


# DNA Methylation: An Epigenetic Risk Factor in Preterm Birth

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## Abstract

Spontaneous preterm birth (PTB; birth prior to 37 weeks of gestation) is a complex phenotype with multiple risk factors that complicate our understanding of its etiology. A number of recent studies have supported the hypothesis that epigenetic modifications such as DNA methylation induced by pregnancy-related risk factors may influence the risk of PTB or result in changes that predispose a neonate to adult-onset diseases. The critical role of timing of gene expression in the etiology of PTB makes it a highly relevant disorder in which to examine the potential role of epigenetic changes. Because changes in DNA methylation patterns can result in long-term consequences, it is of critical interest to identify the epigenetic patterns associated with adverse pregnancy outcomes. This review examines the potential role of DNA methylation as a risk factor for PTB and discusses several issues and limitations that should be considered when planning DNA methylation studies.

## Keywords

prematurity, preterm labor, epigenetics, genetics, DNA

## Preterm Birth is a Global Health Challenge

The preterm birth (PTB) rate continues to rise in the United States, increasing by as much as 30% during the last 25 years. Despite advances in medical care, the World Health Organization recently estimated the global PTB rate for singleton gestation as 9.6%, which corresponds to approximately 13 million preterm babies born every year worldwide; this represents a substantial problem for overtaxed health, education, and social services.<sup>1</sup>

Of neonatal deaths between birth and 7 days not attributable to congenital conditions, 28% result from PTB.<sup>1,2</sup> Children born preterm are more likely to have cerebral palsy, sensory deficits, learning disabilities, and respiratory illnesses.<sup>1,2</sup> Lower gestational age increases the risk of asthma at 6 years of age,<sup>3</sup> and PTB increased the risk of very low birth weight children to be diagnosed with asthma at age 12.<sup>4</sup> Preterm birth also increases the risk of being hospitalized with infections during childhood.<sup>5</sup> Among school aged children, those born preterm show diminished cognitive performance, increased externalizing and internalizing behaviors, and are more likely to develop attention-deficit hypertension disorder.<sup>6</sup> Girls who were delivered preterm have a higher risk of developing anorexia nervosa,<sup>7</sup> and boys who were born small for gestational age (SGA) and preterm are more likely to develop personality and psychotic disorders or be hospitalized for mental illness compared to boys born SGA at term.<sup>8</sup>

Preterm birth and reduced fetal growth have also been linked to a number of important chronic diseases of adulthood.

Preterm birth is associated with the development of type 2 diabetes,<sup>9</sup> independent of fetal growth rates.<sup>10</sup> It is often difficult to delineate the effects of PTB from fetal growth restriction but such adverse intrauterine conditions associate with hypertension, coronary heart disease, and stroke.<sup>11-17</sup> Taken as a whole, it is clear that PTB not only imparts a difficult start to life but may also impart a considerable risk of a disease-burdened life and tremendous economic costs.<sup>1,2</sup>

The 2 major classes of PTB are spontaneous (mostly unknown etiology) and indicated (known risk factors including preeclampsia, multiple gestations, gestational diabetes, and maternal or fetal anomalies).<sup>18</sup> Studies have identified multiple risk factors for PTB including race, socioeconomic factors, psychosocial stressors, behavioral factors, maternal infection, prior history, and genetic variants.<sup>18</sup> Despite the tremendous knowledge gained over the past decade, this information has

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not been translated into screening or effective intervention and has not decreased the rate of PTB.<sup>19–21</sup> Understanding the specific initiators and effectors of the labor process will provide valuable insight into the pathophysiologic pathways that result in preterm labor and may result in novel interventions.

The etiology of PTB remains unclear, and the identification of biomarkers to predict high-risk pregnancies resulting in PTB would represent a significant advancement. In this respect, epigenetics is a particularly attractive area of investigation because of the increasing prevalence of PTB in recent years and the association of PTB with development of adult-onset diseases.

