2020

Longitudinal Analyses Of Epigenetic Correlates Of Externalizing And Internalizing Disorders

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Abstract

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Degree Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Graduate Group

Psychology

First Advisor

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Second Advisor

Ayelet Ruscio

Subject Categories

Psychology

LONGITUDINAL ANALYSES OF EPIGENTIC CORRELATES OF EXTERNALIZING AND INTERNALIZING DISORDERS

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A DISSERTATION

in

Psychology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2020

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ABSTRACT

LONGITUDINAL ANALYSES OF EPIGENTIC CORRELATES OF EXTERNALIZING AND INTERNALIZING DISORDERS

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Statement of Problem: In the field of developmental psychopathology, there has been a historical debate colloquially referred to as "nature versus nurture" in the continued pursuit of understanding the genetic and environmental origins of mental illness. The emergent field of behavioral epigenetics has posited that the underlying dichotomy and conceptual separation between gene and environment influences itself is false. Epigenetic processes show that environmental influences act on genes *mechanistically* as environmental inputs biologically influence the expression of key genes in vital systems. In translating technological advances in epigenetics from the biomedical world, developmental psychopathologists have largely contextualized psychological phenotypes within the same biomedical disease model. However, psychological phenotypes are not automatically amenable to the same methodological framework, as they are uniquely complex in their classification and measurement and are best understood to be calibrated in early life during crucial periods of development. **Methods and Procedures:** Therefore, the central aim of this thesis was to apply epigenetic theory, methodology, and technology to clinically relevant psychological phenotypes in methodologically novel ways that take into account phenotypic complexity and developmental context. Using a longitudinal design from the Avon Longitudinal Study of Parents and Children (ALSPAC), this thesis explored the epigenetic underpinnings (i.e. DNA methylation) of

risk and resilience for internalizing and externalizing disorders during sensitive periods of development. **Results:** Study 1, published in Development and Psychopathology in 2017, used a candidate gene approach examining epigenetic changes in the oxytocin receptor gene (OXTR) to study resilience to prenatal stress. Results showed that children who were resilient in the conduct problem domain only had differential DNA methylation profiles at birth than those who were not resilient. Study 2 used an epigenome-wide approach to explore potential novel epigenetic correlates of depression trajectories in adolescence with follow-up bioinformatic analyses. Results did not show any fetal programming effects when assessing DNA methylation at birth, but several novel genes were identified when DNA methylation was measured in adolescence. **Conclusion:** Because these epigenetic changes are heritable and potentially reversible, insights from epigenetic research have profound implications in the classification, identification, and treatment of mental illness.

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INTRODUCTION

In the field of developmental psychopathology, there has been a historical debate colloquially referred to as "nature versus nurture" in the continued pursuit of understanding the genetic and environmental origins of mental illness. A strong research base of twin and adoption studies supports the heritability of psychological disorders (e.g. Larsson et al., 2014; Lohoff, 2010; Verhulst et al., 2015). At the same time, a wealth of research supports the strong impact of environment (i.e. trauma, maltreatment, poverty) on the development of psychopathology, especially in early sensitive periods of development (e.g. Carr et al., 2013; McCrory et al., 2012; McLaughlin & Lambert, 2017; Reiss, 2013). In the past two decades, the "nature versus nurture" debate has been effectively resolved with the understanding that genes and environment both play vital roles through dynamic interplay with one another. Gene-environment interaction research has shown a moderation relationship between genetic influences and the environment where the presence of a particular genotype influences the impact of an environmental stressor and vice versa (e.g. Brown & Harris, 2008; Koenen et al., 2008; Lau et al., 2007; Nugent et al., 2011). However, the interaction of genes and environmental exposures in these paradigms are purely statistical and still operate under the conceptualization that they are distinct and separate processes. The emergent field of behavioral epigenetics has posited that the underlying dichotomy and conceptual separation between gene and environment influences itself is false. Epigenetic processes show that environmental influences act on genes *mechanistically* as environmental inputs biologically influence the expression of key genes in vital systems such as the stress response and immune

systems. Thus, the theory underpinning behavioral epigenetics suggests that epigenetic mechanisms serve as the mediator of the relationship between life experiences and psychopathology. Because these epigenetic changes are heritable and potentially reversible, insights from epigenetic research have profound implications in the classification, identification, and treatment of mental illness.

Research on epigenetic changes as potential mechanistic explanations for disease began in the biomedical world, particularly in cancer research. In translating technological advances in epigenetics from the biomedical world, developmental psychopathologists have largely contextualized psychological phenotypes within the same biomedical disease model. However, psychological phenotypes are not automatically amenable to the same methodologic framework, as they are uniquely complex in their classification and measurement and are best understood to be calibrated in early life during crucial periods of development. Therefore, the central aim of this thesis was to apply epigenetic theory, methodology, and technology to clinically relevant psychological phenotypes in methodologically novel ways that take into account phenotypic complexity and developmental context. Using a longitudinal design from the Avon Longitudinal Study of Parents and Children (ALSPAC), this thesis explored the epigenetic underpinnings of risk and resilience for internalizing and externalizing disorders during sensitive periods of development. Study 1, published in Development and Psychopathology in 2017, used a candidate gene approach examining epigenetic changes in the oxytocin receptor gene (OXTR) to study resilience to prenatal stress. Study 2 used an epigenome-wide approach to explore potential novel epigenetic correlates of depression trajectories in adolescence.

CHAPTER 1: Epigenetic Molecular Mechanisms and Bioinformatic Methods

Molecular Mechanisms of Epigenetics

The term "epigenetic" means "in addition to changes in genetic sequence" and the term "epi"-genome references the epigenetic signaling layer that sits "on top of" the genome. There has been much debate over the definition of "epigenetic" including what the term means conceptually and what processes it is describing. Over time, it has evolved to a broad definition to include any process that alters gene activity without changing the underlying DNA sequence (Weinhold, 2006).

The term epigenetics was first introduced by Conrad Waddington in the 1940s to describe the process that allows genotypically identical cells to be phenotypically distinct and differentiate into diverse tissue types with specified functions (Waddington, 1957). For example, a neuron is different from a liver cell, which is different from a skin cell, though they all originated from the same identical nucleotide sequence. During embryogenesis, epigenetic mechanisms govern the process of cell differentiation, which results in the permanent and stable specialization of cellular function that is then "stored" in the transcriptional profile of every cell and maintained throughout the lifespan (O'Donnell & Meaney, 2020). During this process, certain aspects of a cell's genome that are not needed for its specialized function are silenced at the level of transcription and subsequent expression due to epigenetic signaling. In this way, epigenetic mechanisms can be thought of as defining the bounds of cellular function for a particular cell. Today, we now understand that in addition to maintenance of cellular identity, epigenetic processes co-ordinate a wide range of biological processes including stress response, immune function, and neurodevelopment (Handy et al., 2011).

