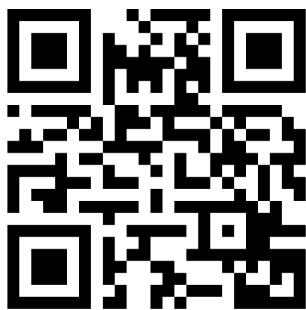REVIEW

Altered placental DNA methylation patterns associated with maternal smoking: current perspectives

Jennifer ZJ Maccani
Matthew A Maccani

Penn State Tobacco Center of Regulatory Science, College of Medicine, Department of Public Health Sciences, Hershey, PA, USA

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Correspondence: Jennifer ZJ Maccani
Tobacco Center of Regulatory Science, Public Health Sciences Department, Suite PSHCI T3425, 500 University Drive, PO Box 850, Hershey, PA 17033-0850, USA
Tel +1 717 531 0003 ext 289575
Fax +1 717 531 0480
Email jmaccani@phs.psu.edu

Abstract: The developmental origins of health and disease hypothesis states that adverse early life exposures can have lasting, detrimental effects on lifelong health. Exposure to maternal cigarette smoking during pregnancy is associated with morbidity and mortality in offspring, including increased risks for miscarriage, stillbirth, low birth weight, preterm birth, asthma, obesity, altered neurobehavior, and other conditions. Maternal cigarette smoking during pregnancy interferes with placental growth and functioning, and it has been proposed that this may occur through the disruption of normal and necessary placental epigenetic patterns. Epigenome-wide association studies have identified a number of differentially methylated placental genes that are associated with maternal smoking during pregnancy, including *RUNX3*, *PURA*, *GTF2H2*, *GCA*, *GPR135*, and *HKR1*. The placental methylation status of *RUNX3* and *NR3C1* has also been linked to adverse infant outcomes, including preterm birth and low birth weight, respectively. Candidate gene analyses have also found maternal smoking-associated placental methylation differences in the *NR3C1*, *CYP1A1*, *HTR2A*, and *HSD11B2* genes, as well as in the repetitive elements LINE-1 and AluYb8. The differential methylation patterns of several genes have been confirmed to also exhibit altered gene expression patterns, including *CYP1A1*, *CYP19A1*, *NR3C1*, and *HTR2A*. Placental methylation patterns associated with maternal smoking during pregnancy may be largely gene-specific and tissue-specific and, to a lesser degree, involve global changes. It is important for future research to investigate the mechanistic roles that these differentially methylated genes may play in mediating the association between maternal smoking during pregnancy and disease in later life, as well as to elucidate the potential influence of emerging tobacco product use during pregnancy, including the use of electronic cigarettes, on placental epigenetics.

Keywords: pregnancy, epigenetics, prenatal, placenta, tobacco

Introduction

According to the developmental origins of health and disease hypothesis, in utero environmental exposures may alter fetal programming and influence the risk of disease in later life,[1–4] including risk for cardiovascular disease, diabetes, asthma, cancer, and other conditions.[5–10] Despite the significant offspring morbidity and mortality associated with maternal cigarette smoking during pregnancy (MCSDP),[11–20] 10%–12% of US women smoke cigarettes during pregnancy.[21,22] This can allow toxicants, including nicotine, to cross the placenta and disrupt placental functioning,[23,24] which may result in fetal programming of later-life disease risk via alterations to normal placental epigenetic mechanisms, whereby changes in gene expression occur without direct changes to the DNA sequence.[25–27]

Four main modes of epigenetic regulation are known, ie, non-coding RNA-mediated regulation, histone modifications, imprinting, and DNA methylation,[26] with DNA methylation being the most extensively studied. When a methyl group is added to the 5′ position of cytosine, DNA takes on a stable, transcriptionally less active and potentially inactive conformation that can repress or silence gene expression, particularly when methylation occurs within gene promoter regions.[28–33] These methylation marks are often found in clusters of cytosine–guanine dinucleotide pairs called CpG islands.[34] Normal methylation patterns are critical to many cellular functions, particularly in the placenta where correct cellular functioning is crucial to fetal development.[35]

