</i> ⁷⁴	
Alcohol Drinking	<i>HERP</i> ⁷⁸	

Persistent organic pollutants. Two studies evaluated the relation between plasma persistent organic pollutant (POP) concentrations and WBC global DNA methylation.^{41,57} In 70 Greenlandic Inuit, a population presenting some of the highest reported levels of POP worldwide, a significant inverse linear relationship was found for DDT, DDE, β -hexachlorocyclohexane, oxychlorane, α -chlordane, mirex, several PCBs and the sum of all persistent organic pollutants with Alu methylation.⁵⁷ However, as the exposure levels in Greenlandic Inuit are much higher than those in the US general population, it is unclear whether the findings apply to other populations with lower exposures. A study in Korea⁴¹ found that most organochlorine (OC) pesticides were inversely and significantly associated with Alu methylation. The strongest OC pesticide associations with methylation were observed with oxychlorane, trans-nonachlor and DDE. Most PCBs and PBDEs showed non-significant inverse trends with Alu methylation. In this same study, POPs were not associated with LINE-1 methylation.⁴¹

Lead. Prenatal maternal lead exposure, measured as maternal tibia and patella lead, was inversely associated with genomic WBC methylation (Alu and LINE-1) in 103 umbilical cord samples from a birth cohort in Mexico City.⁵⁸ Patella, tibia and blood lead

were measured in 517 male participants of the Normative Aging Study and an inverse association was found between patella lead levels and LINE-1 methylation, but not with Alu methylation.⁵⁹

Arsenic. Using the methyl acceptor assay, an unexpected dose-dependent hypermethylation of WBC DNA was found in Bangladeshi adults with chronic arsenic exposure.³⁵ This effect was modified by folate, suggesting that arsenic-induced increases in DNA methylation were dependent upon methyl availability. Arsenic has been associated with gene-specific hypermethylation of *p53* and *p16* promoter regions in blood DNA of subjects exposed to toxic level of arsenic compared to controls.⁶⁰ Hypermethylation of the *p16* promoter was also observed in WBC DNA from 103 patients with arseniasis compared to 110 healthy subjects.⁶¹

Air pollution. The effects of particulate matter (PM) exposure on Alu, LINE-1 and gene-specific methylation was examined in steel plant workers with well-characterized exposure to PM with aerodynamic diameters <10 μ m (PM10).⁶² Long-term exposure to PM10 was negatively associated with methylation in both Alu and LINE-1.⁶² Exposure to black carbon (BC), a marker of traffic particles, was also associated with decreased DNA methylation in LINE-1.⁶³ Another study in the same

cohort looked at the association for six months of exposure to air pollution (PM_{2.5} and two components, BC and sulfate) and DNA methylation. Prolonged exposure to BC and sulfate particles was associated with hypomethylation of both LINE-1 and Alu repetitive elements.⁶⁴ *NOS2* promoter methylation was significantly lower in post-exposure blood samples compared to baseline.⁶²

Behavioral factors. Smoking. The cellular modifications resulting from exposures to the chemicals present in cigarette smoke have been widely investigated and include DNA adducts, gene mutations, micronuclei, chromosome aberrations, sister chromatid exchanges and DNA strand breaks (reviewed in ref. 65). It has also been proposed that DNA methylation changes could result from this exposure. The mechanisms through which smoking might lead to epigenetic changes are not clearly understood. Some research has shown that smokers have lower levels of blood folate than non-smokers.⁶⁶ Additionally, DNA methyltransferases have been found to bind DNA at sites of DNA damage, which results in altered methylation patterns on these regions, suggesting another molecular mechanism for the generation of aberrant DNA methylation by exposures to chemicals such as those present in cigarettes.⁶⁷ Studies looking at genomic DNA methylation levels have not found an association between cigarette smoking and variations in different global DNA methylation measurements. Only a few studies have investigated overall 5-mC content and smoking and found no difference by cigarette smoking patterns.^{18,20,68} More research has focused on the effect of smoking on the methylation levels of repetitive elements. LINE-1, Alu and AluYb8 methylation did not differ between current and never/former smokers.^{17,21,37,41,57,69} Some studies recruited small number of subjects; however, the overall consistency of the results suggests that genomic DNA methylation levels are not affected by active smoking exposure in adulthood.

