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# Maternal Adverse Childhood Experiences and Infant DNA Methylation: Examining an Epigenetic Biomarker of Intergenerational Risk

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Maternal Adverse Childhood Experiences and Infant DNA Methylation:  
Examining an Epigenetic Biomarker of Intergenerational Risk

by

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A THESIS

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## **Abstract**

While “nature” and “nurture” are often viewed as opposing influences on human development, epigenetics is one area of study investigating how these influences work together. Until recently, transmission of intergenerational risk to human development has centred on claims of genetic inheritance, or the persistence of poor social environments such as adverse childhood experiences (ACEs), across generations. Stress occurring during gestation, that affects both the fetus and mother, has also been proposed as a method of transmitting intergenerational risk to offspring. New evidence in animal models suggests that “preconception stress” may also predict DNA methylation (DNAm; one component of epigenetics) in offspring, potentially impacting developmental health outcomes. Thus, the purpose of this study was to investigate the association between human mothers’ preconception stress and differential DNAm patterns in their biological infants. A secondary analysis was conducted, utilizing data obtained from the Fetal Programming (FetalPro) cohort study, a sub-set of participants in the Alberta Pregnancy Outcomes and Nutrition (APrON) study. APrON study participants were voluntary, and all pregnant women were over 16 years old and before 22 weeks of gestation at enrolment. Measures included mothers’ scores on the Adverse Childhood Experiences (ACEs) questionnaire, mental health during pregnancy including the Edinburgh Postnatal Depression Scale and the Symptom Checklist 90 Revised, as well as demographics. Epigenetic data were obtained from buccal epithelial cell (BEC) samples collected from mothers’ 3-month-old infants. Cellular DNA were processed with the Illumina Infinium HumanMethylation450 Bead Chip to investigate DNAm. Relationships were investigated using regression modeling with the Limma function in R-package. Results showed a strong relationship between mothers’ total ACE score and differential DNAm patterning in their infants at eight epigenetic sites out of over 450,000

sites investigated. These findings have implications for the study of DNAm patterning as a biomarker for the transfer of preconception stress in humans and suggest a role for epigenetics in the transfer of intergenerational trauma.

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*For Alisha, as always*

## Table of Contents

Abstract.....	ii
Acknowledgements.....	iv
Dedication.....	vi
Table of Contents.....	vii
List of Tables.....	ix
List of Figures.....	x
Epigraph.....	xi
<b>Chapter 1: Introduction.....</b>	<b>1</b>
1.1 Introduction to Study.....	1
1.2 Overview of Study .....	3
1.3 Format for Thesis.....	4
1.4 Origin of My Interest.....	4
1.5 Literature Review.....	5
1.5.1 Background to Key Concepts and Associations.....	6
1.5.2 ACEs Predict Poor Health and Development.....	9
1.5.3 ACEs and Similar Postnatal Stressors Predict DNAm within Individuals.....	12
1.5.4 Maternal Prenatal Stress and DNA Methylation in Offspring.....	16
1.5.5 Preconception Stress and DNAm in Offspring.....	19
1.5.6 Intergenerational Transmission of Risk: Relevant Theoretical Frameworks.....	21
1.5.7 Intergenerational Trauma is Multi-Faceted.....	24
1.5.8 Intergenerational Transfer of Differential DNAm Patterning.....	25
1.5.9 DNAm and Tissue Specificity.....	26



1.5.10 Summary of Key Findings from Literature Review Guiding this Study .....	27
1.6 Contribution of Authors.....	29
<b>Chapter 2: Submitted Publication.....</b>	<b>30</b>
2.1 Publication Cover Sheet.....	30
2.2 Publication Abstract for <i>Development &amp; Psychopathology</i> .....	31
2.3 Article Submission for <i>Development &amp; Psychopathology</i> .....	32
Background .....	33
Methods.....	45
Results.....	51
Discussion.....	59
Conclusion.....	66
References.....	67
<b>Chapter 3: Further Discussion .....</b>	<b>86</b>
3.1 Expanded Discussion and Consolidation of Learning .....	86
3.1.1 Adhering to Study Guidelines: STROBE and EWAS Einstein .....	86
3.1.2 Epigenomic Plasticity.....	86
3.1.3 Marker Versus Mechanism .....	87
3.2 Nursing Implications of Findings .....	88
3.2.1 Nursing Research .....	88
3.2.2 Nursing Practice.....	89
3.2.3 Nursing Policy .....	90
3.2.4 Nursing Education .....	91
3.3 Conclusion .....	92
Bibliography .....	94

## **List of Tables**

Table 1: Key Terms Associated Study

Table 2: Key Measures and Collection Times

Table 3: Descriptive Statistics of Covariates

Table 4: Frequency and Percentage of ACE Scores

Table 5: Correlations Between Variables

Table 6: Significant Sites for DNAm in Correlation to Total ACE Score (Corrected Model)

Table 7: CpG Site and Related Genes, Location and Function

## **List of Figures**

Figure 1: Theoretical Transmission of Intergenerational Trauma in Humans in Relation to DNA Methylation

Figure 2: Hypothesized Correlation Between Maternal Adverse Childhood Experiences and Differential DNA Methylation Patterns in the Biological Infant

Figure 3: Principal Component Analysis of DNAm and Correlations with Covariates

Figure 4: Histogram Distribution of Total ACE Scores

Figure 5: Volcano Plot of Significantly Differentially Methylated Sites

Figure 6: Display of Correlation Between ACE Standardized Score and DNAm at Significant Sites (nominal  $p < .0001$ ), DNAm Post-Correction for Model Covariates

Figure 7: Comparison Between Zero ACEs and One or More ACEs for Significant Sites (nominal  $p < .0001$ )

The decision to have a child is never made for the sake of the child – for no child then exists. [...] The child is a means to our ends... But morally, the child is first and foremost an end in herself.

Dena Davis, Genetic Dilemmas and the Child's Right to an Open Future<sup>1</sup>

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<sup>1</sup> Davis, D. S. (1997). Genetic dilemmas and the child's right to an open future. *Hastings Center Report*, 27(2), 7-15. Retrieved from: <http://www.jstor.org/stable/3527620>

## Chapter 1: Introduction

### 1.1 Introduction to Study

The possibility that the transmission of epigenetic markers, such as DNA methylation (DNAm), may be associated with the transfer of intergenerational risk for disease has only begun to be examined in humans. Until recently, transmission of intergenerational risk has centred on claims of genetic inheritance or the persistence of poor social environments, such as adverse childhood experiences (ACEs), occurring across generations. Indeed, ACEs, defined as exposure to personal abuse, personal neglect, and household dysfunction (Felliti et al., 1998) prior to 18 years of age are linked to numerous physical and mental health conditions and altered DNAm (Borghol et al., 2011; Mehta et al., 2013; Smith et al., 2011). However, new evidence in animal models suggests that, in addition to gestational stress, early adversity or “preconception stress” may also predict DNAm in offspring. This study is novel in investigating preconception stress in humans, in relation to differential DNAm patterning in biological offspring. Key terms associated with this study are outlined in Table 1 below, familiarity of which is essential for understanding this study.

Table 1

#### *Key Terms Associated with Study*

<b><u>Term</u></b>	<b><u>Definition</u></b>
Adverse Childhood Experiences (ACEs)	Exposure to traumatic events in childhood. The ACE questionnaire has ten items, which address the following types of trauma: personal abuse – physical, emotional and/or sexual (three questions); exposure to personal neglect – physical and/or emotional (two questions); household dysfunction – losing a parent (through divorce, death or abandonment), witnessing mother abused, living with someone with mental illness, living with someone with problematic drug/alcohol use, and living with someone who was incarcerated (five questions; Chapman et al., 2004; Dube et al., 2003; Felitti et al., 1998).

DNA	Deoxyribonucleic acid; a biological molecule found in the cells of all known living organisms, carrying genetic instructions for growth, development, functioning and reproduction. Consists of cytosine/guanine and thymine/adenine dinucleotide pairs (Alberts et al., 2014).
CpG	Refers to the dinucleotide pair consisting of 5'cytosine and 3'guanine on the DNA strand. These pairs are relatively sparse on the genome; areas with high CpG density are referred to as CpG islands (Jones et al., 2015). Specific CpGs are referred to as “sites” or “hits” in studies investigating DNA methylation.
Epigenetics	Modifications to DNA and DNA packaging that do not involve changes to the DNA sequence (i.e. do not involve DNA mutations), and that are potentially transmissible to daughter cells (Bird, 2007). Essentially, they are modifications to genotype that may influence phenotypic changes. Epigenetics has been referred to as the interface between the genome and the environment (Feil & Fraga, 2012).
Epigenetic Inheritance	A key point for epigenetic inheritance is that genetic inheritance is not altered, but genetic expression is (Harper, 2005). It is the transmission to F1 offspring of F0 parental phenotypic responses to environmental challenges, even when the offspring do not experience those particular environmental challenges themselves. Furthermore, F0 methylated DNA may integrate into gametes and be passed on to the F1 embryo of the next generation (Heard & Martienssen, 2014; Van Otterdijk & Michels, 2016).
DNA methylation (DNAm)	The addition of a methyl group (-CH <sub>3</sub> ) to a dinucleotide pair on the DNA strand. The most common form of DNA methylation is the addition of a methyl group to the 5'cytosine end of a CpG dinucleotide pair (Jones et al., 2015). The methyl group acts to block access by molecules involved in gene expression (Cao-Lei, Laplante & King, 2016), and so DNAm is commonly observed to repress gene expression and maintain genome stability (Bird, 2007). CpG sites can be either hypermethylated, or hypomethylated; either of these are referred to as differential methylation.
Intergenerational risk	Intergenerational epigenetic inheritance is the transfer of epigenetic marks from the F0 gametes to the F1 embryo for one generation (Van Otterdijk & Michels, 2016). For in utero inheritance, marks are transferred to F1 and F2 germlines, where male inheritance is transferred only to the F1 germline (Heard & Martienssen, 2014). This should not to be confused with <i>transgenerational</i> transfer, when phenotypic changes are manifested in generations that were not exposed to the initial environmental exposures (Heard & Martienssen, 2014). For transgenerational epigenetic inheritance to occur, epigenetic marks and corresponding phenotypic changes must be transferred across subsequent generations (Van Otterdijk & Michels, 2016).

## 1.2 Overview of Study

This study proposes to examine the association between exposure to preconception adversity, in the form of ACEs in women, and the DNAm of their offspring. The study employs data obtained from individuals in the Fetal Programming cohort (Letourneau et al., 2017), including mothers' scores on the ACEs questionnaire, measures of maternal perinatal stress, demographic data, as well as DNAm patterns obtained from buccal epithelial cell (BEC) samples from their 3-month-old infants. Important measures associated with this study are outlined in Table 2 below. Data were collected from mothers at clinic visits and via questionnaires during pregnancy and ACEs data were collected from mothers postpartum, cross-sectionally, when the youngest children in the cohort were approximately 2 years of age. BECs were collected from infants at three months of age. DNAm data were obtained from buccal swab cell collection procedures and processed using bisulphite conversion and hybridization to an array with the Illumina Infinium HumanMethylation450 Bead Chip (Illumina Inc., San Diego, CA) to investigate DNAm at CpG sites. Background subtraction and colour correction of data was done using Illumina Genome Studio software (Esposito et al., 2016) at the laboratory of Dr. Michael Kobor of the University of British Columbia. Data was available from 139 children's BECs along with data from completed ACEs questionnaires from their biological mothers. Multiple linear regression was employed to explore the relationships between ACEs score and DNAm patterns at each epigenetic site and to consider confounders.

Table 2

### *Key Measures and Collection Times*

MEASURE	TIMING
Sex of child	Birthdate (determined by birth report)

Maternal Depression – EPDS Score >13	Early pregnancy, late pregnancy, 3 months postnatally
Maternal Anxiety – SCL-90R	Early pregnancy, late pregnancy, 3 months postnatally
SES $\propto$ Maternal Education/Income	At enrollment (determined by collapsing variables for education and income)
Maternal Stress $\propto$ EPDS and SCL-90R	Early pregnancy, late pregnancy, 3 months postnatally (utilized highest score for each questionnaire; stress variable obtained through principal component analysis)
Infant Buccal Cell Swabs $\propto$ DNAm	3 months postnatally
Maternal ACEs Questionnaire Score	Between 18 to 24 months postpartum

### 1.3 Format for Thesis

A paper will be submitted to the journal *Development & Psychopathology*, which is included in Chapter 2 of this manuscript and formatted as per the journal's guidelines. As per University of Calgary thesis guidelines (University of Calgary, 2018), a fulsome literature review (section 1.5) was completed prior to initiating this novel study, which describes theoretical and scientific underpinnings leading to the pursuit of this study.

### 1.4 Origin of My Interest

My interest in academic research on intergenerational trauma and risk came from clinical work in addiction and mental health, as an outreach nurse working for organizations like Alberta Health Services and The Alex. My clinical experience has repeatedly shown me that the experience of trauma and risk for adverse health outcomes go hand-in-hand. In my work settings, my clinical and management roles involved caring for adults and seniors experiencing significant mental illness, addiction, homelessness, post-traumatic stress – all ramifications of



intergenerational risk. Much of the research on intergenerational trauma has focused on social and environmental factors contributing to the transfer of risk, and I became interested in how genetics may play a role. Reading about this topic led me to discover *epigenetics* as a field of study, which looks at how the environment influences genetic expression. I was introduced to my supervisor, Dr. Nicole Letourneau, who could provide access to epigenetic data (DNAm data, specifically) from infants and psychosocial data from their mothers. This seed would grow into the interdisciplinary study enclosed in these pages. I speak of intergenerational trauma but will also refer to “intergenerational risk” throughout this manuscript, encompassing the transfer of trauma and the associated health risks.

## **1.5 Literature Review**

An iterative literature review was undertaken to understand and explore associations among key variables for the study as well as key theories to explain intergenerational risk, differential DNAm patterning and association between maternal stress and infant DNAm patterning. Databases searched included PubMed, MEDLINE, CINAHL Plus and PsycINFO, with supplementation through Google Scholar. The following search terms were utilized in various combinations to thoroughly review the existing literature in relation to this study:

- intergenerational trauma
- intergenerational risk
- adverse childhood experiences
- epigenetic transfer
- maternal stress
- prenatal stress
- perinatal stress

- preconception stress
- DNA methylation

Additional articles were accessed by ancestry searches or obtained based on recommendations from committee members. The literature review revealed that ACEs predict poor health and developmental outcomes in adulthood, ACEs are associated with mental illness and addiction, mental illness is associated with differential DNAm patterns, and prenatal maternal stress is associated with differential DNAm patterns in the biological infants. Before reviewing the literature in depth, some background of the key concepts (e.g. Adverse Childhood Experiences, Epigenetics, Intergenerational Risk) is essential.

### **1.5.1 Background to Key Concepts and Associations**

Adverse childhood experiences (ACEs) are defined as exposure to personal abuse, neglect and household dysfunction (Felitti et al., 1998) prior to 18 years of age. The ACE questionnaire was developed through Felitti et al.'s study (1998), initially addressing seven types of childhood adversity: the abuse category included psychological, physical and sexual abuse; household dysfunction category included substance abuse in the home, mental illness in the home, having a mother who was treated violently, and criminal behaviour in the home (Felitti et al., 1998). The ACE questionnaire later included personal neglect as a category consisting of two types, physical and emotional, along with an additional household dysfunction item for losing a parent (Chapman et al., 2004; Dube et al., 2003). It is still widely used, though it has been adapted for different contexts as an epidemiological tool – for example, an Alberta ACE questionnaire removed the question concerning parental criminal behaviour and added a question addressing chronic illness in the home (McDonald & Tough, 2013). The questionnaire has ten items with

“yes” or “no” answers, where one point is given for each “yes” answer, providing a total number of ACEs.

ACEs are linked to: diseases such as cancer, emphysema, diabetes and heart disease; disease risk factors such as smoking, obesity, depression, alcoholism and drug addiction (Chapman et al., 2004; Dube et al., 2003; Felitti et al., 1998); and alterations to the DNA such as methylation (Suderman et al., 2014). Indeed, DNAm may contribute to the association between ACEs and poor health in adulthood. Moreover, mothers’ ACEs are also related to poor health outcomes in their children (McDonnell & Valentino, 2016). In animal models, exposure to early adversity not only predicts DNAm in the same individuals as experienced the adversity (F0), but also their offspring (F1) (Blaze & Roth, 2015), and perhaps even generations F2, F3 and beyond (Zucchi, Yao & Metz, 2012). This is yet to be studied in humans. Thus, rival theories have been proposed to explain the intergenerational transmission of risk in humans, including social conditions occurring across generations and epigenetic transmission (Drake & Liu, 2010). This study will examine the potential of the epigenetic theory of transmission of risk in humans via exploration of DNAm as a biomarker for this transmission.