It has been theorized that the placenta may act as a functional record of in utero environmental quality.[35] Nicotine, for example, crosses the placenta,[11] and some MCSDP-associated perturbations to normal placental epigenetic patterns are also associated with adverse infant health outcomes,[36–42] including preterm birth,[42] birth weight,[43] and neurobehavioral outcomes.[44] MCSDP-associated methylation patterns have also been found in tissues other than the placenta.[36]

This review summarizes what is known about the influence of MCSDP on placental methylation patterns, which may be associated with fetal programming of disease risk in later life.

Cigarette smoking in pregnancy: known risks to fetal health and development

Cigarette smoking is detrimental to health and linked to adverse health outcomes, including lung and other cancers,[45–47] asthma,[48] chronic obstructive pulmonary disease,[48] autoimmune diseases,[49] and adverse fertility outcomes in women[50] and men,[51] both in smokers themselves and those exposed to secondhand smoke. Cigarette smoking is associated with deleterious effects on ovarian steroidogenesis and gametogenesis, oocyte maturity, ovulation, fertilization, and implantation.[52] Animal models of nicotine exposure have revealed associated oocyte apoptosis[53] and reduced sperm quality.[54] MCSDP has also been linked to increased risk of stillbirth[55] and miscarriage.[56] These associations may be biologically explained by the ability of cigarette smoke components to interfere with placental development.

MCSDP negatively affects the processes of trophoblast migration and invasion, which are primarily accomplished by extravillous trophoblast cells[57] and allow the placenta to anchor in the uterine wall.[58] By negatively impacting the function of these placental cell types, MCSDP can increase the risk of placenta previa, placental abruption,[59] and other reproductive problems. MCSDP is associated with detriments to infant neurobehavior[15,16,60–64] and the development of autoimmune diseases.[65–67] Both active[68–73] and passive[71,73–75] MCSDP are linked to small-for-gestational-age infants.

These disruptions to normal placental growth and development can be devastating to fetal growth. The placenta plays a crucial role in providing the fetus with oxygen and nutrients, allowing gas and waste exchange, and producing important hormones and other compounds necessary for fetal development.[35,76–79] The metabolic activity of the placenta also protects the fetus from many potentially harmful environmental toxicants,[76–79] but certain heavy metals,[80,81] cocaine,[82] and nicotine[11] cross this selectively permeable membrane. Changes that affect placental gene expression, such as epigenetic alterations, may have harmful downstream effects not only on placental functioning, but on the health of the developing infant.[35]

Overview of altered DNA methylation patterns associated with MCSDP

DNA methylation patterns are established de novo early in pregnancy following a post-fertilization wave of demethylation in the early embryo.[28,83] DNA methylation involves the addition of a methyl group via a covalent bond to the 5′ position of cytosine, which occurs almost exclusively in the context of CpG dinucleotides.[35] Methylated CpGs, which mostly occur in clusters known as CpG islands, cannot be effectively bound by transcription factors, leading to reduction or silencing of gene expression.[84] This process often occurs in gene promoter regions, where precise control of gene expression is necessary for cellular growth, differentiation, and functioning.[39] Thus, pregnancy and, in particular, the first trimester, is a critical window during which environmental toxicant exposures may elicit detrimental effects on normal DNA methylation and gene expression patterns in multiple tissues. These exposures can have consequences for the developing fetus, which may continue throughout the life course.

Due to the stability of the covalent bond linking methyl groups to cytosine residues, aberrant DNA methylation patterns established early in life may persist into postnatal and adult life. Contrastingly, these aberrant DNA methylation patterns may also comprise an array of biomarkers for adverse early life exposures and serve to identify at-risk infants exposed to MCSDP.[42]

Placental DNA methylation patterns may serve as mechanistic links between in utero exposures and adverse infant

health outcomes.[8,37,42,84–87] Epigenome-wide association studies (EWAS) have observed associations between MCSDP and placental methylation patterns in multiple genomic regions,[37,41,42] although one study found associations between MCSDP and methylation in cord blood only.[88] EWAS studies have also observed loci associated with both MCSDP and known smoking-associated adverse infant health outcomes, including birth weight[37,41] and pre-term birth.[42] These studies may provide mechanistic insight into the links between MCSDP and pre-term birth or low birth weight.[12,14]

Genetic pathways associated with MCSDP

Table 1 describes placental methylation patterns associated with exposure to nicotine or MCSDP.

