The Association Among Maternal Early Life Stress, Prenatal Stress, and Offspring EpiStress Scores

by

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Abstract

Maternal stress (early life stress (ELS) and prenatal stress (PNS)) influence offspring development via the biological embedding of stress. Maternal physiological stress system activation can be inherited by offspring in utero. However, the associations among maternal stress and offspring epigenetic profiles are unclear. This project aims to determine if PNS mediates the association between maternal ELS and offspring DNA methylation (EpiStress scores). A secondary analysis of data from expectant and new mothers (n=129) and their offspring was conducted. Age at sample collection and cell type proportions were highly correlated with offspring EpiStress scores leading to a stratified mediation analysis. Results indicate PNS was not a mediating factor between maternal ELS and offspring EpiStress scores. Maternal ELS negatively predicted newborn- and not infant EpiStress scores. This suggests that the biological embedding of stress from a mother to her newborn is specific to maternal ELS, not prenatal stress.

Keywords: Early life stress; prenatal stress; DNA methylation; glucocorticoid-responsive polyepigenetic score

Dedication

I would like to dedicate this thesis to my past, present, and future self.
To the past for giving me the perspective and the strength to persevere;
To the present for enabling me to successfully complete a Master of Science degree amidst a pandemic and in isolation;
And to the future… whatever it may hold.

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Second, I want to extend a sincere and heartfelt thank you to Dr. Catherine and Dr. Merrill for their undeniable ability to provide quality and effective mentorship. I cannot begin to describe the impact each of you had on both my academic and personal life. Not everyone can be an accomplished scholar, a mentor, and a genuinely outstanding leader; both of you were all of these things and more. For that I am eternally grateful. Thank you one thousand times over.

Third, to my mother who has supported me from day one. You do not always understand my work, but you *always* understand me. I love you to the moon and back.

And finally, to my partner of 70 years, a true partner in this life: thank you. You took care of Link and I while I needed to lock myself away in the office to complete this milestone. You are a rare gem and an incredible man. I am so lucky to be on this journey with you. I love you endlessly and entirely.

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Chapter 1. Introduction

1.1 Early Life Stress (ELS)

Stress early in one's life can impact people in a myriad of ways. Childhood maltreatment (CM; abuse or neglect)) is a significant stressor and has been shown to influence the development of depression (Negele et al., 2015; Cardoso et al., 2017). An estimated 20-25% of children aged 0 to 17 experience maltreatment (Peterson et al., 2018; World Health Organization, 2020). This percentage is striking considering not only the vulnerability of children, but the long-term effects that CM might pose. Experiences of CM, or early life stress (ELS), in which a young person is the victim of sexual, physical, or psychological abuse and/or physical or psychological neglect are known to predict future physiological and psychological disturbances (Heim et al., 2019; Martins et al., 2021). For example, ELS is shown to influence physiological stress response system activation which has been associated with the development of adult mood disorders, such as depression and anxiety (Syed & Nemeroff, 2017; Dahmen et al., 2018). Furthermore, Bronfenbrenner's ecological systems theory posits that in addition to biological influences on development, the environment, including social systems, quality of relationships, sense of community and belonging, and cultural values also impact overall development (Bronfenbrenner, 1992). This theory supports the notion that adverse early life experiences are influenced by a multitude of factors that might trigger various physiological responses from early life and possibly into adulthood. One physiological response that has been previously implicated in stress system activation is the hypothalamic-pituitary-adrenal axis.

1.1.1 The Hypothalamic-Pituitary-Adrenal (HPA) Axis

One of the most highly studied physiological stress systems is the hypothalamic-pituitary-adrenal (HPA) axis (Entringer et al., 2009; Anderson, 2017). Stressors include any perceived threat (real or implied) that can elicit a physiological response within the body. When a stressful event occurs, a physiological response is triggered such that the HPA axis is activated. A cascade of events then follows. Neurons in the hypothalamus of the brain begin to release corticotropin-releasing hormone (CRH) which travels to the pituitary gland, also located in the brain, subsequently releasing adrenocorticotropin

hormones (ACTH) (Zhu et al., 2014; Gerritsen et al., 2017). Once the pituitary gland releases ACTH, the ACTH enters the blood and begins to circulate throughout the body, eventually reaching the adrenal glands which are situated on top of the kidneys. Upon the arrival of ACTH, the adrenal glands release hormones that have been implicated in the stress response: glucocorticoids (GCs; e.g., cortisol) and catecholamines (e.g., adrenaline and noradrenaline) (Zhu et al., 2014). The activation of the HPA axis occurs naturally in response to stressors. However, this response is not perpetually active. Cortisol that is released from the adrenal glands eventually feeds back to the hypothalamus and pituitary gland, effectively inhibiting further release of CRH and ACTH, respectively (see Figure 1) (Gjerstad et al., 2018).

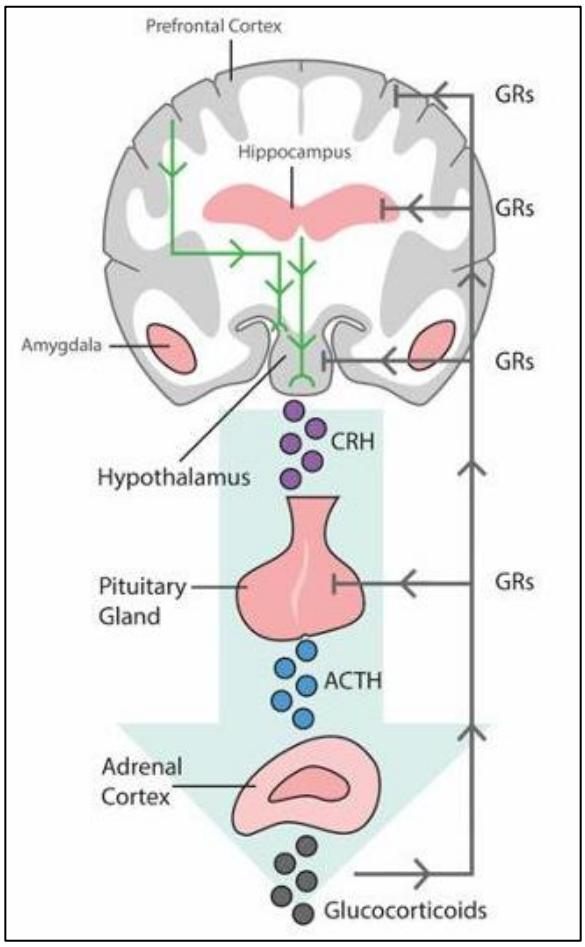


Figure 1: The hypothalamic-pituitary-adrenal (HPA) axis. *The hypothalamus releases corticotropin releasing hormones (CRH; purple) which travel to the pituitary gland. The pituitary gland then releases of adrenocorticotropin hormones (ACTH; blue). The ACTH then enters circulation, reaches the adrenal cortex, and releases glucocorticoids (GCs; grey). GCs then travel throughout the body and the brain where they can bind to GC receptors (GRs) and exert their effects within cells. Image created by Nicholas Stacey (2021) and adapted from https://www.open.edu/openlearn.*

HPA axis inhibition is integral to the negative feedback loop that returns the body to a homeostatic, or balanced, state once the stressor is removed. Chronic stress, however, can lead to persistent HPA axis activity and GC production, impairing the ability of the HPA axis to respond to negative feedback and maintain homeostasis. For example, the experience of ELS has been shown to lead to the development of mood disorders and atypical HPA axis function later in life, leading an individual to become more vulnerable to subsequent stressors (Brand et al., 2009; Gjerstad et al., 2018; Moog et al., 2018). A study by Gerritsen et al. (2017) investigated the influence ELS might have on the HPA axis and subsequent development of mood disorders. They found that genes such as *FKBP5* play a role in the development of major depressive disorders as *FKBP5* is able to modulate GC receptor activity and influence HPA axis functioning (Zannas et al., 2016; Gerritsen et al., 2017). However, not all studies show the same associations, perhaps due to differences in sample size and demographic variables (Buttenschøn et al., 2017; Gerritsen et al., 2017).

Furthermore, chronic production of cortisol has been shown to compromise other bodily systems such as the immune system, as well as to exert adverse effects on the hippocampal, amygdalae, and prefrontal cortex (PFC) regions of the brain, where receptors for GCs are abundant (Taylor, 2010; Gjerstad et al., 2018; Van den Bergh et al., 2019). Hippocampal, amygdalae, and PFC brain regions have been shown to affect behaviour, learning and memory, emotion regulation, cognitive function, and to influence the later development of mood disorders such as depression and anxiety (Van den Bergh et al., 2019). A recent study by Moog et al. (2018) has suggested that maternal ELS might be reflected in the offspring's brain morphology. Moog et al. (2018) examined the effects of maternal ELS on offspring brain volume, including total brain volume (a combination of grey matter, white matter, and cerebrospinal fluid), hippocampal volume, and amygdala volumes, hypothesizing that the effects of maternal ELS on the offspring occur in utero. Moog et al. (2018) found that the newborns (<30 days old) of mothers who experienced ELS had less total brain volume compared to newborns of mothers who did not experience ELS. As well, they found no relationship between maternal ELS and newborn hippocampal and amygdala volumes (Moog et al., 2018).

It is difficult to ascertain when stress transmission occurs, for example, prior to conception or in utero during the prenatal period, especially if mothers were experiencing a mood disorder(s) throughout pregnancy. Moog et al. (2018) offer insight

into the possibility that maternal ELS is transmitted to the child in utero, and that the effects of maternal ELS might be represented through offspring brain morphology. While other studies show that ELS can alter HPA axis functioning and can lead to the development of mood disorders, including throughout pregnancy (Negele et al., 2015; Gerritsen et al., 2017; Thomas et al., 2018). However, results are inconsistent, and how and when stress transmission to the offspring occurs, as well as whether prenatal stress is involved, remains unknown.

1.2 Prenatal Stress

The Developmental Origins of Health and Disease (DOHaD) hypothesis proposes that fetal acquisition of diseases is attributable, in part, to its in utero environment—its mother (O'Donnell & Meaney, 2017). The experiences of ELS and prenatal stress might therefore lead to additive effects of stress across the lifespan, negatively affecting a mother and possibly her fetus. Accordingly, it is important to understand how prenatal stress might reach the fetus. Previous research suggests that prenatal stress alters maternal HPA axis function, leading to dysregulation of maternal GC production (Swales et al., 2018; Epstein et al., 2019). Maternal GC dysregulation has been shown to influence maternal vulnerability to mood disorders, as well as offspring birth weight and offspring susceptibility to altered fetal HPA axis function (Duthie & Reynolds, 2013). One physiological mechanism that has been proposed to play a role in the transmission of maternal stress, as well as a buffer to protect the fetus from maternal GCs, is the placenta.

The placenta is an organ that develops during pregnancy and plays a key role in fetal development by enabling the exchange of essential nutrients and hormones (including cortisol) between the mother and her fetus. As the placenta is the interface between the mother and her fetus, it is here that elevated maternal GCs can exert their effects on the fetus. Maternal cortisol naturally increases during the last trimester of pregnancy and is a necessary component of fetal development, promoting fetal cell differentiation and fetal organ maturation (Krontira et al., 2020). However, chronic stress during pregnancy—and possibly ELS—might lead to an overactive maternal HPA axis. Consequently, elevated prenatal cortisol has been shown to decrease placental growth, and blood vessels are unable to develop as they normally would (Reynolds, 2013;

Ozmen et al., 2017). This could limit the exchange of appropriate nutrients between the mother and fetus, further affecting fetal development (Ozmen et al., 2017).

Fortunately, the placenta contains enzymes that act as a protective barrier between excess maternal cortisol secretion and the fetus. One crucial placental enzyme is 11-beta hydroxysteroid dehydrogenase 2 (11β-HSD2) which transforms excess active maternal cortisol into its inactive form, cortisone, in the placenta before it is able to reach the fetus (Wyrwoll et al., 2011; Napso et al., 2018). However, excess maternal cortisol decreases the ability of placental 11β-HSD2 to convert cortisol to cortisone. When 11β-HSD2 enzymes are decreased, maternal cortisol not only remains active, but it readily passes through the placenta and reaches the fetus (see Figure 2) (Reynolds, 2012). In addition to maternal cortisol affecting maternal psychological health, maternal HPA axis activity, placental 11β-HSD2 enzymes, and fetal HPA axis activity, it has been suggested that stressful life events can become biologically embedded through other mechanisms such as fetal gene transcription, gene expression, and epigenetic mechanisms like DNA methylation (Jensen Peña et al., 2012; Provençal & Binder, 2015; Aristizabal et al., 2020).

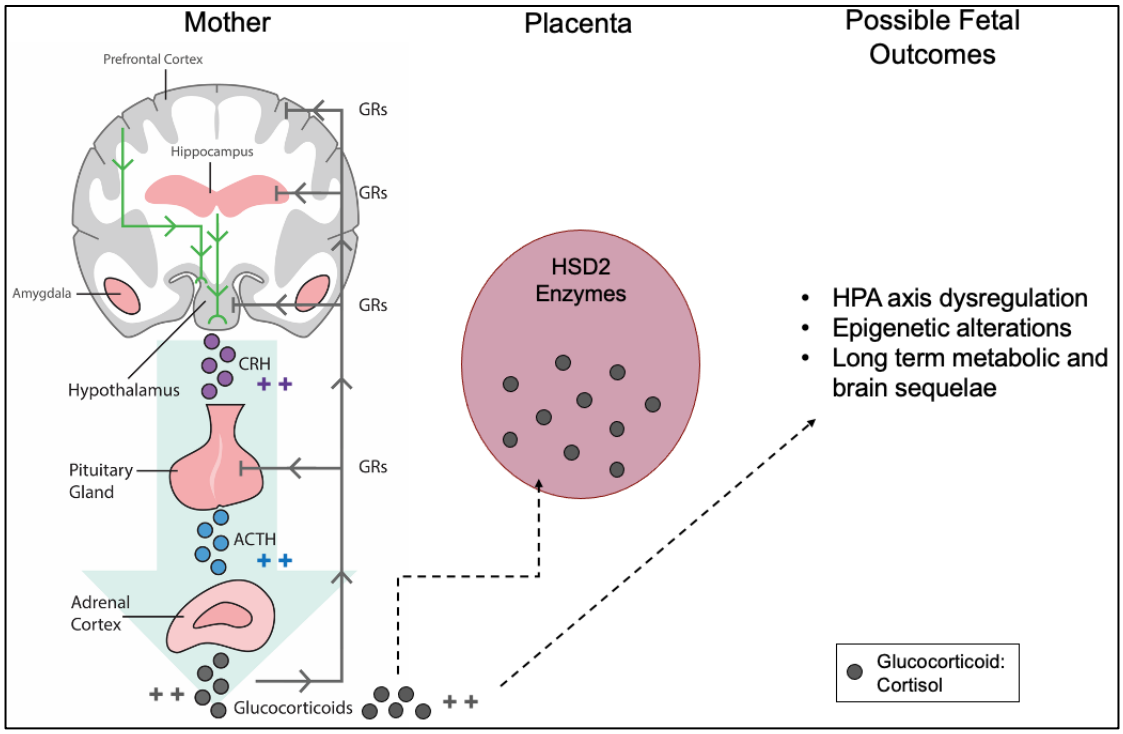


Figure 2: Glucocorticoid (GC) signaling between the mother, placenta, and fetus. *The image depicts how stress experienced by the mother during pregnancy might lead to increases in maternal stress hormone concentrations (GCs). Maternal GC production may lead to overexposure of the fetus to GCs, influencing fetal health outcomes. CRH: Corticotropin releasing hormone; ACTH: Adrenocorticotropin releasing hormone; GRs: Glucocorticoid receptors. Image created by Nicholas Stacey and Karilyn Harris (2021) and adapted from Reynolds (2012), figure used with permission.*

1.3 Biological Embedding of Maternal Stress

The biological embedding of stress is a theory suggesting that stressful life experiences can get “under the skin,” leading to lasting changes in biological processes that alter one’s development, behaviour, and overall health later in life (Boyce & Kobor, 2015; Aristizabal et al., 2020). This theory applies to women who experience ELS and subsequently prenatal stress as it encompasses the broad array of experiences one has throughout their lifetime rather than at a specific time point. Although the exact mechanisms of biological embedding have yet to be elucidated, HPA axis functioning has been proposed as one mechanism, as previously mentioned, as well as epigenetic modifications such as DNA methylation (DNAm) (Aristizabal et al., 2020). Epigenetics, “epi” (meaning above or on top of) and “genetics” (the genome), refers to various molecular processes that influence how our genetic code is expressed without the modification of the genome itself (Greally, 2018). In order to understand DNAm, a brief introduction to the genome, transcription and translation, and the plasma membrane, followed by a discussion of DNAm will be discussed next.