Epigenetic mechanisms regulate the process in which a DNA nucleotide sequence is transcribed and subsequently expressed to produce a phenotype. There are several interrelated molecular processes that fall under the epigenetic umbrella including histone modifications and chromatin remodeling, DNA methylation, and non-coding RNAs (Kim et al., 2008; Peschansky & Wahlestedt, 2014). The best-known epigenetic process by far is DNA methylation, largely because it has been the easiest and least expensive to study with existing technology. DNA methylation refers to the addition of a methyl group (CH3) to a cytosine guanine dinucleotide (CpG) at the 5-position of the pyrimidine ring through a covalent bond (Deaton & Bird, 2011; Klose & Bird, 2006). This process is catalyzed by enzymes known as DNA methyltransferases (DNMTs) that transfer methyl groups to CpG sites within a continuous stretch of DNA. The addition of a methyl group is typically associated with reduced binding access of transcription factors to the DNA sequence and subsequent reduction gene expression or gene "silencing"- though this is not always the case. In the promoter regions that are upstream from a transcription site, DNA methylation leads to gene suppression (Bird, 2002). However, in other regions such as the gene body, DNA methylation increases transcription levels and subsequent expression through processes that are still unknown (Maunakea et al., 2010; Shenker & Flanagan, 2012).

DNA methylation only occurs at the cytosine-guanine dinucleotide (CpG site). CpG sites are rare in the genome due to mutation of methylated cytosine into thymine over evolutionary time (Saxonov et al., 2006). Therefore, CpG sites frequently cluster together in CpG "islands" near promoter regions of genes (i.e. areas that initiate gene transcriptions), particularly near the transcription start sites of housekeeping genes that are necessary for basic functions of the cell (Deaton & Bird, 2011). CpG islands are

defined as regions with at least 500bp, a CG percentage greater than 55%, and an observed-to-expected CpG ratio greater than 65% (Takai & Jones, 2002). CpG islands are largely unmethylated, do not vary across individuals, and their methylation status tends to be more stable over time. DNA methylation is more dynamic (i.e. prone to change) and variant between individuals in regions flanking CpG islands called shores and further out called shelves, where CpG sites are less dense (Irizarry et al., 2009; Ziller et al., 2013). DNA methylation can be extracted from any relevant tissue. In psychological research, peripheral tissues including saliva, buccal epithelial cells and most commonly blood are most typically used as brain tissue availability is limited to post-mortem sampling.

DNA Methylation Bench Science

The most common method to analyze DNA methylation is through the technique of bead-type hybridization using micro-arrays. The Illumina Infinium HumanMethylation Beadchip has become the default array of choice for DNA methylation studies across disciplines. The Illmunia micro-array was designed to detect levels of DNA methylation at CpG sites through quantitative genotyping of C/T polymorphisms (Dedeurwaerder et al., 2014). The following protocol information was obtained from the Infinum HD Assay Methylation Protocol Guide (2015) from Illumina's website. The Beadchip is a hand-sized silicone based array with 12 arrays (6 rows, 2 columns) each array representing one participant's DNA sample. In each individual array, there are tens of thousands of grids. Each grid has beads that are coated with hundreds of thousands of "probes" which are synthetic single strands of DNA fragments, also known as oligonucleotides, each with their own specific DNA sequence that is designed to be complimentary to human genomic DNA. Extracted single strand genomic DNA from an individual will bind to these complimentary probes to create double stranded DNA.

After single strand extraction, a participant's DNA is bisulfite converted, a process that turns all the unmethylated cytosine bases into thymine while all the methylated cytosine bases are protected from the biological reaction and remain unchanged. The treated DNA is then transferred to the Illumina Beadchip for processing of DNA hybridization, where the single strand of human DNA recombines with another single strand of synthetic DNA by complimentary nucleotide bases. The unmethylated cytosines form single nucleotide polymorphisms that are identified by fluorescent staining that represents the level of DNA methylation in that region. The fluorescence is scanned using Illumina IScan and then quantified into beta values that represent the proportion of methylation in that genomic region using a ratio of the methylated probe fluorescent intensity (methylated signal) over the overall intensity (sum of methylated and unmethylated signal). Each CpG site has a corresponding beta value representing a ratio from 0 (no cytosine methylation) to 1 (complete cytosine methylation) (Bibikova et al., 2011).

There are 28 million CpG sites in an individual's epigenome and the Illumina interrogates a small subset of them based on theoretical functional importance selected with the guidance of a consortium comprised of 22 methylation researchers representing 19 institutions worldwide (Bibikova et al., 2011). Illumina is consistently evolving its biotechnology to not only increase the coverage of the array but also to incorporate research findings into choosing more relevant regions to target. The first edition of the array had coverage of 27,000 CpG sites. The 450k array, which was the technology used to generate data for this thesis, covered 485,000 CpG sites. The new EPIC array covers 850,000 CpG sites in the human epigenome. The Illumina 450k focuses on CpG islands (covers 96% of all islands in the human epigenome), shores (92%), and shelves (86%)

and also other functional elements including 3′- and 5′-UTRs, gene bodies, DNAse hypersensitive sites, miRNA promoters and other ncRNAs that also may be important sites for changes in DNA methylation (Bibikova et al., 2011). The 450k also covers 99% of the Reference Sequence (RefSeq) database, which is a comprehensive annotation of all nucleotide sequences and their protein products in the human genome (Pruitt et al., 2005). While whole-genome sequencing techniques are able to interrogate every single CpG site, they are often resource and cost heavy (e.g. $1,000 per participant). In addition, research has shown that the results from the Illumina arrays are correlated $R^2 = .95$ with results drawn from whole-genome sequencing, suggesting that the arrays are efficiently targeting functionally important CpG sites (Bibikova et al., 2011).

Once DNA methylation has been assayed, a matrix data file is created with each CpG site interrogated and its corresponding DNA methylation beta value. The data undergoes pre-processing which includes various quality checks and normalization to correct for batch effects. During the data analysis phase, any differentially methylated CpG sites or "hits" are annotated to their respective genes. In order to examine whether multiple hits are functionally related, the annotated genes are entered into a gene network analysis. More details on quality checks and normalization are available in Chapter 3, Section C. Data analysis of DNA methylation data is described further in subsequent empirical chapters.