EWAS findings have elucidated potentially relevant genes and pathways that may mediate prenatal exposure to MCSDP and disease risk in later life. Many studies have employed the Illumina Infinium HumanMethylation27 BeadArray,[89] which assesses the methylation status of >27,000 CpG loci following DNA bisulfite modification. This method allows for detection of methylated cytosine residues by treating DNA with bisulfite, which converts unmethylated cytosines to uracil; methylation is protective against conversion to uracil. Once converted, DNA samples are hybridized to array probes, and percent methylation at >27,000 loci is

measured in beta values ranging from 0 (absence of methylation) to 1 (complete methylation). The advent of the more comprehensive Infinium HumanMethylation450 BeadArray, which interrogates >450,000 CpG loci,[90] has allowed more extensive epigenome-wide coverage. One such study[91] found placental methylation patterns associated with nicotine exposure during pregnancy in the GTF2H2C and GTF2H2D genes (see Table 1).

MCSDP-associated genes discovered via the Illumina HumanMethylation27 and 450 BeadArrays include RUNX3,[42] PURA,[41] GTF2H2,[41,91] GCA, GPR135, and HKR1[41] (Table 1). Although the placental function of RUNX3 has not been elucidated, RUNX3 is important for cellular differentiation and development in neuronal cells, T-cells, macrophages, and dendritic cells.[92–99] As a tumor suppressor gene, RUNX3 interacts with β-catenin and increases p27, Rb, and TIMP-1 expression when upregulated.[100–102] RUNX3 is associated with numerous cancers,[98,101,103–108] including bladder cancer in smokers.[109] A potential role also exists for RUNX3 to mediate the relationship between MCSDP and asthma and airway hyperresponsiveness,[42,110–116] as has been observed in murine models.[117,118]

Suter et al[41] found MCSDP-associated methylation alterations in a number of genes regulating DNA replication, excision repair, cellular membrane fusion, G-protein coupled receptor activity, and transcriptional regulation,

Table 1 Studies observing differential placental methylation patterns associated with in utero exposure to nicotine or to maternal cigarette smoking during pregnancy

Reference	n	Methodology used to determine placental methylation status	Genes or elements identified	P-values	Associated health outcomes
Appleton et al[121]	444	Bisulfite pyrosequencing	HSD11B2	<0.05, <0.10	NA
Chhabra et al[91]	80	Illumina HumanMethylation450 BeadChip array	GTF2H2C, GTF2H2D	2.87×10^{-06}, 3.48×10^{-05}	NA
Maccani et al[42]	206	Illumina HumanMethylation27 BeadChip array; bisulfite pyrosequencing	RUNX3	0.04	Preterm birth
Paquette et al[120]	444	Bisulfite pyrosequencing	HTR2A	0.0008–0.02	Infant neurobehavior (NICU Network Neurobehavioral Scales [NNNS])
Suter et al[40]	34	Bisulfite sequencing	CYP1A1	0.027	NA
Suter et al[41]	36	Illumina HumanMethylation27 BeadChip array; bisulfite sequencing	STX5, FUT11, TUSC3, FAN1, and ZNF671 associated with both smoking and birth weight; PURA, GTF2H2, GCA, GPR135, and HKR1 associated with smoking	7.66×10^{-10}, 1.48×10^{-06}	Birth weight reduction
Stroud et al[119]	45	Bisulfite pyrosequencing	NR3C1	0.024	Infant basal and reactive cortisol over the first postnatal month
Wilhelm-Benartzi et al[37]	380	Bisulfite pyrosequencing; Illumina HumanMethylation27 BeadChip array	LINE-1; AluYb8	0.01, <0.0001	Birth weight percentile