## Epigenetics and Fetal Development

Epigenetics refers to structural modification of DNA sequence that produces stable, but potentially reversible, alterations in the transcriptional potential of a cell, without changes in the DNA sequence. Cytosine methylation is one such modification that occurs at position 5' of the cytosine pyrimidine ring in CpG dinucleotides. Methylation of CpG sites in the promoter regions of genes may disrupt transcription factor binding or attract methyl-binding proteins. These changes to the promoter structure correlate with chromatin remodeling and transcriptional silencing.<sup>22</sup>

Epigenetics play an important role in fetal development. The literature on epigenetic regulation during embryogenesis has been reviewed by Shi and Wu.<sup>23</sup> Following fertilization, areas that are not protected by imprinting undergo both active and passive demethylation.<sup>24,25</sup> DNA methylation patterns then begin to differentiate by developmental stage and by tissue.<sup>26</sup> Because fetal development is a period of extensive cellular replication and growth, environmentally induced epigenetic changes may result in stable patterns of gene expression and phenotypic differences among exposed individuals.<sup>27–29</sup> In contrast, the rate of DNA synthesis is much lower during adulthood, and the epigenome may be more resilient to environmental insults.

## DNA Methylation as a Risk Factor for PTB

Studies suggest that nutritional deficiencies and imbalances, psychosocial stressors, behavioral factors such as cigarette smoking, maternal infection (bacterial vaginosis or intra-amniotic infection), and race all increase the risk of PTB,<sup>18</sup> and epigenetic mechanisms such as DNA methylation may partially underlie many of these risk factors. Therefore, a comprehensive risk model of PTB should incorporate the factors outlined in Figure. 1. Indeed, many ongoing studies are investigating their numerous interactions.

Environmental factors that alter the intrauterine environment may induce epigenetic-mediated changes in gene expression that result in increased susceptibility to adverse pregnancy outcomes.<sup>27,30</sup> In humans, differential methylation of storkhead box 1 gene (STOX1) has been associated with preeclampsia.<sup>31,32</sup> In rats, intrauterine growth restriction (IUGR) has been

associated with decreased p53 methylation, decreased CpG island and genome-wide methylation, and decreased expression of DNA methyltransferase 1 (*DNMT1*).<sup>33,34</sup> *DNMT1* is a maintenance methyltransferase that plays a role in placental development and can disrupt this process in the first trimester of pregnancy.

A review by Suter and Aagaard-Tillery<sup>12</sup> discussed the environmental factors that can influence the epigenome during pregnancy, and indeed several studies have supported that environmental risk factors influence DNA methylation patterns during pregnancy. For example, animal studies show that insulin growth factor 2 (*IGF2*) imprinting in the placenta is altered by *Campylobacter rectus* infection during pregnancy,<sup>35</sup> and it is likely that methylation patterns may differ depending on the type of infectious agent.

Diet can also modify the epigenome, and deficiency of methyl donors such as folate is directly correlated with changes in DNA methylation.<sup>36–40</sup> Several studies have demonstrated the impact of maternal diet on offspring outcomes via epigenetic mechanisms. In a mouse model, a maternal high-fat diet increased the risk of offspring obesity by altering offspring preference for palatable foods via changes in DNA methylation and messenger RNA (mRNA) expression of dopamine and opioid-related genes.<sup>41</sup> Using a protein restriction model in rats, Sohi et al<sup>42</sup> recently reported that epigenetic changes mediate the association between maternal undernutrition and offspring cholesterol dysregulation. Consistent with this finding, IUGR, induced by a low-protein maternal diet through pregnancy and lactation, decreases the ratio of offspring liver to body weight and results in higher levels of hepatic cholesterol; the authors concluded that this change in circulating cholesterol associated with histone modifications in the promoter region of *Cyp7a1* associated with chromatin silencing and reduced *Cyp7a1* expression.<sup>42</sup> Modifications of gene expression at imprinted loci and global and local placental DNA methylation patterns are also reported to result from a high-fat diet.<sup>43</sup> These reports underscore the importance of a balanced diet during pregnancy to avoid epigenetic modifications that can contribute not only to pregnancy complications such as IUGR or PTB but also to long lasting effects in the offspring.<sup>44</sup>