Research focusing on DNA methylation at specific genomic regions has found differential effects of smoking status on these epigenetic marks. A discovery study looked at blood DNA of smokers and non-smokers using the Illumina Infinium HumanMethylation27 array, allowing for the identification of specific loci that showed significant differences between smokers and non-smokers.¹⁰ Using two different analytical methods, the investigators isolated *F2RL3*, a gene that encodes thrombin protease-activated receptor-4 (PAR-4), as the most relevant epigenetic modification resulting from smoking exposure. In the same study, they were able to replicate this finding in a different population. Along with PAR-1, PAR-4 is essential in thrombin-mediated platelet aggregation and overall maintenance of vascular integrity.⁷⁰ This locus had not been previously studied in relation to smoking and its discovery can be a useful step in identifying proteins involved in smoking induced molecular changes. In a study looking at differences between male and female smokers, analysis of methylation of the *MAOA* 5' untranslated region in lymphocytic cell lines (LCL) and blood from smokers and non-smokers found that while males had lower levels of LCL methylation at this locus, females only showed a difference in blood.⁷¹ In a study of African-Americans, *COMT* promoter

methylation levels did not differ by smoking status. However, in a more detailed analysis statistically significant differences were uncovered for position -193 of this promoter. To further understand this association, the authors replicated the study in another set of individuals finding consistent results.⁷²

It has been suggested that chemicals affecting epigenetic marks could have a larger impact when exposure occurs in utero or earlier in life, a time when epigenetic modifications are being established. Overall levels of genomic DNA methylation have been investigated in several studies in children and adults. Prenatal exposure to cigarette smoke has been associated with an increase in the overall blood levels of DNA methylation in adulthood.⁵³ Other studies have focused on individual repetitive element methylation. LINE-1, Sat2 and Alu methylation was measured in adults and children prenatally exposed to smoking. LINE-1 and Sat2 levels were lower in exposed individuals when compared to unexposed ones.⁷³ However, other elements such as LINE-1 do not seem to be affected by exposure to cigarette smoke. Specific sequences have also been identified in association with in utero smoking exposure. The 5' untranslated region of the *BDNF* promoter was hypermethylated in a group of exposed young adults.^{74,75} Several genes were investigated in blood samples from adults in a cohort of individuals born preterm that were exposed to smoking, with lower levels found for *IGF2* methylation. However, no associations were found for *GNASAS*, *INSIGF* and *LEP*.⁷⁵ Similarly, environmental tobacco smoke (ETS) exposure in childhood has been associated with methylation changes in adult life. ETS exposure was found to be related to an increased methylation in Sat2.⁷³ It is important however to consider that in utero and childhood ETS exposure information is collected retrospectively in most studies, which can limit the validity of these findings.

Alcohol. Alcohol consumption may decrease DNA methylation in hepatic tissue by disrupting folate metabolism and/or methionine synthesis, which might decrease levels of the universal methyl donor S-adenosylmethionine (SAM).⁷⁶ Excessive alcohol consumption has been shown to alter DNA methylation in humans. Compared with controls, patients suffering from alcoholism had 8–10% higher methylation in CCGG sequences.^{77,78} Associations between alcohol drinking and repetitive element DNA methylation levels have shown inverse associations between Alu and alcohol drinking.³⁷ However, several studies did not find an association between alcohol consumption and LINE1 methylation.^{21,37,41,79} Gene-specific DNA methylation has not been widely investigated in association with alcohol consumption. One study found a significant increase in *HERP* (homocysteine-induced ER protein) promoter methylation in patients with alcohol dependence.⁷⁸

Body mass index. Studies investigating the association between DNA methylation and body mass index (BMI) are limited to measures of repetitive element methylation. A large study carried out by Zhu et al. found that in WBC DNA, LINE-1 and Alu methylation levels were not correlated with BMI.³⁷ Similar results have been reported in two other studies, one investigating LINE-1 and Alu levels and another one looking at LINE-1 only.^{41,79} In contrast, in a study of women of child bearing age,

Piyathilake et al. found that higher BMI was associated with lower LINE-1 methylation.⁸⁰

Physical activity. Only one study has investigated the association between physical activity and DNA methylation. Using a cross-sectional design, Zhang and colleagues reported a trend of higher levels of LINE-1 methylation with higher levels of physical activity.⁷⁹