Most early DNA methylation studies in developmental psychopathology used a candidate gene approach where specific genes were chosen a priori due to known biological, physiological, or functional relevance based on theoretical importance and empirical evidence from genetic studies. A candidate gene approach is useful because it allows for researchers to understand the functional elements of epigenetic changes in a

larger system. The design allows a drill down approach where all parts of the biological cascade including DNA methylation, access to transcription factors, and gene expression can be studied in unison. However, a candidate gene approach is limiting in that it is only able to explore already identified and well-known genes. A complimentary approach is the epigenome-wide association study (EWAS), a "hypothesis-free" approach, where an individual's whole epigenome is interrogated and CpG sites or regions that are differentially methylated across individuals or "hits" are annotated to nearby genes. This approach allows for the discovery of potentially novel biological correlates and can contribute to a more comprehensive and holistic understanding of etiology of psychopathology.

CHAPTER 2: Application of Epigenetics to Etiology of Psychopathology

Role of Epigenetics in Risk for Psychopathology

Epigenetic mechanisms are normative background processes that are essential to many organism functions. Researchers across disciplines are studying when and how these processes are altered to function improperly and confer risk for disease. Environmental inputs have shown to be an important trigger for altered epigenetic signaling. It has been well documented that the epigenome is highly susceptible to environmental input and certain environmental exposures including cigarette smoking (Lee & Pausova, 2013), pollutants or toxins (Ho et al., 2012), exercise (Voisin et al., 2015), and diet (Anderson et al., 2012) alter DNA methylation patterns. Importantly for psychologists, thousands of studies in the field of behavioral epigenetics have also demonstrated the link between psychosocial stressors and DNA methylation across developmental stages (for a review of maternal prenatal stressors see Sosnowski et al., 2018; for a review of child maltreatment see Lutz & Turecki, 2014).

Due to the vital role that adverse life experiences play in the development of mental illness, epigenetic processes present a novel way to unify the biological and environmental origins of risk and resilience to psychopathology. Epigenetic changes including DNA methylation have emerged as potential mechanisms that can both capture the effects of stressful early life experiences and explain their propagation into adulthood. The promise of a mediational model suggests that exposure to stressful early life experiences during crucial periods of development sets individuals on relatively stable biological trajectories via epigenetic mechanisms that then alter key systems such as the stress response and immune systems, that result in the development and maintenance of psychopathology later in life.

A seminal study using an animal model done by Weaver and colleagues (2004) demonstrated the potential of this mediational framework in describing how early life experiences can lead to behavioral phenotypes via epigenetic mechanisms. Results showed that pups who were raised by anxious, low nurturing mothers (i.e. characterized by decreased pup licking, grooming, and arched-back nursing) were more likely to demonstrate a stable, anxious phenotype into adulthood than pups who were raised by nurturing mothers. Pups who were neglected had higher levels of DNA methylation in the region of the glucocorticoid receptor gene (NR3C1), had decreased transcription factors, and subsequent gene expression in the hippocampus. NR3C1 is integral in the modulation of the HPA axis and a key player in the stress response system. This was the first study to demonstrate the link between early life experiences and an animal model of psychopathology via epigenetic mechanisms.

Prenatal Calibration of Risk: Fetal Programming Hypothesis

It is clear that adverse environmental inputs early in the lifespan have long range health consequences into adulthood. In the past couple of decades, researchers have been asking the question of exactly how early in development can risk be embedded. A vast empirical base suggests that prenatal exposure to environmental stress can set a child on disease trajectories with long lasting effects into adulthood before the child is even born. A seminal example of this is findings from the Dutch Hunger Winter cohort ($N = 2141$), conceived during a famine in the Netherlands in 1944 as a result of WWII and followed up in adulthood. Results showed that prenatal exposure to famine in general lead to higher rates of Type II diabetes in middle adulthood and exposure to famine particularly in early gestation was linked to coronary heart disease, breast cancer, increased stress responsiveness, and higher rates of obesity (Roseboom et al., 2006).

The long-lasting effects of prenatal exposures have been explained by the "fetal programming hypothesis" which posits that risk for psychopathology later in the lifespan is biologically programmed in the uterine environment during a period of rapid neurodevelopment when the fetus is particularly susceptible to environmental influences (Barker, 1995). The fetus incorporates signals about the maternal environment including diet and stress into its developmental trajectory via epigenetic changes related to neurodevelopment of key brain areas and networks in an effort to most adaptively match development to future postnatal environment (Barker, 1995; Gluckman, Hanson, Cooper, & Thornburg, 2008). Within this framework, vulnerability to psychopathology has been described as the "three hit hypothesis." Genetic predisposition is hit 1, the prenatal environment is hit 2, both calibrate susceptibility to hit 3, life experiences and exposures postnatally (Daskalakis et al., 2013). In recent follow up studies of the Dutch Hunger Winter, researchers found that even 60 years after the event, siblings who were prenatally

exposed to the famine showed lower DNA methylation of the insulin like growth factor 2 (IGF2), a gene essential in fetal growth and development, compared with their unexposed same-sex sibling (Heijmans et al., 2008). Using an epigenome-wide approach, researchers identified additional differentially methylated genes associated with prenatal famine exposure including the insulin receptor gene (INSR), a gene involved in prenatal growth and insulin signaling, and the carnitine palmitoyltransferase 1A gene (CPTA1), which is involved in fatty acid oxidation (Tobi et al., 2014).

The fetal programming hypothesis has also been used to understand the early embedding of risk for psychopathology as well. A large number of studies have shown that exposure to prenatal maternal psychopathology such as depression and anxiety is associated with both internalizing and externalizing behavioral outcomes in children such as depression, anxiety, ADHD symptoms, and conduct problems, above and beyond the quality of the child's postnatal environment (Barker et al., 2011; O'Connor et al., 2002; O'Donnell et al., 2014; Van den Bergh & Marcoen, 2004). Prenatal exposure to maternal mood disorders may account for 10–15% of the variance in children's behavior problems, accounting for concurrent levels of maternal mood symptoms (Glover, 2015). A wealth of research has emerged studying the process by which prenatal stressors via DNA methylation confer risk for psychopathology later in the lifespan. One study found that higher perceived maternal stress in the second trimester was associated with higher DNA methylation of hydroxysteroid 11-beta dehydrogenase 2 (HSD11B2), a gene that is integral in the deactivation of cortisol, which was in turn associated with a lower score on an index of fetal neurodevelopment in the third trimester (Monk et al., 2016). Another found that decreased prenatal maternal mood was related to increased DNA methylation in NR3C1 in the newborn, which was associated with increased cortisol response at 3

months (Oberlander et al., 2008). Utilizing the ALSPAC sample, researchers found variation in DNA methylation at seven loci across the epigenome in cord blood differentiated children who go on to develop early-onset conduct problems in middle childhood (Cecil et al., 2018). The prenatal period may be an essential stage of development in which to study epigenetic processes as embedding risk for psychopathology as the rate of DNA synthesis is high and the epigenetic marks needed for normal tissue differentiation and development are being established (Dolinoy et al., 2007).