Social factors offered to explain the observation that parents at risk for poor health tend to have children who are also at risk for poor health include: poverty, parental risk-taking behaviours, lower education, and poor parenting behaviours. The transfer is often called intergenerational transmission of risk (Serbin & Karp, 2003). An example may serve to illustrate: consider the situation of a parent who experienced trauma as a child and as an adult engages in impulsive and addictive behaviours to manage and cope with their early adversity. Their impulsivity and addictions affect the quality of their parenting towards their own child. Combined with role modeling and social learning, the parents’ behaviours result in the child

experiencing trauma and addiction at an early age, and thus the cycle continues (Bombay, Matheson & Anisman, 2009). However, animal models have shown DNAm patterns are linked to ACEs-like factors in previous generations, and may even persist across generations (Babenko, Kovalchuk & Metz, 2015; Zucchi, Yao & Metz, 2012). The purpose of my project is to explore how this may happen in humans as part of the intergenerational transmission of risk, going beyond social factors.

While "nature" and "nurture" were often viewed as opposing influences on human development, epigenetics is one of several paradigms that look at how these two influences work together. The above example of social transfer shows how "nurture" plays a role with intergenerational risk. What is less understood is the idea that stress experienced by a human mother translates into changes in the child at a genetic level (Bowers & Yehuda, 2016).

“Epigenetic inheritance” has been defined as the transmission to offspring of parental phenotypic responses to environmental challenges, even when the offspring do not experience the challenge themselves (Harper, 2005). Many differences in phenotypic expression can be explained in part because of different environmental exposures and experiences activating or altering levels of gene expression (Harper, 2005).

One of the epigenetic changes that may contribute to altered gene expression is DNAm. The most common form of DNAm occurs at the 5’cytosine/3’guanine dinucleotide pairings, or CpG pairings, of the DNA strand (Robertson, 2005). The methyl group attaches to 5’cytosine of the CpG pairings, and acts to block access by molecules involved in gene expression (Cao-Lei, Laplante & King, 2016). The blocking mechanism by the methyl group prevents gene transcription, meaning that DNAm is involved in gene regulation mostly through the silencing of gene promotion activity (Cao-Lei et al., 2016; Jones et al., 2015). In this way, DNAm when

located in gene regulatory regions commonly represses gene expression and maintains genome stability (Bird, 2007). It is important to note that the interplay between DNAm and gene expression is more complex than simply silencing gene expression, and gene expression may actually regulate DNAm in some cases (Jones, Fejes & Kobor, 2013). Nevertheless, altered DNAm levels could be at least partly associated with the development of disease later in life, including cancer, diabetes, psychiatric conditions, and a propensity for addictive behaviours (Chiarella, Tremblay, Szyf, Provencal & Booij, 2015; Curley, Jensen, Mashoodh & Champagne, 2011; Fagiolini, Jensen & Champagne, 2009). DNAm does not necessarily result in the expression of pathological traits, but may render the individual more susceptible when faced with stressful events (Bombay et al., 2009). For a listing of key terms, refer to Table 1.

A substantial amount of literature regarding DNAm patterns in infants related to maternal prenatal stress in humans is available, and this body of literature continues to grow. Many human studies have shown increased DNAm in association with early life adversity, in candidate genes like *NR3C1* (Turecki & Meaney, 2016). The current evidence for traumatic stress and human DNAm is weakened by methodological issues and lack of consistency between studies. Inconsistencies are frequently due to sample sizes, tissue-type selection, and aggravation by publication bias; and so, the role of DNAm as an epigenetic mechanism remains unclear in the path from environmental exposure to phenotypic response (Vinkers et al., 2015). Furthermore, there appears to be a lack of studies looking at the transmission of intergenerational risk in humans with relation to epigenetic markers such as DNAm.

### **1.5.2 ACEs Predict Poor Health and Development**

Even before Felitti et al. (1998) released their landmark study of ACEs and health outcomes in adulthood, it was known that childhood adversity had some contributing negative impact on

adult life. For example, lack of care from a parent during childhood and childhood physical abuse increased the likelihood of homelessness (Herman, Susser, Struening & Link, 1997), suggesting that childhood adversities were strong predictors for pathologies associated with adult homelessness. A study of American male war veterans (n=46) with substance abuse disorders investigated the relationship between childhood trauma and substance abuse, controlling for war-related post-traumatic stress disorder (PTSD) as diagnosed by the DSM-III (Triffleman et al., 1995). The total number of lifetime substance abuse disorders was positively associated with total childhood trauma exposure. This relationship remained significant after controlling for demographics, family history of alcohol problems, combat exposure, combat-related PTSD, and lifetime PTSD (Triffleman et al., 1995).

Felitti et al. (1998) demonstrated a graded relationship between the number of categories of ACEs with adult health risk factors and diseases. Disease risk factors assessed included smoking, severe obesity, physical inactivity, depressed mood, suicide attempts, alcoholism, drug abuse, parenteral drug use, high number of sexual partners and a history of sexually transmitted diseases. The study by Felitti et al. (1998) demonstrated a dose-response relationship between the number of ACEs an individual had and the propensity for heart disease, cancer, emphysema, hepatitis and skeletal fractures. Individuals with greater than or equal to four ACEs had odds ratios of 1.6 for diabetes, 3.9 for emphysema, 1.6 for skeletal fractures, and 2.3 for hepatitis when compared with individuals who had no ACEs. Dube et al. (2003) continued the ACEs study and showed that each ACE increased the likelihood of early initiation of illicit drug use between two- and four-fold. Individuals with greater than or equal to five ACEs had between seven and ten times more likelihood of drug problems compared to those with no ACEs,

demonstrating a strong graded relationship between ACEs and drug use problems, drug addiction and parenteral drug use (Dube et al, 2003).

The number of ACEs an individual had was shown to have a graded relationship to alcoholism and depression in adulthood, independent of whether the parent abused alcohol or not (Anda et al., 2002). Childhood emotional abuse increased the risk for lifetime depressive disorders, and the number of ACEs had a graded relationship to both lifetime and recent depressive disorders in adulthood (Chapman et al., 2004). It is important to note that childhood emotional abuse exhibited the strongest relationship to measures of depressive symptoms, suggesting this type of ACE may pose particularly detrimental consequences on adult mental health (Chapman et al., 2004). Lower mean scores on mental health assessments have been shown to be associated with a higher number of ACEs, and more so with an increased number of different categories of ACEs (Edwards, Holden, Felitti & Anda, 2003).

Exposure to ACEs is associated with depression and anxiety (Lindert et al., 2014), heart disease (Dong et al., 2004), cancer (Brown, Thacker & Cohen, 2013) and obesity (Edwards et al., 2003). Individuals with six or more ACEs have been shown to die nearly twenty years earlier on average than those with no ACEs; after multivariable adjustment, adults with greater than or equal to six ACEs were 2.4 times more likely to die before they reached the age of 65 years (Brown et al., 2009). The proposed mechanism of early mortality is that ACEs lead to social, emotional and cognitive impairment; this leads to health-risk behaviours and disease, disability and social problems, resulting in early death (Felitti et al., 1998). Interestingly, the increased risk of mortality is only partly explained by ACE-related health and social problems, suggesting other mechanisms by which ACEs contribute to early death (Brown et al., 2009).

Maternal ACEs have been shown to predict higher levels of perinatal depressive symptoms, and maternal childhood maltreatment has specifically predicted higher levels of maladaptive infant socioemotional functioning at six months of age (McDonnell & Valentino, 2016). This finding hints that maternal ACEs may predict or influence the transmission of intergenerational risk. A mechanism of this transfer might be gene alterations, such as DNAm. Maternal ACEs have also been shown associated with prenatal and perinatal infant health complications (Madigan et al., 2017). In a study of 501 mother-infant pairs utilizing the ACEs questionnaire, it was found that four or more ACEs related to a two-fold increased risk of biomedical prenatal and perinatal complications, and a five-fold increased risk of psychosocial prenatal and perinatal complications. There was a linear association demonstrated between the number of maternal ACEs reported and the extent of cumulative biomedical and psychosocial risk in the infant (Madigan et al., 2017). These findings suggest maternal ACEs confer vulnerability to adverse health outcomes in the prenatal, perinatal and postnatal time periods.

### **1.5.3 ACEs and Similar Postnatal Stressors Predict DNAm within Individuals**

While data from the ACE questionnaire revealed a lot about the impact of a host of adversities on health and development, to date, researchers have not examined DNAm linked specifically to the ACE questionnaire responses, nor can the ACE questionnaire be applied to non-human animal models. However, researchers have examined variables captured on the ACE questionnaire in humans and other animals.

*Animal Studies.* A study of macaques demonstrated no difference in epigenome-wide DNAm between individuals experiencing early life stress, characterized by exposure to difficult conditions for foraging, and the control group exposed to low foraging demands (Kinnally et al., 2011). However, the greatest risk of maladaptive behavioural responses posed by early life stress



was for individuals with higher epigenome-wide DNAm (Kinnally et al., 2011), suggesting that differential epigenome-wide methylation patterns may correlate with a background of risk in individuals that experience life stress. Despite the finding of no significant difference in DNAm patterns between groups in this study, many rodent studies have shown associations between early life stress and maladaptive or pathological outcomes, possibly related to differential DNAm patterns.

Prolonged maternal separation, akin to ACEs, has been associated with increased anxiety and depression-like behaviours in rodents, as well as increased stress-induced corticosteroid release and alterations in memory and passive stress coping, possibly explained by DNAm patterns (Boccia & Pedersen, 2011; Curley et al., 2011; Murgatroyd et al., 2009). Individual variations in maternal care during the immediate postpartum period in rats are associated with changes in activity in the hypothalamus-pituitary-adrenal (HPA) axis of the offspring (Champagne et al., 2006; Meaney, 2001; Weaver et al., 2004). Conversely, increased maternal care behaviours of licking and grooming have been associated with blunted reactivity of the HPA axis, and decreased anxiety-like behaviours (Caldji et al., 1998; Liu et al., 1997; Meaney et al., 1996). A key regulator of the HPA axis is the negative feedback action of glucocorticoids, mediated by glucocorticoid receptors (GRs). GRGR is coded by expression of *NR3C1*, with potentially more expression after exposure to early life stress due to differentially methylated CpGs in its promoter region (Bockmuhl et al., 2015). In rats, increased DNAm of the brain-derived neurotrophic factor promoter site in the pre-frontal cortex has also been associated with exposure to abusive maternal care (Fagiolini et al., 2009). These effects emerged in infancy and were sustained into adulthood (Fagiolini et al., 2009).

*Human studies.* Rodent models of early life adversity are limited, as the study of psychological and sexual abuse is beyond the reach of rodent models (Lutz & Turecki, 2014). Social experiences are also particularly influential on the developing human brain, with continued plasticity into adolescence and adulthood (Curley et al., 2011). Epigenetic studies in humans have identified numerous epigenetic associations with early life stress and adversity (Provencal & Binder, 2015); one study by Essex et al. (2011) demonstrated a prospective association between family stress, which is associated with ACEs as household dysfunction, and differential DNAm patterns in adolescents. Studies have shown global DNAm differences for human individuals exposed to early adversity, suggesting alterations in early childhood environment have the ability to cause changes in the methylation status of numerous genes, including those involved in the development and function of nervous and immune systems (Borghol et al., 2011; Mehta et al., 2013; Smith et al., 2011). DNAm appears to be a crucial mediator of human early life experiences, maintaining life-long neurobiological consequences and strongly determining psychopathological risk (Lutz & Turecki, 2012). These associations can be seen as biomarkers of pathological risk, but methylation may also be involved in the disease state through actions on the HPA axis and immune system, for example (Miller, Fisk, Modi & Glover, 2005).

Childhood abuse has been linked to 997 differentially methylated gene promoters, which were enriched for genes involved in key cell signaling pathways for transcriptional regulation and development (Suderman, et al., 2014). Infant DNAm patterns are demonstrated to have a relationship with lower levels of maternal responsiveness and greater maternal depressive symptoms (Conradt et al., 2016). Interestingly, higher maternal responsiveness acted as a buffer for the negative developmental effects of greater depressive symptoms (Conradt et al., 2016). A

study of seven- to ten-year-old children who were in institutional care showed hypermethylation of 28 genes involved in brain development and function, including genes implicated in the HPA axis and stress response (Naumova et al., 2012). Children who have experienced maltreatment displayed increased methylation of the *NR3C1* promoter gene (Romens, McDonald, Svaren & Pollak, 2014). In humans, this gene codes for the glucocorticoid receptor, and “is the prime candidate gene for epigenetic influence on outcomes as it plays a key regulatory role in HPA axis functioning” (Cao-Lei et al., 2016, p. 17). DNAm of the *NR3C1* gene is implicated in prolonged increase of circulating glucocorticoids (Braithwaite et al., 2015). It has received considerably more attention than other genes in the regulation of fetal stress response, possibly because it influences the dysregulation of an infant’s HPA axis (Cao-Lei et al., 2016).

A study of 468 adolescents with mean age of 16.1 years showed stressful early life events and traumatic youth events (e.g. maternal psychological problems during pregnancy, pre-term delivery, maternal alcohol use or smoking during pregnancy, sexual abuse or physical abuse as an adolescent) are also associated with higher methylation of *NR3C1* (Van der Knaap et al., 2014). Stressful events in adolescence were associated with higher *NR3C1* methylation, independent of childhood stressful events (Van der Knaap et al., 2014). This supports the idea of the epigenome being susceptible to changes in DNAm during periods of rapid development in adolescence, similar to the period of fetal development.

Altered *SLC6A4* methylation is also prominent in the literature of early life stress, which encodes the gene for serotonin transporter. Higher DNAm of *SLC6A4* is significantly associated with childhood adversities, as well as worse clinical presentations of depression and anxiety (Kang et al., 2013). Exposure to childhood abuse has been associated with hypermethylation of *SLC6A4* (Beach et al., 2010). Importantly, the total number of past-traumatic events – ranging

from abuse and maltreatment, sudden death in the family, or serious injury – is significantly associated with *SLC6A4* methylation in adults (Koenen et al., 2011).

In contrast to animal studies, most clinical and epidemiological work on human epigenetics does not include brain or nervous tissue samples (Provencal & Binder, 2015). However, one particular study utilized the hippocampi of adult men who experienced childhood abuse and later completed suicide; the study found hypermethylation of 248 gene promoters and associated decreases in expression of these promoters (Labonte et al., 2012). The most differentially methylated genes were responsible for cellular and neuronal plasticity (Labonte et al., 2012). Maternal stressors in infancy, and paternal stressors in the preschool years – akin to ACEs – were shown to be associated with differential DNAm patterns (Essex et al., 2011). In a study on bullying victimization, Ouellet-Morin et al. (2013) measured DNAm of the serotonin-transporter gene in monozygotic human twin pairs, ideal for examining unique influence of the environment on the same genotype. Bullied twins had higher DNAm at age ten compared to their non-bullied co-twins, and this difference cannot be attributed to the twin pairs' genetic makeup or shared familial environments (Ouellet-Morin et al., 2013).

#### **1.5.4 Maternal Prenatal Stress and DNA Methylation in Offspring**

Many studies in both humans and animal models have pointed out that stress has multiple effects on DNAm (Babenko, Kovalchuk & Metz, 2015). Stress can play an important regulatory role in both brain development and disease etiology, with many previous clinical and animal studies suggesting a causal association between an adverse prenatal environment and an elevated risk of psychiatric diseases later in life (Babenko, Kovalchuk & Metz, 2015). A family history of stress may program central and peripheral pathways regulating gestational length, as a history of maternal stress has been associated with preterm birth, a known contributor to poor health

outcomes in infants (Yao et al., 2014). Gestational experience modulates gene expression with potential phenotypic consequences (Zucchi et al., 2013); one of the mechanisms by which gene expression is altered through gestational experience may be DNAm. Prenatal maternal distress and altered post-natal maternal behaviours might be a result of the mother's own DNAm patterns from early life stress, and altered maternal behaviours likely leads to differential DNAm patterns in the infants; in other words, stress responses in mothers might be programmed by DNAm inheritance across generations (Ward et al., 2013).