Smoking has also been linked to PTB, and 1 study found suggestive evidence that genome-wide methylation is decreased in adults who were exposed prenatally to cigarette smoke.<sup>45</sup> In placental samples obtained from smokers and nonsmokers, prenatal tobacco exposure was associated with hypomethylation of the *CYP1A1* promoter region and increased *CYP1A1* expression.<sup>46</sup> Recent data suggest a potential mechanism for these changes as both markers of oxidative stress and apoptosis are increased in normal human fetal membrane cultures that were exposed to cigarette smoke extracts.<sup>47</sup>

The burden of PTB is disproportionately concentrated in Africa and Asia and is especially high for those of African descent in developed countries.<sup>1</sup> Pregnancies in which either parent is of African descent are at increased risk of PTB,<sup>48</sup> and this effect appears to be largely independent of measured environmental risk factors.<sup>49</sup> Consistent with the hypothesis that racial

disparity in PTB may result from genetic or epigenetic changes, differences in genetic polymorphisms have been associated with PTB as well as biomarkers of infection and inflammation associated with PTB (tumor necrosis factor- $\alpha$  and interleukin 6 concentration) among African American and Caucasian mothers.<sup>50–53</sup> Further, DNA methylation patterns vary in the umbilical cord blood of African American newborns.<sup>54</sup>

Although epidemiologic studies support the association of several environmental risk factors with adverse pregnancy outcome, no studies to date have directly examined DNA methylation as a molecular mediator between the environmental risk factors and PTB. Such studies will be required to determine whether epigenetic changes play a role in the relationship between environmental risk factors and PTB.

## Developmental Origin of Adult Health and Diseases

The work of Barker and colleagues,<sup>15,16</sup> who observed that impaired placental growth was associated with chronic heart failure in later life, stimulated interest in the hypotheses known as the developmental origins of adult health and diseases hypothesis. Environmental insults during prenatal development, such as restricted maternal nutrition, can result in growth restrictions and long-term consequences for affected offspring, and epigenetic changes may serve as mediators between environmental insults and the development of adult-onset diseases. Support has been provided by plant and animal studies that document epigenetic inheritance in the context of their potential contribution to adult health and diseases.<sup>55–57</sup>

Studies in rats have shown that nutritional deprivation during pregnancy results in intrauterine and postnatal growth restriction.<sup>58,59</sup> Notably, IUGR pups born to food-restricted mothers but cross-fostered by normally fed mothers demonstrated accelerated growth that resulted in increased weight and body fat, suggesting that restricted maternal nutrition may contribute to obesity in later life.<sup>58,59</sup>

Similarly, among people born during the Dutch famine, exposure to famine during early gestation was associated with coronary heart disease, a more atherogenic plasma lipid profile, disturbed blood coagulation, increased stress responsiveness and higher rates of obesity. Those exposed to famine in midway through gestation had increased rates of microalbuminuria and obstructive airways disease, and those exposed to famine at any stage of gestation were more likely to have impaired glucose tolerance.<sup>60</sup> These findings show that maternal undernutrition during gestation affects health in later life, but these effects are dependent on the timing of the exposure. While timing of the exposure is clearly important, these studies collectively suggest that epigenetic modifications resulting from such intense stressors may result in long-term consequences for an individual.<sup>60,61</sup>