Applying Epigenetics to Psychological Phenotypes

The study of epigenetic alterations as mechanisms for disease was first appreciated in cancer research where DNA methylation is among the most common somatic errors involved in carcinogenesis and accounts for a high proportion of tumor suppressor gene inactivation. Momentum has also been building in studying DNA methylation in the context of aging and other disease including autoimmune disorders, cardiovascular diseases, diabetes, and neurodegenerative disorders. Epigenetic research in the biomedical world commonly employs the case-control study design where a population is divided into a "disease" group and a "non-disease" group to examine epigenetic differences between the two. In translating technological advances in epigenetics from the biomedical world, developmental psychopathologists have largely contextualized psychological phenotypes within the same biomedical disease model, frequently using convenience sampling of adults (i.e. dividing a sample into individuals that meet criteria for depression and comparing their epigenetic profiles to controls). Due to their heterogeneity and comorbidity, psychological phenotypes are uniquely complex in their classification and measurement and are not automatically amenable to the same

methodologic framework. Many psychological disorders are better understood as occurring on a continuity of "normative" human experiences rather than discrete disease entities and psychological disorders have more complex continuity and discontinuity over time.

It is clear that the roots of both psychological and biomedical disorders can be traced back to early life development. However, unlike most biomedical disorders studied through the lens of epigenetic alterations, many psychological disorders have their initial onset in childhood. According to the World Health Organization, externalizing or impulse-control disorders across the world have the earliest onsets with median 7-9 years for ADHD, 7-15 for Oppositional Defiant Disorder (ODD), 9-14 years for conduct disorder. Research has shown that specific phobias tend to being in early to middle childhood, social anxiety in early to mid-adolescence, obsessive compulsive disorder in mid to later adolescence, and panic disorder in early adulthood (Kessler et al., 2005). Studies on depression have shown that the prevalence rates are generally low in children and grow to near-adult prevalence levels in adolescence (Merikangas et al., 2010). Because most adult psychopathology has its origins in childhood, it is imperative that in seeking biological mechanisms for the origins of psychological disease, researchers must examine epigenetic mechanisms early in the lifespan and in the developmental contexts in which these disorders initially occur. Utilizing longitudinal designs not only allows researchers to study epigenetic changes in a relevant sensitive period, they also have the advantage of establishing temporal order of epigenetic changes and disease outcome. In case-control designs, DNA methylation and disease outcome is usually measured concurrently, which limits interpretations of epigenetic changes as causative as it is equally reasonable to assume that epigenetic changes can be interpreted as a biomarker of

"wear and tear" of a debilitating and often chronic mental illness rather than a causal mechanism that explains its origin.

Therefore, the central aim of this thesis was to apply epigenetic theory, methodology, and technology to clinically relevant psychological phenotypes in a longitudinal framework that take into account phenotypic complexity and developmental context.

CHAPTER 3: Description of the Study Population

Description of the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort

The *Avon Longitudinal Study of Parents and Children* (ALSPAC) also known as "Children of the 90s" is an ongoing epidemiological study of children born in the city of Bristol in the United Kingdom in the 1990s (see cohort profiles Boyd et al., 2013; Fraser et al., 2013). The recruitment sample (N=14, 541) was all women residing in the Avon county catchment area with an expected date of delivery between April 1, 1991 and December 31, 1992. Data collection is still ongoing as ALSPAC participants transition into adulthood as well as efforts to gain information on the next generation.. Recruitment for the multi-generational cohort ALSPAC-G2 or "Children of Children of the 90s" began in 2012 and aimed to recruit all children of the (now adult) original ALSPAC children to continue recurring biological, psychosocial, and physiological assessments on the next generation and examine intergenerational connections to health and disease.

The families have been followed up with frequently, with 68 data collection time points between birth and age 18 including self, mother, father, and teacher reports. Self-reported questionnaire measures focused on mother's health included mental health (focusing largely on anxiety and depression), reproductive health (e.g. contraception,

menstrual patterns, repeatedly assessed pregnancies), cardiometabolic health (e.g. hypertension, cholesterol, musculoskeletal health (e.g. falls and fractures, arthritis), respiratory health (e.g. asthma, bronchitis), and health-related behaviors including substance use, diet, physical acitviity. Environmental measures include life course characteristics (e.g. retrospective measures of their child abuse and/or neglect, parental socioeconomic status and childhood housing conditions) and current life stressors including social support networks and romantic relationship functioning.

Self-reported child questionnaires included environmental measures assessed throughout the lifespan include diet, physical activitiy, hosuing, socioeconomic background, life stressors, air polluants (e.g. cigarette smoking in the home, home close to heavy traffic), and school environment. Physiological measures include anthropometry, blood pressure, pulse rate, lung function, fitness, skin, eye, and dental observations. Cognitive measures include IQ, speech and language ability, motor skills, and reading abiliy. As the ALSPAC children matured into pre-adolescennce, puberty and mensutration measures were collected. Psychosocial questionnaires focused on gender bhavior, self-esteem, peer relationships, romantic relationships, eating disorders, and alcohol and drug use. Psychopathology information was also collected assessing internalizing symptoms (e.g. mood and anxiety disorders), externalizing symptoms (e.g. ADHD, conduct and impulse control diosrders), as well as symptoms related to bipolar disorder and psychosis. The study website contains details of all the data that is available through a fully searchable data dictionary: http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/.

Like with all longitudinal cohorts, attrition over the years has been a challenge. Attrition rates were highest in the early neonatal stage of the study (66%) and decreased

over time, ranging from 48%-60% in childhood, 49% in adolescence, and 39.44% in transition to adulthood. However, mean attrition rates largely exaggerate the numbers of pariticpants lost to follow up due to the high volume and frequency of data collection points and different response patterns across nearly three decades. For example, some participants return to the study after years of no follow up. In fact, it seems that ALSPAC has a core of 3,000 devoted families that have completed all possible assessments, with close to 5,777 families completing 75% of all assessments throughout a 30 year period.