*Human studies.* Associations between human prenatal maternal stress and DNAm are well-studied. Maternal prenatal stress has been shown to result in lasting DNAm signatures in several tissues of the offspring, affiliated with 957 genes predominantly related to immune function (Cao-Lei et al., 2014), likely contributing to decreased immune function. When human mothers had lifetime history of depression (a known outcome of exposure to ACEs), there were DNAm changes in their offspring's blood leukocytes across the genome (Nemoda et al., 2015). These patterns were detectable at birth in the infant immune system through samples of neonatal cord blood, and persisted through to adulthood in the brain, supporting the hypothesis that system-wide epigenetic changes influence life-long responses in human offspring to maternal depression (Nemoda et al., 2015). The study by Nemoda et al. (2015) is uncommon for including data from 62 post-mortem hippocampal samples of adult humans. Genome-wide DNAm has been shown in one-year-old infants of mothers with major depressive disorder (Cicchetti, Hetzel, Rogosch, Handley & Toth, 2016), and higher maternal depressive symptomatology is related to differential infant DNAm (Conradt et al., 2016); depressive symptoms in adulthood are also associated with a higher number of ACEs as described earlier, and so there might be an association between differential infant DNAm patterning and maternal ACEs.

*NR3C1* is a prominent candidate site for DNAm studies of human maternal prenatal stress, in addition to DNAm studies of individual ACEs (discussed earlier). Human prenatal depressive symptoms significantly predicted increased DNAm of the *NR3C1* gene in male infant BECBEC samples (Braithwaite et al., 2015). Maternal cigarette smoking has shown to alter DNAm patterns in placental *NR3C1* (Green & Marsit, 2015). Chronic prenatal stress and war trauma has shown to cause widespread DNAm on HPA axis genes in infants, including *NR3C1*, in samples of infant blood leukocytes, umbilical cord blood, and placental tissue (Kertes et al., 2016). Yehuda et al. (2014) demonstrated a link between maternal and paternal post-traumatic stress disorder in holocaust survivors and DNAm of the *NR3C1* site in their offspring's blood leukocytes. Mothers who had been exposed to the Tutsi Genocide in Rwanda showed higher DNAm within the *NR3C1* coding sequence twenty years after the event; these mothers were pregnant at the time of the event, and their children also showed differential DNAm patterns for the *NR3C1* site of blood leukocyte samples (Perroud et al., 2014). This indicates the effects of prenatal stress can have a long-lasting impact on DNAm patterns in biological offspring. A meta-analysis by Palma-Guidel et al. (2015) combined evidence from seven studies and data from 977 individuals to demonstrate a statistically significant correlation between DNAm levels on the *NR3C1* promoter site and different forms of prenatal stress. It is important to note that studies included in a review of socioenvironmental stress and DNAm of *NR3C1* were often plagued with small effect sizes (Turecki & Meaney, 2016).

Another candidate gene prominent in the literature is *IGF2*. This gene is important for fetal growth (Green & Marsit, 2015). DNAm is involved in gene regulation mostly by silencing gene promoter activity (Cao-Lei et al., 2016), and so increased DNAm on the *IGF2* gene may contribute to low birth weight and premature development in the fetus (Green & Marsit, 2015).

Maternal blood cortisol levels, scores on the Edinburgh Depression Scale and Pregnancy-Related Anxiety Questionnaire, as well as maternal exposure to bisphenol-A have all been shown to be associated with DNAm of *IGF2* (Green & Marsit, 2015; Vangeel et al., 2015). In summary, ample evidence suggests that prenatal stress affects DNAm of offspring. What is not known is whether adversity experienced by parents prior to pregnancy, called “preconception stress” affects the DNAm of their offspring. Indeed, as discussed next, emerging evidence suggests that some of the epigenetic changes associated with exposure to stress within an individual may be inherited by the next generation.

### **1.5.5 Preconception Stress and DNAm in Offspring**

The body of literature investigating the human transfer of DNAm patterns intergenerationally is in its infancy. Reasonable predictions can be made, however, drawing on animal studies.

*Animal studies.* Animal models of maternal and paternal stress exposures have examined offspring phenotypes programmed either during pregnancy or prior to breeding, and have identified novel epigenetic processes by which neurodevelopmental changes occurred (Bale, 2014). A rodent study by Bock et al. (2016) found adversity experienced by a mother before her pregnancy resulted in highly complex changes in neuronal structure in the medial prefrontal cortex of her offspring. Another study of preconception adversity in female rats found changes in affective and social behaviours in their adult offspring, when compared to the offspring of females who were not in the stressful preconception condition (Shachar-Dadon, Schulkin & Leshem, 2009). Chronic unpredictable stress leads to increased corticotropin releasing factor Type 1 (CRF1), which is a key component in the stress response (Zaidan, Leshem & Gaisler-Salomon, 2013). Interestingly, increased CRF1 expression was found in the brain, but also in the

ova of female offspring, suggesting a possible mechanism of transgenerational transmission (Zaidan et al., 2013).

Leshem & Schulkin (2012) provide insight into how epigenetic inheritance is plastic; their rodent study of female rats with preconception stress demonstrated that preconception enrichment for the mothers and post-conception enrichment for the offspring both mitigate the transmission of risk. This suggests the effects of preconception stress on offspring can be buffered with a supportive environment (Leshem & Schulkin, 2012).

*Animal studies of father's role in preconception stress.* Discussion up this point has centred on maternal preconception stress, but there is a growing body of animal studies investigating paternal epigenetic transfer of risk. Certain paternal traits are acquired in response to ancestral exposures such as mental stressors, suggesting epigenetic inheritance can occur through the sperm (Chen, Yan & Duan, 2016). DNAm patterns might be maintained in the sperm, resulting in persistence of those patterns in the offspring (Feng, Jacobsen & Reik, 2010). Evidence for RNA-mediated inheritance of ancestrally acquired traits is now expanding (Chen et al., 2016). Chronic stress may modify epigenetic expression of genes in maturing spermatozoa, impacting gene expression and development in the embryo after fertilization (Mychasiuk, Harker, Ilnytsky & Gibb, 2013). In response to preconception paternal stress, global DNAm was increased in the hippocampi of both male and female rat offspring (Mychasiuk et al., 2013). Male offspring of chronically stressed male mice have shown core symptoms of major depressive disorder, including social withdrawal, anhedonia and increased anxiety behaviours (Dietz et al., 2011). Stress-induced DNAm and altered expression of sperm microRNAs may help explain not only an intergenerational transmission of risk, but a transgenerational transmission of risk (Bowers & Yehuda, 2016; Pang, Short, Bredy & Hannan, 2017). Chronic stress that may modify epigenetic



expression of genes in maturing spermatozoa, thus impacting gene expression and development in the embryo (Mychasiuk et al., 2013) may help explain these findings.

*Human studies.* Preconception stress in humans is not as well-studied as in animal models. Harper (2005) outlines several characteristics of stressful conditions resulting in epigenetic inheritance: the stressors appear cyclically and are severe, they endure for more than one generation, and they are recurring but not predictable; in human history, war and famine have satisfied these conditions, and so Holocaust survivors are ideal study subjects. There is some support for the epigenetic transfer of Holocaust-related trauma on survivors' children (Blaze & Roth, 2015). One review of earlier studies of Holocaust survivors found no link between parents' experience in the Holocaust and offspring behavioural outcomes (Blaze & Roth, 2015). However, Yehuda et al. (2014) more recently found significant epigenetic association in the offspring of Holocaust survivors, specifically differential DNAm patterns to the glucocorticoid receptor gene *NR3C1*. This finding supports the idea of altered epigenetic and behavioural effects as a result of preconception stress in humans.

#### **1.5.6 Intergenerational Transmission of Risk: Relevant Theoretical Frameworks**

*Social learning theory.* Since ACEs result in health and social problems in adulthood, children may be exposed to their adult parents' health and social problems, and a social transfer of the parents' adversity is plausible. This is in line with Bandura's Social Learning Theory (1977), which posits how children learn vicariously by observing and imitating their parents. Indeed, the mechanisms of intergenerational risk transmission in humans garnering the strongest support are social mechanisms; however, other explanations beg exploration.

*Biological embedding theory.* Hertzman (1999) proposed a hypothesis that the gradient between socioeconomic status and health status is the result of interaction between the

developmental status of people and the material and psychosocial conditions they encounter over their life course. Socioeconomic differences in the quality of early life experiences is given special consideration towards health status through socioeconomic differences in brain sculpting (Hertzman, 1999). Four characteristics define biological embedding: life experience must translate into neurobiological adaptations, adaptations are variable according to the intensity or quality of life experiences, such variable effects must be stable and long-term, and the effects must influence behavioural patterns or mental health outcomes over the lifetime (Hertzman, 2012).

*Fetal origins hypothesis.* Barker (1990) linked type 2 diabetes, hypertension and coronary artery disease to in utero undernutrition, giving rise to the “Barker Hypothesis” that these diseases originate as a result of developmental plasticity in response to under-nutrition. The hypothesis has led to research on the developmental origins of health and disease, or the “fetal origins hypothesis” emphasizing influences of early life exposures on adult physiology (Barker et al., 2007; Wadhwa et al., 2009). Much of the research in this area has focused on the epigenetic contribution of prenatal maternal stress towards offspring behaviour and psychopathology, focusing on the highly malleable developmental period of fetal growth. Prenatal maternal stress and fetal DNAm is one such epigenetic process discussed in detail earlier. The transfer of DNAm patterns across generations might also play a role in the transmission of risk.

*Three-hit concept of vulnerability and resilience.* Barker’s original concept likely underestimates the role of post-natal environment on the development of disease (Kim, Bale & Epperson, 2015). Risk could be attributed to three factors outlined in the “three-hit hypothesis of disease vulnerability and resilience” (Daskalakis, Bagot, Parker, Vinkers & de Kloet, 2013):

genetic predisposition, prenatal environment, and altered gene expression leading to phenotypes with differing susceptibility to later life experiences (Daskalakis et al., 2013). Prenatal programming may lead to a “mismatch” between the offspring’s biological systems (including central nervous system activity), and mental states or behaviours required to function optimally in the future environmental conditions they are exposed to (Lewis, Galbally, Gannon & Symeonides, 2014).

*Intergenerational trauma.* Interpersonal trauma exposure has an impact on prenatal attachment, and most research suggests that traumatized adults may be emotionally or functionally unavailable for their infant, resulting in ACEs and continued pathology into the next generation (Schwerdtfegger & Goff, 2007). Impaired self-esteem with persistent identity problems can emerge in the children of traumatized parents; for example, pressure to achieve more than the parents and preoccupation with what cannot be controlled causes persistent anxiety (Kellermann, 2001). Social learning and socialization in the context of traumatized parents can result in exaggerated family attachment or dependency, or exaggerated independence and difficulties maintaining meaningful relationships (Kellermann, 2001).

Much of the research on intergenerational trauma in humans is from Holocaust survivors and their families, showing children of survivors are more likely than controls to develop post-traumatic stress disorder and depression when faced with stressful events (Bombay et al., 2009). A qualitative study by Lev-Wiesel (2007) investigated the trauma experiences of three different types of trauma over three generations – the families of a Holocaust survivor, an individual who resided in a transit camp after immigrating, and an individual who was forced to dislocate due to war. The transmission of intergenerational trauma depended on the type of trauma, and had different meanings and impacts on psychological well-being (Lev-Wiesel, 2007). This may also

be in part due to sociocultural influences; Japanese Americans who experienced incarceration during the second World War often engaged in family avoidance of discussing the subject, resulting in a “conspiracy of silence” that is unique from the experience of trauma in other cultures (Nagata, Kim & Nguyen, 2015). Incarceration was not talked about directly, and this contributed to greater perceived familial distance. In families where it was communicated more, there were increased feelings of sadness and anger in the children coinciding with feeling closer to the parents (Nagata et al., 2015).

Menzies (2006) has identified that post-traumatic stress disorder as a diagnosis does not take into account unique social and cultural experiences. He developed an “Intergeneration Trauma Model” from qualitative interviews of homeless Aboriginal men (2010; 2006), investigating indicators of trauma in personal, family, community and national systems. The model is predicated on the assumption that public policies have disrupted relations between the four systems, resulting in negative social conditions for homeless Aboriginal men (Menzies, 2006). Through this model, negative social conditions are held responsible for the transmission of intergenerational trauma.

### **1.5.7 Intergenerational Trauma is Multi-Faceted**

Likely, the transmission of intergenerational trauma is multi-faceted and involves diverse processes explored above, going beyond only social factors (Bombay et al., 2009). Behavioural disturbances occur with traumatic experiences, such as depression, anxiety, post-traumatic stress and substance abuse (Bombay et al., 2009). Trauma also increases the response to additional stressors, where non-traumatic stressors can have additive health effects. Vulnerability to pathology as a result of trauma can be influenced by age, gender and the presence of illness co-morbidities (Bombay et al., 2009). Child maltreatment in one generation may predispose to the

occurrence of child maltreatment in the next generation, and it is often accompanied by poverty, poor mental health, substance abuse, poor coping and vulnerability to stress (Bombay et al., 2009). With regards to epigenetics, DNA methylation in particular may play a significant role in the transmission of intergenerational risk, as supported by numerous animal and human studies discussed previously.

### **1.5.8 Intergenerational Transfer of Differential DNAm Patterning**

The transfer of intergenerational trauma in association with DNAm patterning requires understanding the intergenerational transfer of DNAm patterning itself. While the mammalian epigenome is reset between each generation by genome-wide DNA demethylation, there is evidence that some methylation escapes this process (Blaze & Roth, 2015). Both DNA and DNAm patterns are inherited by daughter cells during cell division, allowing differentiated cells to transmit their phenotype to the next generation of cells (Fukuda & Taga, 2005). DNAm changes must survive two phases of epigenetic reprogramming during embryonic development. In the first phase, shortly after fertilization, the majority of DNAm is removed and novel epigenetic modifications are established; in the second phase, occurring in the germline, parental somatic marks are erased, and the inactive X chromosome is reactivated in females (Choi et al., 2010; Morgan, Santos, Green, Dean & Reik, 2005). Epigenetic imprints that are parent-specific are established during the second phase, but some epigenetic marks survive these reprogramming events in the form of epialleles, transgenes and paramutations (Daxinger & Whitelaw, 2010). Epialleles in particular are resistant to DNAm erasure during reprogramming events (Daxinger & Whitelaw, 2010). Crews (2008) suggests classifying epigenetic changes as either heritable or context-dependent, where heritable changes occur across the germline (i.e. across generations),

and context-dependant changes occur in somatic cells and persist only for the organism's lifetime (i.e. within generations).

### **1.5.9 DNAm and Tissue Specificity**

DNAm patterns in humans have been investigated using several different tissue types, and it is feasible there are tissue-specific differences in susceptibility to the effects of environmental stress on DNAm levels, due to tissue-specific variation in exposure levels and responses to the same risk factors (Foley et al., 2009; McKay et al., 2011). It is also important to note that differences in tissues, and differences in cell types within tissues, are the largest predictors of differential DNAm patterns (Edgar et al., 2017; Farre et al., 2015; Jiang et al., 2015). A correlation analysis done by Lokk et al. (2014) of DNAm patterning in seventeen different human somatic tissue types appeared to show higher correlation between tissues of similar biological function. Many epidemiological studies have measured DNAm in blood leukocytes, and BECs are also being utilized as they are more readily available (Wu et al., 2014). Epigenome-wide DNAm levels in BECs were shown to be lower compared to blood leukocytes, in a study of 57 human females aged 6-15 years (Wu et al., 2014). Overall DNAm was also lower in BECs than in blood leukocytes in a sample of 330 adult human males and females (Ashbury et al, 2014), and more variable than DNAm patterns in blood ((n = 25; Jiang et al., 2014). Though BECs have been suggested as more suitable for population epigenetic studies, blood has also been suggested as a better choice due to its functional relation to many major diseases (Jiang et al., 2014).

Compared to venous cells, DNAm patterns of BECs have been identified as better related to the central nervous system and brain tissue as well as being more similar to patterns from several brain regions compared to blood leukocytes (Smith et al., 2011). Buccal samples may be more

informative for epigenome-wide association studies as they may show significantly greater overlap in DNAm patterns with other human tissues (Lowe et al., 2013). Contrarily, the variability of DNAm between brain and blood cells has also been shown as moderately concordant (Farre et al., 2015), and tools can be used to better interpret blood samples in relation to brain tissue (the BECon, for example; Edgar et al., 2017). Currently, it is unclear whether BECs or blood cells are more representative of brain tissue, in the context of DNAm studies.