1.3.1 The Genome

The genome holds the instructions for cellular and organism development and is located in cells throughout the human body. The genome consists of three main components: a sugar group and a phosphate group (the sugar-phosphate backbone), and a nitrogenous base (Haddad, 2021). These three components make up one single nucleotide. Each sugar and phosphate group are chemically bound to one of four nitrogenous bases: adenine (A), thymine (T), guanine (G), or cytosine (C) (Haddad, 2021). Adenine and thymine pair together, and guanine and cytosine pair together via two or three hydrogen bonds, respectively, and are called base pairs (Clancy & Brown, 2008). The nitrogenous bases contain the information needed to produce messenger RNA strands. Messenger (mRNA) is a complement of the DNA strand, except that the thymine nitrogenous base is converted to uracil in mRNA (Clancy & Brown, 2008). This conversion is a preliminary step in the overall process leading to protein production. Protein production is the result of two key molecular processes: transcription and translation.

1.3.2 Transcription and Translation

During transcription, an enzyme called RNA polymerase, as well as transcription factors, bind to the promoter region of the DNA and begin to create a complementary mRNA strand, one base at a time (Lee & Young, 2013). Once the mRNA strand has been transcribed, it exits the nucleus of the cell into the cytoplasm (the liquid that fills each cell) where translation begins. Three nitrogenous bases in a row, called triplet codons, enable the mRNA code to produce specific amino acids once transcription is complete (Lee & Young, 2013). Translation is the process in which the mRNA triplet codons are 'read' by molecular machinery (transfer RNA (tRNA) that contains anticodons, and two ribosomal subunits), and a polypeptide chain is formed (see Figure 3) (Clancy & Brown, 2008; Lee & Young, 2013). The resulting polypeptide is important because proteins are involved in numerous biological processes and are essential for human development and function. For example, proteins influence biochemical reactions within cells and aid in the organization and maintenance essential for cell structure and function (Clancy & Brown, 2008).

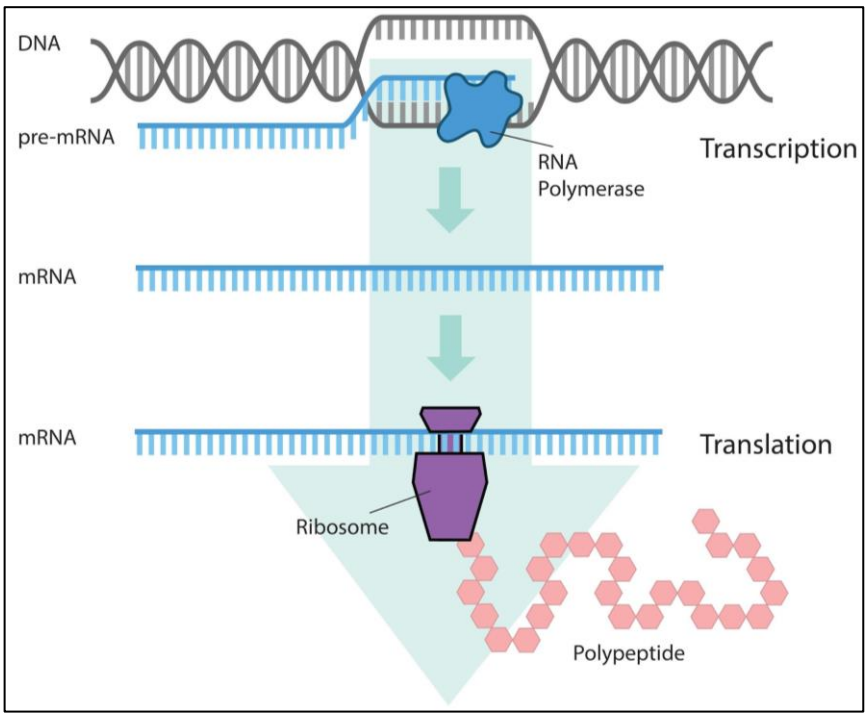


Figure 3: Transcription and translation of a single messenger RNA (mRNA) strand from a double-stranded piece of DNA. *RNA polymerase uses the existing double-stranded DNA as a template to produce a single-stranded mRNA molecule that will eventually be translated into a polypeptide chain (Clancy & Brown, 2008). Image created by Nicholas Stacey (2021) and adapted from Clancy and Brown (2008).*

1.3.3 The Plasma Membrane

The plasma membrane is composed of a phospholipid bilayer with hydrophilic heads and hydrophobic tails that provides a protective barrier between the cytoplasm and the extracellular fluid. Proteins that are embedded in the cell membrane aid in controlling the transport of molecules into and out of the cell, allow the binding of molecules to receptors which can induce intracellular effects, enable enzyme activity that transforms molecules into other forms within the body, and finally, acts as a link between intracellular and extracellular structures (see Figure 4) (Clancy & Brown, 2018). There are exceptions, however, such that not all molecules require a protein channel for transport into the cell. Steroid hormones like cortisol and synthetic GCs like Dexamethasone (DEX) can readily permeate the cell membrane.

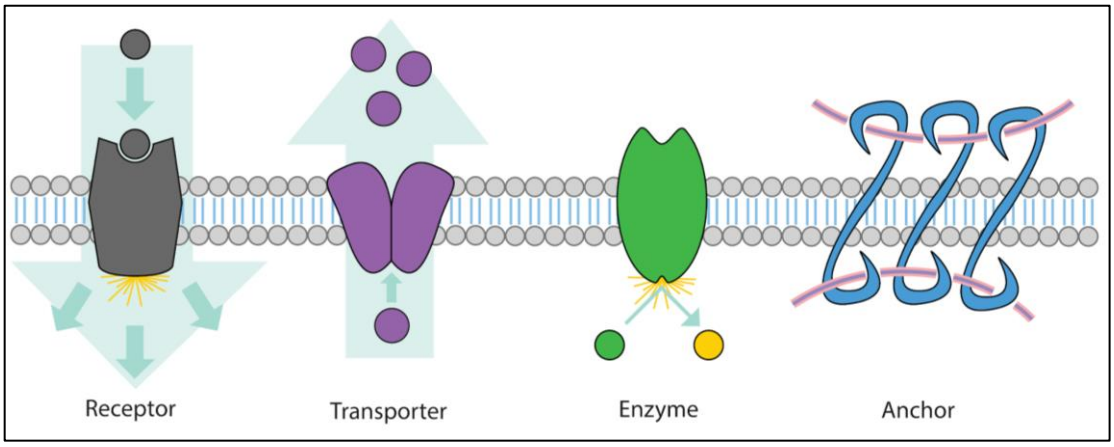


Figure 4: Transporters, receptors, enzymes, and anchors depicting the various ways that proteins can influence biological processes within or outside of the cell. *The plasma membrane consists of a phospholipid bilayer, indicated by the hydrophilic heads (light grey circles) and hydrophobic tails (vertical blue lines between the hydrophilic heads). Molecules bind to receptors to induce intracellular effects; transporters move molecules out of cells; enzymes change existing molecules via chemical reactions; and anchors act as links between intracellular and extracellular structures. Image created by Nicholas Stacey (2021) and adapted from Clancy and Brown (2008).*

Once a GC enters the cell, it can bind to a GC receptor (GR) creating a GC-GR complex (Spengler & Binder, 2016). The GC-GR complex can then readily permeate the nuclear membrane, enter the nucleus of the cell (where the DNA is located), and initiate intracellular effects like gene transcription (Spengler & Binder, 2016). In order for GRs to act as transcription factors and initiate gene transcription, they must bind to the genome. This is accomplished when the GC-GR complex binds to glucocorticoid responsive elements (GREs); GREs are DNA sequences along the genome (Spengler & Binder,

2016). Once the GC-GR complex is bound to GREs, it can activate gene transcription and lead to protein production (Spengler & Binder, 2016). This process is illustrated in Figure 5. Because GCs and GRs are ubiquitous throughout the brain and body, the production and regulation of these hormones and receptors is essential for healthy and adaptive human functioning. Transcription factors that bind to the GC-GR complex impair its ability to bind to GREs. For example, the binding of the transcription factor nuclear factor-kappa B (NF-*κ*B) to the GC-GR complex inside the nucleus prevents the GC-GR complex from binding to the DNA which inhibits protein synthesis (van der Goes et al., 2014). Additionally, protein synthesis can be influenced by epigenetic modifications such as DNAm.

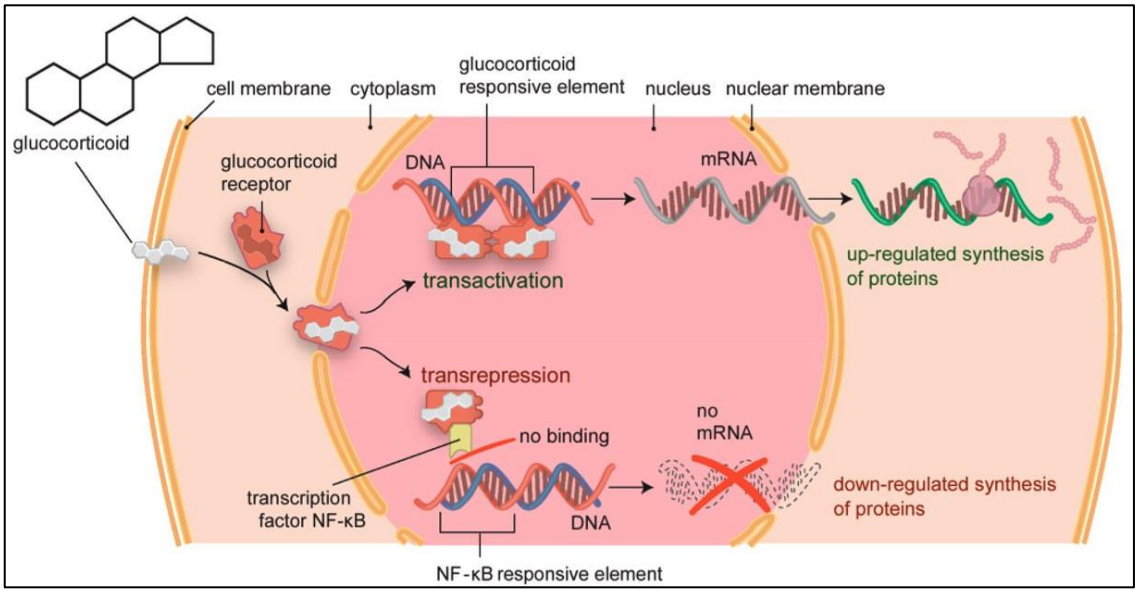


Figure 5: Mechanisms by which glucocorticoids (GCs) enter the cell and the cell nucleus. *GCs readily pass through the cell membrane and bind to GC receptors (GRs) in the cytoplasm (left). The GC-GR complex readily permeates the nuclear membrane where it binds to the promoter region of GC responsive elements (GREs) along the genome which can activate transcription ("transactivation"). Transactivation leads to and up-regulation of protein synthesis. When transcription factors such as* NK-κB *shown here bind to the complex, transcription is repressed ("transrepression"). Transrepression leads to a down-regulation of protein synthesis (bottom right). Image from van der Goes, Jacobs, and Bijlsma (2014) and used with permission.*

1.3.4 DNA methylation

DNA methylation (DNAm) occurs when a methyl group (CH_3) is chemically added to cytosine nitrogenous bases that are followed by guanine bases (see Figure 6) (Lyko, 2018). These cytosine-guanine regions are known as CpG sites. The location of DNAm along the genome will determine whether protein synthesis can occur. When methylation occurs in regions such as gene bodies, it has been suggested that gene expression can proceed (Jones, 2012). Whereas when CpG sites in the promoter region of genes are methylated, gene expression is repressed because transcription factors are blocked from binding to the DNA by the methyl group. In contrast, when CpG sites in the promoter region of the DNA are not methylated, gene expression can proceed (see Figure 7) (Lyko, 2018). DNAm occurs through chemical processes involving enzymes called DNA methyltransferase (Dnmt) 1, -3A, and -3B which play individual roles in the establishment (Dnmt3 enzymes) and maintenance (Dnmt1) of DNAm patterns during embryonic development (Stewart et al., 2015; Lyko, 2018).

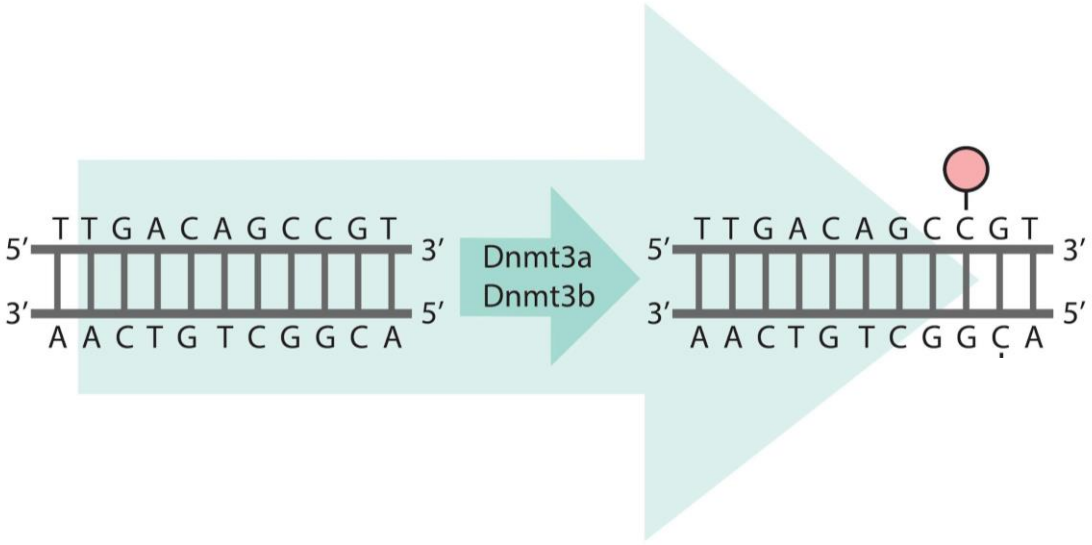


Figure 6: DNA methylation. *DNA methyltransferases (Dnmt3a and Dnmt3b) aid in the transfer of methyl groups (CH_3; pink circle) onto the double-stranded DNA at the cytosine base at the cytosine-guanine sites along the genome. Unmethylated DNA is shown on the left; methylated DNA, with the help of Dnmt3a and Dnmt3b, is shown on the right. Actions of DNMT1 are not shown here. Image created by Nicholas Stacey (2021) and adapted from Moore, Le, & Fan (2013).*

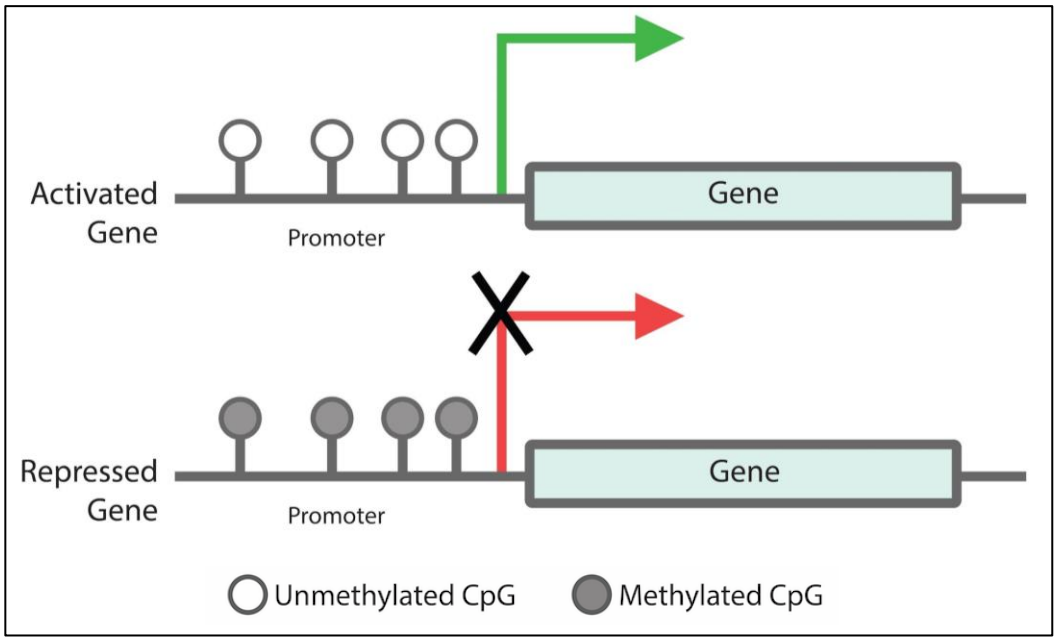


Figure 7: The effects of DNA methylation in the promoter region of the gene. *The top image depicts an unmethylated promoter region representing an active gene (indicated by the green arrow), whereas the bottom image depicts a methylated promoter region representing a repressed gene (indicated by the red arrow). Image created by Nicholas Stacey (2021) and adapted from www.kkorthauer.org, figure used with permission.*

During embryonic development when cells are rapidly dividing, methylated DNA plays a major role in determining cell type. For example, cells develop from stem cells and each stem cell has its own DNAm profile (Jones et al., 2018). Specific DNAm patterns are seen in all cell types and within different tissues, contributing the variation seen when measuring DNAm (Jones et al., 2018). For example, differential DNAm has been implicated in autoimmune disorders via the regulation of gene expression, and previous research has suggested that maternal stress can be epigenetically embedded via DNAm (Provençal & Binder, 2015; Heim et al., 2019; Mazzone et al., 2019).