The ALSPAC cohort presents an unprecedented breadth and depth in assessment of health across the lifespan in an epidemiological cohort. One major limitation of the cohort, however, is the under-representation of non-White minority ethnic groups (2.2% of ALSPAC mothers were non-white) which limits external validity when generalizing findings to other populations. Under-representation is laregly due to the demographic profile of the original catchment area of Avon (4.4% non-White mothers) as well as the effects of differential attrition based on socioeconomic status. Children lost to follow-up are more likely to have a lower educational attainment, are more likely to be eligible for free school meals, and are more likely to be male. In order to address differential attrition and enhance the data resource, researchers used data linkage to link participants to publically available public health and administrative records including medical records, education records, economic, emplyment and social support records, criminal convictions, and neighborhood data.

Description of the Data Resource Profile: Accessible Resource for Integrated Epigenomic Studies (ARIES)

In 2012, a sub-project of ALSPAC named the Data Resource Profile: Accessible Resource for Integrated Epigenomic Studies (ARIES), was established to create a

population-based resource of DNA methylation data with the aim to understand the role of DNA methylation in health and development in the ALSPAC cohort (see cohort profile Relton et al., 2015). Based on available retrospective DNA sampling, 1,018 mother-child dyads (50% female offspring) from the original ALSPAC cohort were chosen to obtain epigenome-wide DNA methylation samples using the Illumina Infinium HumanMethylation450 BeadChip (450 K) array. DNA samples for offspring were available at three separate time points: at birth and extracted from cord blood drawn from the umbilical cord upon delivery, at mean age 7.5 years, and at mean age 15.5 years, both extracted from peripheral blood. Maternal samples were collected during pregnancy at mean 26 weeks gestation and again approximately 15-17 years later. The ARIES sub-sample is considered to be reasonably representative of the main study population. Though mothers included in ARIES were slighltly older, more likely to have a non-manual occupation, and are less likely to have smoked during pregnancy.

The ALSPAC cohort sample is one of a kind as its large scale and in-depth rich analysis of mental and physical health across the lifespan is unprecedented in epidemiological studies. Due to recurrent measuring of key phenotyoes and genetic and biological samples at multiple time points, this data set is uniquely suited to explore questions of epigenetic origins of risk and resilience for psychopathology within a developmental and ecological framework.

Processing of the ARIES DNA Methylaton Data

DNA methylation data processing using the Illumina 450k array was conducted at the University of Bristol. DNA samples from all participant ages (cord, middle childhood, adolescence) were distributed across slides using a semi-random approach to minimize the possibility of confounding due to batch effects. DNA methylation data went

through several quality checks to ensure accuracy. In order to remove sample mismatches, genotype probes were compared with SNP data from the same indidivudal in the ALSPAC sample and samples were flagged if there was a sex mismatch based on X-chromosome methylation. The Illumina 450k array also has 850 quality control probes on each array that asses various aspects of the data collection process to determine the status of staining, extension, hybridization, target removal, bisulfite conversion, specificity, non-polymorphic and negative controls. Samples failing quality control were repeated and, if unsuccessful, excluded from further analysis. Micro-arrays have been shown to have very strong batch effects that can mask true biological differences between samples. Statistical controlling for batch effects during the data analysis phase is not sufficient and data needs to be initially normalized to remove any artificial variation between samples. There are several statistical methods that can be used to normalize the 450k array using packages in R.

There are two confounders present in the set-up of the Illumina microarray: polymorphic probes and cross-reactive probes. Polymorphic probes are probes that target CpG sites that are on or near SNPs. Since the Illumina platform uses quantitative genotyping of C/T SNPs, probes with polymorphisms at the target C or G have the potential to confound a difference in genotype rather than a difference in DNA methylation. By utilizing genotype and DNA methylation information from the same individuals, researchers have found that DNA methylation profiles of probes located on or near a SNP were typically explained by patterns of the SNP genotype and do not reflect any underlying epigentic mechanisms (Chen et al., 2013; Price et al., 2013). Cross-reactive probes are probes that hybridize to multiple genomic locations that are similar. The level of DNA methylation at these probes likely reflects a combination of

DNA methylation at the various locations to which they hybridize (Chen et al., 2013; Price et al., 2013) . Both polymorphic and cross-reactive probes have been annotated by researchers who recommend removal of these probes for analyses examining differences in DNA methylation.

Like most exisitng cohort studies, ALSPAC has been utilizing stored whole blood samples for DNA methylation analyses. Whole blood is a heterogenuous collection of different cell types, which carry their own unique DNA methylation profile as a blueprint for cell differentiation (Reinius et al., 2012). For each individual, the proportion of cell types within their specific blood sample can vary widely, which in turn can confound DNA methylation measurement. The overwhelming majority of the ALSPAC DNA samples did not have whole blood cell counts assessed prior to DNA extraction and therefore a post hoc correction is necessary. First, the fraaction of CD8, CD4, NK, B, monocyte, and granulocyte cells in each individual is estimated and those estimated cell type proportions serves as covariates in any subsequent analyses. For cord blood obtained at birth, the Bakulsi method was used (Bakulski et al., 2016). For peripheral blood obtained in adolescence, the Houseman method was utilized (Houseman et al., 2016).

Introduction to Empirical Chapters

The first study describes a manuscript titled “Variation in DNA Methylation of the Oxytocin Receptor Gene Predicts Children's Resilience to Prenatal Stress” published in Development and Psychopathology in 2017. It is a candidate gene approach examining epigenetic changes in the oxytocin receptor gene (OXTR) as it relates to resilience to prenatal stress. It is an early training step into behavioral epigenetic research, theory, and methods. The second, not yet published, study represents a more in-depth and

methodologically sophisticated epigenome-wide approach examining epigenetic correlates of depression trajectories in adolescence and follow up gene annotation, gene network analyses, and regional analyses.

CHAPTER 4: Variation in DNA Methylation of the Oxytocin Receptor Gene Predicts Children's Resilience to Prenatal Stress

Introduction

Resilience is defined as successful emotional, behavioral, or social adaptation or adjustment despite experience of significant adversity, stress, or trauma (Luthar et al., 2000; Rutter, 2012). In children, researchers have operationally defined this adaptation in a myriad of ways such as mastering normative developmental tasks (Luthar et al., 2015), absence of psychopathological outcomes (Martinez-Torteya et al., 2009) or functioning that is “better than expected” given a level of exposure to risk (Kim-Cohen et al., 2004). Some children show resilience across multiple domains of functioning. More commonly, however, at-risk children show resilience in one domain of functioning but not in others or they may be resilient at one time period but not another (Masten, 2013). Thus, resilience as a construct may be better defined as a dynamic process (not a trait or characteristic) that depends on the balance of risk and protective factors available to an individual at a given point in time (Jaffee et al., 2007; Rutter, 2006, 2012). Understanding the mechanisms that promote resilient functioning in addition to the processes that confer risk for psychopathology, and the dynamic balance between them, is essential to understanding how normative and maladaptive developmental trajectories form.