#### **1.5.10 Summary of Key Findings from Literature Review Guiding this Study**

- Adverse childhood experiences (ACEs) predict poor developmental outcomes
- ACEs predict physical and mental health problems in adulthood, including depression and addiction concerns
- Early adversity can predict differential DNAm patterns in individuals
- Maternal prenatal and perinatal stress can predict differential DNAm patterns in the offspring
- Preconception stress may also predict DNAm patterns in future offspring
- Intergenerational trauma is mostly explained by either social or biological/genetic factors
- Intergenerational trauma is likely a result of both social and biological/genetic factors
- Epigenetics looks at the interplay between socioenvironmental exposures and genetic expression
- Differential DNAm patterning might be an epigenetic biomarker associated with the transfer of intergenerational risk
- A theoretical model of intergenerational trauma with relation to DNAm in humans is proposed in Figure 1 below
- The hypothesized correlation underlying this study is proposed in Figure 2 below

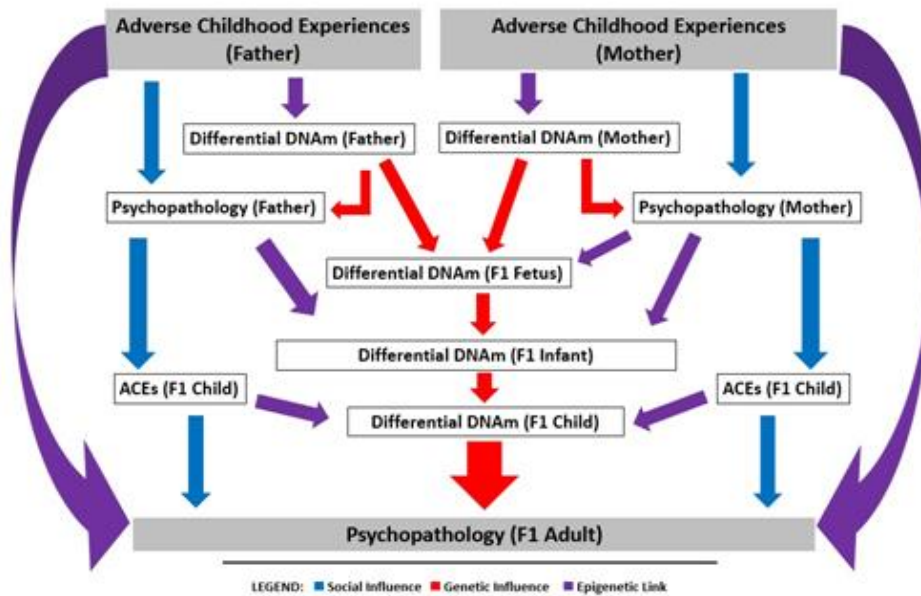


Figure 1. Theoretical transmission of intergenerational trauma in humans in relation to DNA methylation.

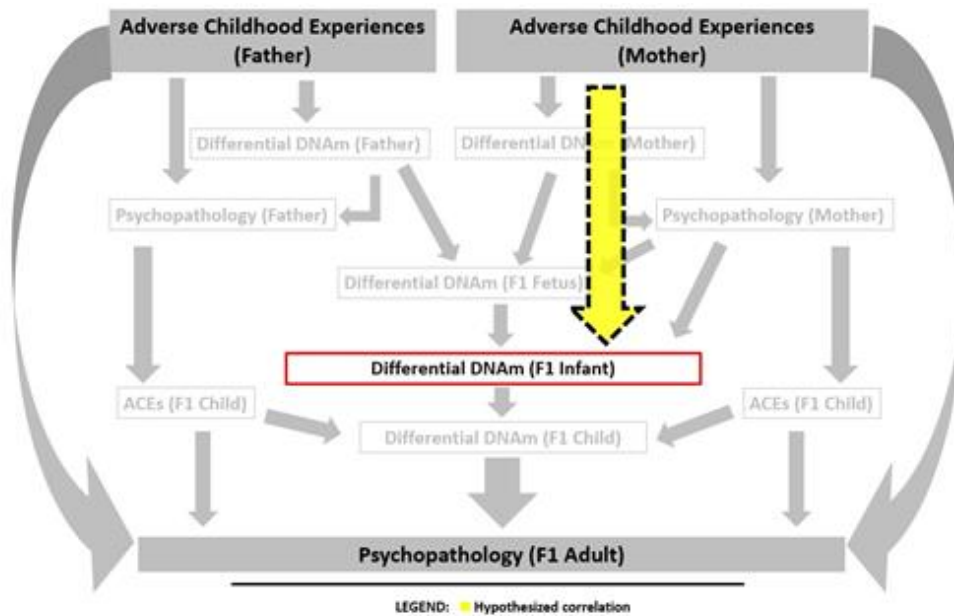


Figure 2. Hypothesized correlation between maternal adverse childhood experiences and differential DNA methylation patterns in the biological infant.



## **1.6 Contribution of Authors**

Dr. Sarah Moore completed the biostatistical analysis and ran the regression model in the study, applying statistical, mathematical and other formal techniques to analyze the data. She also generated the majority of figures showing results and contributed to writing the results, while reviewing the paper as a whole for publication. Dr. Nicole Letourneau, my thesis supervisor, suggested the examination of how parental ACEs relate to children's DNAm, helped to formulate the study concept, shared the data, and provided guidance and oversight of my project. She also provided resources and network contacts so that I could complete this study and assisted in writing and reviewing the publication draft and this thesis manuscript. Dr. Meaghan Jones provided revisions to the paper, supplementary education regarding DNA methylation, as well as feedback for the statistical analyses. Dr. Michael Kobor, whose lab completed the epigenetic data preparation, offered expertise in epigenetics and key connections to experts in his laboratory. Along with Dr. Letourneau, Dr. Gerald Giesbrecht provided the Fetal Programming sample data (e.g. on covariates and demographics) and ensured that all data were used appropriately and robustly. Thesis committee members (Dr. Letourneau, Dr. Kobor and Dr. Giesbrecht) each reviewed my writing, and provided guidance toward strengthening the work.

## Chapter 2: Submitted Publication

### 2.1 Publication Cover Sheet

Working Title: Maternal Adverse Childhood Experiences and Infant DNA Methylation

Short Title: Maternal ACEs and Infant DNA Methylation

Target Journal: *Development & Psychopathology*

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## 2.2 Publication Abstract for *Development & Psychopathology*

### Abstract

Adverse Childhood Experiences (ACEs) are known to associate with adverse adult health outcomes in humans, and ACE-like stressors (i.e. early exposure to adversity and stressful environments) have been shown to associate with differential DNA methylation (DNAm) profiles in animal and human studies. Maternal prenatal and perinatal stress associations with infant DNAm profiles in humans are well-studied; less-so is the association between maternal *preconception stress* and infant DNAm profiles. We employed an epigenetic-wide association study (EWAS) to investigate DNAm profiles of human infants in association with their biological mothers' ACEs questionnaire score. Confounders of maternal perinatal stress, household socioeconomic status, and infant biological sex and ethnicity were controlled for through multiple regression modeling. Our findings suggest an increase in maternal ACEs is associated with an increased differential DNAm profile (i.e. higher or lower DNAm) in the child. To our knowledge, this is the first EWAS investigating human preconception stress in the form of the ACEs questionnaire, and its association with the epigenetic marker DNAm in infants. Our findings support future studies investigating human preconception stress, as further studies are required to explore whether DNAm may serve as a biomarker for the transfer of intergenerational trauma in humans.

*Word Count:* 194 / 200

*Keywords:* adverse childhood experiences, DNA methylation, preconception stress, intergenerational trauma, intergenerational risk, EWAS, epigenetics

### **2.3 Article Submission for *Development & Psychopathology***

Maternal Adverse Childhood Experience and Infant DNA Methylation

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### **Background**

Felliti et al. (1998) defined adverse childhood experiences (ACEs) as exposure to personal abuse, personal neglect, and household dysfunction prior to 18 years of age. A higher score on the ACEs questionnaire has been linked to numerous mental health and addiction concerns, including psychiatric conditions such as depression, as well as numerous chronic illnesses (Chapman et al., 2004; Dube et al., 2003; Felitti et al., 1998). Moreover, the ACEs of mothers have been linked to adverse developmental outcomes in their children (McDonnell & Valentino, 2016), suggesting a transfer of risk in line with theories of intergenerational trauma. Explanations for the transfer of intergenerational trauma in humans have typically focused on socioenvironmental conditions occurring across generations, but increasingly are focusing on biological or genetic transmission (Buss et al., 2017; Drake & Liu, 2010).

Poverty, parental risk-taking behaviours, lower education, and poor parenting behaviours are all social factors offered to explain the observation that parents at risk for poor mental health outcomes tend to have children who are also at risk for poor mental health outcomes. The transfer has often been called “intergenerational transmission of risk” (Serbin & Karp, 2003). Bandura’s Social Learning Theory (1977) posited that the social transfer of parental adversity is plausible if children are exposed to their parents’ health and social problems and engage in observing and imitating their parents. A systematic review of intergenerational trauma in refugee families identified psychosocial transfer in the forms of parent-child attachment, parental engagement, child maltreatment, and family communication problems (Sangalang & Vang, 2017). Empirical literature included in the review were fairly consistent in describing parenting and family interactions as playing significant roles in the transmission of intergenerational trauma. However, a retrospective cohort study (n = 1172) investigating the impact of maternal

interpersonal trauma on developmental risk in children discovered only 24% of the observed effect was transmitted through maternal psychosocial factors only (e.g. maternal depression; Folger et al., 2017). Other factors involved in the transfer of intergenerational risk need to be explored, including biological and genetic mechanisms which have not been as strongly focused on (Buss et al., 2017).

Although the relative influences of "nature" and "nurture" were historically debated, it is now appreciated that human development is attributed to the complex interdependencies of biology and environment (Meaney, 2010). One area of study investigating the relationship between biology and environment on developmental outcomes is *epigenetics*.

Socioenvironmental influences are well studied in intergenerational trauma; less understood is the idea of how stress experienced by a human mother translates to biological changes in the child (Bowers & Yehuda, 2016). The transmission to offspring of parental phenotypic responses to environmental challenges, when the offspring does not experience the challenges directly, has been defined as "epigenetic inheritance" (Harper, 2005). Differences in phenotypic responses may be explained in part because of different socioenvironmental experiences altering levels of gene expression via epigenetic alterations (Harper, 2005).

DNA methylation (DNAm) is one of the epigenetic changes that in some contexts has correlated with gene expression, commonly studied at the 5'cytosine/3'guanine dinucleotide pairings (CpG pairings) in DNA (Robertson, 2005). Molecules promoting gene transcription may be blocked at gene promoting sites, by attachment of a methyl group to the 5'cytosine of the CpG dinucleotide (Cao-Lei, Laplante & King, 2016). However, gene expression may actually regulate DNAm itself, and the correlation between DNAm levels and gene expression is dependent on genomic location, so it is important to note that the interplay between DNAm and

gene expression is more complex than simply silencing gene expression (Jones, Fejes & Kobor, 2013). Much of the existing literature has made causal statements about DNAm and decreased gene expression; however, increased methylation has also been associated with increased gene expression in certain cases (Jones et al., 2013). Furthermore, changes in gene expression itself may also result in changes in DNAm patterns over time (Jones et al., 2013). The role of DNAm as a mechanism involved in genetic expression and phenotypic responses is not clear.

Nevertheless, differential DNAm levels could be a marker for disease risks: altered DNAm profiles have been associated with psychiatric conditions, addictive behaviours, cancer and diabetes (Chiarella, Tremblay, Szyf, Provencal & Booij, 2015; Curley, Jensen, Mashoodh & Champagne, 2011; Fagiolini, Jensen & Champagne, 2009).

Animal models have shown ACE-like factors (e.g. chronic and unpredictable maternal separation of rodent offspring) are linked to DNAm in offspring and may even persist across generations (Babenko, Kovalchuk & Metz, 2015; Zucchi, Yao & Metz, 2012), and ACEs in humans have been associated with alterations in DNAm patterns (Suderman et al., 2014). In animal models, exposure to early adversity has predicted differential DNAm in the individual that experienced the adversity (F0), but also their offspring (F1) (Blaze & Roth, 2015), and possibly even generations F2, F3 and beyond (Zucchi, Yao & Metz, 2012).

The experience of significant stress by mothers prenatally and perinatally has been shown related to differential DNAm patterns in their children (Franklin et al., 2010; Kinnally et al., 2011; Yehuda & Bierer, 2009). Relative to offspring exposures, the body of literature looking at the transmission of intergenerational risk (i.e. preconception stress) in relation to DNAm in humans is just emerging. To our knowledge, the link between parental exposure to early adversity, as measured by the ACEs questionnaire, and offspring DNAm is yet to be studied in

humans. This study aimed to explore the relationship between maternal childhood adversity and DNAm patterns in offspring, an association suggestive of the transfer of intergenerational trauma (as preconception stress) in humans. In the following, we review the relationship between ACEs and developmental outcomes, as well as links between early life adversity similar to ACEs and DNAm. Finally, confounders in the relationship between maternal ACEs and infant DNAm are explored, and support for the investigation of differential DNAm patterns in infants with relation to their mothers' ACEs is provided.

### **ACEs Predict Poor Health and Development**

Felitti et al. (1998) demonstrated there is a dose-response relationship between the total number of ACEs and the propensity for many poor health outcomes (Felitti et al., 1998). Individuals with four or more ACEs on the questionnaire exhibited odds ratios of 1.6 for diabetes, 3.9 for emphysema, 1.6 for skeletal fractures, and 2.3 for hepatitis compared to individuals without ACEs (Felitti et al., 1998). After multivariable adjustment, adults with greater than or equal to six ACEs were 2.4 times more likely to die before they reach the age of 65 years, and more likely to die nearly twenty years earlier on average than those lacking ACEs (Brown et al., 2009). ACEs have been associated with the development of heart disease (Dong et al., 2004), cancer (Brown, Thacker & Cohen, 2013) and obesity (Edwards, Holden, Felitti & Anda, 2003) in adulthood. It has been proposed that the mechanism of early mortality associated with a high ACE score is that ACEs lead to social, emotional and cognitive impairment, in turn contributing to health-risk behaviours and disease, disability and social problems (Felitti et al., 1998).

There are similarly detrimental mental health outcomes associated with ACEs. A higher number of ACEs was associated with lower mean scores on mental health assessments (Edwards



et al., 2003), with the number of ACEs having a graded relationship with depressive disorders in adulthood (Anda et al., 2002; Chapman et al., 2004), and higher ACEs associated with clinical depression and anxiety (Lindert et al., 2014). Specific to the emotional abuse items on the ACE questionnaire, childhood emotional abuse increased the risk for lifetime depressive disorders (Chapman et al., 2004). The number of ACEs a person was exposed to also demonstrated a graded relationship with alcoholism in adulthood, and this relationship was independent of having a parent who abused alcohol (Anda et al., 2002). Each ACE increased the likelihood of early illicit drug use between two- and four-fold (Dube et al., 2003). Individuals with greater than or equal to five ACEs had between seven and ten times more likelihood of problems associated with drug use, drug addiction and parenteral drug use (Dube et al., 2003).

With respect to the impact of ACEs on mothers and their infants, ACEs predicted higher levels of prenatal depressive symptoms, and childhood maltreatment specifically predicted higher levels of maladaptive infant socioemotional functioning at six months of age (McDonnell & Valentino, 2016). Maternal ACEs have also been associated indirectly with internalizing and externalizing problems in children (Letourneau et al., 2018). Finally, maternal ACEs associated to prenatal and perinatal infant health complications, and there was a linear association between the number of maternal ACEs and the extent of cumulative biomedical and psychosocial risk to infants (Madigan et al., 2017). These findings hint that maternal ACEs may predict or influence the transmission of intergenerational risk, and the processes may extend beyond social learning to underlying epigenetic biomarkers. Altered DNAm profiles, in particular, have been shown to associate with ACE-like stressors.

**ACE-Like Stressors Relate to Differential DNAm Patterns**

Early adversity variables similar to ACEs have been examined in numerous animal and human studies in relation to DNAm. In rats, increased DNAm of the brain-derived neurotrophic factor promoter site in the pre-frontal cortex associated with exposure to abusive maternal care, with effects emerging in infancy and sustaining into adulthood (Fagiolini et al., 2009). A study of macaques demonstrated no significant difference in epigenome-wide DNAm between controls and individuals experiencing early life stress, characterized by exposure to difficult conditions for foraging (Kinnally et al., 2011). However, individuals with higher global DNAm were at the greatest risk of maladaptive behavioural responses posed by early life stress (Kinnally et al., 2011), suggesting differential DNAm patterns may correlate with a background of risk in individuals who have experienced early life stress.

Rodent models of early life adversity are limited, in part because animal models for the experiences of psychological and sexual abuse do not exist (Lutz & Turecki, 2014). Social experiences are also particularly influential on the developing human brain, with continued plasticity into adolescence and adulthood (Curley et al., 2011) and epigenetic studies in humans identified several epigenetic associations with early life stress and adversity (Provencal & Binder, 2015). Studies have shown global DNAm differences for human individuals exposed to early adversity, including those involved in the development and function of nervous and immune systems (Mehta et al., 2013; Smith et al., 2011). Early-life poverty has been associated with differential DNAm patterning, irrespective of current socioeconomic status (Lam et al., 2012). DNAm appears to be an important biomarker of human early life experiences, and may even predict psychopathological risk (Lutz & Turecki, 2014). These associations may also be involved in the disease state through actions on the HPA axis and immune system, for example

(Miller, Fisk, Modi & Glover, 2005), though – as discussed earlier – the evidence for DNAm as a mechanism involved in producing disease states is weak.