Interestingly, methylation marks that have been suggested to underlie the biological embedding of stress are erased in germ cells (Lyko, 2018). This begs the question of how stress could be transmitted to subsequent generations. First, female offspring are born with all of their oocytes (Tilly et al., 2004). This is important because fetal oocytes may therefore be directly exposed to maternal stress in utero, or via maternal germ line alterations that occur during the mother's life. Additionally, genomic imprinting of DNAm can occur, such that DNAm is inherited in a parent-of-origin specific manner in both human and rodent models (Li et al., 1993; Ferguson-Smith, 2011; Carpenter et al., 2018). Second, paternal sperm micro RNAs (miRNA) may be implicated

in physiological alterations to the offspring. For example, Rodgers et al. (2013) used rodent models to test whether stress exposure alters miRNA in the sire's sperm and reprograms the HPA axis of the offspring. The offspring of the sires who were exposed to six weeks of stress during puberty and adulthood and prior to breeding both exhibited reduced HPA axis responsivity, increased GC-responsive genes, and altered sperm miRNA (Rodgers et al., 2013). This study not only implicates miRNAs as a potential mechanism of stress transmission, but also illuminates the role that fathers might play in offspring health which is lacking from the literature.

In line with the biological embedding theory of stress, then, maternal stress might get "under the skin" of her offspring as a result of maternal HPA axis alterations, offspring exposure to maternal GCs via the placenta, through epigenetic mechanisms such as DNAm, immune alterations, maternal germ line alterations, or perhaps via paternal stress exposure that alters the miRNA content of sperm. In an attempt to further quantify how maternal stress, specifically, might impact her offspring, Provençal et al. (2020) created an offspring EpiStress score.

1.5 Offspring EpiStress Scores

Individuals are differentially responsive to the stressful experiences that occur over the course of their lives, and it is unknown how these experiences are transmitted to subsequent generations. Provençal et al. (2020) created a glucocorticoid-responsive polyepigenetic score (EpiStress score) as a tool to measure these effects. The EpiStress score is a methylation risk score (i.e., scores that can be used to predict disease risk) that show differential responses to GCs (Hüls & Czamara, 2020; Provençal et al., 2020). An elastic net regression was performed on overlapping CpG sites from both adult blood samples from the Max Planck Institute of Psychiatry (MPIP) and an immortalized human hippocampal progenitor cell line to create the EpiStress scores (Provençal et al., 2020). The current project utilizes the EpiStress scores not only to measure prenatal stress, but also to determine if maternal ELS might influence the offspring's DNAm.

With the potential to use the newly created EpiStress score as a biological marker of maternal stress exposure in offspring, further validation of the EpiStress score was required. An additional study by Provençal et al. (2020) was conducted using the Prediction and Prevention of Preeclampsia and Intrauterine Growth Restriction Cohort

(PREDO). The PREDO cohort was previously established between 2005 and 2009 and is prospective study of Finnish women and their newborn children (Girchenko et al., 2017). This cohort was designed to help identify risk factors and biological markers in vulnerable, pregnant women who might be at risk of preeclampsia and intrauterine growth restriction (Girchenko et al., 2017). Utilizing the PREDO cohort, Provençal et al. (2020) sought to find associations between mothers who were clinically diagnosed with depression or anxiety throughout pregnancy and offspring EpiStress scores. The results suggested that prenatal depression and prenatal anxiety were both negatively and significantly associated with the offspring's EpiStress scores (Provençal et al., 2020).

1.6 Hypothesis

Maternal early life stress (ELS), defined as the experience of abuse and/or neglect during childhood, is negatively associated with offspring EpiStress scores, and this relationship is mediated by prenatal stress (either prenatal depression or anxiety).

1.7 Rationale

Until now, research has attempted to draw connections between maternal ELS, prenatal stress, and DNAm of candidate genes that might play a role in the development of poor child health outcomes. Recently, Provençal et al. (2020) has shown significant associations between offspring EpiStress scores and prenatal stress. These findings suggest a possible link between prenatal stress and DNAm levels in offspring from a human hippocampal progenitor cell line and an adult whole blood sample after DEX exposure. What is currently unknown is the influence of maternal ELS on the molecular biology of her child, and whether prenatal stress mediates the relationship between maternal ELS and offspring EpiStress scores.

The current study will utilize the EpiStress score to explore associations between maternal ELS and offspring DNAm levels. A negative association between maternal stress variables and offspring EpiStress scores is predicted because as maternal ELS and prenatal stress exposure increase, it is expected that offspring EpiStress scores will decrease. The current study attempts to further characterize the EpiStress score in terms of its application in both newborns (<30 days old) and infants (>=30 days old) in relation to demographic measures, brain morphology, and cell type. This characterization is fundamental for supporting the notion that the EpiStress score can be used as a biomarker of stress exposure in newborns and/or infants in future studies.

1.8 Research Aim

The research aim of this project is to determine if maternal ELS (experiences of childhood abuse and neglect) is associated with EpiStress scores in offspring (where the data has not yet been stratified), in newborns (<30 days old) and in infants (>=30 days old), and if this relationship is mediated by prenatal stress (prenatal depression or anxiety). A summary of the proposed aims is provided in the Figure 8 where "EpiStress Scores" include all three groups (offspring, newborn, and infant) that will be tested.

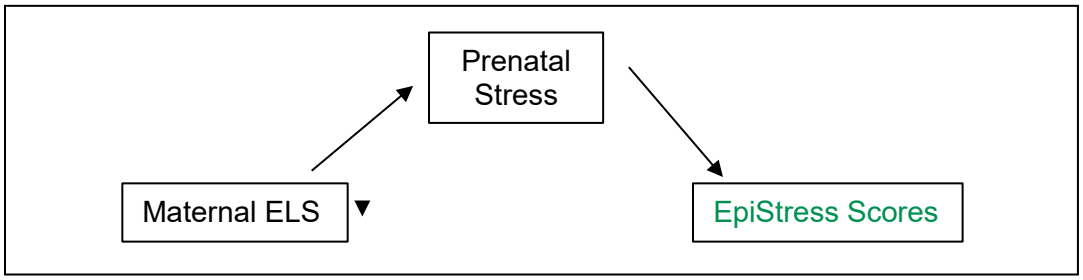


Figure 8: Summary of the proposed aims of the research study. *Maternal ELS is directly associated with EpiStress scores, as well as indirectly associated with EpiStress scores via exposure to prenatal stress (prenatal depression or anxiety).*

Chapter 2. Methods

2.1 University of California, Irvine (UCI) Cohort

This project utilizes an existing prospective longitudinal study from the University of California, Irvine (UCI) for secondary data analysis. The longitudinal design allows for the identification of stress exposure (early life and/or prenatally) in the mother that might be biologically transmitted to her child (Caruana et al., 2015). The UCI cohort contains data collected from N=131 expectant mothers who were receiving prenatal care at the university, as well as at institutions and clinics that are affiliated with the university (Moog et al., 2018). Women were recruited during the first trimester of pregnancy and received depression and anxiety assessments once per trimester and once postpartum (Moog et al., 2018). Additionally, the Childhood Trauma Questionnaire (CTQ) was administered to the women to determine if any of them had experienced abuse or neglect throughout childhood and adolescence (Moog et al., 2018).

A sample of expectant mothers (n=129) and their offspring from the UCI cohort was used in this study. The women had singleton, intrauterine pregnancies, and no known cord, placental, uterine abnormalities, fetal malformations, or conditions associated with atypical neuroendocrine function or corticosteroid use (Moog et al., 2018). Mothers were aged 18-41, with no obstetric complications. Mothers were excluded if they reported using corticosteroids, illicit drugs, or antidepressant or anxiety medication during pregnancy (n=2), and offspring were excluded if they were less than 34 weeks' gestation or if they had any congenital, genetic, or neurological disorders at birth (Moog et al., 2018). Upon birth or shortly thereafter, blood was drawn by a small needle prick in the offspring's foot (Moog et al., 2018). Maternal ELS measures were collected retrospectively via the CTQ, and prenatal stress (maternal depression and maternal anxiety) measurements were collected three times throughout pregnancy (Moog et al., 2018). This allows for the establishment of temporal precedence such that maternal ELS might influence prenatal stress which might ultimately influence the offspring's neurological and stress profile development. See Tables 1-1 to 1-4 for a summary of maternal and offspring measures.

2.2 Research Ethics and Data Access

The UCI institutional review board previously approved the study procedures; all participants provided written informed consent (Moog et al., 2018; Czamara et al., 2019). Ethical approval was also obtained from Simon Fraser University's Office of Research Ethics (SFU ORE).

2.3 Measures

2.3.1 Maternal Measures

Maternal Early Life Stress (ELS): In this thesis, maternal ELS was a dichotomous zero-one variable for the presence or absence of childhood maltreatment (CM). Assessments for maternal ELS were conducted using the Childhood Trauma Questionnaire (CTQ). The CTQ is a reliable and valid 28-item retrospective questionnaire that assesses CM prior to the age of 18, including: emotional-, physical-, and sexual abuse, and emotional- and physical neglect in childhood and adolescence (Bernstein et al., 2003). Cut-off values for each dimension of CM (emotional abuse ≥13, physical abuse ≥10, sexual abuse ≥8, emotional neglect ≥15, and physical neglect ≥10) were used to create a binary variable representative of CM exposure (Moog et al., 2018). Mothers who were exposed to one or more type of CM, indicated by reaching or exceeding the cut-off score for at least one type of CM, were given a score of one compared to mothers who did not reach a cut-off score for CM and who were given a score of zero (Moog et al., 2018). The CTQ has been validated in diverse populations across the United States, including African-American, Asian-Pacific Islander, Hispanic, non-Hispanic Caucasian, and Caucasian participants, as well as in individuals struggling with substance abuse, psychiatric patients, and normative communities (Bernstein et al., 2003). For further details, see Bernstein et al. (2003) and Moog et al. (2018).

Prenatal Depression: Clinical assessments for prenatal depression were conducted using the Centre for Epidemiologic Studies Depression Scale (CESD). The CESD is a scale used to measure depression across ages and cultures (Radloff, 1977). This is a 20-item self-report tool measuring changes in depression levels the week prior to administration of the CESD (Moog et al., 2018). Responses are measured on a scale of 0-3 and depend on how frequently the symptom(s) occurs. The cut-off score for

clinical depression is 16 and above, where higher scores correlate to higher depressive symptomatology (Radloff, 1977). The total possible range of scores is between 0-60 (Radloff, 1977). Mean CESD scores were calculated by adding each score per trimester and dividing by three trimesters; this was done to account for missing data.

Prenatal Anxiety: Clinical assessments for prenatal anxiety were conducted using the State-Trait Anxiety Inventory (STAI). This is an inventory used to measure current anxiety symptoms (Julian, 2011). The state scale from the STAI was administered; it is a 20-item self-report tool in which the items are rated on a 4-point Likert scale. Responses vary from 1) almost never, 2) sometimes, 3) often, to 4) almost always (Julian, 2011). The cut-off score for clinical anxiety is 39 and above, where higher scores correlate with higher anxiety symptomatology (Julian, 2011). The total possible range of scores is between 20-80 (Julian, 2011). Mean STAI scores were calculated by adding each score per trimester and dividing by three; this was done to account for missing data.

Smoking Status: Maternal smoking was a dichotomous zero-one variable for the presence or absence of smoking at any time throughout pregnancy. Smoking throughout pregnancy was assessed via maternal self-report measures and further verified by measuring cotinine concentration in participants' urine (Czamara et al., 2019). Cotinine was collected once per trimester using the Nicotine/COT(Cotinine)/Tobacco Drug Test Urine Cassette (Czamara et al., 2019). A predetermined cut-off value for the presence of smoking was set at ≥200 ng/ml and was coded as 1, and absence of smoking was coded as 0 (Czamara et al., 2019).

2.3.2 Offspring Measures

2.3.2.1 Demographic Measures

Age at Sample Collection: Obtained from hospital records. Age at sample collection was stratified into infant- (≥30 days old) and newborn (<30 days old) samples due to the differences in cell type proportions during the early stages of immune development (Jacob, 2016).

Sex: Obtained from hospital records.

Genotype: Blood was drawn from the offspring via a small needle prick in their heel (Czamara et al., 2019). DNA was extracted from those blood samples and was used for genetic analysis (Czamara et al., 2019). Genotyping was performed on Illumina Human Omni Express Arrays containing 713,014 single nucleotide polymorphisms (SNPs) (Czamara et al., 2019). SNPs refer to any single variation of a nucleotide along the genome (International Human Genome Sequencing Consortium, 2001). DNA samples had a call rate above 97% (Czamara et al., 2019). Call rates refer to the proportion of samples that were assigned a genotype when a particular assay was used (Gardner et al., 2013). Imputation was performed using Positional Burrows-Wheeler Transform (BWT) (Czamara et al., 2019). Imputed SNPs were removed if they had an information metric of <0.8 or if they had a minor allele frequency (MAF) of <0.01; duplicate and ambiguous SNPs were removed (Czamara et al., 2019). After Quality Control (QC), 602,807 SNPs were available (Czamara et al., 2019). For further details, refer to Czamara et al. (2019).

Gestational Age: Obtained from hospital records.

Birthweight: Obtained from hospital records. Offspring were weighed at birth and their birthweights were subsequently converted into percentiles (Moog et al., 2018).

Age at MRI Scan: Offspring whose mother's provided consent were administered an MRI scan five to 64 days after birth, with 67.5% of offspring scanned within the first 30 days (Moog et al., 2018).

DNA Methylation (DNAm): DNAm was analyzed using the Infinium Illumina MethylationEPIC BeadChip (EPIC) array using the DNA obtained from a heel prick of the offspring's foot (Czamara et al., 2019). The EPIC array detects the presence of methylation at CpG sites along the genome via probes which correspond to either a methylated or an unmethylated CpG site; eight samples can be tested at one time covering a total of ~850,000 CpG sites using this technology (Illumina Array Technology). Functional normalization (*funnorm*) was performed on methylation beta-values with the goal to remove unwanted variation due to control probes that are present on the array (Fortin et al., 2014; Czamara et al., 2019). Data was adjusted for technical factors (array row, experimental batch, and sample plate) using *ComBat*, QC was performed using the *minfi* package in R, and no outliers were detected (Czamara et al.,

2019). Samples had a call rate of 95% or above (Czamara et al., 2019). Any CpGs with a detection value of $p < 0.0001$, those with probes missing more than three beads in >5% of the cohort, and those with non-specific or cross-hybridizing and SNP probes were removed (Czamara et al., 2019). The final dataset contained 768,910 CpGs (Czamara et al., 2019).

EpiStress Scores: Provençal et al. (2020) created an EpiStress score utilizing adult whole blood samples from the Max Planck Institute of Psychiatry (MPIP) cohort (n=24,423) and progenitor cells from a human hippocampal progenitor cell line (n=6,096). The MPIP cohort consisted of 200 male participants, of which 81 received a clinical diagnosis of depression, and 97 female participants, of which 49 received a clinical diagnosis of depression (Provençal et al., 2020). A baseline measurement of whole blood samples from MPIP cohort participants was taken at 6:00pm, two hours after fasting and abstaining from coffee and physical activity (Provençal et al., 2020). At that time, participants received 1.5mg of DEX administered orally. A second blood sample was taken at 9:00pm to measure any deviations from baseline (Provençal et al., 2020).