Abbreviations: NA, not applicable; NICU, Neonatal Intensive Care Unit; n, sample size.

potentially highlighting the placental genomic damage incurred by exposure to MCSDP. The array-based findings in both studies were validated by gold-standard bisulfite pyrosequencing.[41,42]

The findings of differential *GTF2H2* methylation by Suter et al were confirmed in 2014 by Chhabra et al[91] (see Table 1), who observed differential *GTF2H2C* and *GTF2H2D* methylation associated with in utero nicotine exposure.[91]

Candidate gene studies have also elucidated links between MCSDP and altered placental methylation patterns. Most studies have utilized bisulfite pyrosequencing to interrogate the methylation status of candidate gene regions of interest. Candidate genes of potential interest that have been associated with MCSDP include *NR3C1*,[119] *CYP1A1*,[40] *HTR2A*,[120] and *HSD11B*.[121] *NR3C1*, better known as the glucocorticoid receptor gene, and *HSD11B2*, the 11β-hydroxysteroid dehydrogenase type 2 (11-β-HSD2) gene, play important roles in stress response.[122] Placental methylation status of *NR3C1* has been previously associated with infant birth weight[43] and neurobehavior,[123,124] and placental methylation status of *11-β-HSD* has been associated with infant growth[125] and neurobehavior.[124,125] The 11-β-HSD2 enzyme catalyzes the conversion of active cortisol into inactive cortisone, thus regulating the availability of glucocorticoids to the glucocorticoid receptor.[122] Placental cortisol is also associated with postnatal weight gain,[126] underscoring the potential for this pathway as a marker of infant health outcomes. The relationship between placental *NR3C1* methylation, MCSDP, and birth weight[41,43,127] is likely a complex one and birth weight may be a proxy measure for multiple interplaying in utero factors that can influence fetal growth and development.

CYP1A1 is a xenobiotic-processing enzyme known to be involved in the phase I metabolism of potentially carcinogenic compounds found in cigarette smoke, including polycyclic aromatic hydrocarbons.[40] Suter et al[40] found that *CYP1A1* expression is upregulated by MCSDP via a mechanism of placental *CYP1A1* promoter hypomethylation, suggesting important roles for placental methylation alterations in the physiological response to this exposure.

HTR2A, or the serotonin receptor gene, is expressed in placental tissue and is regulated by DNA methylation.[120,128] Although its functional role in placental tissue has yet to be fully elucidated, Paquette et al[120] recently observed MCSDP-associated placental *HTR2A* methylation, adding to a growing literature linking placental *HTR2A* to placental implantation[129] and neurodevelopment.[130,131]

In addition to these candidate gene studies, Wilhelm-Benartzi et al[37] observed associations between MCSDP and methylation of the repetitive elements LINE-1 and AluYb8 (see Table 1). This partly confirmed findings by Moore et al,[132] who showed that cytosine methylation levels differ according to smoking status. The methylation levels of these repetitive elements were, in turn, associated with epigenome-wide placental methylation patterns as measured by the 27K array platform.[37] Methylation of repetitive elements, which comprise roughly 50% of the human genome, is important for the maintenance of genomic stability.[133,134] These findings suggest that placental methylation may be an indicator of underlying functional alterations to normal placental development that can be perturbed by environmental toxicant exposures, such as exposure to MCSDP.