Researchers have long posited the importance of adverse life events during early critical periods in understanding risk and resilience. The prenatal period, specifically, is one in which the fetus is especially vulnerable to a wide range of environmental

exposures that have the potential to confer risk for emotional, cognitive, and behavioral problems in childhood (Braithwaite et al., 2014; Rice et al., 2007). A large number of studies have shown that exposure to prenatal maternal psychopathology such as depression and anxiety is associated with both internalizing and externalizing behavioral outcomes in children such as depression, anxiety, ADHD symptoms, and conduct problems (Barker et al., 2011; O'Connor et al., 2002; O'Donnell et al., 2014; Van den Bergh & Marcoen, 2004). Prenatal exposure to maternal stressful life events, such as death of a close relative or friend, divorce, marital problems, and job loss, has also been linked to ADHD, behavioral problems, and internalizing symptoms (Laucht et al., 2000; Pawlby et al., 2009; Ronald et al., 2010). It is clear that a range of stressors during the prenatal period increases risk for child psychopathology. However, not all children exposed to environmental stressors in utero go on to develop psychopathology and some children seem to be less vulnerable than others. There are several hypotheses that can potentially account for this differential vulnerability. One possibility is that a supportive postnatal environment can attenuate or reverse the effects of prenatal stress. For example, researchers have found that sensitive caregiving moderates the effect of prenatal maternal stress on infant fearfulness (Bergman et al., 2008) and cognitive outcomes (Bergman et al., 2010)

A second possibility is that individual differences in genotype confer protection against prenatal stressors. For example, researchers have found that variation in the glucocorticoid receptor gene (*NR3C1*), a gene integral to the functioning of the hypothalamic-pituitary-adrenal (HPA) axis which is involved in stress reactivity, moderated the effect of prenatal maternal psychological symptoms on later emotional and behavior problems, such that children whose mothers were depressed or anxious when

they were pregnant with the child had an increased risk of emotional and behavioral problems at age 3 if they possessed the minor allele C (CC or CG), but not if they were homozygous for the major allele (GG) (Pluess et al., 2011). Using data from 1513 children in the Generation R cohort, Pluess and colleagues (2011) found that infants whose mothers were more anxious during pregnancy had higher scores on a measure of negative emotional temperament than infants whose mothers were not anxious and this effect was significantly stronger for infants who carried the short 's' form of the serotonin transporter gene (5-HTTLPR) compared with infants who carried two copies of the long 'l' form of the gene. In addition, Oberlander and colleagues (2010) found that prenatal exposure to maternal anxiety predicted internalizing symptoms in children with 2 copies of the 5-HTTLPR 's' allele (but not in children who carried the 'l' allele). In contrast, a mother's anxiety during pregnancy predicted her child's externalizing problems only if her child had 2 copies of the 'l' allele and not if the child carried at least one 's' allele (Oberlander et al., 2010).

In addition to identifying structural variants in the genome that buffer against the effects of prenatal stress, new research in the field of behavioral epigenetics has started to elucidate the underlying biological mechanisms of the relationship between stress exposure and later developmental outcomes, including emotional and behavioral problems. Epigenetic research sits at the intersection of social and biological explanations for developmental psychopathology and has enormous potential for describing how stressful life events "get under the skin" and have lasting effects on mental and physical health. The epigenome describes the chemical switches that sit on top of genes and modulate gene expression. Stress-induced epigenetic modifications are typically measured by examining DNA methylation, where methyl groups are added to cytosine-

guanine-phosphate (CpG) sites on the regulatory or promoter regions of genes to silence transcription factors or block access to recognition elements of a gene (Bick et al., 2012). DNA methylation is typically related to lower gene expression in promoter regions. However, DNA methylation in other genomic regions can have the opposite effects on expression (e.g. gene body) and there is little known about the functional role of DNA methylation in other locations such as the intergenic region (Jones, 2012). Studies have shown that DNA methylation patterns are under significant control – as evidenced by the discovery of a large number of methylation quantitative trait loci (mQTL; Gaunt et al., 2016; Jones, 2012) – but are also sensitive to environmental influences (McGowan & Roth, 2015). Although the environment modifies the epigenome throughout the lifespan, there is some evidence that the in utero environment has the largest effect (Billack et al., 2012). These prenatal effects have been largely interpreted in terms of the fetal programming hypothesis in which the fetus adapts its phenotype – such as stress reactivity or metabolism – to what it anticipates its postnatal environment to be on the basis of the biological cues from the mother's environment (Gluckman et al., 2008b).

The majority of studies investigating the association between prenatal exposure to maternal stress and methylation have focused on *NR3C1*. Prenatal stressors such as maternal depression (Conradt et al., 2013a; Hompes et al., 2013), exposure to intimate partner violence (Radtke et al., 2011) and exposure to war (Mulligan et al., 2012) have been associated with increased methylation of *NR3C1* at birth. There is, however, variability in these methylation profiles, even among newborns whose mothers reported high levels of stress and this variability may be predictive of children's risk for emotional or behavioral health problems versus their resilience. To date, few biologically informed

prospective studies have explored gene-specific methylation patterns in the context of resilience. We focus on methylation of the oxytocin receptor gene (*OXTR*).

Oxytocin is an essential neuropeptide and hormone in the regulation of social and affiliative behavior such as empathy, attachment, bonding, emotion recognition, and processing of social stimuli (Jack et al., 2012). Oxytocin has also been shown to have anxiolytic effects by dampening physiological, hormonal, and brain-level responses to stressful or aversive signals (Heinrichs et al., 2009). Thus, stress-related epigenetic changes in the oxytocin system may confer risk for the development of psychopathology by shaping socio-emotional, socio-cognitive, and stress response systems that underlie temperament and children's relationships with peers and adults.

It is biologically plausible to predict that prenatal (or postnatal) stressors would be associated with increased DNA methylation and, in turn, increased emotional and behavioral problems. That is, if DNA methylation acts as a gene silencer, increased methylation in the promoter region of the gene would result in lower messenger RNA (mRNA) levels, blocking of transcription factors, and in turn, decreased gene expression and decreased circulating oxytocin in the blood. Indeed, a number of studies have found evidence for these relationships. For example, increased *OXTR* methylation leads to decreased *OXTR* mRNA expression in hepatoblastoma human cells (Kusui et al., 2001) and in murine cells (Mamrut et al., 2013) in the promoter region of the gene. Gregory and colleagues found increased promoter region *OXTR* methylation in peripheral blood as well as in temporal cortex tissue in individuals with autism compared with controls. Increased methylation resulted in a 20% reduction in mRNA expression (Gregory et al., 2009) .