Evaluating the impact of early environmental exposures on the fetal epigenome may offer insight into both regulatory processes inherent in development and adult-onset diseases. Exposure to environmental chemicals can alter the fetal

epigenome, thereby changing gene expression patterns.<sup>29</sup> Neonatal exposure to both estradiol and bisphenol A, an additive for many plastics including food containers and baby bottles, may cause multiple gene-specific changes in DNA methylation in the rat prostate, including hypomethylation of the phosphodiesterase type 4 variant 4 (*PDE4D4*), which has been associated with prostate cancer risk.<sup>62</sup> Also, prenatal exposure to diethylstilbestrol (DES) results in hypomethylation in 2 critical DNA control regions.<sup>63</sup> Individuals treated with DES during pregnancy had increased rates of reproductive disorders and clear cell adenocarcinoma of the vagina,<sup>63</sup> and both DES-exposed mothers and their children were more likely to report lupus, arthritis, asthma, and respiratory tract infections in the 1985 National Health Interview Survey.<sup>64</sup>

Direct evidence implicating environmentally induced DNA methylation changes as a causal factor in PTB has yet to emerge. While the purpose for this review is to promote this line of research, future research will be required to delineate whether DNA methylation changes represent a cause or consequence of PTB.

## A Widening Perspective on DNA Methylation

While many studies to date have focused on the examinations of 5-methylcytosine (5mC), few studies have been able to distinguish 5mC from its modification, 5hmC. 5-Hydroxymethylcytosine plays an intermediate role in oxidative demethylation pathways<sup>65</sup> and is integral in the self-renewal and maintenance of embryonic stem cells<sup>66</sup> from several mammalian species.<sup>67</sup> 5hmC is associated across the genome with increased transcription, and variations in expression due to 5hmC may influence the processes of pluripotency and lineage commitment.<sup>68</sup>

Similarly, while most conventional studies of methylation focus on CG methylation, almost 25% of DNA methylation in embryonic stem cells occurs at non-CG dinucleotides. This non-CG methylation is enriched in gene bodies and depleted in protein-binding sites and enhancers. It is also enriched near genes involved in pluripotency and differentiation. However, non-CG methylation is not evident after cellular differentiation occurs.<sup>69</sup>

Although 5hmC and methylation of non-CG dinucleotides have yet to be extensively evaluated in the context of human disease, the enrichment and likely regulatory roles of 5hmC and non-CG methylation in embryonic stem cells suggest that they could be relevant to early-life and developmental disorders. Hence, both 5hmC and non-CG methylation represent a previously unexplored aspect of epigenetic regulation that may contribute to PTB.

## Genome-Wide Epigenetic Studies

Methods to conduct DNA methylation studies have been reviewed extensively,<sup>70,71</sup> so here we will focus on the evolving field and the issues to consider before undertaking such studies. As with studies of sequence variants, the initial foray

into examining epigenetic changes associated with PTB has focused on specific candidate genes. These studies are dependent on the choice of candidate genes and may lead to findings that have small effect sizes and do not replicate consistently. However, the recent availability of inexpensive commercial methylation microarrays has facilitated genome-wide methylation studies, which have already met with some success.<sup>72-75</sup> Although genome-wide studies of DNA methylation have much in common with both genetic association studies and gene expression studies, at this early stage a consensus has not been reached about the optimal study design or analysis methods for methylation studies.

The appropriate analytical approaches for methylation studies will depend on the technology used. For array-based platforms that rely on bisulfite treatment of DNA such as the Illumina GoldenGate and Infinium chips that provide estimates of the methylation proportion with single-CpG resolution, it is common to perform simple tests of association between the methylation proportion and the phenotype, adjusting for multiple testing via Bonferroni or false discovery rate procedures. Platforms that enrich methylated DNA through the use of methylation-specific restriction enzymes (McrBC) or methylated DNA immunoprecipitation (MeDIP) require more complex statistical approaches to estimate the absolute methylation level while accounting for sequence-dependent biases due to CpG density and other factors. To address this, strategies that model the dependence of enrichment on CpG density have been proposed for MeDIP<sup>76,77</sup> and McrBC approaches.<sup>78</sup>