Functional consequences: changes in gene expression and implications for future disease risk

Several studies[40,43,120,123,135] have found MCSDP-associated placental gene expression patterns, and these findings are supported by studies of placental methylation changes occurring concomitantly with changes in expression of relevant genes. In particular, one study found 241 genes to be differentially expressed in the placentas of infants born to smoking mothers, many of which were related to xenobiotic metabolism, collagen, coagulation and thrombosis.[135] Another genome-wide study found 174 genes to be differentially expressed in the placenta, including *CYP1A1* and *CYP19A1*, perhaps indicating a response to the oxidative stress induced by MCSDP.[136] A third study[137] found 329 genes to be differentially expressed in the placentas of infants exposed to MCSDP, including the additional cytochrome P450 family gene *CYP1B1*. These findings are consistent not only with other studies linking active and passive MCSDP with oxidative stress[138–141] and the induction of the hypoxia-sensitive protein HIF1α,[142] but also with the findings of Suter et al,[40,41] who noted that placental methylation and expression changes occurred within gene regions related to xenobiotic processing, oxidative stress response, and hypoxia.

Other groups have also demonstrated MCSDP-related changes in gene expression associated with alterations in placental methylation. One study[120] observed both placental methylation and expression alterations in the *HTR2A* gene, while Stroud et al[119] found *NR3C1* placental methylation alterations associated with MCSDP and altered cortisol levels. Additional work has suggested that *NR3C1* methylation status is correlated with glucocorticoid receptor expression.[43,123] Taken together, these studies suggest that methylation alterations in these genes within placental tissue

may have functional consequences for important placental pathways.

MCSDP is associated with a host of diseases and disorders in infancy, childhood, and later life.[11–19,22] These include pre-term birth,[11,12] fetal growth retardation and intrauterine growth restriction,[13,14] adverse neurobehavioral outcomes,[15,16] obesity,[17–19] and asthma.[10,84,110–116] In fact, even grand-maternal smoke exposure has been associated with an increased risk of asthma in grandchildren,[10] although a recent study of children from the Avon Longitudinal Study of Parents and Children did not reveal such an association.[143] Nonetheless, epigenetic mechanisms have been implicated in the relationship between grand-maternal and maternal smoking during pregnancy and risk of asthma and airway hyper-responsiveness in offspring.[84,144] EWAS have borne out this finding, showing that alterations in placental methylation associated with both MCSDP and adverse infant health outcomes, such as pre-term birth, occur in genes associated with asthma and airway hyper-responsiveness (such as *RUNX3*).[42] These findings suggest that epigenetic mechanisms may underlie MCSDP-associated adverse health outcomes.

Conclusion and future directions

Although a growing body of literature exists on MCSDP-associated alterations in placental methylation, there is much work yet to be done to elucidate the specific signaling pathways and mechanisms involved in mediating the relationship between MCSDP and disease risk in later life. Ongoing cohort studies may help to further discern the risks posed by MCSDP to infant and child health outcomes, including risks for respiratory disorders,[110,111,113,114,116] adverse neurodevelopmental outcomes,[15,16,60,61,64,85,145] and obesity,[17–19] all of which have been linked to MCSDP.

One such cohort study is the Rhode Island Child Health Study (RICHS), a population-based birth cohort enrolling mother-infant pairs at the Women and Infants' Hospital in Providence, RI, USA.[146] RICHS recruits newborn infants and their mothers following delivery and seeks to examine how the prenatal environment may influence postnatal health and neurobehavioral outcomes. Several important findings describing epigenetic links between the prenatal environment and postnatal outcomes have already been published from this cohort[44,146–150] and work is ongoing to examine additional mechanistic links between prenatal exposures and a variety of postnatal outcomes.

The Norwegian Mother and Child Cohort Study has also published several key studies on MCSDP and DNA methylation patterns in cord blood,[151,152] fetal loss,[153] plasma lipid

levels in adult offspring,[154] and infant behavioral outcomes.[155] This cohort will likely continue to produce important data on MCSDP-associated disease risk in later life in the coming years.