The human hippocampal progenitor cell line came from an immortalized, multipotent human fetal hippocampal progenitor cell line from a 12-week-old fetus and was proliferated using growth factors (EGF, FGF) and 4-hydroxytamoxifen (4-OHT) (Provençal et al., 2020). The progenitor cells were treated with 1μM of DEX or a vehicle (ethanol) in four different experiments: three days of proliferation, during proliferation and seven days of differentiation (growth factors and 4-OHT were removed), during proliferation and differentiation in which cells were cultured for 20 additional days without DEX or ethanol, and finally, after proliferation and differentiation for 10 days (followed by 20 days of washout to compare effects between treatments (see Provençal et al., 2020 for more information).

Using both the MPIP cohort whole blood samples and the human hippocampal progenitor cell line, an enrichment analysis revealed 496 DEX-responsive CpG sites that overlapped in each group (Provençal et al., 2020). Provençal et al. (2020) subsequently narrowed down those 496 sites to 24 CpG sites using an elastic net regression. Once the 24 CpG sites were identified, the sum of the methylation values across those 24

CpG sites was multiplied by a weight (representative of changes in DEX concentrations) to create the EpiStress scores (Provençal et al., 2020).

2.3.2.2 Brain Morphology

Brain Morphology: Offspring magnetic resonance imaging (MRI) scans were performed during natural sleep with a Siemens 3T scanner (Moog et al., 2018). Images were captured using a three-dimensional magnetization-prepared rapid gradient echo sequence (spatial resolution was a 1 x 1 x 1 mm voxel for T1-weighted images) and a turbo spin echo sequence (spatial resolution was a 1 x 1 x 1 mm voxel with a 0.5 mm interslice gap for T2-weighted images) (Moog et al., 2018). Hippocampal and amygdalae images were captured using a multi-modality, multi-template based automatic method that combines T1- and T2-weighted images, and manual corrections were performed on those images using ITK-Snap (Moog et al., 2018). Intracranial volume (ICV) consisted of three components: grey matter (GM), white matter (WM), and cerebrospinal fluid (CSF), and was defined using the Advanced Neuroimaging Tools toolkit. For further details, refer to Moog et al. (2018).

2.3.2.3 Cell Type

Cell Type: Offspring blood cell proportions were estimated for CD4T cell-, CD8T cell-, B cell-, natural killer cell-, monocyte-, granulocyte-, and nucleated red blood cell proportions (Czamara et al., 2019). Cell type proportions were estimated using the Houseman method in which differentially methylated regions (DMRs) of the DNA were used to identify immune cells (Houseman et al., 2012). Houseman et al. (2012) used DNAm as a highly correlated measure of the distribution of white blood cells. Cell type has been previously shown to differ between newborns and infants and therefor was included in the analysis (Hermansen, 2001; Houseman et al., 2012; Jacob, 2016). For further details, refer to Houseman et al. (2012).

2.3.3 Covariates

Offspring sex and the first two principal components (PCs) of the offspring's genotype were included as covariates because they have previously been shown to influence DNAm levels (Barfield et al., 2014; Saurez et al., 2020).

2.4 Statistical Analysis

2.4.1 Descriptive Statistics

Descriptive statistics (range, median, mean, and standard deviation) were conducted on maternal measures (see Table 1-1) as well as offspring demographic measures, brain morphology, and cell type (see Table 1-2). Descriptive statistics were also conducted separately for infant- (see Table 1-3) and newborn (see Table 1-4) demographic measures, brain morphology, and cell type due to significant associations between age at sample collection with offspring EpiStress scores and offspring cell type.

2.4.2 Characterization Analysis of Offspring Measures

2.4.2.1 Investigation of Offspring Demographic Measures

Demographic measures (age at sample collection, gestational age, maternal smoking status, birthweight, genotype (the first two PCs), and sex) potentially associated with offspring EpiStress scores were analyzed via bivariate analyses in R (version 4.0.2). A characterization analysis was conducted using the Epigenome-Wide Association Study (EWAS) Atlas from the National Genomics Data Centre (NGDC). The EWAS Atlas is a digital tool that enables the identification of methylation at CpG sites of interest that have been previously implicated in disease(s) (Xiong et al., 2020). The purpose of the characterization analysis was to explore the possible relationship(s) between methylation levels at the 24 CpG sites used to create the EpiStress score and whether those sites have been previously established the literature. If any of the 24 CpG sites of interest correspond to a previously identified disease in the EWAS Atlas, the EWAS Atlas will reveal the trait, type, and occurrence of the disease(s) (see Appendix A).

2.4.2.2 Investigation of Offspring Brain Morphology

Brain morphology (right- and left hippocampal volume, right- and left amygdala volumes, and total ICV) potentially associated with offspring EpiStress scores was analyzed via bivariate analyses in R. A characterization analysis was conducted using the Blood-Brain Epigenetic Concordance (BECon) tool. BECon is a digital tool that enables the comparison of CpG sites of interest with a pre-existing database of human brain and blood methylation in individuals who are now deceased (Edgar et al., 2017).

The purpose of the characterization analysis was to explore the possible relationship(s) between methylation levels at the 24 CpG sites used to create the EpiStress score against an existing database of human brain and blood. BECon produces a summary of metrics from each CpG site entered into the tool. This includes chromosome location, gene coordinates, genes, gene regions, variability and Spearman correlations as they compare to Brodmann's Area (BA) 10, 20, and 7, and in the blood. These are brain regions that correspond with the prefrontal cortex, the temporal cortex, and the parietal lobes (Edgar et al., 2017).

BECon metrics are calculated from 16 deceased adults who were healthy upon their deaths and who donated their tissues to science (Edgar et al., 2017). The amount of variability refers to the range of the beta methylation values from the HumanMethylation450 BeadChip array (450K array) that fell between the 10th and 90th percentile (Edgar et al., 2017). In other words, variability is the difference in DNAm at a specific CpG site across individuals in each tissue (BA 10, 20, and 7, and in the blood) (Edgar et al., 2017). The percentiles are used to limit outlier effects at each CpG site, as outliers could potentially give an incorrect estimate of the total amount of variability (Edgar et al., 2017). Finally, Spearman correlations represent the correlations between methylation values at CpG sites and human brain methylation of BA 10, 20, and 7 (Edgar et al., 2017).

2.4.2.3 Investigation of Offspring Cell Type

Cell type (estimated CD4T-, CD8T-, B cell-, natural killer cell-, monocyte-, granulocyte-, and nucleated red blood cell proportions) potentially associated with offspring-, infant-, and newborn EpiStress scores were analyzed via bivariate analyses in R.

2.4.3 Mediation analysis

Two mediation analyses were performed: model 1 was a multivariate analysis of the association between maternal ELS and infant- and newborn EpiStress scores using prenatal depression as the mediating variable, and prenatal anxiety as the mediating variable for model 2. These relationships were examined using the *Mediate* function in the *Mediation* package in R. This package calculates the direct effect, indirect effect, total effect, and proportions mediated. 1,000 Bootstrap simulations were run due to the sample size of n=129. Bootstrapping is a sampling technique that assumes that the sample data is a proxy of the population and repeatedly resamples from that data to enable users to make inferences about the general population (Efron & Tibshirani, 1994). Mediation effects were examined separately for infants and newborns (see Table 1-6). The rationale for this analysis was to examine whether prenatal stress explained some or all of the association between maternal ELS and infant- or newborn EpiStress scores. It is expected that there will be negative associations because it has been suggested that as stress exposure increases, EpiStress scores are expected to decrease (Provençal et al., 2020).

2.4.4 Missing Data

Prior to testing, independent variables, mediating variables, and dependent variables were evaluated for missing data. According to Bennet (2001) results can be biased if more than 10% of the data is missing. As the independent variable (maternal ELS) was only missing five data points out of 129 (0.38%), no further methods were used to rectify this. To account for missing data for the mediating variables (prenatal depression and prenatal anxiety), a mean sum score was calculated by adding the total data points in each trimester and dividing by three trimesters. Using the mean sum scores, n=129 for both prenatal depression and prenatal anxiety.

Chapter 3. Results

3.1 Descriptive Statistics

Table 1-1 and 1-2 show descriptive statistics for maternal measures and offspring measures, respectively. Cut-off scores for prenatal depression are 16 and above and cut-off scores for prenatal anxiety are 39 and above. The mean sum scores for both measures of prenatal stress are below the cut-off scores, indicating that the average number of participants did not reach the threshold for a clinical diagnosis. Lower offspring EpiStress scores indicate higher glucocorticoid/stress exposure and because the weights of the scores are all negative, samples with higher methylation tend to have a more negative EpiStress score (-2.45 versus -0.84) (Provençal et al., 2020). Tables 1-3 and 1-4 show descriptive statistics for infant- and newborn demographic measures, brain morphology, and cell type.

Table 1-1. Descriptive statistics for maternal measures.

Measures	Final N (%)	Range (%)	Median	Mean	Standard Deviation
Maternal Early Life Stress*	124 (96.12%)	0 (63.7% did not experience ELS) or 1 (36.3% did experience ELS)	-	-	-
Prenatal Maternal Depression (sum scores)	129	1.33 to 43	12.33	13.98	8.14
Prenatal Maternal Anxiety (sum scores)	129	20 to 59.5	32.33	33.6	8.62
Maternal Smoking Status*	119 (92.2%)	0 (91.6% did not smoke) or 1 (8.4% did smoke)	-	-	-

*Categorical variable
Proportions are indicated in percentages (%)

Table 1-2. Descriptive statistics for offspring demographic measures, brain morphology, and cell type.					
Demographic Measures	**Final N = 129 (%)**	**Range (%)**	**Median**	**Mean**	**Standard Deviation**
EpiStress Scores	129	-2.45 to -0.84	-1.96	-1.95	0.22
Age at Sample Collection (days)	129	2 to 68	26	27.37	13.10
Sex	129	1 (53.5% male) or 2 (46.5% female)	-	-	-
Genotype (PC1)	129	-0.10 to 0.18	0.0028	0.0016	0.085
Genotype (PC2)	129	-0.50 to 0.085	0.024	0.00012	0.085
Gestational Age	129	242 to 293	276	274.38	10.04
Birthweight (percentile)	122 (94.6%)	2 to 99	48	47.16	27.78
Age at MRI Scan (days)	81 (62.8%)	5 to 64	24	26.49	13.11
Brain Morphology (cm^3)	**Final N = 129 (%)**	**Range (%)**	**Median**	**Mean**	**Standard Deviation**
Right Hippocampal Volume	81 (62.8%)	931 to 1587.5	1195.5	1209.58	143.82
Left Hippocampal Volume	81 (62.8%)	916.5 to 1488.5	1142.5	1171.79	131.96
Right Amygdala Volume	81 (62.8%)	221 to 358	280.5	281.1	32.02
Left Amygdala Volume	81 (62.8%)	195 to 360.5	271	271.88	31.92
Intracranial Volume	79 (61.2%)	355859 to 639807	483473	487635.5	58337.73
Cell Type	**Final N = 129 (%)**	**Range (%)**	**Median**	**Mean**	**Standard Deviation**
CD4T Cell Proportions*	120 (93.0%)	0.14 to 0.74	0.45	0.45	0.097
CD8T Cell Proportions*	120 (93.0%)	0.014 to 0.27	0.11	0.12	0.059
B Cell Proportions*	120 (93.0%)	0.005 to 0.39	0.16	0.16	0.084
Natural Kill Cell Proportions*	120 (93.0%)	0.0 to 0.13	1.83e-18	0.0022	0.013
Monocyte Proportions*	120 (93.0%)	0.0 to 0.17	0.042	0.047	0.039
Granulocyte Proportions*	120 (93.0%)	0.0 to 0.71	0.18	0.19	0.11
Nucleated Red Blood Cell Proportions*	120 (93.0%)	0.0 to 0.021	0	0.00057	0.0029

*Estimated cell type proportions

Table 1-3. Descriptive statistics for infant demographic measures, brain morphology, and cell type.					
Demographic Measures	**Final N = 48 (%)**	**Range (%)**	**Median**	**Mean**	**Standard Deviation**
EpiStress Scores	48	-2.45 to -1.56	-2.08	-2.06	0.17
Age at Sample Collection (days)	48	30 to 68	38	40.96	9.90
Sex	48	1 (39.6% male) or 2 (60.4% female)	-	-	-
Genotype (PC1)	48	-0.098 to 0.16	0.026	0.010	0.088
Genotype (PC2)	48	-0.50 to 0.085	0.023	-0.0079	0.10
Gestational Age	48	242 to 288	275	273.06	11.57
Birthweight (percentile)	45 (93.75%)	2 to 99	51	49.44	25.91
Age at MRI Scan (days)	27 (56.25%)	8 to 58	32	33.33	13.61
Brain Morphology (cm^3)	**Final N = 48 (%)**	**Range (%)**	**Median**	**Mean**	**Standard Deviation**
Right Hippocampal Volume	27 (56.25%)	1015.5 to 1587.5	1210.5	1214.20	169.57
Left Hippocampal Volume	27 (56.25%)	916.5 to 1488.5	1202.5	1204.85	146.53
Right Amygdala Volume	27 (56.25%)	226 to 343.5	281.5	284	29.17
Left Amygdala Volume	27 (56.25%)	214.5 to 360.5	271	272.89	30.35
Intracranial Volume	27 (56.25%)	409005 to 639807	482965	496876.6	59599.61
Cell Type	**Final N = 48 (%)**	**Range (%)**	**Median**	**Mean**	**Standard Deviation**
CD4T Cell Proportions*	47 (97.9%)	0.28 to 0.74	0.47	0.48	0.098
CD8T Cell Proportions*	47 (97.9%)	0.024 to 0.27	0.10	0.11	0.060
B Cell Proportions*	47 (97.9%)	0.096 to 0.39	0.22	0.22	0.060
Natural Kill Cell Proportions*	47 (97.9%)	0.00 to 0.023	2.28e-18	0.00071	0.0036
Monocyte Proportions*	47 (97.9%)	0.00 to 0.13	0.014	0.025	0.032
Granulocyte Proportions*	47 (97.9%)	0.00 to 0.36	0.12	0.14	0.074
Nucleated Red Blood Cell Proportions*	47 (97.9%)	0.00 to 0.0046	0	9.82e-05	0.00067

*Estimated cell type proportions

Table 1-4. Descriptive statistics for newborn demographic measures, brain morphology, and cell type.					
Demographic Measures	**Final N = 81 (%)**	**Range (%)**	**Median**	**Mean**	**Standard Deviation**
EpiStress Scores	81	-2.38 to -0.84	-1.90	-1.88	0.22
Age at Sample Collection (days)	81	2 to 29	20	19.32	6.37
Sex	81	1 (61.7% male) or 2 (38.3% female)	-	-	-
Genotype (PC1)	81	-0.10 to 0.18	-0.022	-0.0035	0.083
Genotype (PC2)	81	-0.48 to 0.080	0.024	0.0049	0.073
Gestational Age	81	248 to 293	276	275.16	8.99
Birthweight (percentile)	77 (95.1%)	2 to 99	43	46.82	28.89
Age at MRI Scan (days)	54 (66.7%)	5 to 64	19.5	23.07	11.53
Brain Morphology (cm^3)	**Final N = 81 (%)**	**Range (%)**	**Median**	**Mean**	**Standard Deviation**
Right Hippocampal Volume	54 (66.7%)	931 to 1464	1171.75	1193.77	127.87
Left Hippocampal Volume	54 (66.7%)	924 to 1472	1136.75	1155.26	122.13
Right Amygdala Volume	54 (66.7%)	221 to 358	280.5	279.65	33.52
Left Amygdala Volume	54 (66.7%)	195 to 343	270.75	271.38	32.94
Intracranial Volume	52 (64.2%)	355859 to 608543	487200	482837.2	57666.86
Cell Type	**Final N = 81 (%)**	**Range (%)**	**Median**	**Mean**	**Standard Deviation**
CD4T Cell Proportions*	73 (90.1%)	0.14 to 0.67	0.43	0.43	0.091
CD8T Cell Proportions*	73 (90.1%)	0.014 to 0.27	0.13	0.13	0.058
B Cell Proportions*	73 (90.1%)	0.0050 to 0.38	0.11	0.13	0.077
Natural Kill Cell Proportions*	73 (90.1%)	0.00 to 0.13	1.51e-18	0.0032	0.017
Monocyte Proportions*	73 (90.1%)	0.00 to 0.17	0.064	0.061	0.037
Granulocyte Proportions*	73 (90.1%)	0.00 to 0.71	0.22	0.23	0.11
Nucleated Red Blood Cell Proportions*	73 (90.1%)	0.00 to 0.021	0	0.00087	0.0036

*Estimated cell type proportions

3.2 Statistical Analysis

3.2.1 Characterization Analysis of Offspring Measures

3.2.1.1 Investigation of Offspring Demographic Measures

A bivariate analysis of offspring EpiStress scores and demographic measures revealed that the offspring EpiStress scores were significantly and negatively correlated with age at sample collection (r = -0.46, p = 0.000000048) (see Figure 9). Offspring EpiStress scores were not correlated with offspring gestational age, maternal smoking status, offspring birthweight, offspring genotype (the first two PCs), or offspring sex (see Figure 9 and 10). The EWAS Atlas revealed traits that have been previously linked with the 24 CpG sites used to create the offspring EpiStress scores. The trait, type, and occurrence (out of 24 CpG sites) are depicted in Table 1-5. The two traits with the highest occurrence are multiple sclerosis (MS) which is present in 12 out of the 24 CpG sites (50%) and systemic lupus erythematosus (SLE) which is present in 15 out of the 24 CpG sites (62.5%). The MS samples came from CD19+ B cells of those diagnosed with MS (mean age 40.7 years old) and controls (mean age of 43.3 years old), while the SLE samples came from whole blood of those diagnosed with SLE (mean age of 47 years old) and controls (mean age of 47.1 years old) (Xiong et al., 2020).