Childhood abuse was linked to 997 differentially methylated gene promoters involved in key cell signaling pathways for transcriptional regulation and development (Suderman, et al., 2014). Early experiences with parent attachment, such as lower levels of maternal responsiveness and greater maternal depressive symptoms, have demonstrated a relationship with infant DNAm patterns (Conradt et al., 2016). One study of seven- to ten-year-old children in institutional care showed increased DNAm of 28 genes involved in brain development and function, including genes implicated in the hypothalamus-pituitary-adrenal (HPA) axis and stress response (Naumova et al., 2012). Another study showed 30 CpGs were more methylated in 19 genes of individuals who experienced early childhood adversity in other countries prior to adoption, when compared to American-born individuals (Esposito et al., 2016). Early life adversities seem to be associated with differential DNAm patterns.

Human DNAm studies have emphasized genes implicated in the HPA axis, especially the *NR3C1* gene (Braithwaite, Kundakovic, Ramchandani, Murphy & Champagne, 2015; Kertes et al., 2016; Monk et al., 2016; Palma-Guidel, Cordova-Palmera, Eixarch, Deuschle & Fananas, 2015; Perroud et al., 2014; Yehuda et al., 2014). There was demonstrated increased DNAm of the *NR3C1* promoter gene in children who have experienced maltreatment (Romens, McDonald, Svaren & Pollak, 2015). A study of 468 adolescents (age  $M = 16.1$  years) showed stressful early life events and traumatic youth events were also associated with hypermethylation of *NR3C1* (Van der Knaap et al., 2014). Interestingly, stressful events in adolescence were also associated with higher *NR3C1* methylation, independent of childhood stressful events (Van der Knaap et al., 2014). The involvement of this gene in the human “flight or fight” response may have

implications for human phenotypic outcomes, especially if transcription is altered at an early developmental stage. This could also be true of other candidate genes.

Altered *SLC6A4* methylation is also prominent in the literature of early life stress, which encodes the gene for serotonin transporter. Childhood adversities and more severe clinical presentations of depression and anxiety have been associated with higher DNAm of *SLC6A4* (Kang et al., 2013). Exposure to childhood abuse associated with hypermethylation of *SLC6A4* (Beach et al., 2010). Importantly, the total number of types of past traumatic events was significantly associated with *SLC6A4* methylation, ranging from abuse and maltreatment, sudden death in the family, or serious injury (Koenen et al., 2011).

Most clinical and epidemiological work on human epigenetics has not included brain or nervous tissue samples (Provencal & Binder, 2015). However, one particular study of the hippocampi of adult men, who experienced childhood abuse and completed suicide in adulthood, showed increased DNAm of 248 gene promoters and associated decreases in expression of these promoters (Labonte et al., 2012). Further, the genes with the most differential DNAm were responsible for cellular and neuronal plasticity (Labonte et al., 2012). Maternal stressors in infancy, and paternal stressors in the preschool years also associated to global differential DNAm patterns in adolescents (Essex et al., 2013). A twin study is ideal for examining unique influences of the environment on the same genotype; in a monozygotic human twin study on bullying victimization, bullied twins showed increased global DNAm at age ten compared to their non-bullied co-twins, and the difference could not be attributed to the twin pairs' genetic makeup or shared familial environments (Ouellet-Morin et al., 2013). This finding is important, as genetic differences accounted for less association with differential DNAm patterns, and socioenvironmental exposure to stress appeared to have a stronger association.

ACE-like stressors have been shown to associate with altered DNAm profiles in animal and human studies, but investigations of this relationship have largely remained within the same generation (i.e. most studies looked at the effects of ACEs on that individual's DNAm profile, not on the next generation's DNAm profile). Though there is a paucity of human research investigating preconception stress on offspring DNAm patterns (which our study aims to do), a more recent finding by Yehuda et al. (2014) showed differential DNAm patterns at the *NR3C1* gene in the offspring of Holocaust survivors, supporting the idea of altered DNAm profiles with relation to preconception stress. Ahead of investigating the association between human maternal preconception stress and infant DNAm, confounders which associate with differential DNAm patterns needed exploration. An important confounder is maternal perinatal stress: if ACEs lead to poor health outcomes in adulthood, stress experienced by the adult mother in the perinatal period may impact DNAm in the infant. Indeed, associations between maternal *prenatal* stress and infant DNAm are well-studied in humans.

### **Maternal Prenatal Stress and DNAm in Offspring**

Maternal prenatal stress resulted in lasting DNAm signatures in several tissues of the offspring, affiliated with 957 genes predominantly related to immune function (Cao-Lei et al., 2014). An outcome of exposure to ACEs was a lifetime history of depression, and when human mothers had lifetime history of depression, there were DNAm changes in multiple tissues across the lifespan (Nemoda et al., 2015). In a study of 62 people whose mothers had a history of depression, differential DNAm patterns were detectable through samples of their neonatal cord blood and found to persist through to their adult brains, with evidence derived from post-mortem hippocampal samples (Nemoda et al., 2015). It is still unclear if the DNAm patterns contributed

to altered genetic expression and phenotypes, or if they were simply reflective of current stress states which were transferred through socioenvironmental means.

Differential DNAm patterning was related to higher maternal depressive symptoms (Conradt et al., 2016), and epigenome-wide DNAm alteration was present in one-year-old infants of mothers with major depressive disorder (Cicchetti, Hetzel, Rogosch, Handley & Toth, 2016). Human prenatal depressive symptoms significantly predicted increased DNAm of the *NR3C1* gene in male infant buccal epithelial cells (BECs) (Braithwaite et al., 2015), and maternal cigarette smoking related to altered DNAm patterns in placental *NR3C1* (Green & Marsit, 2015). Chronic prenatal stress and war trauma were associated with increased DNAm on HPA axis genes in infants, including *NR3C1*, in several tissue samples (Kertes et al., 2016). Yehuda et al. (2014) demonstrated a link between post-traumatic stress disorder in Holocaust survivors (both maternal and paternal) and DNAm of the *NR3C1* site in the offspring's blood leukocytes. Mothers exposed to the Tutsi Genocide in Rwanda showed higher DNAm within the *NR3C1* coding sequence 20 years after the event (Perroud et al., 2014). The mothers in this study were pregnant at the time of the event, and their now-grown children also showed increased DNAm within *NR3C1* of blood leukocyte samples (Perroud et al., 2014). A meta-analysis combining evidence from seven studies and data from 977 individuals demonstrated a statistically significant correlation between DNAm levels on the *NR3C1* promoter site and different forms of prenatal stress (Palma-Guidel et al., 2015).

Another candidate gene prominent in the literature on prenatal stress is *IGF2*, important for fetal growth. Maternal blood cortisol levels, scores on the Edinburgh Depression Scale and Pregnancy-Related Anxiety Questionnaire, as well as maternal exposure to bisphenol-A have all associated with increased DNAm of *IGF2*, which may have further associated with low birth

weight and premature development in the fetus (Green & Marsit, 2015; Vangeel et al., 2015).

Ample human evidence has suggested prenatal stress is associated with differential DNAm of offspring; an investigation of preconception stress associations with DNAm profiles would require controlling for maternal prenatal stress.

### **DNAm and Biological Sex**

DNAm patterns are different for males and females, and the mechanisms are still largely unknown (Jones, Moore & Kobor, 2018). In female mammals, before implantation in the uterine wall, the paternal X-chromosome is preferentially inactivated, which involves differential methylation of the chromosomes and the expression of RNA (Clerc & Avner, 2000). Inactivated X-chromosome shows relatively more methylation in certain DNA regions (Cohen & Lee, 2002). Observed differences between male and female global DNAm patterning may be attributed to the process of X-chromosome inactivation in males, the presence of an additional X-chromosome in females, or to the downstream effects of sex determination (El-Maarri et al., 2007). Male mammals tend to show higher differential DNA methylation across the epigenome when compared to females (Bock, 2016; El-Maarri et al., 2007). Differences in DNAm patterns may reflect the many physiological differences between males and females (Jones, Moore & Kobor, 2018), and so biological sex is another important covariate when investigating maternal ACES and infant DNAm profiles.

### **DNAm and Socioeconomic Status**

Socioeconomic status also requires consideration for its association with DNAm; lower socioeconomic status (SES) in childhood (but not adulthood) associated with specific gene expression (Miller et al., 2009), and with global differential DNAm patterning in blood cells (Lam et al., 2012). Poverty has associated with poor health outcomes, and covaried with

differential DNAm patterns as well. Early life poverty correlated with higher differential DNAm patterning, and there appears to be a general trend toward correlation between DNAm and variables of early-life SES (Lam et al., 2012). Low childhood SES and low adult SES both associated with differential DNAm in stress-related genes and inflammation-related genes across multiple ethnicities ( $n = 1,264$ ; Needham et al., 2015). SES associated with DNAm of genes involved in inflammation, supporting the hypothesis that a social environment related to SES can modify epigenetic patterns in a manner that is relevant to the physical consequences of socioeconomic deprivation (Stringhini et al., 2015). One prospective longitudinal study utilized epigenetic, neuroimaging and behavioural data from adolescents ( $n = 132$ ) to show changes in DNAm patterns were associated with lower SES, and these DNAm changes predicted changes in risk-related brain functions (Swartz et al., 2017).

### **DNAm and Other Confounders**

There are differences in global DNAm patterning between ethnicities (Zhang et al., 2011); consequently, DNAm analysis models have typically included ethnicity as a covariate. DNAm similarities within ethnicities may be due in part to shared genetic ancestry, but also to shared cultures and environments (Jones et al., 2018). Cell-type differences, diet and lifestyle could all contribute to patterns seen in ethnic groups (Jones et al., 2018). DNAm is not stable over the lifetime, in part because it is experience and environment dependant, but also because there is an overall decrease in DNAm over time (Jones et al., 2018), so the timing of samples requires attention. Lastly, genetic variation is another consideration, as specific sites with DNAm have been shown as highly associated with nearby genetic variants (Jones et al., 2018). Genetic variation is often the most inherently difficult confounder to control for in empirical studies.



**The Current Study: Maternal Preconception Stress and Infant DNAm**

As reviewed above, a higher score on the ACEs questionnaire has been associated with poor developmental health outcomes, and measures of early life adversity have associated with differential DNAm patterning in animal and human models. Maternal preconception stress, in the form of ACEs, may also associate with differential DNAm patterning in the next generation. Buss et al. (2017) propose a transdisciplinary framework for the intergenerational transmission of risk, suggesting transmission through psychological, behavioural and biophysical outcomes of maternal childhood maltreatment (forms of which can be ACEs). Epigenetic transmission may play a role in this framework, and our study investigates a potential epigenetic relationship between maternal ACEs and infant DNAm, in the context of their framework. We hypothesized that compared to infants of mothers with lower ACE questionnaire scores, infants of mothers with higher scores would have differential DNAm profiles. We sought to answer the following question: What is the association between maternal ACEs and DNAm in offspring, controlling for perinatal exposure to maternal stress and other confounders?

**Methods**

This secondary analysis utilized data obtained from participants in a sub-study of the APrON study (Alberta Pregnancy Outcomes and Nutrition; Kaplan et al., 2014), which ended enrollment in 2012. Data were collected from mothers at clinic visits and via questionnaires during pregnancy, and ACEs data were collected from mothers postpartum, cross-sectionally, when the youngest children in the cohort were approximately two years of age. Participants were all pregnant women over 16 years old, before 22 weeks of gestation. Women were excluded if they were unable to answer questions in English or if they planned to move out of the region during the timeframe of the study (Kaplan et al., 2014). Data were available from 139 mother-

infant pairs. An epigenome-wide association study (EWAS) design was employed, as this approach holds great promise for understanding the role of epigenetic variation in health and disease, and has increasingly replaced candidate gene approaches (Michels et al., 2013). Though much of the previous literature has utilized candidate gene approaches, many have erroneously resulted in ideas of causality, and so choosing candidate targets would have limited our investigation. EWAS allowed examination of DNAm at multiple CpGs and assessment of any potential sites associated with a trait or phenomenon (Michels, et al., 2013).

## Measures

**Predictor: ACEs questionnaire.** The primary predictor of maternal childhood adversity was measured using the ACE questionnaire, developed by Felitti et al. (1998) for their landmark study. It is a ten-item scale with “yes” or “no” categorical responses, yielding a total score ranging from zero to ten. Five of these questions address personal trauma (three for personal abuse, two for personal neglect), and five questions address household dysfunction related to family members. The questionnaire was administered to mothers in the study ranging from 18 to 24 months postpartum.

**Outcome: DNA methylation.** DNAm patterns in humans have been investigated using several different tissue types. There are likely tissue-specific differences in susceptibility to effects of environmental stress on DNAm levels, due to variations in exposure levels and responses to the same exposures (Foley et al., 2009; McKay et al., 2011). DNAm patterning in 17 different human somatic tissue types appeared to show higher correlation between tissues of similar biological function (Lokk et al., 2014). BECs are being utilized as they are more readily available for DNAm studies (Wu et al., 2014). Epigenome-wide DNAm levels were shown to be lower in BECs compared to blood leukocytes, in a study of 57 human females aged 6-15 years

(Wu et al., 2014) and in a sample of 330 adult human males and females (Ashbury et al, 2014), as well as more variable than DNAm patterns in blood (Jiang et al., 2014)) Compared to blood leukocytes, buccal samples may be more informative for EWAS as they have shown significantly greater overlap in DNAm patterns with other human tissues (Lowe et al., 2013). DNAm patterns of BECs might be better related to the central nervous system and brain tissue, as well as more similar to patterns from several brain regions (Smith et al., 2011), which is of particular interest when investigating psychopathological outcomes.

BECs were collected from infants at three months of age using cytology brushes and were stored at a temperature of -80°C. Cell swabs were placed in a cell lysis mixture and purified using the Gentra Puregene Kit (Qiagen, Venlo, Limburg, Netherlands). Cell lysis buffer and proteinase K were added to the samples, and they were incubated with occasional mixing. Samples were centrifuged, and the cytology brush was removed from the tube. The samples were processed for DNA purification using the Autopure method (Qiagen, Venlo, Limburg, Netherlands), and processed further using the cell lysate program. Samples were left open to air allowing for evaporation of excess ethanol, and low-TE buffer was added to the tubes. The samples were then centrifuged, rehydrated and transferred into microtubes for long-term storage at 4°C at the Alberta Children's Hospital Genetics Laboratory.

We employed sodium bisulphite treatment which is the most common method of measuring DNAm in human samples. This converted unmethylated cytosines into thymidines, while leaving methylated cytosines intact and quantifies the proportion of thymidines to the remaining methylated cytosines (i.e. the proportion of methylated cytosines to unmethylated cytosines; Clark, Harrison, Paul & Frommer, 1994). Bisulphite conversion of DNA was performed with the EZ-DNA methylation kit (Zymo Research, Irvine, CA, USA) and bisulphite

converted DNA was processed using the Illumina Infinium HumanMethylation450 Bead Chip Kit (Illumina Inc., San Diego, CA). Background subtraction and colour correction of data was done using Illumina Genome Studio software (Esposito et al., 2016). Prior to data analysis, initial quality controls included examination for obvious outliers or failed samples, and checking the expected biological sex of the participant.

To reduce the risk for cross-contamination of BECs with leukocytes (e.g. during stress or illness), we included a surrogate cell-type proportion variable (SV1), to help ensure findings at any epigenomic site are related to ACEs, and not due to cell-type differences. Of eight well-known and utilized methods for cell-type correction, including reference-based method, surrogate variable analysis is a method that has performed well in EWAS (McGregor et al., 2016). This method is also preferable to reference-based methods for this study, as reference-based methods were developed on adult methylomes that may not necessarily be appropriate for infant samples (Hattab et al., 2017; McGregor et al., 2016). SV1 was calculated using the surrogate Variable Analysis (SVA) package in R (Leek, Johnson, Parker, Jaffe & Storey, 2012; Leek et al., 2017).

**Covariates.** Covariates in our analysis included maternal stress, infant biological sex, infant ethnicity, and socioeconomic status. Detailed discussion of these variables follows.

**Maternal Stress.** Stressors occurring during and soon after the period of gestation have a more pronounced effect on infant DNAm patterns; therefore, current maternal stress was included in the model. The maternal stress variable (PC-S) in the regression model was defined through a principal component analysis (PCA) of measures for maternal anxiety and maternal depression, performed using the “prcomp” package in R (Ritchie et al., 2015). Participant mothers completed the Edinburgh Postnatal Depression Scale (EPDS; Cox et al., 1987; Jomeen

& Martin, 2007) and the Symptom Checklist-90-R (SCL-90-R; Derogatis, 1994) in early pregnancy (within first two trimesters), late pregnancy (in third trimester), and three-months post-partum. For both instruments, each individual's highest reported score from the three time points was utilized in the PCA for this variable.