Both epigenome-wide and candidate-based studies of placental methylation patterns have yielded intriguing results for genes of potential biological interest that should be further investigated in future studies, including *RUNX3*,[42] *CYP1A1*,[40] *NR3C1*,[119] *HTR2A*,[120] *HSD11B2*,[121] *PURA*,[41] *GTF2H2*,[41,91] *GCA*, *GPR135*, and *HKR1*.[41] The methylation status of genomic repetitive elements, such as LINE-1 and AluYb8, has also shown promise as a potential biomarker of MCSDP.[37] It is important to note that an additional gene, the aryl hydrocarbon receptor repressor (*AHRR*), has also been recently investigated with respect to exposure to MCSDP, but while an association was found within this gene in cord blood mononuclear cells, a similar association was not found in placental tissue.[156]

Cumulatively, these results suggest that MCSDP-associated placental methylation is gene-specific, and perhaps, to a lesser degree, can also occur epigenome-wide, a conclusion previously drawn by Suter and Aagaard.[133] These patterns also appear to be tissue-specific, as studies investigating MCSDP-associated methylation patterns have observed alterations in genes with only partial overlap in various tissues of interest. These genes include *FRMD4A*,[157,158] *C11orf52*,[157] *AHRR*,[151,152] *CYP1A1*, *GFI1*,[152] *ATP9A*, *GALNT2*, and *MEG3*.[158] Consideration should also be given to the role that additional in utero factors may play in placental methylation patterns. The studies described above have largely attempted to control for or match samples on potential confounders, including infant sex,[37,40–42,120,121] maternal age,[37,41,42,121] maternal pre-pregnancy body mass index,[37,41,121] birth weight,[42,120] delivery method,[42] gestational age or birth weight percentile,[41,120,121] maternal education,[120] race/ethnicity,[37,41,121] and other maternal factors,[37,40] including smoking status for analyses of methylation patterns associated with infant health outcomes.[37,119,120] Future studies may reveal concordance in genes identified, although to date only *GTF2H2*[41,91] has been differentially methylated in association with MCSDP or nicotine exposure in multiple studies.

Concordance of methylation patterns between tissues and life stages should also be investigated. For example, two studies[159,160] observed smoking status-associated peripheral blood methylation patterns in adults, but these findings have yet to be confirmed in the placenta. Investigation of these findings, as well as epigenome-wide analyses of placental methylation patterns differing between infants exposed to MCSDP throughout pregnancy versus mothers who quit, would help to

elucidate whether placental methylation patterns are reversible with smoking cessation. As methylation is known to exhibit a degree of plasticity with respect to environmental and stochastic factors,[87] demonstration of reversible methylation with smoking cessation would have implications for variations in MCSDP-associated health risks.

It will be important for future studies to focus on the use of emerging tobacco products as unique prenatal exposures that may be associated with unique gene-specific and tissue-specific methylation patterns. Such emerging tobacco products include electronic (e-)cigarettes, electronic nicotine delivery devices which, as of February 2015, are under consideration for regulation at the federal level by the US Food and Drug Administration.[161] Some types of e-cigarettes are capable of producing nicotine yields at levels comparable with those in traditional cigarettes,[162] but e-cigarette liquids and vapors contain different compounds, such as propylene glycol and specific flavors, not found in traditional cigarettes.[163–165] E-cigarette flavors may also be formulated with other compounds that are not found in traditional cigarettes. The influence of prenatal exposure to e-cigarettes on the placenta and developing fetus remains unknown, and it is important to investigate such exposures during pregnancy or in in vitro models. It will also be important to investigate placental methylation alterations associated with prenatal e-cigarette exposure and, if they exist, to compare these e-cigarette exposure-associated placental methylation profiles with the placental methylation profiles previously associated with MCSDP.

In conclusion, while a growing literature exists on MCSDP-associated placental methylation, work remains to be done to fully investigate the gene-specific and tissue-specific mechanisms that underlie the relationship between MCSDP and disease in later life. This knowledge will help identify at-risk infants exposed to MCSDP and hopefully help to formulate effective interventions to improve infant health. Future studies should examine placental methylation alterations associated with prenatal exposure to emerging tobacco products as well, so that information on potential health effects can be disseminated to women who are pregnant or of child-bearing age. Collectively, such efforts will help to further understand links between prenatal tobacco exposure and infant and child health outcomes, with the goal of better elucidating the greater developmental origins of health and disease.

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Disclosure

The authors have no conflicts of interest to disclose.

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