Consistent with the possibility that *OXTR* methylation may be a mechanism by which prenatal exposures increase risk for psychopathology, Dadds and colleagues (2014) found that elevated methylation in *OXTR* in a sample of 4- to 16-year-olds was associated with lower levels of circulating oxytocin and higher levels of callous-unemotional traits. Similarly, in a sample of youth with early-emerging and persistent conduct problems, Cecil and colleagues found that higher methylation at birth at the *OXTR* locus was associated with higher levels of callous-unemotional traits at age 13, although the effect was only observed in youth with low levels of internalizing problems (Cecil et al., 2014). Moreover, mothers' reports of behaviors that might have caused stress to themselves or the fetus (e.g., their own criminal behavior, their partner's criminal behavior, their own psychopathology and substance use) were associated with elevations in *OXTR* methylation at birth.

Despite the plausibility of a pathway by which prenatal stressors lead to increased *OXTR* methylation, a number of studies have found the inverse relationship between *OXTR* methylation and prenatal stress as well as psychological outcomes. For example, one study focusing on prenatal stress found that the more life-changing stressful events a mother experienced when she was pregnant, such as being a victim or witness of assault or experiencing the severe illness or death of a loved one, the *lower* the *OXTR* methylation levels in cord blood at birth (Unternaehrer et al., 2015). Reiner and colleagues found that depressed women had lower *OXTR* exon 1 DNA methylation levels compared to non-depressed women (Reiner et al., 2015). Moreover, Ziegler and colleagues found in a sample of adults that decreased *OXTR* methylation was associated with a diagnosis as well as symptoms of social anxiety disorder, increased cortisol responses to a stress test, and increased amygdala responsiveness during social anxiety

word processing (Ziegler et al., 2015). In addition, in a brain imaging study, researchers found that higher *OXTR* methylation was related to *increased* brain activity in areas associated with social perception such as the temporoparietal junction and the dorsal anterior cingulate cortex (Jack et al., 2012). Thus, the evidence is mixed as to whether prenatal stressors are associated with increased or decreased *OXTR* methylation and whether individual differences in *OXTR* methylation are associated with positive or negative child (or adult) outcomes. Direction of effects could be highly dependent on the location of the probes examined.

The present study is the first to make use of a longitudinal design to examine if *OXTR* methylation at birth can differentiate resilient and non-resilient youth as measured by hyperactivity, conduct problem, and emotional problem outcomes in mid-childhood. Additionally, a strength of the study is that only children with pre- and post-natal adversity were included which ensures that resilience is not driven by differences in the quality of the postnatal environment. Given mixed findings in the literature about the direction of the relationship between stress in pregnancy and methylation levels at birth as well as the relationship between methylation levels and later behavior, we do not propose a directional hypothesis. Understanding plasticity at this critical period in development can help us examine how early stress can "get under the skin" and alter developmental trajectories. We hypothesize that this variability will be predictive of which newborns grow up to have low levels of psychopathology, despite their exposure to prenatal risk factors.

Methods

Participants

The *Epigenetic Pathways to Conduct Problems Study* consists of a subsample of youth (n = 339, 50% female) nested within ALSPAC and ARIES with established trajectories of conduct problems from ages 4 to 13 (Barker & Maughan, 2009) and have epigenetic data at birth and/or childhood. This subsample is comparable to the full trajectory sample (n = 7,218) in terms psychiatric comorbidity (Barker et al., 2010). DNA methylation measures were available for 326 youth at birth. Children with missing ethnicity information were removed, leaving a total sample of 321. Except for factor analyses, in which we used data from all youth, the present study only included youth who scored above the sample average on our measure of prenatal and postnatal (birth to age 7) environmental risk exposure. Although exposure to prenatal risk was the focus of our study, we wanted to ensure that differences in the postnatal environment did not account for any observed associations between methylation profiles at birth and resilience in middle childhood. These measures of prenatal and postnatal risk are described below in the section "Environmental Risk." The final analytic sample was n = 91, all of whom had complete data including DNA methylation at birth, had been exposed to pre- and post-natal adversity, and for whom information on emotional and behavioral outcomes was collected. See Figure 1 for a flow chart representing which youth were included in the analysis sample.

Measures

DNA Methylation data at Birth. DNA methylation was assayed according to standard protocol described in the Introduction section of this thesis (p. 19-22). Samples were quantile normalised using the *dasen* function within the *wateRmelon* package (wateRmelon_1.0.3; 19) in R and batch corrected using the ComBat package (Johnson et al., 2007).

We extracted data for probes located within the *OXTR* CpG island (*n* = 12), as this area has been previously investigated and shown to play a key role in modulating the transcriptional activity of *OXTR* (Kusui et al., 2001). For each probe, methylation levels were indexed by beta values (corresponding to the ratio of methylated signal divided by the sum of the methylated and unmethylated signal). Factor analysis was used to reduce the 12 *OXTR* probes into a smaller set of factors, which accounted for shared variance between them. A 3-factor solution showed the best fit to the data as well as good temporal stability. See Supplement 1 and 2. We present findings relating specifically to Factor 1, three probes located in the 5'UTR region – Probe 1 (cg00078085), Probe 5 (cg03987506), and Probe 10 (cg12695586) – because Factor 2 and 3 scores were not significantly associated with any type of resilience.

Environmental Risk. The prenatal risk score comprised items that were reported by mothers and summed to create 4 conceptually distinct but related domains: (i) *Life events* (e.g. death in family, accident, illness), (ii) *Contextual risks* (e.g. poor housing conditions, financial problems), (iii) *Parental risks* (e.g. maternal psychopathology, criminal involvement and substance use), (iv) *Interpersonal risks* (e.g. intimate partner violence, family conflict). Measures of post-natal environmental risk were created for the early childhood (birth-age 7) and middle childhood (age 8-9) periods. These included all domains represented in the prenatal risk composite as well as a measure of *Direct victimization* (e.g. child bullied by peers or physically hurt; available only postnatally).

Risk domains were positively and significantly correlated, both within and between developmental periods, with the majority of correlations ranging from *r* =.20-.40. For the prenatal and postnatal periods, we used confirmatory factor analyses (CFAs) to assess the internal reliability of the risk domains and to extract one global cumulative

risk score for each developmental period, showing good model fit. Higher scores indicate greater environmental risk exposure. See Supplement 3 for full item descriptions, details of inter-correlations between risk domains and factor analysis fit indices. To ensure that youth who were defined as resilient or non-resilient had been exposed to at least some moderate level of environmental risk, the sample was restricted to youth who scored above the mean on the measures of prenatal and postnatal cumulative environmental risk, as described in the Participants section.