Although the basic quality control criteria leading to the removal of specific data points, samples, or CpG sites are standard for commercially available methylation arrays, there is currently no consistently used approach to normalization of methylation data. Methods to normalize signals abound in the gene expression literature,<sup>79,80</sup> but these methods are based on the assumption of no global differences between samples, which is often inappropriate for methylation data.<sup>78</sup> The absence of differences in the overall signal is generally thought to be an appropriate assumption for expression data, but global differences may be observed in methylation data when comparing cancer versus healthy tissue,<sup>81</sup> different tissue types,<sup>82</sup> or even individuals of different ages.<sup>83,84</sup> If global methylation patterns vary with respect to the phenotype studied, the application of standard normalization methods such as Loess and quantile normalization could drastically reduce power by removing true differences between samples and thus are likely inappropriate for analysis of methylation data. The use of subset quantile normalization,<sup>85</sup> which equalizes the quantiles of negative control probes instead of the signal probes, has been proposed for methylation microarray data.<sup>78</sup> However, subset quantile normalization requires a large number of negative control features whose signals cover the entire range of the signal probes; hence, for experiments with only a small or moderate number of negative control probes, the development of further approaches would be valuable.

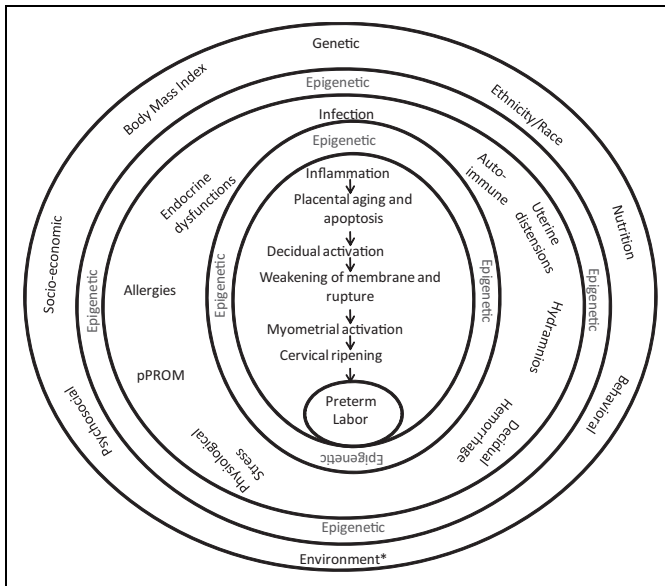
Population stratification, a well-documented issue in studies of sequence variants,<sup>86</sup> is also likely to impact the studies of

DNA methylation. Recent studies have reported differential methylation of genes between African American and Caucasian umbilical cord blood samples,<sup>54</sup> and evaluation of participants of varying ancestry suggest that genetic and epigenetic variation may be somewhat correlated.<sup>87</sup> It is well known that the frequency of sequence-based polymorphisms varies across different ethnic backgrounds, and the similarity of methylation patterns in families<sup>83</sup> and within twin pairs<sup>88</sup> suggests that sequence variation may impact methylation patterns. In fact, evidence of allele-specific methylation has been reported<sup>89-91</sup> and may be partially responsible for differences in methylation patterns associated with race or disease state. These results emphasize the importance of controlling for ancestry in epigenetic studies as well as in studies of sequence variants, ideally by careful ancestry-matching of cases and controls as part of the study design and inclusion of covariates representing self-reported or genetic ancestry.

Cell type heterogeneity between samples is an issue analogous to population stratification that may affect both gene expression and methylation studies but not studies of sequence variants. The cell type composition of blood is known to be altered in a variety of disease states<sup>92-97</sup> and can vary with other health-related phenotypes such as exercise level<sup>98</sup> and obesity.<sup>99</sup> Differences in methylation between cell types have been reported for specific genes<sup>100,101</sup> and genome-wide.<sup>102</sup> Because cell type composition is correlated with both individual health and methylation, the association between DNA methylation and an outcome of interest may be confounded by variations in the proportion of cell types within a sample or tissue. Hence, it is important to control for cell type differences within a study; however, attempts to sort tissues into subpopulations may limit the amount of DNA available for the study. As an alternative, the development of statistical approaches to account for differences in cell type proportions across samples would be valuable.