***Ethnicity.*** Ethnic differences in DNAm profiles may be the most profound, so it was essential to control for the effect of participant ethnicity on global DNAm patterns. Data on the ethnicities of infants in the study were available through infant birth reports and were categorized as “Caucasian” and “non-Caucasian.”

***Infant Sex.*** There are also differences between biological male and female epigenome-wide DNAm profiles. Data for the biological sex of the infants in the study were available through infant birth reports.

***Socioeconomic Status.*** In the past, SES has been a composite measure determined by combinations of variables, typically including one or more of: income, education, marital status, occupational prestige, and financial assets (Letourneau, Duffet-Leger, Levac, Watson & Young-Morris, 2013). Income and education data were available for study participants, and utilized for a composite SES measure. Income was reported as \$0 - \$70,000 or more than \$70,000; education was reported as less than post-secondary degree or having at least a post-secondary degree. These two variables were collapsed to define the SES variable by utilizing the sum of the two variables to obtain a score of 0, 1 or 2, from low to high SES.

## **Analysis**

Prior to analysis, pre-processing involved removing a subset of probes including those on the X or Y sex chromosome and probes with missing values, polymorphic probes, and those shown to cross-hybridize (Moore et al., 2017; Price et al., 2013). The remaining probes required

normalization, as the Illumina Bead Chip Kit includes two probe types with differing distributions (Moore et al., 2017). Normalization was necessary for the two types to have similar, comparable distributions of  $\beta$ -values, and so the beta mixture quantile method was employed (Moore et al., 2017; Teschendorff et al., 2013). PCA was then performed on genome wide DNAm as a means of data reduction, and the top principal components (PCs) were correlated with covariates for descriptive purposes. By correlating PCs which account for the majority of variability in DNAm with covariates of interest, the potential confounding effects of covariates could be observed.

The sample was described with measure of central tendency and frequencies. To test the study hypothesis, multiple linear regression modeling was performed using the R function “lmfit,” in the Limma package (Ritchie et al., 2015). For a more conservative approach to analysis, all continuous scale variables were standardized. Six of the cases had incomplete data for income, education and ethnicity variables, with one case missing a score for the anxiety measure. To keep all cases in the analysis and conserve power, the missing variables were substituted in the following way: (1) If missing only income, SES was scored the same as education, and vice versa; (2) The sample average was imputed for one case missing a score for anxiety measure; and (3) Two cases were missing data for income, education and ethnicity, and given this small amount of missingness, these were substituted with the means for the sample.

Because there were over 450,000 CpG sites measured, typical methods to correct for false positives such as the Bonferroni method could be overly conservative (Narum, 2006), and so the Benjamini-Hochberg correction was more appropriate (Benjamini & Hochberg, 1995; Jones, Moore & Kobor, 2018). A reference range of DNAm values between 5<sup>th</sup> percentile and 95<sup>th</sup> percentile was determined to remove outliers and manage the DNAm data more robustly.

The  $\Delta\beta$  is defined as the mean  $\beta$ -value difference between two groups, calculated as a subtraction of one group's  $\beta$ -value from the other, resulting in both positive and negative values. A  $\Delta\beta$ -value of 0.05 has been considered a biologically significant effect (Esposito et al. 2016; Ladd-Acosta et al., 2014; Lam et al. 2012); however, effect sizes of smaller magnitude have often been reported in epigenetic studies and may have implications for healthy child development (Breton et al., 2017). In the initial EWAS, m-values (a log transformation of  $\beta$ ) were utilized because the distribution of  $\beta$ -values would violate the assumptions of our regression model (i.e. m-values were more normally distributed). We converted m-values back to  $\beta$ -values, as these are interpretable biological effects; denoted as  $\Delta\beta$ , they represent the difference in  $\beta$ -values when increasing ACE scores by one standard deviation. Finally, volcano plots were employed to show the  $\Delta\beta$ , or measure of effect, and the statistical significance at  $-\log_{10}$  of the  $p$ -value. A supplementary analysis of the significant CpGs was completed to help decipher the results; namely, whether one or more maternal ACEs had an association with altered infant DNAm at the specific CpGs when compared to zero maternal ACEs. Genes associated with the significant CpGs were investigated with USCS Genome Browser, February 2009 assembly (Kent et al., 2002), GeneCards (Safran et al., 2010), and ENCODE (Encode Project Consortium, 2012).

## Results

### Sample Description

Table 3 presents descriptive data. Mothers were an average of 31 years old and the highest ACE score reported is 8/10, with the highest reported score in the “household dysfunction” category (ACE score  $M = 0.99$ ,  $SD = 1.68$ ). The percent of mothers with 0 ACEs was 60.4%; Table 4 shows the frequency and percentage of total ACE scores from the sample. Collapsing income and education variables revealed over half of the sample was in the highest

SES category. Only 15.8% were in the lowest category, suggesting the sample was negatively skewed towards higher socioeconomic individuals.

Principal Component Analysis

Figure 3 illustrates effects of variables on DNAm data after accounting for technical batch effects (array, and row that the sample was located on the array) using ComBat in R. As expected, SV1 (cell-type correction variable) accounted for the largest amount of variability in the data, given the primary role of DNAm profiles in determining the fate of specific cell types. The effects of covariates and ACEs are also apparent in the data, with the exception of PC-S (current maternal stress variable).

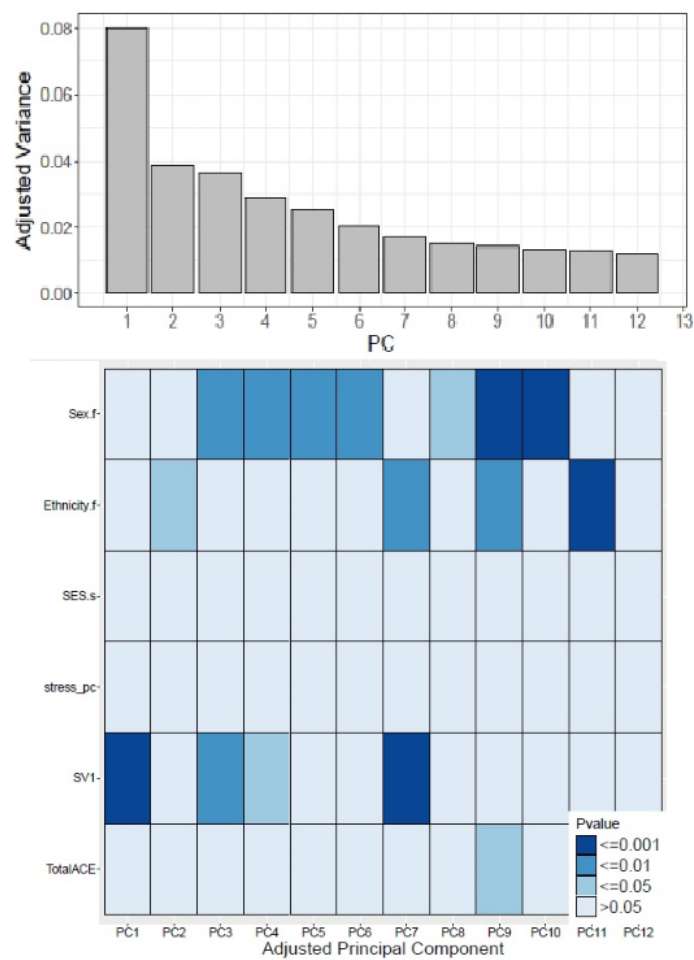


Figure 3: Principal Component Analysis of DNAm and Correlations with Covariates.



CAPTION: “PCA was performed on genome wide DNAm as a means of data reduction, and the top principal components (PCs) were correlated with covariates to observe the potential confounding effects of covariates. The cell-type proportion variable (SV1) accounted for the largest amount of variability in the data. Covariates and ACEs effects were also present, except for the maternal stress (PC-S) and SES variables.”

Table 3: Descriptive Statistics of Demographic Variables Covariates

		Maternal Age	Income	Education	Ethnicity	Sex	Anxiety Score (SCL-90-R)	Depression Score (EPDS)	Total ACEs
N	Valid	139	133	135	135	139	138	139	139
	Missing	0	6	4	4	0	1	0	0
Mean		31.31	---	---	---	---	4.2899	7.6187	.99
Std. Deviation		3.645	---	---	---	---	5.06916	4.65847	1.675
Minimum		22	---	---	---	---	.00	.00	0
Maximum		40	---	---	---	---	30.00	22.00	8

Table 4: Frequency and Percentage of ACE Scores

	ACE Score	Frequency	Percent	Cumulative Percent
Valid	0	84	60.4	60.4
	1	19	13.7	74.1
	2	18	12.9	87.1
	3	7	5.0	92.1
	4	3	2.2	94.2
	5	3	2.2	96.4
	6	1	.7	97.1
	7	3	2.2	99.3
	8	1	.7	100.0
	Total	139	100.0	

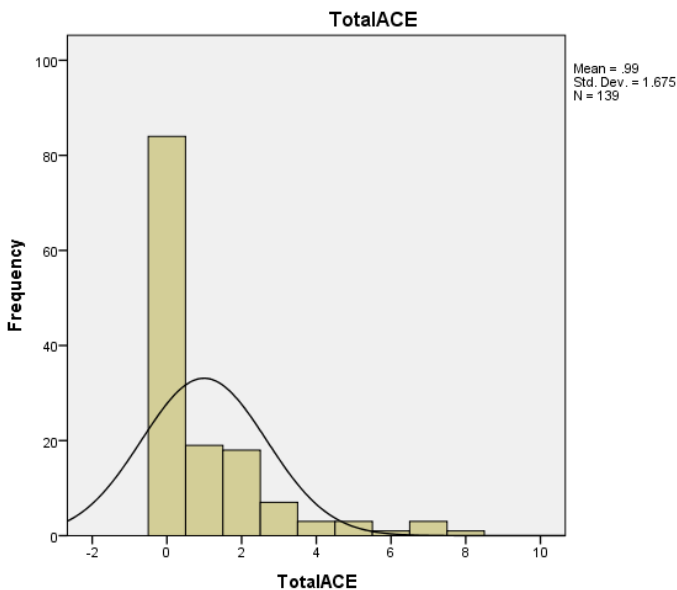
Table 5: Correlations Between Variables

		Maternal Age	Income	Education	Ethnicity	Sex	Anxiety	Depression	Total ACEs
Maternal Age	Pearson Correlation	1	---	---	---	---	---	---	---
	Sig. (2-tailed)		---	---	---	---	---	---	---
Income	Pearson Correlation	.033	1	---	---	---	---	---	---
	Sig. (2-tailed)	.709		---	---	---	---	---	---
Education	Pearson Correlation	-.210*	-.305**	1	---	---	---	---	---

	Sig. (2-tailed)	.014	.000		---	---	---	---	---
Ethnicity	Pearson Correlation	.048	.137	-.112	1	---	---	---	---
	Sig. (2-tailed)	.582	.115	.197		---	---	---	---
Sex	Pearson Correlation	-.069	.080	-.022	.077	1	---	---	---
	Sig. (2-tailed)	.422	.359	.803	.376		---	---	---
Anxiety	Pearson Correlation	-.211*	-.181*	.152	-.138	.063	1	---	---
	Sig. (2-tailed)	.013	.038	.080	.112	.462		---	---
Depression	Pearson Correlation	.036	-.135	.188*	-.120	.016	.541**	1	---
	Sig. (2-tailed)	.674	.120	.029	.167	.848	.000		---
TotalACE	Pearson Correlation	.019	-.151	.091	.004	.082	.145	.132	1
	Sig. (2-tailed)	.821	.083	.295	.962	.338	.090	.123	

\*. Correlation is significant at the 0.05 level (2-tailed).

\*\*. Correlation is significant at the 0.01 level (2-tailed).



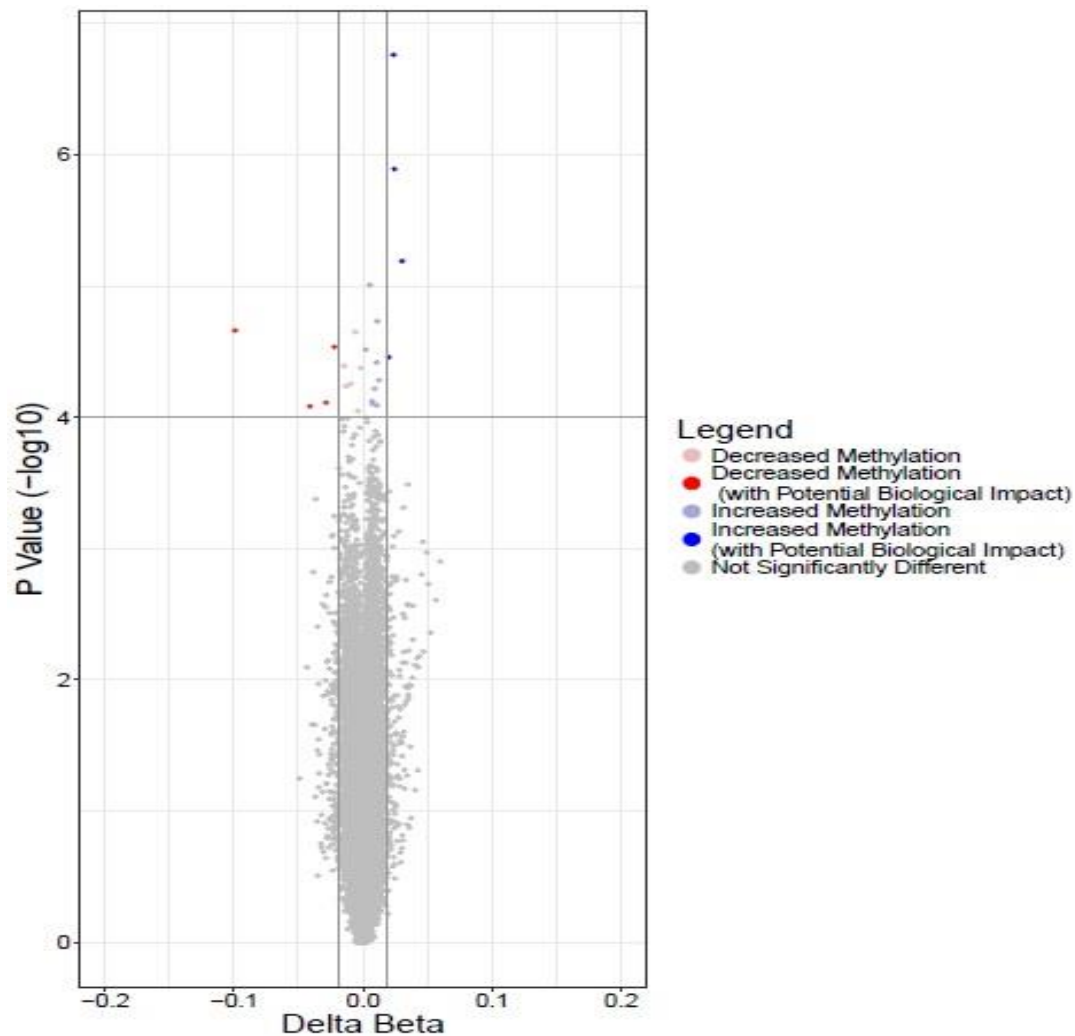
*Figure 4: Histogram Distribution of Total ACE Scores*

CAPTION: “Total reported ACE scores were positively skewed, indicating much of the sample did not report trauma in the form of early childhood adversity, as measured by the ACE questionnaire.”

### Epigenome Wide DNAm Analysis

Volcano plots are a type of scatter plot that can allow for easy visual comparison of different conditions (e.g. EWAS results from other studies, showing the extent of global differential DNAm). We produced a volcano plot of CpGs with nominally significant differential DNAm (Figure 4) utilizing the final model:

$$\text{DNAm} = \beta_0 + \beta_1 \times \text{ACE Score} + \beta_2 \times \text{SES} + \beta_3 \times \text{PC-S} + \beta_4 \times \text{SV1} + \beta_5 \times \text{Sex} + \beta_6 \times \text{Ethnicity} + \beta_7$$



*Figure 5: Volcano Plot of Significantly Differentially Methylated Sites*

CAPTION: “This volcano plots depicts the relationship between maternal ACE questionnaire scores and DNAm patterns in their infants. DNAm was either decreased or increased in relation to higher ACE scores. Eight CpGs were identified with nominally significant differential methylation in relation to higher standardized scores on the ACE questionnaire.”