Dietary folate. Folate is required for the synthesis of methionine, a precursor of the universal methyl donor SAM. SAM provides methyl groups in a variety of biochemical reactions, including methylation of DNA. One would expect that a deficiency of methyl groups in the diet might lead to hypomethylation of DNA in several tissues, including blood. However, associations between global DNA methylation in blood and dietary folate and plasma levels of folate have not been generally detected in observational studies. Serum folate, red blood cell 5-methyltetrahydrofolate, red blood cell non-methylfolate and red blood cell total folate were not associated with 5-mC content in blood.⁸¹ Another study did not observe any association between 5-mC content in blood and nutrients including folate, vitamin B1, B2, B3, B12 and total protein.¹⁸ Individuals with lower dietary folate showed no difference in LINE-1 methylation compared with individuals with higher dietary folate intake.^{21,79} Although there is little data to date investigating dietary factors and gene-specific WBC methylation, there was an intriguing report studying maternal diet, as measured by exposure to famine, and gene-specific methylation patterns in *IGF2*.⁸² Although this study investigated overall maternal undernutrition, the association was robustly tested using both time and sibling controls and provides evidence that it may be important to consider the timing of exposures when examining DNA methylation patterns.

Studies Examining Changes in WBC DNA Methylation after Risk Factor Changes

Given the limitations arising from drawing inferences from cross-sectional studies, reports that investigate whether changes in exposures are related to changes in DNA methylation patterns are particularly compelling. For example, a randomized estrogen replacement therapy trial in postmenopausal women found that conjugated equine estrogen was associated with a statistically significant increase in percentage of 5-mC in peripheral mononuclear cells compared with women provided with placebo,⁸³ though there was no difference in promoter methylation in the *ER α* , *ER β* and *p16*.⁸³

Changes in global WBC methylation from folate intake have been more frequently investigated in controlled feeding or randomized placebo-controlled intervention trials with seven studies reported, primarily using the [³H]-methyl acceptor assay.^{54,84-89} The controlled feeding studies start with a folate depletion diet of around 120 $\mu\text{g}/\text{day}$ for, most frequently, 7 weeks. This is then followed by high doses ranging up to 800 $\mu\text{g}/\text{day}$. In the five controlled feeding studies, only one found a significant decrease in WBC methylation during the depletion phase (See Table 3 for summary). During supplementation, two studies found an increase in methylation.^{84,87} Two double-blind randomized trials

used doses as high as 2,000 $\mu\text{g}/\text{day}$, but no significant change was observed.^{85,88} These studies are limited by the relatively small number of subjects, which ranged from 8 to 61, and the relatively short time frames of the trials.

Methodological Challenges

The main challenge in making inferences from studies examining risk factor associations and DNA methylation is that most of the studies are cross-sectional in nature and mainly focus on risk factors later in life. With cross-sectional studies it is difficult to understand the direction of the association and causality cannot be determined. Studies focusing on early life factors such as prenatal smoke exposures and prenatal famine exposure are few but intriguing, in that they suggest that some early life factors may have persistent associations with global and gene-specific methylation patterns in adulthood.^{53,73,74,75,82} Prospective studies, like the folate intervention studies described above, which can investigate a contrast or change in exposure and its association with a change in DNA methylation levels, can provide particularly compelling data. Another challenge with most of the existing studies is that many of them are small in sample size, which limits the ability to examine effect modification with any precision. For example, *MTHFR* 677TT has been investigated as a potential modifier between folate and DNA methylation, but the results were not consistent.^{87,89} These studies point to the importance of considering potential modifying effects of genes in one-carbon metabolic pathways when investigating the effect of folate on DNA methylation, but also reveal the need for studies of sufficient sample size as well as a more systematic approach to the evaluation of gene-environment interactions when considering risk factor associations with DNA methylation levels.

Source of WBC

Using data from multiple DNA sources from the same individuals, we have observed variation in genomic DNA methylation within specific WBC types.⁹⁰ 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 investigating DNA methylation in WBC. It is well known that there are differences in total WBC counts in healthy individuals, with a range of 5,000–10,000/ μl , but also differences in cell populations. No association was found between the total number of WBC and either Alu or LINE-1 methylation, or between the differential blood count and Alu methylation.³⁷ Percent neutrophils and lymphocytes exhibited a positive and negative association with LINE-1 methylation, respectively.³⁷ It is also clear that during acute infections or inflammation and in cancer, an abnormally large increase in the number of WBCs in blood (leukocytosis) is observed. Other illnesses, such as neutrophilia, lymphocytosis and granulocytosis, target specific types of WBC. Drugs such as NSAIDs can also cause leukocytosis. Exercise has impacts on neutrophilia,⁹¹ while a meta-analysis found that depression and stress (e.g., a standard life event scale for naturalistic stressors or an