Because DNA methylation varies with cell type, it is important to select a relevant tissue in which to study PTB. Placental tissue, fetal membranes, and umbilical cord blood have obvious biological relevance and can be obtained through noninvasive methods. Umbilical cord blood has the added advantage of being amenable to longitudinal studies in which blood sampling may occur at birth and then regularly over development. However DNA samples obtained from saliva are highly heterogeneous with the proportions of leukocytes and epithelial cells ranging from 20% to 80% among individuals. The sample volumes, especially from infants, may be too limited for cell-sorting methods so this may not be a feasible approach until statistical approaches are available to adjust for cell type proportions in different samples. As both maternal and fetal factors can contribute to PTB, one should also consider sampling maternal blood samples, cervical or myometrial tissues.

Another important confounding variable in DNA methylation studies is age. Recent studies have reported widespread age-related methylation differences at CpG sites across the genome.<sup>72,83,84,103</sup> These results underscore the importance of



**Figure 1.** This figure provides a comprehensive schema of the processes that impact preterm birth (PTB) from a general level (outer circle) to the final steps (inner circle). These factors can potentially function independently or can interact to affect PTB. Each risk factor operates via multiple related clinical manifestations (middle circle), encompassing overlapping and often redundant biomolecules, culminating in a final effector cascade (innermost circle). Preterm birth is effectively a syndrome of multiple disease processes. These risk factors and disease processes are mediated by immune and inflammatory processes that produce uterotonins (Prostaglandins - PGs) and stimulate decidual activation, myometrial contractility, fetal membrane weakening and rupture, and cervical ripening and dilatation, culminating in premature labor and delivery.<sup>5</sup> These pathologic processes are initiated by well-characterized risk factors (outer circle). \*The environment alone may consist of multitude of exogenous factors not limited to a single factor. Environmental risk factors may lead to epigenetic changes, resulting in long-term alterations in gene expression that increase the risk of adult-onset diseases. Epigenetic changes can also occur as a result of risk associated clinical manifestations and also due to interventions, if any, at this stage. Unlike static genetic risk factors, epigenetic changes can happen at multiple levels as indicated in the figure.

matching for age in case-control studies of DNA methylation. To avoid confounding due to differences in the age distribution, cases and controls should be matched by age whenever possible. Adjustment for age as a covariate is also useful but may not be sufficient if the age distributions differ substantially between cases and controls. Moreover, if age is correlated with case-control status, its inclusion as a covariate may reduce power. Conversely, in well-matched samples, age will be independent of the phenotype of interest, so including age as a covariate can lead to increased power since it will explain the methylation differences that would otherwise be attributed to noise.

## Summary

Preterm birth is a complex disease with many known epidemiologic and genetic risk factors, and awareness of the impact of

prenatal conditions on an individual's long-term health has focused more attention on PTB. Epigenetic changes induced by various risk factors for pregnancy may influence the risk of PTB or induce changes in the fetal epigenome that predispose a neonate to adult-onset diseases. Although epigenetic changes, such as DNA methylation, have been associated with many complex disease outcomes, their potential influence on PTB remains unexplored. Although many epidemiologic factors as listed in Figure 1 are associated with PTB, a link between these factor-induced DNA methylation changes and its association with adverse pregnancy outcome or long-term consequences during the life course are still lacking. Animal model studies clearly support the rationale for such phenomena and motivate future research in this area. Future PTB studies should focus on genome-wide epigenetic changes in human pregnancies, ideally in the context of longitudinal designs to assess the role played by DNA methylation in both short- and long-term outcomes related to PTB.

This review is meant to provide an overview of the potential role of DNA methylation in adverse pregnancy outcomes as well as to discuss the issues and limitations that should be considered when planning DNA methylation studies. Evaluating the potential role of epigenetic changes in PTB is a timely goal, and it is hoped that this review will aid researchers who are contemplating or designing these studies.

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