After model corrections, correlations were plotted between total ACE scores and nominally significant DNAm sites. Table 5 outlines the 8 nominally significant CpGs for differential DNAm. Effect sizes ranged from -0.10 to 0.03 (Figure 5).

Table 6: Significant Sites for DNAm in Correlation to Total ACE Score (Corrected Model)

Epigenomic Site	logFC	<i>T</i>	<i>p</i> - value	Adj <i>p</i> -value	Effect ( $\Delta\beta$ )
Cg22307029	0.167426	5.540233	1.51E-07	0.032873	0.023801
Cg24324837	0.2928	5.10616	1.09E-06	0.118344	0.024392
Cg22590775	0.194001	4.700772	6.28E-06	0.455439	0.030307
Cg07792871	-0.8227	-4.4707	1.63E-05	0.595236	-0.09895
Cg00458927	-0.13434	-4.37906	2.36E-05	0.595236	-0.02206
Cg00753924	0.292754	4.25403	3.88E-05	0.740937	0.019828
Cg04044836	-0.19791	-4.11534	6.67E-05	0.763151	-0.02854
Cg09605634	-0.25671	-4.05413	8.43E-05	0.851475	-0.04103

Table 7: CpG Site and Related Genes, Location and Function

Epigenomic Site	Gene Name	CpG Relation	Regulatory Group (i.e. Promoter/Coder)
Cg22307029	CCDC155 / KASH5	Island	Unclassified
Cg24324837	CCDC155 / KASH5	Island	Unclassified
Cg22590775	CCDC155 / KASH5	Island	Unclassified
Cg07792871	HCG9	N Shore	*
Cg00458927	CCT4	S Shore	*
Cg00753924	RXRA	N Shore	Unclassified
Cg04044836	STRN	*	*
Cg09605634	DCLK1	*	*

\*Information unavailable

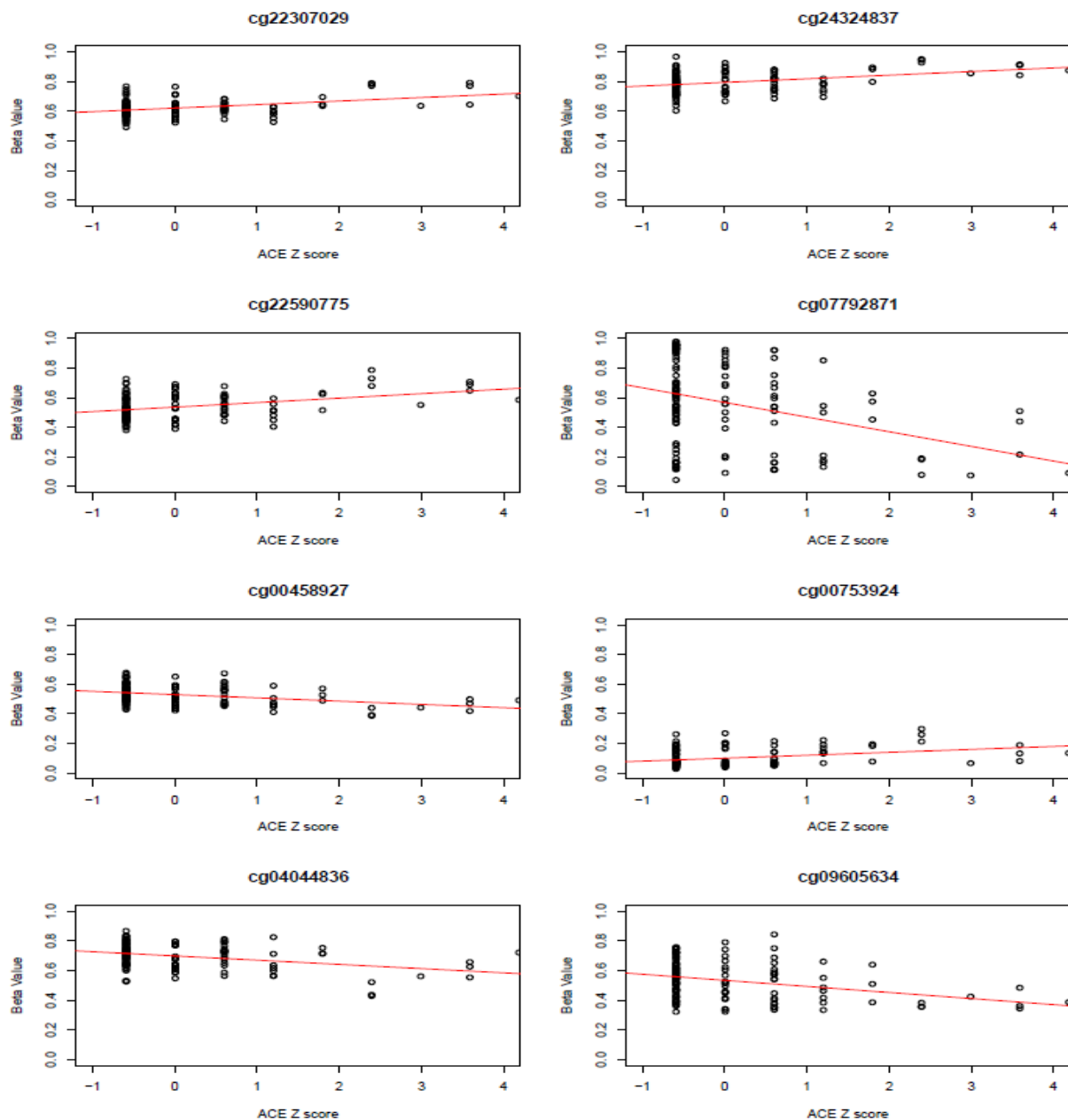
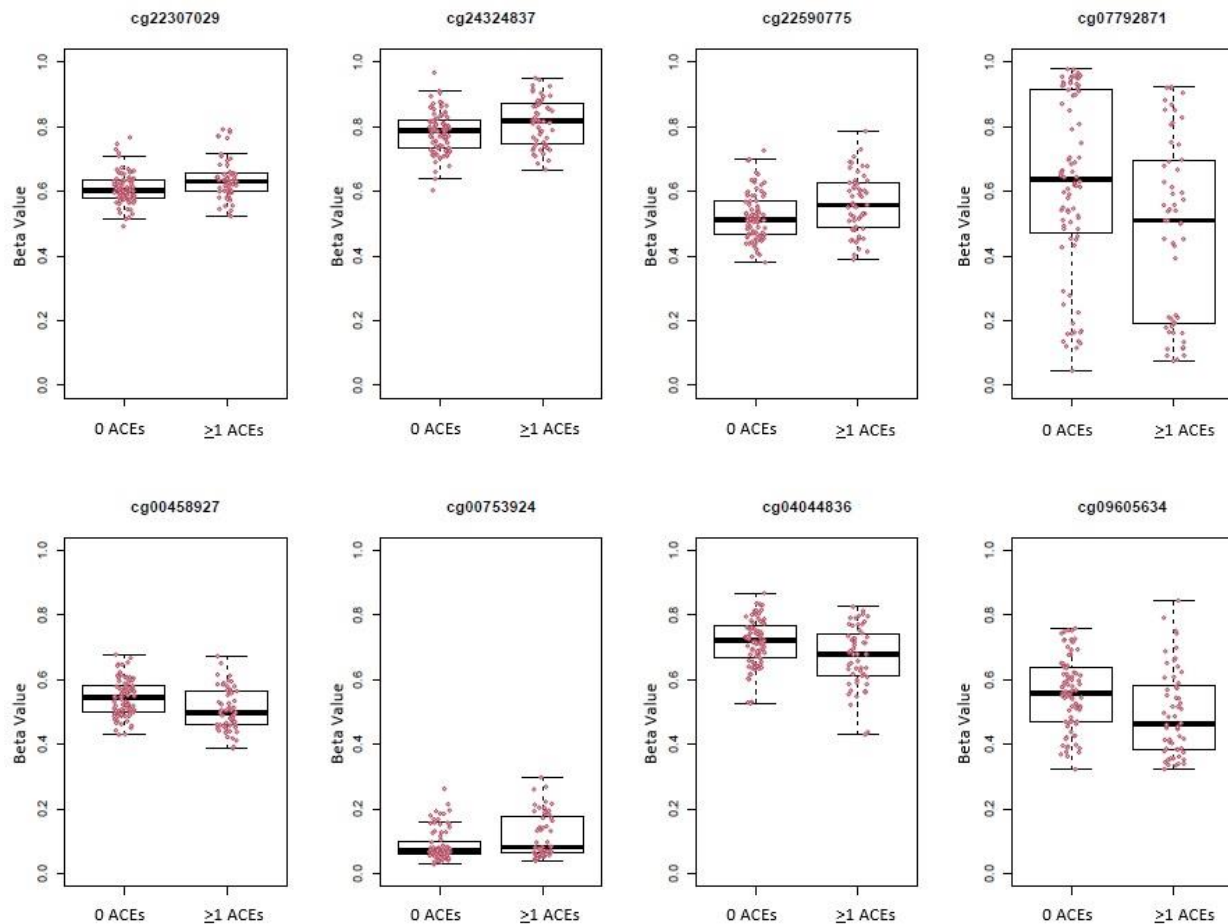


Figure 6: Display of Correlation Between ACE Standardized Score and DNAm at Significant Sites (nominal  $p < .0001$ ), DNAm Post-correction for Model Covariates

CAPTION: “The 8 sites showing nominally significant correlation with standardized ACE score are displayed here. Effect sizes ( $\Delta\beta$ ) ranged from -0.10 to 0.03.”

A comparison of individuals with no ACEs and individuals with one or more ACEs at the significant CpGs reveals generally, there is more variance in the  $\beta$ -values for the group with ACEs.



*Figure 7: Comparison Between Zero ACEs and One or More ACEs for Significant Sites*  
(nominal  $p < .0001$ )

CAPTION: “This shows the distribution of  $\beta$ -values for the 8 significant CpGs, with respect to two categories: those with no ACEs and those with one or more ACEs. All of the sites show less variance for the group with no ACEs, with the exception of *cg07792871*. This site shows not only higher variance for the group with no ACEs, but very large variance for both groups overall.”

## Discussion

Our study sought to explore the association between maternal childhood adversity and biological infant DNAm patterns. Our results showed that ACE score had a positive or negative association with DNAm; as there was an increase in ACE scores, we saw lower or higher levels of DNAm at particular CpGs (i.e. there is more differential DNAm). Our results showed eight significantly differentially methylated CpGs out of over 450,000 sites investigated through EWAS. For these CpGs, there was generally more variance in the  $\beta$ -values for the group that had ACEs compared to those with no ACEs.

The present study employed data from individuals in a sub-study of the APrON study (Letourneau et al., 2017; Kaplan et al., 2014), investigating an association between maternal scores on the ACEs questionnaire (Felitti et al., 1998) and infant DNAm patterns. To our knowledge, this was the first EWAS to investigate differential DNAm patterns in human infants in relation to their biological mothers' ACEs. Our approach was distinguished by considering current maternal stress and socioeconomic status as covariates in regression modeling, as well as the nonbiased exploration of CpGs across the genome.

### ACE Score and Epigenome-Wide Association Study

Through an EWAS approach, our statistical modeling yielded eight nominally significant sites out of over 450,000 CpGs investigated. Our study did not show high overall global differential DNAm patterns. This may be in part due to characteristics of our sample, which consists largely of individuals with low reported trauma, low stress, and high SES.

**Trauma and DNAm.** Overall, maternal ACEs were low in our study sample, with 61.4% reporting zero ACEs (i.e. no childhood adversity). Referring to our proposed relationship between ACEs and DNAm, it is reasonable to suggest significant DNAm sites may have been

more numerous if ACEs scores were more variable. Similarly, there were also overall low levels of maternal depression (EPDS; Cox et al., 1987) and anxiety (SCL-90-R screen; Derogatis, 1994) in our sample. This is consistent with previous literature, linking adversity in childhood to higher instances of depression and anxiety in adulthood. Because current stress (maternal prenatal stress) levels were overall low, were controlled for in statistical models, and did not demonstrate a signal in DNAm data, we can be more confident that our results are not due to the confounding influence of perinatal maternal stress during pregnancy on infant DNAm

**High Socioeconomic Status and DNAm.** SES is associated with a variety of health outcomes, and generally research has shown higher association between low SES and poor health outcomes (Baum, Garofalo & Yali, 1999). Sources of stress and adversity associated with low SES may include social discrimination, inadequate or inconsistent access to health care, poor residential environments, health risk behaviours and lower quality of nutrition (Baum et al., 1999). 51.5% of our study sample was in the highest socioeconomic status category (determined by income and education). This may have impacted the results, where potential differential DNAm outcomes measured in the infants were mitigated in the presence of higher socioeconomic conditions. In other words, exposure to higher SES may have buffered the effects of maternal ACEs on infant DNAm. Much of the literature has supported an association between low SES and increased differential DNAm, and so it is reasonable to suggest our high SES population may have associated with lower global differential DNAm. Again, this would suggest the current socioenvironmental conditions may have a stronger relationship with DNAm than maternal preconception stress.



### Significantly Differentially Methylated CpG Sites

Exploration of the genes associated with nominally significant CpG sites was undertaken, though it must be reinforced that differential methylation of these sites does not infer any causation or epigenetic mechanism resulting in phenotypic responses. CpG sites identified with association to functional elements of the genome are still correlational findings. Three CpGs were related to the *KASH5* gene, a protein coding gene related to calcium ion binding and dynein complex binding. This gene appears to play an important role in mammalian meiosis through involvement in meiotic chromosome movement (Morimoto et al., 2012). Thus, it can be reasoned it may be involved in gametogenesis (i.e. the creation of spermatocytes and oocytes in mammals). *RXRA* is also a protein coding gene, related to transcription of androgen receptor regulated genes, as well as transcription factor regulation in adipogenesis; increased methylation of this promoter site has been associated with increased childhood adiposity, and suggests some component of metabolic disease risk has a prenatal developmental basis (Godfrey et al., 2011). *CCT4* is a protein-coding gene as well, with pathways related to organelle biogenesis and maintenance. With regards to mental health outcomes, there were CpGs related to genes involved with neuronal development and dopamine receptor signaling. *DCLK1* is a protein-coding gene involved in several different cellular processes involving neurons, such as neuronal migration, neuronal apoptosis and neurogenesis. *DCLK1* has been shown as important to human memory and cognitive functions through upregulation of brain-derived neurotrophic factors, with associations to IQ scores and verbal memory function (Le Hellard et al., 2009). *STRN* is also a protein-coding gene, with pathways related to dopamine receptor signaling. Differential expression of this gene has been associated with symptomatic depression and major depressive disorder (Yang et al., 2017). *HCG9* is the only site with a non-coding class; it is an RNA gene

and has been identified through multi-tissue DNAm analysis as having an association with bipolar disorder (Kaminsky et al., 2012). Some of the genes associated with the significantly differentially methylated CpGs have been shown to have direct association with cognitive and mental health outcomes, while others are involved in broad cellular processes including meiosis, and may also contribute to developmental health outcomes in adult humans.

**Comparing CpG Sites for Zero ACEs and More Than One ACE.** The majority of the sample reported zero ACEs, and so a supplementary analysis comparing those with no maternal ACEs and those with one or more maternal ACEs was valuable to further understand if ACEs had an impact on DNAm profiles. The eight significant CpGs identified through our EWAS were investigated for variance in  $\beta$ -values, comparing the group with no ACEs against the group reporting one or more ACEs. For seven CpGs, there was more variance in the  $\beta$ -values for the group with ACEs, suggesting higher ACEs relate to more differential DNAm profiles.

### **Implications of Findings for Psychopathology**

The majority of CpGs showing significant DNAm in our study are associated with protein-encoding genes, involved in important cellular mechanisms. Further investigation is required to assess the role of these genes in neurophysiological development and mental health outcomes. Of the significant sites investigated, one has been shown to associate with bipolar disorder (Kaminsky et al., 2012), and another plays an important role in the dopamine system. It is well-studied how the dopamine system plays a role in mental illness, dysregulation of which may underlie the pathophysiology of depression and schizophrenia (Grace, 2016), addiction (Volkow, Wise & Baler, 2017), ADHD (Li, Sham, Owen & He, 2006), and novelty-seeking in decision making (Costa, Tran, Turchi & Averbeck, 2014) associated with personality disorders.

The concept of epigenomic plasticity may be supported by our finding that current environmental conditions of support (i.e. high SES and low maternal stress) may relate to lower infant global differential DNAm (i.e. only eight CpGs of the EWAS). If DNAm is a biomarker for mental health risk, further investigation is needed to see if positive socioenvironmental exposures (such as high SES and low maternal stress as experienced by infants in our study sample) mitigates differential DNAm patterning associated with psychopathological outcomes and the transfer of intergenerational trauma. Our findings support an association between maternal ACEs and differential DNAm profile in infants; however, our current understanding of DNAm does not allow for any strong causative statements of its impact on psychopathology in the context of our study.