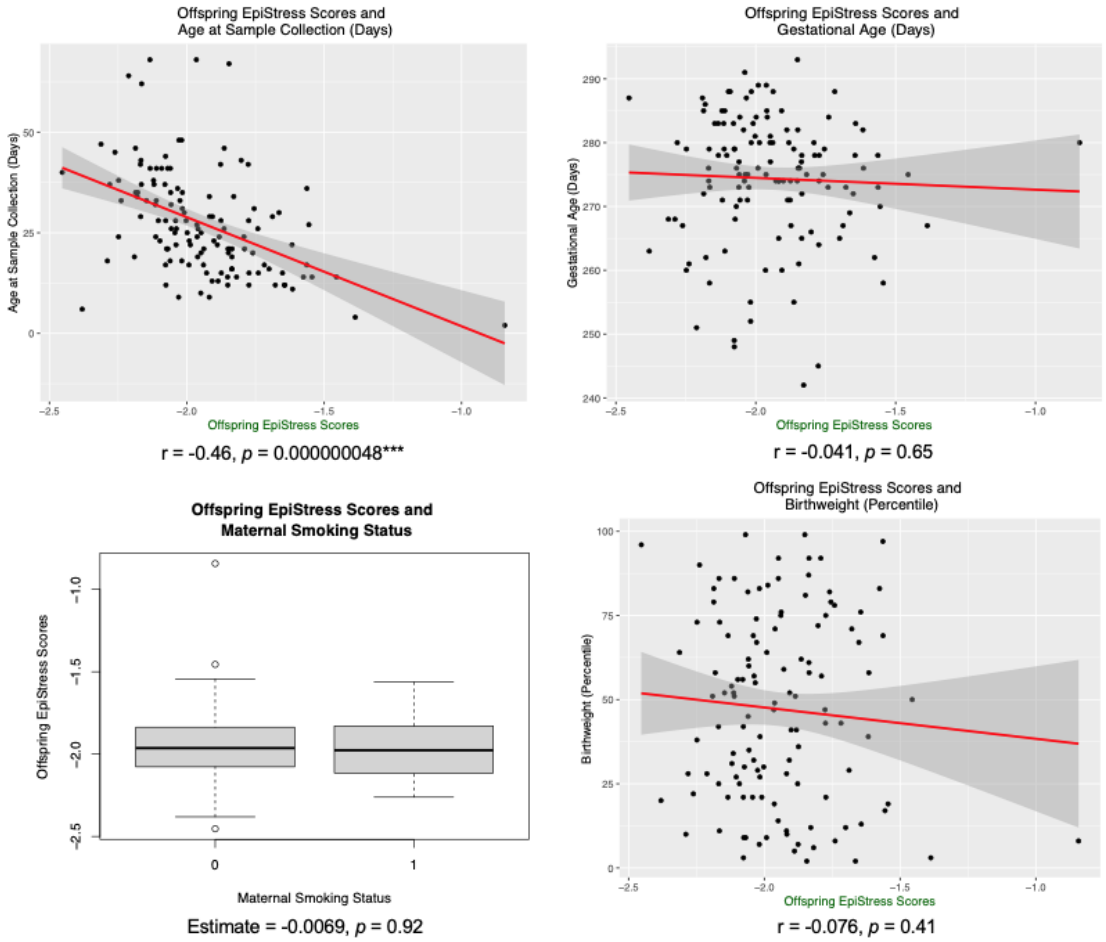


Figure 9: Bivariate analyses using offspring EpiStress scores and demographic measures. *Demographic measures include age at sample collection (days), gestational age (days), maternal smoking status, and birthweight.*

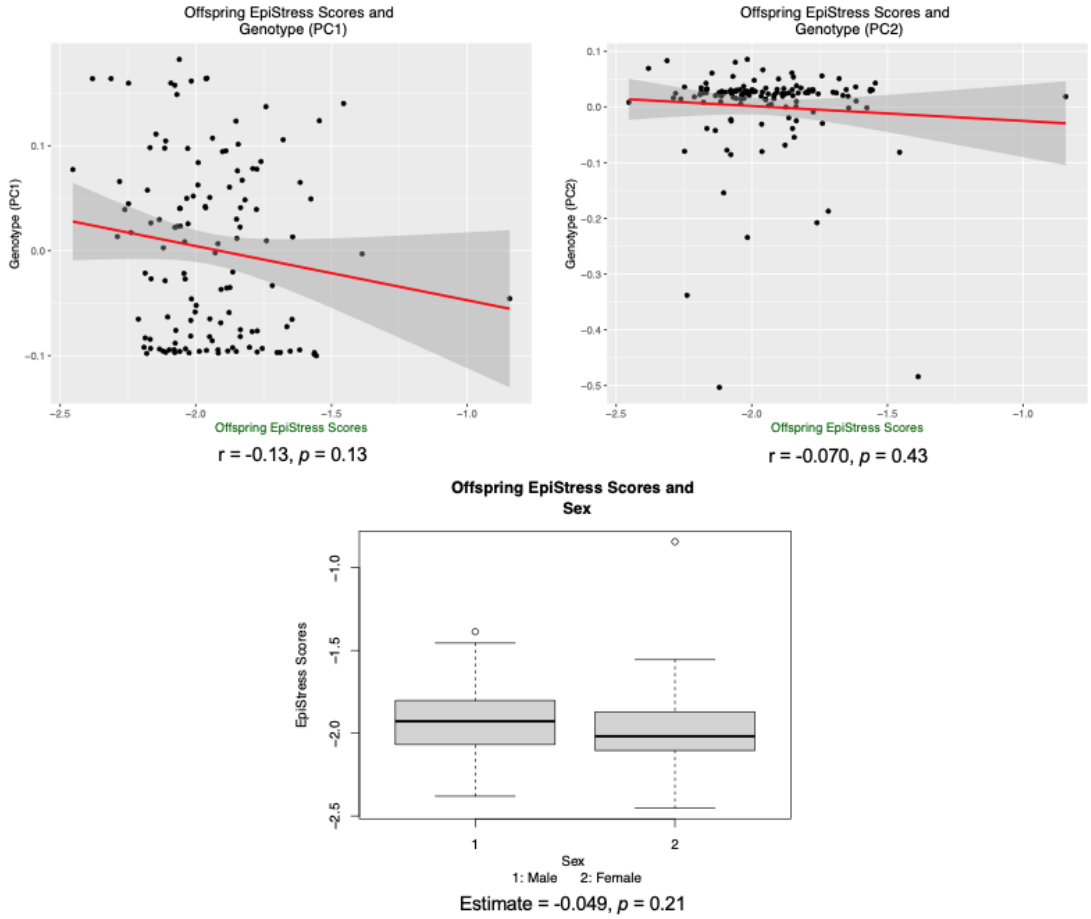


Figure 10: Bivariate analyses using offspring EpiStress scores and demographic measures. *Demographic measures include the first two genotype PCs and sex.*

Table 1-5. EWAS Atlas summary. A summary of 15 traits and the type of factor or illness associated with the 24 CpG sites used to create the EpiStress score. The occurrence of each trait per CpG out of a possible 24 CpGs is listed on the right.

Trait	Type	Occurrence
Air pollution (NO2)	Environmental factor	5/24 (20.8%)
B acute lymphoblastic leukemia	Cancer	2/24 (8.3%)
Breast cancer	Cancer	3/24 (12.5%)
Chronic fatigue syndrome	Non-cancerous systemic intolerance	2/24 (8.3%)
Crohn's disease	Autoimmune disorder	4/24 (16.7%)
Fatigue	Phenotype	1/24 (4.17%)
Fractional exhaled nitric oxide	Phenotype	1/24 (4.17%)
Gulf War illness	Non-cancerous systemic intolerance	2/24 (8.3%)
Hepatocellular carcinoma	Cancer	5/24 (20.8%)
Inflamed Crohn's disease	Autoimmune disorder	1/24 (4.17%)
Multiple sclerosis	Autoimmune disorder	12/24 (50%)
Osteoarthritis	Degenerative disease	1/24 (4.17%)
Preterm birth	Phenotype	4/24 (16.7%)
Systemic lupus erythematosus	Autoimmune disorder	15/24 (62.5%)
Vitamin B12 supplement	Environmental factor	5/24 (20.8%)

3.2.1.2 Investigation of Offspring Brain Morphology (cm^3)

A bivariate analysis of offspring EpiStress scores and brain morphology (right- and left hippocampal volumes, right- and left infant amygdala volumes, and total intracranial volume) did not reveal any significant correlations (see Figure 11). Utilizing the Blood-Brain Epigenetic Concordance (BECon) tool by Edgar et al. (2017), the 24 CpG sites found by Provençal et al. (2020) were individually examined for correlations between Brodmann's Area's (BA) 10, BA 20, and BA 7 in the brain and blood (see Figure 12). Four CpG sites (cg02862467, cg01400750, cg16141752, and cg20977312) that also correspond to MS and SLE from the EWAS Atlas were revealed to be variable in the blood, but not variable in BA 10, 20, nor 7. Two CpG sites (cg21344746 and cg04674060) were variable in all regions and the blood except BA 7, while one CpG site (cg12157761) was variable in all regions and the blood except BA 10 (see Figure 12). The CpG sites mentioned here show predominantly negative correlations with the brain regions, where a small portion show positive correlations. Of the positive correlations revealed by BECon, two CpG sites show correlations above 0.70 (cg23987336 and cg07052737). There are no other highly correlated regions. Extending these results to the 16 subjects who donated their tissues to science, beta values from the HumanMethylation 450K array indicate variable methylation between subjects and CpG sites, especially the in blood (see Figure 13).

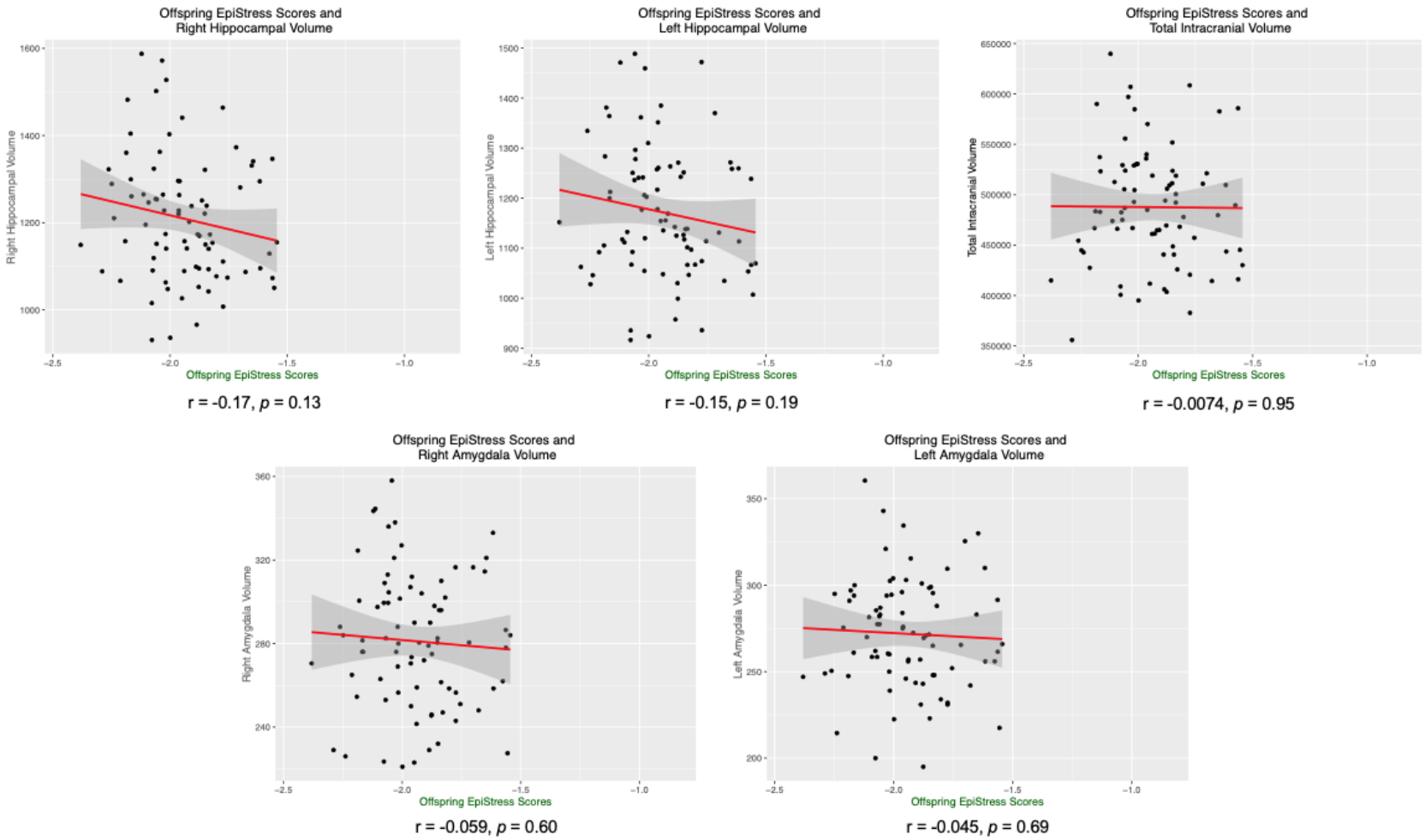
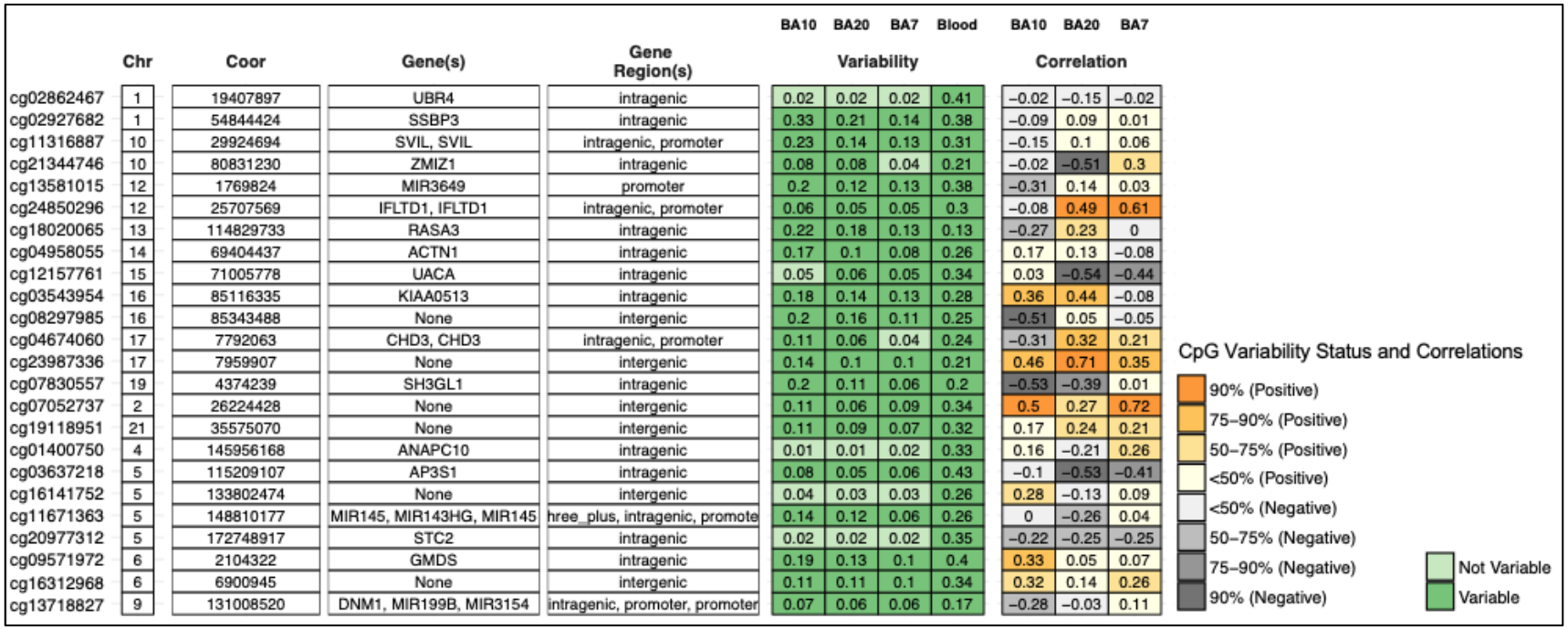