Table 3. Folate intervention studies

Author	Subjects	Study	Folate Intake	Duration	Analysis Method	Change from Baseline
Jacob et al. ⁸⁴	8 F (49–63 years)	Controlled feeding study	56–510 µg/d	91 days	[³ H]-methyl	Significant decrease during depletion Significant increase during supplementation
Fenech et al. ⁸⁵	34 M 30 F (18–35 years)	Double-blind, randomized, placebo-controlled	700 µg/d	12 weeks	[³ H]-methyl	No significant change
			2,000 µg/d	12 weeks		No significant change
Rampersaud et al. ⁸⁶	33 F (60–85 years)	Controlled feeding study	118 µg/d	7 weeks	[³ H]-methyl	Significant decrease during depletion
			200 or 415 µg/d	7 weeks		12 weeks
Shelnutt et al. ⁸⁷	41 F (20–30 years)	Controlled feeding study	115 µg/d	7 weeks	[³ H]-methyl	No significant change
					5-mC	No significant change
					[³ H]-methyl	No significant change
			400 µg/d	7 weeks	5-mC	Significant increase during supplementation only in MTHFR 677TT genotype
Basten et al. ⁸⁸	30 M 31 F (20–60 years)	Double-blind, randomized, placebo-controlled	1,200 µg/d	12 weeks	[³ H]-methyl	No significant change
Axume et al. ⁵⁴	28 F (18–45 years)	Controlled feeding study	135 µg/d	7 weeks	CCGG methylation	No significant change
			400 or 800 µg/d	7 weeks		No significant change
Axume ⁸⁹	43 F (18–45 years)	Controlled feeding study	135 µg/d	7 weeks	CCGG methylation	No significant change
			400 or 800 µg/d	7 weeks		Significant decrease during supplementation only in MTHFR 677TT genotype

*p < 0.05.

experimental stressor) affects cell numbers and ratios.⁹² 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.⁹³ Martin⁹⁴ suggested that definitive analyses require specific methodologies to account for shifts in cellular population heterogeneity.

Most studies of DNA methylation in blood have been carried out using total WBC isolated from the simple centrifugation of blood and collection of the “buffy coat”, since it is the easiest process and provides the greatest amount of DNA. Centrifugation of blood over a density gradient allows the separation of the more abundant granulocytes (~85% of total WBC) from the mononuclear cells, which are comprised of monocytes and lymphocytes. Many publications refer to the mononuclear cell fraction as lymphocytes even though they contain ~15%

monocytes. The isolation of pure lymphocytes or specific lymphocyte subpopulations, requires additional steps, such as flow cytometric separation using cell surface receptor antibody binding, and is thus rarely, if ever, done in epidemiologic studies. Given the lack of cell purification in most studies and the variation in cell populations in individual samples, care should be taken in interpreting results.

Overall Summary

Epidemiologic studies investigating associations between WBC DNA methylation and different disease endpoints suggest the potential for measuring WBC DNA methylation as a biomarker of risk. Nevertheless, many studies have been retrospective in nature and require confirmation in prospective designs to ensure that the biomarker is not influenced by the disease process.

A number of risk factors have been associated with WBC DNA methylation both globally and gene-specifically, although few consistent patterns have emerged. Interestingly, when patterns exist with a given risk factor and DNA methylation levels, they appear to differ across markers (e.g., lower Alu methylation with increasing age and lower LINE-1 methylation in females). Findings that suggest correlations between early life exposure to environmental factors and DNA methylation are intriguing but need to be replicated in larger, prospective studies. Inconsistent patterns arise due to the challenges of interpreting results from different assays and from different sources of DNA. Intervention studies examining whether changes in exposures within individuals are associated with changes in DNA methylation offer a

robust way to test specific hypotheses. More data needed to support the use of monitoring biomarkers; there are still no epidemiologic studies examining whether changes in WBC methylation over time are associated with changes in disease endpoints. Large, prospective studies that focus on specific windows of susceptibility will be needed to understand whether DNA methylation patterns measured in WBC represent a useful biomarker on the pathway linking environmental exposures to disease endpoints.

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