### **Future Considerations**

Moving forward, a big challenge in human DNAm research will be to utilize DNAm as a biomarker in the diagnosis of illness and prediction of intervention response (Perroud et al., 2013). Human research on stress and DNAm lacks experimental methods that are present in animal counter-parts, due to ethical issues surrounding the creation of stress in human mothers (Cao-Lei et al., 2014). These ethical issues are likely the driving force for human studies of prenatal, perinatal or preconception stress and DNAm being observational in nature. There are also many challenges to investigating human epigenetic processes, the biggest issue being the retrospective nature of these studies (Lutz & Turecki, 2014).

Future studies investigating preconception stress and DNAm patterning in association with the transfer of intergenerational trauma may implement a more numerous and ethnically diverse sample, as well as a sample with a more diverse trauma history. Essentially, more variance in the data is required, as the data from our sample did not demonstrate normal

distributions for measures of maternal childhood trauma, SES and current maternal stress. Other tissue samples could also be utilized along with buccal swabs, such as blood which tends to be more homogenous, so tissue-specific differences in phenotypic outcomes can be accounted for (Chen, Breeze, Zhen, Beck & Teschendorff, 2016; Smith et al., 2015).

Longitudinal studies collecting multi-tissue samples during childhood, adolescence, adulthood, perinatally, and in the infant of the next generation might be the strongest way to assess if differential DNAm patterning is indeed transferred intergenerationally in humans, or if it is plastic and susceptible to socioenvironmental influences at each period of measurement. However, strong measures for socioenvironmental exposures and conditions would need to be assessed simultaneously. Ideally, a prospective longitudinal study could be initiated where maternal mood and socioeconomic conditions are assessed concurrently with tissue sample retrieval across developmental periods. This time-ordering could help elucidate whether DNAm is associated with the transfer of intergenerational trauma, or rather represents a biomarker for current adversity states. There are other potential benefits from a study of this design – for example: if the ACEs questionnaire were administered during adolescence, adulthood and perinatally, there is an opportunity to rigorously assess the test/re-test reliability of this commonly used epidemiological tool.

### **Limitations**

Limitations for this study included the use of a retrospective self-report of preconception stress (i.e. the ACEs questionnaire), the relatively low-risk nature of the sample as well as limited variability in ACE scores and SES of participants. The cohort in our study under-represented women in the lowest education and income categories, as well as women from rural areas and non-Caucasian women (Leung et al., 2016). A significant limitation of EWAS design

is the investigation of methylation from all possible developmental origins, including those patterns which are inherited, prenatally set, and programmed environmentally (Jones & Takai, 2001; Kaminsky et al., 2009; Schalkwyk et al., 2010; Weaver et al., 2004). This makes it inherently difficult to separate the effects of preconception and perinatal stress.

BECs were utilized in our study, but currently it is unclear whether BECs or blood cells are more representative of brain tissue, and blood has also been suggested as a better choice for EWAS due to its functional relation to many diseases (Jiang et al., 2015). The variability of DNAm between brain and blood cells has been shown as moderately concordant (Farre et al., 2015) and a tool has been created to better interpret results from blood samples in relation to brain tissue (Edgar et al., 2017). It is important to note that tissue specificity is by far the largest predictor of differential DNAm patterns, and cell-type differences within similar tissues becomes the second biggest predictor of variation in DNAm (Farre et al., 2015).

The influence of genetic differences between individuals was not accounted for in our study. Teh et al. (2014) found the best explanation for 75% of differentially methylated CpGs in their study was the interaction of genotype with different in utero environments (such as maternal smoking, maternal depression, and maternal body mass index). Though our study accounted for some environmental exposures, it was not possible to account for all the potential differences in environmental exposures; nor was it possible to account for all the genetic differences between individual children.

Overall, a total of eight nominally significant CpGs appears quite small for an EWAS investigating over 450,000 sites; more CpGs may have shown biologically significant DNAm patterns if the data on ACE scores had more variance. Finally, there was a lack of power in our study, given the smaller sample size. Data from 139 participants did not meet suggested sample

sizes to achieve 80% power for both case-control and disease-discordant EWAS designs (Tsai & Bell, 2015), and the low power may have been a reason why additional CpGs were not found to be differentially methylated.

### **Conclusion**

Through this study, maternal ACE scores were associated with nominally significant differential DNAm at eight CpGs on the epigenome, obtained from BECs of the biological infants. An increase in standardized maternal ACE score associated with increased infant differential DNAm (i.e. either higher or lower methylation) at these CpGs, and the variance in DNAm was generally higher for the infants of mothers with one or more ACEs compared to those with zero ACEs. This suggests human maternal ACEs associate with differential DNAm profiles in the infants, and supports future EWAS designs investigating human preconception stress and DNAm in offspring. DNAm may serve as a biomarker for developmental health risk, but further studies are needed to determine if there is a transmission of differential DNAm patterns associated with the transmission of intergenerational risk, or intergenerational trauma in humans. Longitudinal studies profiling human DNAm patterns over generations and through different environmental exposures would aid in this endeavour.

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## **Chapter 3: Further Discussion**

### **3.1 Expanded Discussion and Consolidation of Learning**

Through this thesis study, there were additional learnings concerning the area of epigenetics that are worth noting, obtained through the nature of interdisciplinary work between epigeneticists, biostatisticians and Registered Nurses (RNs).

#### **3.1.1 Adhering to Study Guidelines: STROBE and EWAS Einstein**

The STROBE guideline was utilized when reporting the findings of this study (Von Elm et al., 2007). This guideline was developed for observational studies, and the majority of items on the checklist were included in the publication; however, epigenetic studies such as EWAS do not follow established guidelines at this time. Co-authors specializing in this area (namely Dr. Kobor, Dr. Jones and Dr. Moore) encouraged an overview of the “EWASscore” available on the internet at <http://epgntxeinstein.tumblr.com>; this EWAS checklist was developed out of a review by Lappalainen & Grealley (2017). The area of epigenetics does not have a defined reporting guideline. It is a relatively new area of research, subject to scrutiny from experts in the field as the area of study continues to evolve. A goal of this thesis study is knowledge translation, and so the study team and thesis committee members consulted one another on which journal to pursue, how to organize the publication, and which elements of both these checklists were most important to support this goal.

#### **3.1.2 Epigenomic Plasticity**

Our sample overall demonstrated low levels of trauma and stress, and higher socioeconomic status, associated with lower epigenome-wide differential DNAm. Leshem & Schulkin (2012) provide insight into how epigenetic inheritance is plastic; their rodent study of female rats with preconception stress demonstrates that preconception enrichment for the

mothers and post-conception enrichment for the offspring both mitigated the transmission of risk. This suggests the negative effects of preconception stress on offspring may be buffered with a supportive environment (Leshem & Schulkin, 2012). If DNAm is associated with this risk transfer, it is feasible there would be lower infant global differential DNAm found through our study; the mothers in the study represent a low-trauma, low-stress sample, and the infants were exposed to socioenvironmental conditions related to a high SES. This idea is highly speculative, but may have implications for nursing as it supports the idea of socioenvironmental interventions to buffer the effects of perinatal stress and preconception stress in humans.

### **3.1.3 Marker Versus Mechanism**

One of the most significant learnings from the co-authors specializing in epigenetics is the idea that DNAm in the literature has not been strongly supported as an epigenetic mechanism or process that contributes to phenotypic outcomes (i.e. disease states; Lappalainen & Greally, 2017). Rather, DNAm should be discussed as an epigenetic biomarker. This distinction is important as epigenetic studies continue to explore the direction of the relationship between differential DNAm, socioenvironmental exposures, and phenotypic outcomes such as disease states.

This study revealed low global differential DNAm in infants with relation to maternal ACEs; DNAm is likely a biomarker for current stress exposure, largely re-set each generation – at least in somatic cells such as the BEC samples collected from infants in this study. The findings may provide some hope to mothers who have had past trauma, knowing with appropriate socioenvironmental conditions their children can still be healthy. If “nature” and “nurture” work together, DNAm might be a clue to how. But more research is needed to explore the direction of the relationships: does DNAm influence developmental outcomes, or does it

simply reflect current states? RNs are well-positioned to explore this further, which is discussed next in the implications for RNs.

### **3.2 Nursing Implications of Findings**

#### **3.2.1 Nursing Research**

RNs have a unique skillset to lend the area of epigenetics research, and can help inform questions to the field of epigenetics rooted in clinical and public health outcomes. Florence Nightingale's own research focused on the importance of a healthy environment for physical and mental health (Daack-Hirsch, 2016). RNs are well-educated on the social and biological determinants of health, and therefore are well-prepared to integrate epigenetics into research and apply their findings to improve health (Daack-Hirsch, 2016). They are ideal professionals to explore the direction of the relationship between DNAm profiles, socioenvironmental conditions, and health outcomes.

Most findings through epigenetic studies have been associative, and do not necessarily support the idea that early adversity causes epigenetic effects such as DNAm (Park & Kobor, 2015). The reverse is also true; the idea that epigenetic changes cause illnesses has not been strongly supported. Though RNs have researched much on the effects of early childhood adversity and the transfer of intergenerational trauma, developmental changes predicted on the basis of these can turn out to be inappropriate because the environment changes from one generation to the next (Hanson & Gluckman, 2011). Researchers have begun designing longitudinal studies, across generations, incorporating multiple tissue-types for epigenetic markers (Park & Kobor, 2015). RNs are in the ideal position to do carry out this research: they work in clinical settings where biological samples can be collected, in academic roles to develop research initiatives, as well as in management roles to support these research initiatives.

Building on the findings from this thesis study, RNs can incorporate repeated sample collections with repeated data collection on socioenvironmental factors and current health states, utilizing multiple tissue types for DNAm profiles, to determine whether the transfer of DNAm patterns associates with the transfer of intergenerational trauma. RNs have begun to make important contributions to the area of genetics (Alexander, 2015), and it is important they start contributing to the area of epigenetics as well.

An inherent challenge for epigenetic research is knowledge translation (Park & Kobor, 2015). Understanding the biological underpinnings of epigenetic science is difficult for those who are not trained in the subject, and most scientists are not trained in knowledge translation (Park & Kobor, 2015). However, RNs are very skilled at this, as they constantly seek to translate research findings into both clinical practice and healthy policy. RNs are trained in the biological and social sciences, and the profession is well-prepared to conduct and integrate epigenetic research into clinical practice and health policy (Clark, Adamian & Taylor, 2013).

### **3.2.2 Nursing Practice**

Long before the Human Genome Project, nurses were addressing the social and environmental structures that impact human health (Daack-Hirsch, 2016), and the influence of environment on health is a foundational paradigm for the RN profession (DeSocio, 2016). The findings from this thesis study may support the idea of early modifications to social and environmental support for newborns, young children and their families – modifications RNs have been making to optimize health and healing since Florence Nightingale (DeSocio, 2016). Specifically, this study may support the idea of RNs screening expectant mothers for ACEs and providing appropriate social supports and interventions, for optimal physical and mental health outcomes in their babies.

Epigenetic studies in the future may elucidate targets for social and environmental interventions, helping to pave the way to more effective and customized approaches in improving health across an individual's lifespan (Hanson & Gluckman, 2011). Epigenetic modifications such as DNAm may even have the potential to guide diagnostic testing in the future (Clark et al., 2013). The study of epigenetics compliments existing nursing practice as well. Mental health RNs, for example, develop competencies in providing psychoeducation, psychotherapy and psychopharmacology as part of their "integrated practice" (DeSocio, 2016). Epigenetics is essentially a scientific explanation for the synergistic mechanisms that underlie a holistic approach to care utilizing integrated practice and healthy lifestyle modification (DeSocio, 2016). Further epigenetic research will likely support holistic RN practice. It is compelling how in the postgenomic era, attention is returning to socioenvironmental influences on health (Daack-Hirsch, 2016). RNs are at the frontline of patient care, fueled by multiple roles and responsibilities, providing interventions in the bio-psycho-social realms, while working with multiple disciplines and various professionals. They are an ideal group of medical professionals to incorporate epigenetic research directly into clinical practice and health policy (Clark et al., 2013).

### **3.2.3 Nursing Policy**

Research in genetics, epigenetics and neurophysiology garner significant support in terms of funding and are powerful in supporting the creation of public health policy. Early intervention for at-risk families requires revisions to public health policy, with implications for health, education and "empowerment of women and children in particular" (Hanson & Guckman, 2011, p. S3). The findings from this thesis study are useful for policy development supporting early interventions for families at risk. Epigenetic marks such as DNAm may serve as a biomarker of

early risk exposure in families (Park & Kobor, 2015). Providing further empirical, quantitative evidence of epigenetic markers associated with the transfer of intergenerational trauma may result in the creation of community-based health and social supports that are family-centered, customized and which address healthy childhood development.

The majority of Canadian provincial health regions self-report “healthy child development” as the determinant of health that receives their greatest attention (Frankish et al., 2007). Developing public policies targeting healthy child development in Canada is important and epigenetic research such as this thesis study can provide evidence for pursuing this endeavour, while influencing the development and creation of policies targeting maternal perinatal and infant/child health (Park & Kobor, 2015). Though RNs have long known early socioenvironmental exposures influence health outcomes, having a biological explanation for why prenatal and early childhood socioenvironmental exposures are important can strengthen the existing evidence and further substantiate what is already known (Park & Kobor, 2015). Studies such as this one may help change individual perceptions on the importance of socioenvironmental factors surrounding healthy child development. With a sufficient number of adopters, critical mass may be achieved and there could be a shift in public opinion across Canadian society, leading to implementation of public healthy policies benefiting young families and their children (Park & Kobor, 2015).

### **3.2.4 Nursing Education**

The field of epigenetics is complimentary to RN practice, as discussed earlier, because it demonstrates associations related to the synergy of “nature” and “nurture,” as well as holistic and integrated practice. Discoveries in epigenetics shed new light on how the relationship between genes and environments influences human health (Daack-Hirsch, 2016). However, research has

shown practicing RNs have inadequate genetic and epigenetic knowledge (Camak, 2016). This is largely because genetic and epigenetic understanding is lacking in nursing education (Camak, 2016). A significant barrier to the integration of genetic and epigenetic content in RN curricula is the lack of understanding of relevancy to nursing practice (Camak, 2016). The implications of epigenetics for RN practice have been discussed earlier – this type of information should be shared with RN students before they enter practice. Nurse educators play an important role in preparing the next RN graduates to care for patients (Alexander, 2015), and should integrate some epigenetic content into curricula.

Epigenetic inquiry is especially relevant to RNs, as the profession has traditionally emphasized the interaction between environment and individual (Daack-Hirsch, 2016). The findings from this thesis study can further substantiate what RNs are already learning about ACEs, the Socioeconomic Determinants of Health, the Population Health Promotion Model, upstream and preventative care, as well as the interplay of the bio-psycho-social realms of care. RNs are increasingly being taught social interventions alongside medical ones, and studies like this may reinforce the importance of both. While the RN profession more recently has been focused on care provided in hospitals and long-term care facilities, education on epigenetics may allow future RNs to re-visit what entails nursing care and focus again on socioenvironmental factors that interact with the genome to affect human health and development (Daack-Hirsch, 2016).

### **3.3 Conclusion**

ACEs are known predictors of poor health outcomes, and early adversity is also known to predict differential DNAm patterns in humans. Human maternal perinatal stress also predicts differential DNAm in the child, and there is animal evidence that preconception stress may also



predict differential DNAm in the offspring. The transfer of intergenerational risk is mostly explained by either social factors, or biological factors; however, it is likely the result of an interplay between socioenvironmental exposures and genetic expression. This study investigated preconception stress of mothers in the form of ACEs, and DNAm patterning in their infants, as an epigenetic biomarker possibly associated with the transfer of intergenerational risk.

Our study revealed an association between maternal ACE scores and infant DNAm profiles. There was overall low global differential DNAm patterns associated with maternal ACEs through EWAS, with only eight significant sites out of over 450,000 investigated. The results show with each increase in standardized ACE score, the sites are more differentially methylated. Findings from this study and similar epigenetic studies have implications for nursing research, practice, policy and education. This study supports the design of future studies investigating the intergenerational transfer of differential DNAm patterning, in conjunction with socioenvironmental factors. Longitudinal studies would be ideal for this exploration, utilizing multiple tissue types and simultaneous data on social and environmental conditions. The RN profession is positioned ideally to develop such longitudinal epigenetic studies, due to a strong background in both biological and social sciences, as well as significant presence in clinical, management and academic roles.

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