Figure 11: Bivariate analyses using offspring EpiStress scores and brain morphology ***(cm³).*** *Brain morphology includes right- and left hippocampal volumes, right- and left amygdala volumes, and total intracranial volume (cerebrospinal fluid, grey matter, and white matter). No significant correlations were found.*



	Chr	Coor	Gene(s)	Gene Region(s)	Variability				Correlation		
					BA10	BA20	BA7	Blood	BA10	BA20	BA7
cg02862467	1	19407897	UBR4	intragenic	0.02	0.02	0.02	0.41	−0.02	−0.15	−0.02
cg02927682	1	54844424	SSBP3	intragenic	0.33	0.21	0.14	0.38	−0.09	0.09	0.01
cg11316887	10	29924694	SVIL, SVIL	intragenic, promoter	0.23	0.14	0.13	0.31	−0.15	0.1	0.06
cg21344746	10	80831230	ZMIZ1	intragenic	0.08	0.08	0.04	0.21	−0.02	−0.51	0.3
cg13581015	12	1769824	MIR3649	promoter	0.2	0.12	0.13	0.38	−0.31	0.14	0.03
cg24850296	12	25707569	IFLTD1, IFLTD1	intragenic, promoter	0.06	0.05	0.05	0.3	−0.08	0.49	0.61
cg18020065	13	114829733	RASA3	intragenic	0.22	0.18	0.13	0.13	−0.27	0.23	0
cg04958055	14	69404437	ACTN1	intragenic	0.17	0.1	0.08	0.26	0.17	0.13	−0.08
cg12157761	15	71005778	UACA	intragenic	0.05	0.06	0.05	0.34	0.03	−0.54	−0.44
cg03543954	16	85116335	KIAA0513	intragenic	0.18	0.14	0.13	0.28	0.36	0.44	−0.08
cg08297985	16	85343488	None	intergenic	0.2	0.16	0.11	0.25	−0.51	0.05	−0.05
cg04674060	17	7792063	CHD3, CHD3	intragenic, promoter	0.11	0.06	0.04	0.24	−0.31	0.32	0.21
cg23987336	17	7959907	None	intergenic	0.14	0.1	0.1	0.21	0.46	0.71	0.35
cg07830557	19	4374239	SH3GL1	intragenic	0.2	0.11	0.06	0.2	−0.53	−0.39	0.01
cg07052737	2	26224428	None	intergenic	0.11	0.06	0.09	0.34	0.5	0.27	0.72
cg19118951	21	35575070	None	intergenic	0.11	0.09	0.07	0.32	0.17	0.24	0.21
cg01400750	4	145956168	ANAPC10	intragenic	0.01	0.01	0.02	0.33	0.16	−0.21	0.26
cg03637218	5	115209107	AP3S1	intragenic	0.08	0.05	0.06	0.43	−0.1	−0.53	−0.41
cg16141752	5	133802474	None	intergenic	0.04	0.03	0.03	0.26	0.28	−0.13	0.09
cg11671363	5	148810177	MIR145, MIR143HG, MIR145	hree_plus, intragenic, promote	0.14	0.12	0.06	0.26	0	−0.26	0.04
cg20977312	5	172748917	STC2	intragenic	0.02	0.02	0.02	0.35	−0.22	−0.25	−0.25
cg09571972	6	2104322	GMDS	intragenic	0.19	0.13	0.1	0.4	0.33	0.05	0.07
cg16312968	6	6900945	None	intergenic	0.11	0.11	0.1	0.34	0.32	0.14	0.26
cg13718827	9	131008520	DNM1, MIR199B, MIR3154	intragenic, promoter, promoter	0.07	0.06	0.06	0.17	−0.28	−0.03	0.11

Figure 12: Results from the Blood-Brain Epigenetic Concordance (BECon) tool showing the 24 CpG sites used to create the EpiStress score (left). *Also shown is the gene(s) where the CpG site has been found, the gene region(s), the variability of each CpG site in one of three brain regions (Brodmann's Area (BA) 10, 20, and 7) and in the blood, and Spearman correlations (right) between each CpG site and BA 10, 20, and 7. Chr: Chromosome of the CpG; Coor: Genomic coordinate of the CpG. Image created by Karilyn Harris (2021) using the BECon tool.*

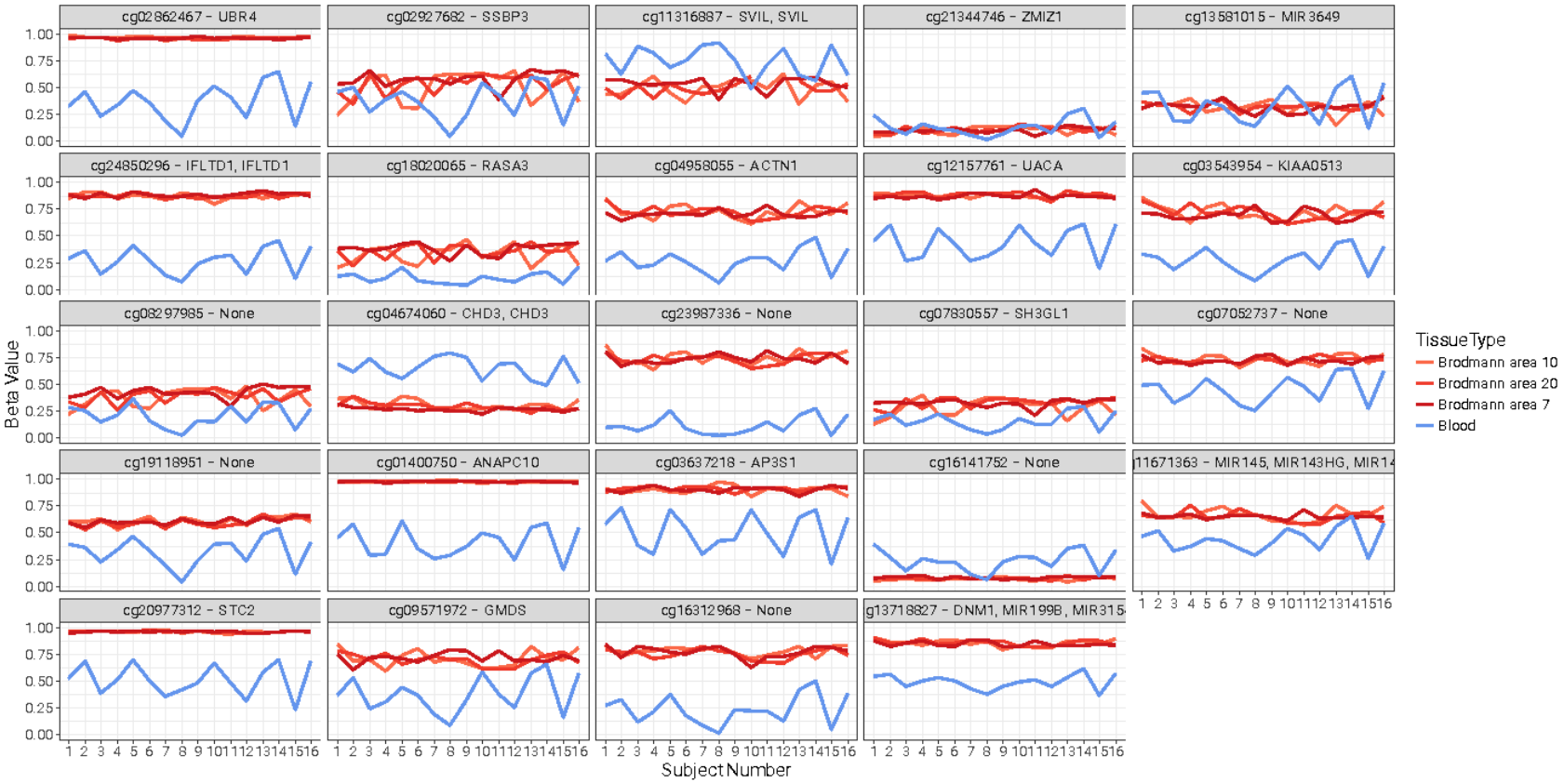


Figure 13: Results from the Blood-Brain Epigenetic Concordance (BECon) tool showing the beta values (methylation level) from the 450K array of the 24 CpG sites used to create the EpiStress score (y-axis). *Also shown are the 16 subjects whose data was collected (x-axis) and who also show methylation at these same CpG sites in one of three brain regions (Brodmann's Area (BA) 10, 20, and 7), and in the blood. Image created by Karilyn Harris (2021) using the BECon tool.*

3.2.1.3 Investigation of Offspring Cell Type

A bivariate analysis of offspring EpiStress scores and DNAm estimated cell type (CD4T cell-, CD8T cell-, B cell-, monocyte-, granulocyte-, natural killer cell-, and nucleated red blood cells (nRBC) proportions) revealed that offspring EpiStress scores were significantly negatively correlated with estimated CD4T cell- (r = -0.72, p = <2.2e-16***) and B cell (r = -0.56, p = 3.18e-11***) proportions (see Figure 14). Offspring EpiStress scores were also significantly and positively correlated with estimated monocyte- (r = 0.59, p = 1.044e-12***) and estimated granulocyte cell proportions (r = 0.96, p = <2.2e-16***), with estimated granulocyte cell proportions revealing an almost perfect correlation (see Figure 15). As well, non-significant correlations were found between offspring EpiStress scores and both the estimated CD8T cell- (r = -0.12, p = 0.18) and nRBC proportions (r = 0.25, p = 0.0052*) (see Figures 14 and 15). No correlations were found between offspring EpiStress scores and estimated natural killer cell proportions (see Figure 15).

3.2.1.4 Investigation of Infant and Newborn Cell Type

Because of the significant associations between offspring EpiStress scores and age at sample collection, and offspring EpiStress scores and estimated cell type proportions, the offspring EpiStress scores were stratified into infant- and newborn EpiStress scores. The purpose of this stratification was to determine if infant- and newborn EpiStress scores were differentially associated with estimated cell type proportions (CD4T cell-, CD8T cell-, B cell-, monocyte-, granulocyte-, natural killer cell-, and nRBC proportions). Cell type proportions change with age, and therefore it is hypothesized that the EpiStress scores are highly associated with age at sample collection because of their association with estimated cell type proportions (Jones et al., 2018).

Significant and negative associations were found between infant EpiStress scores and estimated CD4T cell- (r = -0.73, p = <6.4e-09***), while significant and positive associations were found between infant EpiStress scores and estimated monocyte- (r = 0.50, p = 0.00034***) and estimated granulocyte cell proportions (r = 0.96, p = <2.2e-16***). No significant associations were found between infant EpiStress

scores and estimated CD8T cell-, B cell-, natural killer cell, and nRBC proportions (see Figures 16 and 17).

Significant and negative associations were found between newborn EpiStress scores and estimated CD4T cell- (r = -0.70, p = 8.674e-12***) and estimated B cell (r = -0.60, p = 2.289e-08***) proportions, while significant and positive associations were found between newborn EpiStress scores and estimated monocyte- (r = 0.50, p = 8.837e-06***), granulocyte- (r = 0.96, p = <2.2e-16***), and nucleated RBC proportions (r = 0.26, p = 0.027*). No significant associations were found between newborn EpiStress scores and estimated CD8T cell- nor estimated natural killer cell proportions (see Figure 18). Notably, a differential association was revealed between the estimated B cell proportions of infants and newborns. B cells are white blood cells found in the adaptive immune system that produce antibodies via exposure to pathogens (Alberts et al., 2002).

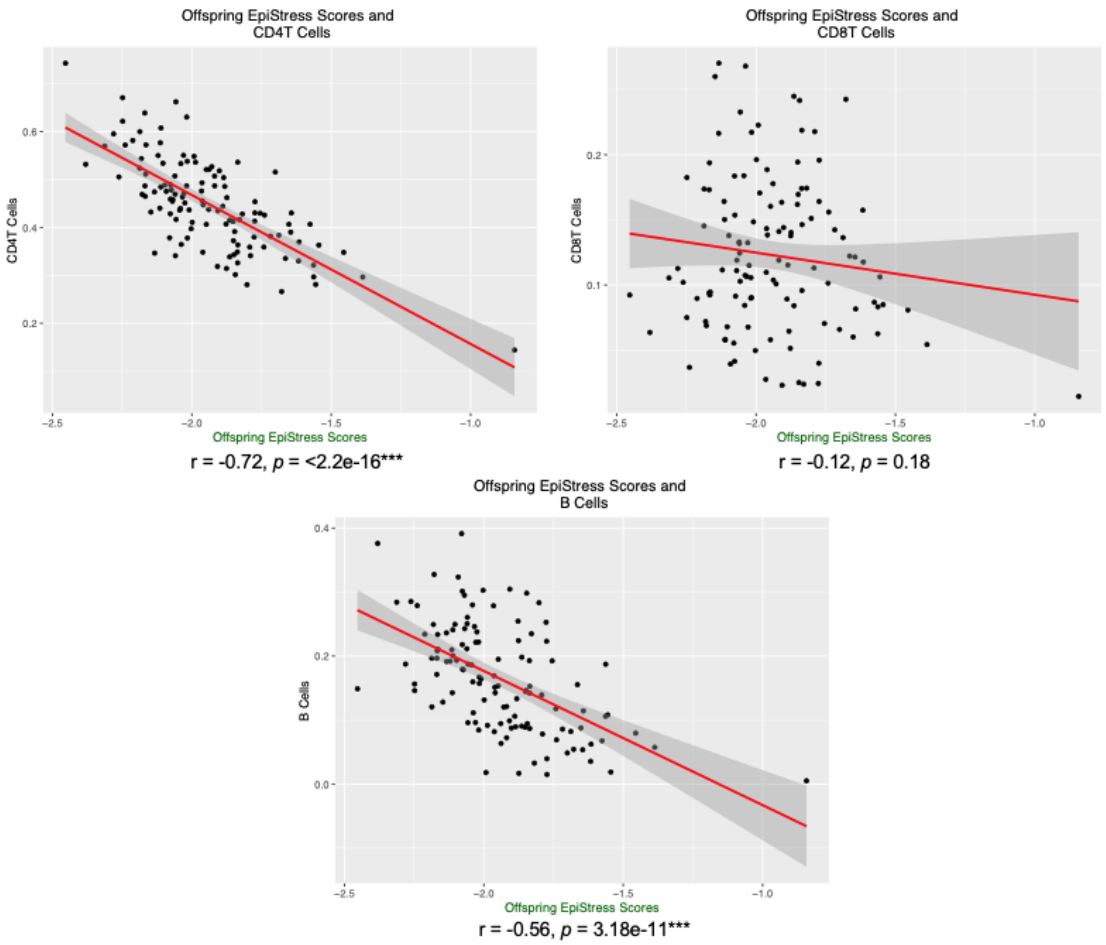


Figure 14: Bivariate analyses using offspring EpiStress scores and estimated CD4T cell-, CD8T cell-, and B cell proportions. *Associations between offspring EpiStress scores and cell type proportions of interest.*

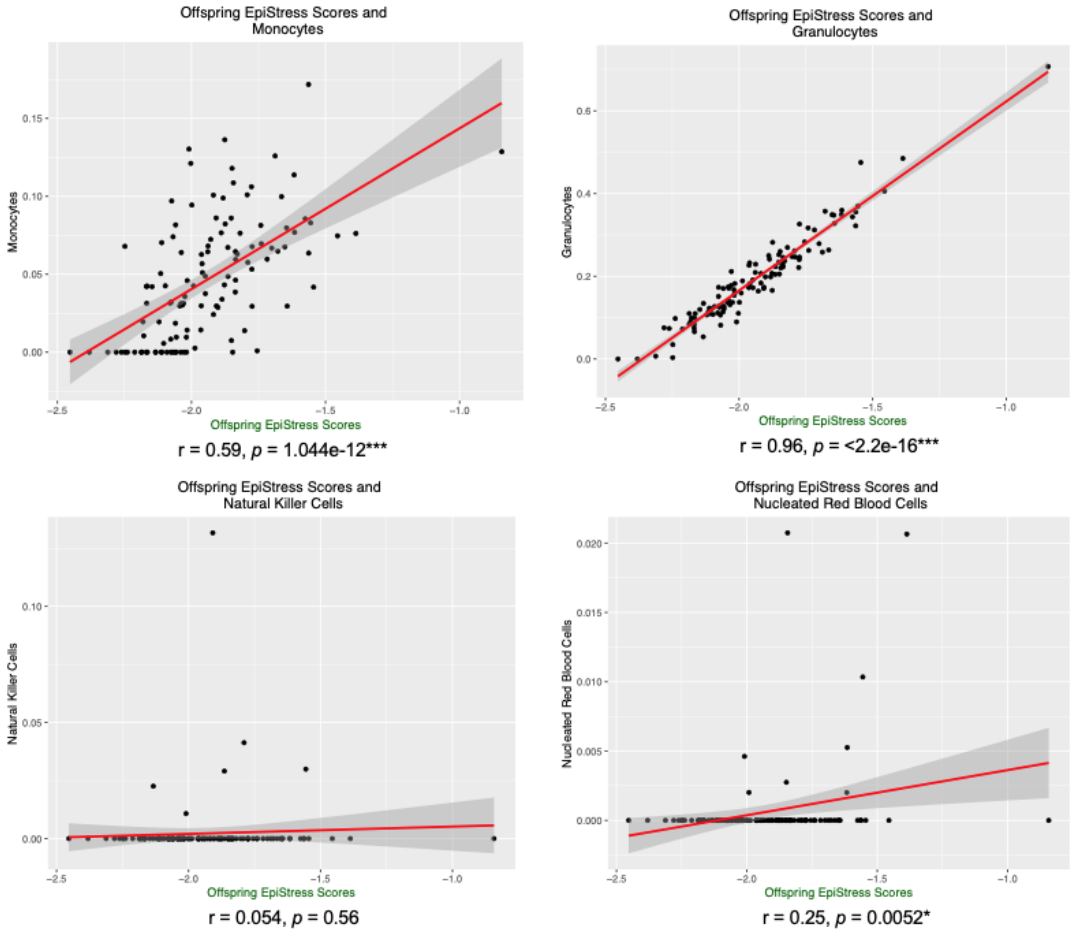


Figure 15: Bivariate analyses using offspring EpiStress scores and estimated monocyte-, granulocyte-, natural killer-, and nucleated red blood cell proportions. *Associations between offspring EpiStress scores and cell type proportions of interest.*

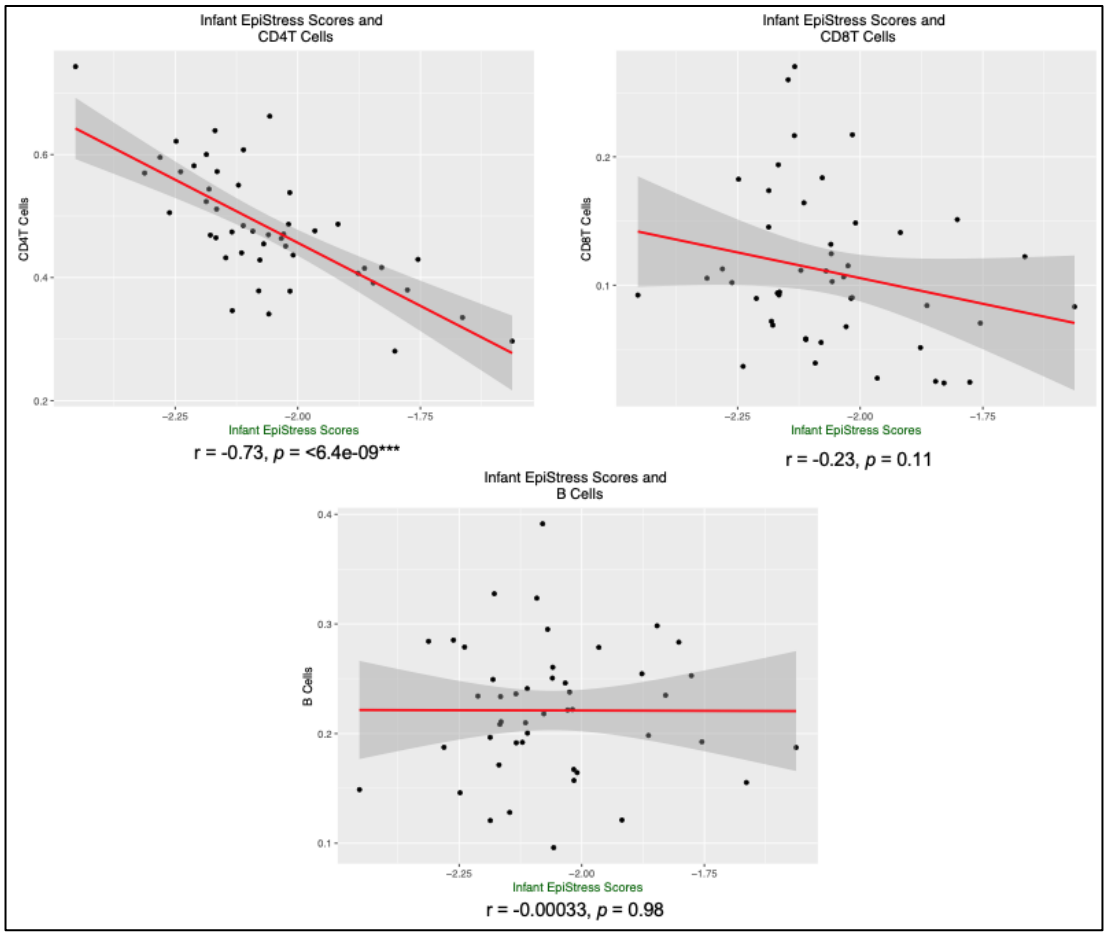


Figure 16: Bivariate analyses using infant EpiStress scores and estimated CD4T cell-, CD8T cell-, and B cell proportions. *Associations between infant EpiStress scores and cell type proportions of interest.*

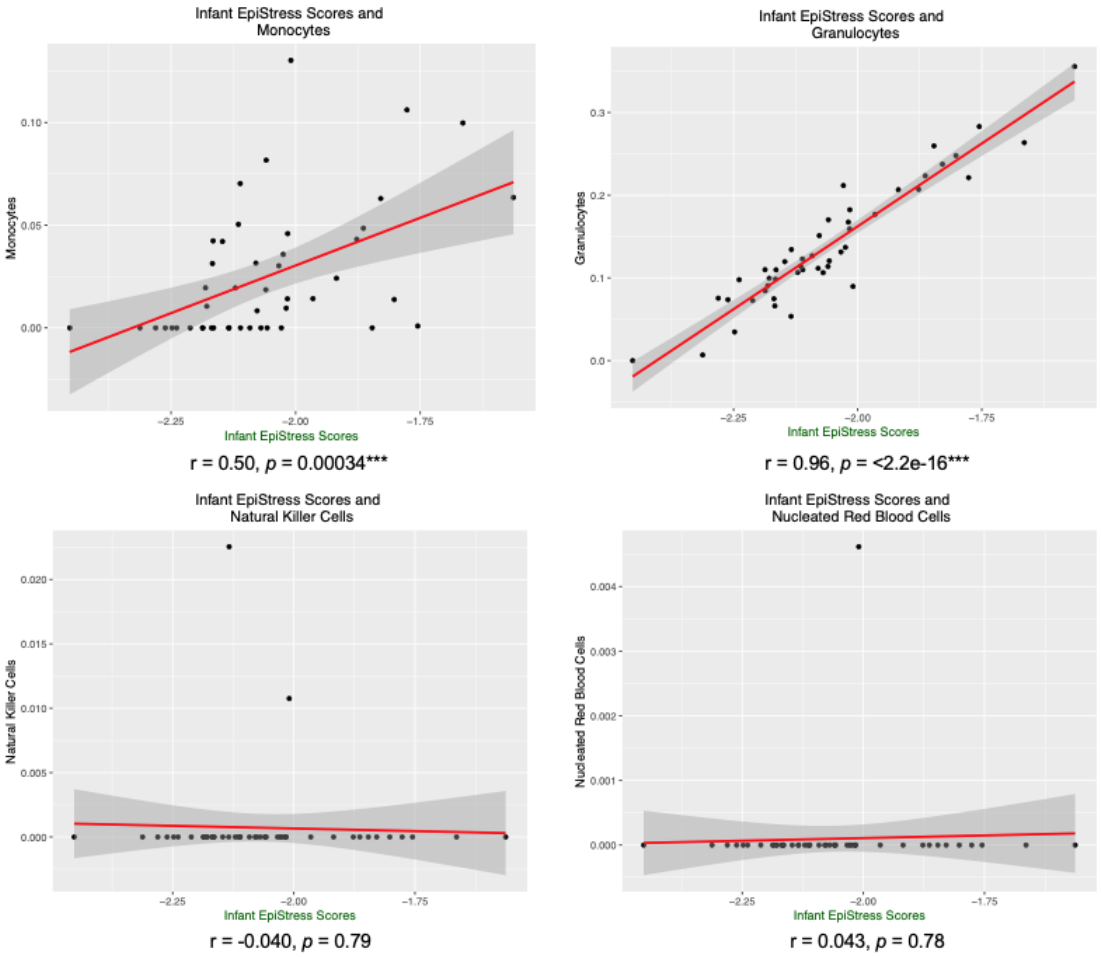


Figure 17: Bivariate analyses using infant EpiStress scores and estimated monocyte-, granulocyte-, natural killer-, and nucleated red blood cell proportions. *Associations between infant EpiStress scores and cell type proportions of interest.*

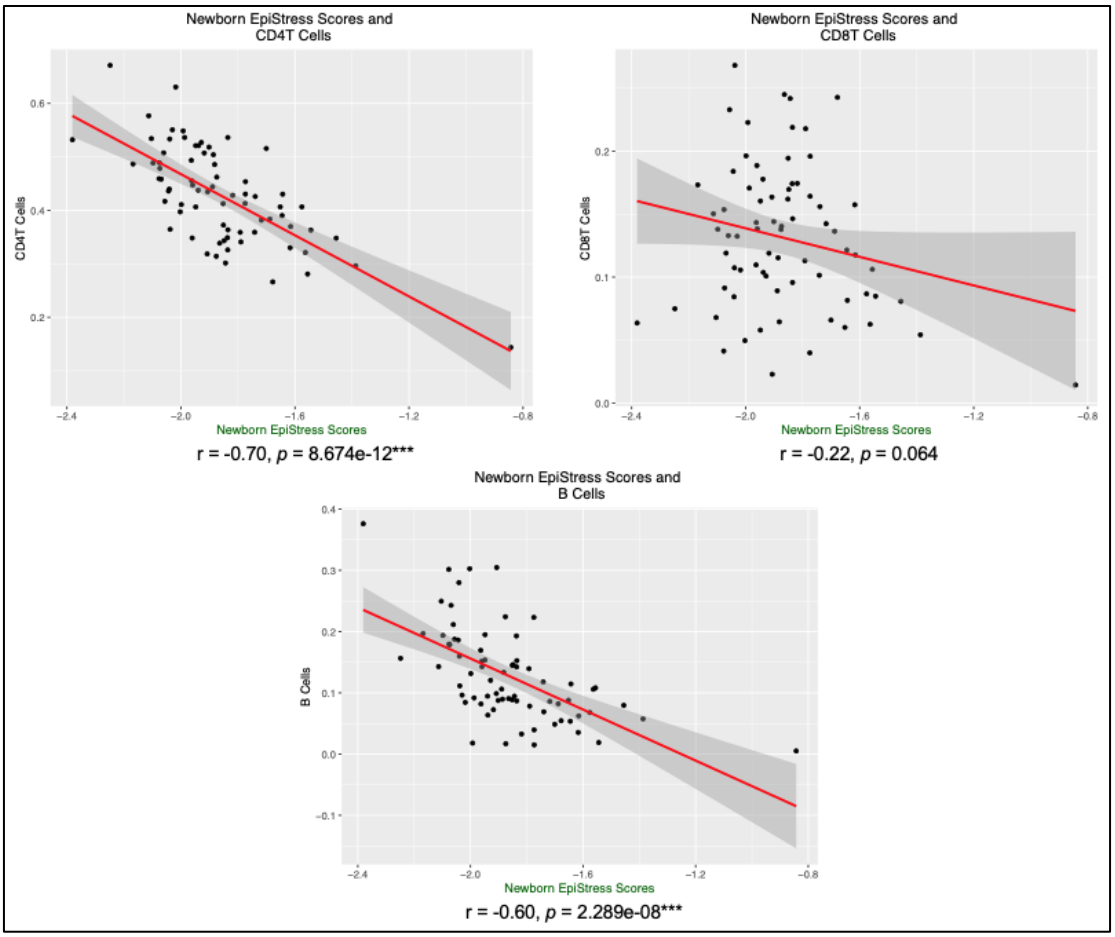


Figure18: Bivariate analyses using newborn EpiStress scores and estimated CD4T cell-, CD8T cell-, and B cell proportions. *Associations between newborn EpiStress scores and cell type proportions of interest.*

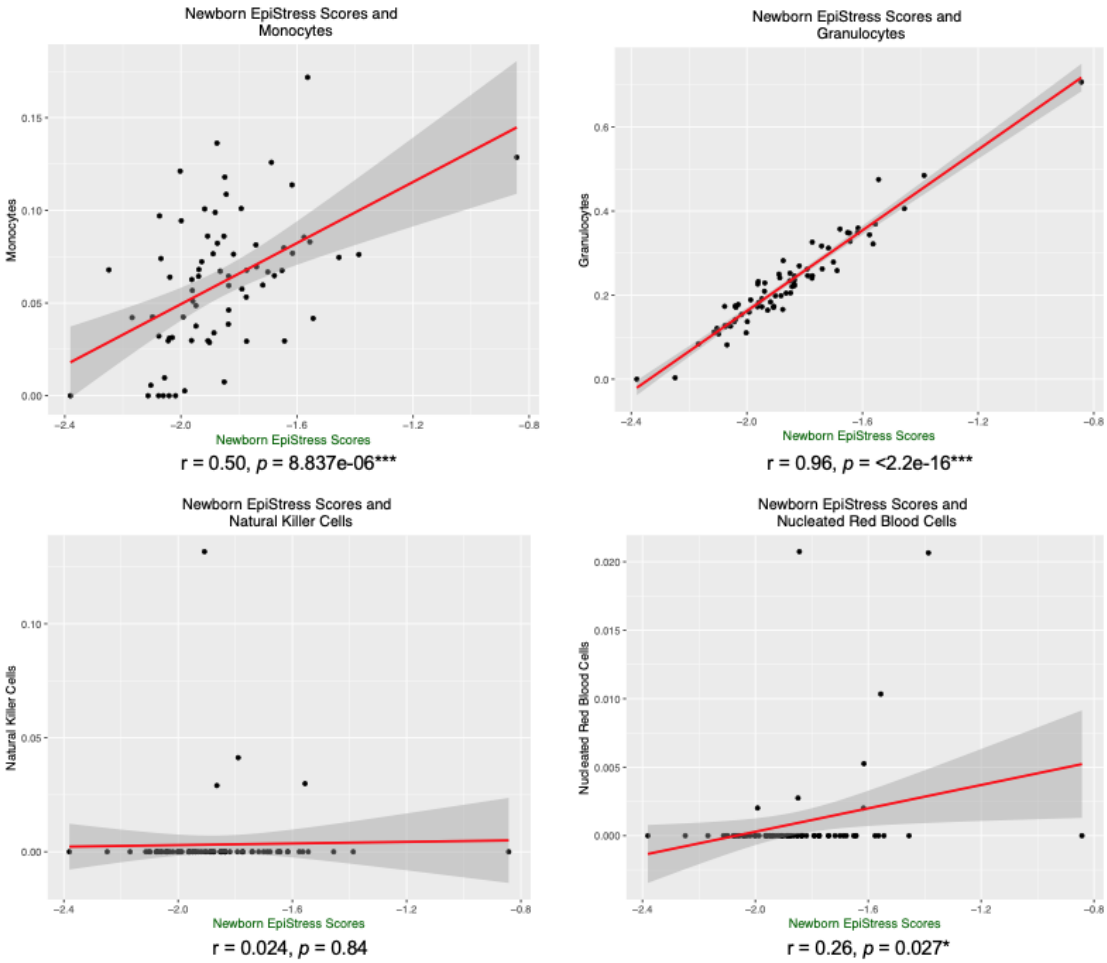


Figure 19: Bivariate analyses using newborn EpiStress scores and estimated monocyte-, granulocyte-, natural killer-, and nucleated red blood cell proportions. *Associations between newborn EpiStress scores and cell type proportions of interest.*

3.2.2 Mediation Analysis

Given the known differences in cell type composition during the newborn and infant development period, and because age at sample collection was confounded with offspring EpiStress scores and cell type, a stratified mediation analysis was developed (Hermansen, 2001; Jacob, 2016). The goal of the stratified mediation analysis was to see if there were significant associations between maternal stress and infant EpiStress scores (>=30 days old), and maternal stress and newborn EpiStress scores (<30 days old). 1,000 Bootstrap simulations were run, and the mediation effects were examined separately for newborns and infants. Overall, no significant mediation (indirect) effects of either prenatal depression or prenatal anxiety were revealed. Infant mediation models did not reveal any significant direct- or indirect effects. Newborn mediation analyses, however, showed significant, negative direct and total effects when prenatal depression and prenatal anxiety were accounted for (see Table 1-6).

Table 1-6. Stratified mediation analysis. The table shows the indirect effects, direct effects, and total effects from the stratified mediation analysis for newborn and infant data where prenatal depression and prenatal anxiety are the mediating variables.

Infants	Mediating Variable: Prenatal Depression			Mediating Variable: Prenatal Anxiety		
	p-value	Estimate	95% Confidence Interval	p-value	Estimate	95% Confidence Interval
Indirect Effect	0.78	-0.0078	-0.062 to 0.08	0.14	-0.048	-0.14 to 0.01
Direct Effect	0.20	0.087	-0.055 to 0.19	0.07	0.13	-0.014 to 0.24
Total Effect	0.08	0.079	-0.010 to 0.18	0.098	0.079	-0.028 to 0.17

Newborns	Mediating Variable: Prenatal Depression			Mediating Variable: Prenatal Anxiety		
	p-value	Estimate	95% Confidence Interval	p-value	Estimate	95% Confidence Interval
Indirect Effect	0.37	-0.011	-0.051 to 0.01	0.32	0.016	-0.0079 to 0.06
Direct Effect	0.016*	-0.10	-0.18 to -0.02	<2e-16***	-0.13	-0.21 to -0.04
Total Effect	0.018*	-0.12	-0.20 to -0.02	0.012*	-0.16	-2.39 to 0.03

Chapter 4. Discussion

4.1 Characterization Analysis: Summary

4.1.1 Utility of Offspring EpiStress Scores

A study by Suarez et al. (2020) examined associations between the EpiStress score at birth and future mental and behavioural disorders or total behavioural problems when the children reached 7 to 11 years of age. This study used the PREDO cohort, and fetal cord blood samples were obtained for genetic and epigenetic analyses. Child mental and behavioural disorder data was obtained from the Care Register for Health in Finland, and total behavioural problems were obtained from the mothers via a Child Behaviour Checklist (Saurez et al., 2020). Saurez et al. (2020) found that EpiStress scores were not significantly associated with the development of a mental or behavioural disorder during childhood, nor total behavioural problems. However, they did find that lower EpiStress scores were significantly associated with the number of days of inpatient or outpatient treatment for child mental or behavioural disorders (Saurez et al., 2020). This suggests that although children did not receive a formal mental or behavioural disorder diagnosis, there was a level of dysfunction that led to inpatient or outpatient treatment. In this case, and as with the current study, lower EpiStress scores could potentially identify children who might be *at risk* for future inpatient or outpatient treatment due to an array of adverse health outcomes.

4.1.2 Age at Sample Collection and Autoimmune Disorders

An investigation of demographic measures was conducted to inform the ways in which the EpiStress scores were associated with variables that are commonly influenced by DNA methylation. Of these variables, offspring EpiStress scores were significantly and negatively correlated with the offspring's age at sample collection, while none of the other demographic variables were correlated. This correlation provoked the question about whether offspring EpiStress scores might be differentially associated with maternal stress in infants (>=30 days old) or newborns (<30 days old) and subsequently led to a stratified mediation analysis (refer to section 4.2). Offspring EpiStress scores would be expected to differ with offspring age since DNAm has previously been shown to be

lowest in newborns (Jones et al., 2015). Additionally, DNAm is differentially associated with cell type and immune development at this age (Jacob, 2016).

Findings from the EWAS Atlas showed that MS and SLE, both non-cancerous, autoimmune disorders, have been previously associated with some of the CpG sites that the EpiStress scores are derived from. These disorders are the result of an abnormal bodily response to its own immune system but the causes of MS and SLE are both unknown. Two additional traits out of the 15 identified by the EWAS Atlas have also been associated with autoimmune disorders in adults: Crohn's- and inflamed Crohn's disease ("Crohn's"), albeit at lower percentages than MS and SLE. Crohn's is an autoimmune disorder that causes chronic inflammation of the gastrointestinal tract (Crohn's and Colitis Foundation of America, 2014). It is suspected that genetic influences, environmental influences, and immune abnormalities play a role in their development (Axisa & Hafler, 2016; Charras et al., 2021). Although the autoimmune disorders listed do not have a known cause, the samples taken from the adults came from CD19+ B cell proportions, whole blood, and colon samples. This draws a parallel to the EpiStress scores which are partially derived from whole blood, and which show associations with estimated B cell proportions, indicating that EpiStress scores might represent immune differences rather than DNAm differences.

4.1.3 Offspring EpiStress Scores are not Correlated with Brain Morphology

An investigation of brain morphology information was conducted to inform the ways in which the offspring EpiStress scores were associated with brain morphology measurements in children. Right- and left hippocampal volumes, right- and left amygdala volumes, and total intracranial volume were considered in these analyses. Of these variables, offspring EpiStress scores were not significantly correlated with any of the brain morphology data, indicating that there was no association between offspring EpiStress scores and brain morphology in the UCI cohort. Offspring EpiStress scores showed no association with brain morphology, including in the hippocampal regions, even though the EpiStress scores were partially derived from an immortalized human hippocampal progenitor cell line. Immortalized cell lines allow for constant cell profileration in controlled environments without the need to recruit new participants and obtain new samples (Carter & Shieh, 2015). Regardless, many brain regions work together to process information, and brain cells (e.g., neurons and glia) each have their

own distinct DNAm profile (Jones et al., 2018). It is possible that a different cohort with a larger sample size might indeed show associations with brain morphology.

Findings from the BECon tool revealed that four out of 24 CpG sites of the EpiStress scores were variable in the blood but were not variable in BA 10, 20, or 7, that two out of 24 CpG sites were not variable in BA 7, and that one out of 24 CpG site was not variable in BA 10. The remaining 16 sites were variable in all regions. Because the variability refers to the level of methylation per subject per CpG, these findings might indicate that the EpiStress scores are measuring a component(s) of the blood rather than the brain. This is especially relevant for the estimated cell type proportions and because the offspring EpiStress scores did not correlate with any of the brain morphology measures. The BECon tool revealed correlations between the 24 CpG sites and BA 10, BA 20, and BA 7. 30/72 of the CpG sites were negatively correlated and no correlations were stronger than -0.54; 40/72 of the CpG sites were positively correlated and two of the CpG sites were strongly correlated (e.g., above 0.70) with either BA 20 (temporal cortex) or BA 7 (parietal lobes); and 2/72 of the CpG sites showed no correlation. The majority of CpG sites showed positive correlations with BA 10, BA 20, and BA 7; however, the modest correlations overall might indicate that DNAm of the CpG sites from the EpiStress scores are not necessarily influencing methylation in the prefrontal cortex, temporal cortex, or parietal lobes.

4.1.4 Differential Infant- and Newborn Cell Type

An investigation of cell type was conducted to inform the ways in which the EpiStress scores were associated with estimated immune cell proportions (monocyte-, granulocyte-, natural killer cell-, CD4T cell-, CD8T cell-, B cell-, and nRBC proportions). A technical variable (age at sample collection) rather than a biological variable was the only difference between infant- and newborn EpiStress scores when analyzing cell type proportions. This is expected because, as mentioned previously, cell type composition is undergoing rapid changes in newborns as their immune system develops. The results showed a highly positive correlation with offspring-, infant-, and newborn EpiStress scores and estimated granulocyte proportions. In other words, offspring-, infant-, and newborns with higher EpiStress scores tend to have greater estimated granulocyte cell proportions, possibly indicating that their innate immune systems are functioning normally to protect against pathogens early in life.

As well, differential associations between estimated B cell proportions and infant- and newborn EpiStress scores such that newborns had higher estimated B cell proportions and lower EpiStress scores. Rather than the EpiStress scores measuring associations between offspring DNAm and maternal stress, they might in fact be measuring offspring immune cell proportions that have been influenced by maternal stress. Glucocorticoids have been shown to have both anti-inflammatory and pro-inflammatory effects and might explain the differences seen in the immune cell proportions in this dataset (Cruz-Topete & Cidlowski, 2015; Ronchetti & Riccardi, 2018). If the EpiStress scores indeed represent cell type proportions, and four out of 15 traits identified by the EWAS have been previously associated with autoimmune disorders in adults, consideration should be paid to the possibility that the EpiStress scores are representing offspring immune differences through DNAm.

4.2 Mediation Analysis

A stratified mediation analysis of newborns and infants was conducted because the investigation of offspring demographic measures and offspring cell type revealed that offspring EpiStress scores were significantly and highly correlated with age at sample collection and cell type (CD4T cell-, B cell-, monocyte-, granulocyte-, and nRBC proportions). Offspring were stratified into newborns (<30 days old) and infants (>= 30 days old) as cell type proportions are a major driver of DNAm differences, and these differences are seen in the first month of life.

Infant mediation models did not reveal any significant mediation, direct, or total effects, whereas newborn mediation models revealed direct and total effects, and no mediation effects. A study by Sammallahti et al. (2021) demonstrated similar findings such that prenatal anxiety, specifically, did not correlate with DNAm in cord blood. It was expected that prenatal stress would mediate the relationship between maternal ELS and offspring EpiStress scores because exposure to stress is expected to associate with decreased EpiStress scores (Provençal et al., 2020). However, prenatal stress was not a mediating factor in the association between maternal ELS and infant- or newborn EpiStress scores, so it is possible that an alternative mechanism was at play. For example, it is possible that maternal ELS could be associated with socioeconomic status, employment and education status, and access to healthcare—sources of stress that are not purely psychological.

4.3 Limitations

This study is not without its limitations. Associating maternal ELS with infant and newborn DNAm is a challenging and ongoing task because the mechanisms by which stress exposure is transmitted remain largely unknown (Aristizabal et al., 2019). Identifying biological marks in newborns and infants that is a result of transgenerational inheritance is challenging, and no causal claims can be made at this time. Further, researchers must be cognizant of the definitions that are used to characterize epigenetic mechanisms. Lappalainen and Greally (2017) and Greally (2018) caution against broad definitions that might equate epigenetics with the roles of transcriptional regulators. Epigenetic mechanisms like DNAm refer to gene activation and silencing; transcriptional regulators also accomplish this task. It is important, then, to clearly define how these terms are being used to ensure uniformity across studies. Longitudinal studies that use consistent definitions of methylation risk scores and molecular mechanisms are recommended.

Additionally, the small sample size in the stratified sample (n=48 infants and n=81 newborns) presents issues adequately addressing power, and it is unclear whether maternal ELS or another factor(s) such as the postnatal environment is influencing the outcomes. For example, the mothers were not assessed for nursing status, i.e., breastfed or formula-fed babies. EpiStress scores have only been used in two studies to date which limits the generalizability of findings across populations, and the ethnicity of each participant is unclear in this analysis. The inability to appropriately correct for cell type due to the strong correlations found with the EpiStress scores is also a limitation in this study, and cell type proportions were estimated, not measured via cell sorting methods. As well, retrospective measures of maternal ELS may result in reporting bias due to one’s inability to accurately remember past events, especially if the mothers were experiencing depression and anxiety at the time of assessment (Reuben et al., 2016).

Overall, these limitations make it difficult to determine if infant- and newborn biology, by way of cell type or DNAm, is indeed inherited from the mother via HPA axis alterations, GC exposure, epigenetic mechanisms, or if some other factor is at play. Further investigation and replication are required.

4.4 Conclusion

Studying DNAm in infants and newborns which might be the result of maternal ELS exposure has the potential to identify mechanisms involved in the transgenerational transmission of stress from mother to offspring, specifically, newborns. Interestingly, prenatal stress was not shown to mediate the relationship between maternal ELS and infant- and newborn EpiStress scores, indicating that stressful life experiences prior to conception might have a greater impact on the offspring. The identification of biomarkers such as DNAm in newborns as a means of detecting transgenerational trauma is a daunting but important task. The hope is to hinder disease progression and maximize opportunities for children to thrive—in any environment. Children represent the future, and it is everybody's responsibility to ensure their development is optimal.

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Appendix

Table A.1.: Detailed EWAS Atlas Results from the EWAS Atlas. The table depicts the 24 CpG sites used to create the EpiStress score and the trait and type of factor or illness that the CpG site has previously been associated with. The "Publications" column shows the number of times the CpG site has been implicated in previous publications.

CpG Site	Trait	Type	Number of Publications
cg01400750	Air pollution (NO2) Preterm birth Systemic lupus erythematosus (SLE) Gulf Warm illness	Environmental factor Phenotype Autoimmune disorder Systemic intolerance	4 7 6 1
cg02862467	Multiple sclerosis (MS) SLE	Autoimmune disorder Autoimmune disorder	7 6
cg02927682	MS SLE	Autoimmune disorder Autoimmune disorder	7 6
cg03543954	Chronic fatigue syndrome SLE	Systemic intolerance Autoimmune disorder	3 6
cg03543954	Preterm birth SLE	Phenotype Autoimmune disorder	7 6
cg04674060	Preterm birth SLE	Phenotype Autoimmune disorder	7 6
cg04958055	No matching records	N/A	N/A
cg07052737	B Acute Lymphoblastic Leukemia MS SLE	Cancer Autoimmune disorder Autoimmune disorder	1 7 6
cg07830557	B Acute Lymphoblastic Leukemia MS SLE	Cancer Autoimmune disorder Autoimmune disorder	1 7 6
cg08297985	Chronic fatigue syndrome Osteoarthritis SLE Fractional exhaled nitric oxide Gulf War illness	Systemic intolerance Degenerative disease Autoimmune disorder Phenotype Systemic intolerance	3 3 6 1 1
cg09571972	Fatigue Preterm birth SLE Inflamed Crohn's disease	Phenotype Phenotype Autoimmune disorder Autoimmune disorder	1 7 6 1
cg11316887	Air pollution (NO2) Crohn's disease (CD) SLE	Environmental factor Autoimmune disorder Autoimmune disorder	4 4 6
cg11671363	Air pollution (NO2) Crohn's disease (CD) SLE	Environmental factor Autoimmune disorder Autoimmune disorder	4 4 6
cg12157761	Air pollution (NO2) Crohn's disease (CD) SLE	Environmental factor Autoimmune disorder Autoimmune disorder	4 4 6
cg13581015	Air pollution (NO2) Crohn's disease (CD) SLE	Environmental factor Autoimmune disorder Autoimmune disorder	4 4 6

Table A.1. continued…			
CpG Site	**Trait**	**Type**	**Number of Publications**
cg13718827	Breast cancer MS	Cancer Autoimmune disorder	13 7
cg16141752	Breast cancer MS	Cancer Autoimmune disorder	13 7
cg16312968	Breast cancer MS	Cancer Autoimmune disorder	13 7
cg18020065	Hepatocellular carcinoma (HCC) MS Vitamin B12 supplement	Cancer Autoimmune disorder Environmental factor	7 7 1
cg19118951	HCC MS Vitamin B12 supplement	Cancer Autoimmune disorder Environmental factor	7 7 1
cg20977312	HCC MS Vitamin B12 supplement	Cancer Autoimmune disorder Environmental factor	7 7 1
cg21344746	HCC MS Vitamin B12 supplement	Cancer Autoimmune disorder Environmental factor	7 7 1
cg23987336	HCC MS Vitamin B12 supplement	Cancer Autoimmune disorder Environmental factor	7 7 1
cg24850296	SLE	Autoimmune disorder	6