Internalizing and Externalizing Problems. Repeated assessments of conduct problems, hyperactivity, and emotional problems were made at ages 4, 7, 8, 10, 12, and 13 via maternal reports on the Strengths and Difficulties Questionnaire (SDQ) (Goodman, 1997). The SDQ is a widely used screening instrument with reliability and validity demonstrated in a large national sample (Goodman, 2001). The SDQ comprises five subscales, each consisting of five items rated by mothers as 'certainly true', 'somewhat true', or 'not at all true'. In the current study, we utilized the conduct problems subscale (e.g., 'often fights with other children or bullies them', 'often lies or cheats'), the hyperactivity/inattention subscale (e.g., 'restless, overactive, cannot stay still for long', 'constantly fidgeting or squirming') and the emotional problems subscale (e.g., 'often unhappy, down-hearted or tearful', 'many worries, often seems worried'). In order to obtain more robust and reliable estimates of symptomatology, we performed a confirmatory factor analysis for each of the three subscales that included data from age 4 to 13, so as to generate a single factor score for each subscale that accounted for shared variance across time points. We also created a 'global symptomatology' factor score combining all three SDQ subscales as a measure of more general overall functioning. See Figure 2 for summary statistics as well as full details of the confirmatory factor analysis.

Psychosocial Functioning. We used factor scores from the peer problems (e.g. 'rather solitary and tends to play alone', 'generally liked by other children') and prosocial behavior (e.g. "considerate of other people's feelings', 'kind to younger children') subscales from the SDQ. We also utilized a six item callous-unemotional traits questionnaire completed by mothers when the child was 13 (e.g., 'makes a good impression at first but people tend to see through him/her after they get to know him/her', and 'shallow or fast changing emotions') (Moran et al., 2008). Items were rated on a three-point scale ranging from 'not true' to 'certainly' true. Social cognition was assessed using the 12-item Social Communication Disorder Checklist (Skuse et al., 2005) completed by mothers when the child was 7 years old. Items included for example: 'not aware of other people's feelings', 'does not notice the effect of his/her behavior on other members of the family'. Higher scores indicate lower social cognition.

Classification of Resilience. In order to classify the sample into resilient (1) and non-resilient (0) groups, we conducted four ordinary least squares regressions to predict (i) global, (ii) conduct problems, (iii) hyperactivity, and (iv) emotional problems, respectively, from the prenatal cumulative risk factor score. We utilized residuals from these regressions to classify youth into resilient and non-resilient groups in each domain. Specifically, youth with negative residual scores were classified as resilient (indicating that they had lower-than-predicted levels of psychopathology, given their exposure to prenatal risk) and youth with non-negative residual scores were classified as non-resilient (indicating that they had predicted or higher-than-predicted levels of psychopathology, given their exposure to prenatal risk). One subject with conduct, hyperactivity, and global symptomatology residual scores > 3 s.d. from the mean was removed from all the analyses. Retention of the outlier results in a non-normal distribution of resilience

residuals although findings remain unchanged with the subject's inclusion. For all domains, the distributions of the residuals were normal. See Figure 3 for resilience classification.

For resilience as defined by global problems, n = 44 (48%) youths were classified as resilient and n = 47 (52%) were not resilient. For resilience as defined by conduct problem scores, n = 44 (48%) youth were classified as resilient and n = 47 (52%) were not resilient. For resilience as defined by hyperactivity scores, n = 50 (55%) youth were classified as resilient and n = 41 (45%) were not resilient. Finally for resilience as defined by emotional problem scores, n = 50 (55%) youth were classified as resilient and n = 41 (45%) were not resilient.

Data Analysis

Factor analyses were conducted in Mplus version 6.1.128 and all other analyses in SPSS 21. Regression analyses were conducted to test whether resilience (defined globally and in terms of specific domains) was associated with the Factor 1 methylation score. Post-hoc analyses were conducted to test whether resilience was associated with the individual probes (Probes 1, 5, 10) that make up Factor 1. Covariates in all models included sex and cell-type composition, estimated using the approach described in Houseman and colleagues (2012). Analyses were bootstrapped 10,000 times. Bootstrapping is advantageous with small samples as it derives an approximation of the sampling distribution via repeated resampling of the available data to yield bias corrected 95% confidence intervals (CI). Significant associations were only presented if they survived bootstrapped confidence intervals. Then, further analysis on any resilient domains that had significant methylation results was conducted. Resilient and non-

resilient groups in that domain were compared on additional psychosocial functioning factors.

Results

As shown in Table 1, children who had lower conduct problem scores than predicted given their exposure (to pre-natal environmental risk; i.e. resilient group) had a higher *OXTR* methylation Factor 1 score than non-resilient children. In contrast, when resilience was defined globally or in terms of hyperactivity or emotional problems, resilience scores were not associated with *OXTR* methylation.

When examining the individual probes that make up the *OXTR* methylation factor (Probes 1, 5, and 10), we found that youth who were resilient in terms of conduct problems had significantly higher methylation levels across all three probes compared to the non-resilient group. Interestingly, resilience as defined in terms of global problems and hyperactivity problems predicted increased methylation only within one probe (Probe 5). Figure 4 highlights percent methylation differences across groups who were resilient versus non-resilient in terms of conduct problems.

Table 2 provides descriptive information regarding the groups who were resilient and non-resilient in terms of conduct problems. The groups did not differ in terms of gender or in environmental risk at any developmental period (prenatal – age 9). Furthermore, in an ANCOVA controlling for sex, we found that youth who were resilient in terms of conduct problems also had lower hyperactivity, emotional problems and callous-unemotional traits, higher prosocial behavior and better social cognition as compared to youth who were non-resilient in terms of conduct problems. Thus, youth who were resilient to prenatal risk in terms of having relatively low levels of conduct

problems were functioning well across multiple domains that are typically compromised when youth have conduct problems.

Post-hoc Analysis: Exploring Potential Genetic Influences. We explored potential genetic factors that may influence the DNA methylation sites associated with resilience to conduct problems. Because our sample was underpowered to directly examine genetic polymorphisms (SNPs) affecting DNA methylation, we used the mQTLdb resource (http://www.mqtldb.org/) to search for known methylation quantitative trait loci (mQTLs) associated with our methylation sites of interest. The mQTLdb database contains the results of a large-scale study based on the ARIES sample in ALSPAC (from which our subsample is derived), characterizing genome-wide significant *cis* effects (i.e. SNP within ±1000 base pairs of the DNA methylation site) and *trans* effects (i.e. ±1 million base pairs) on DNA methylation levels across Illumina 450k probes at five different life stages, including cord blood DNA methylation at birth (Gaunt et al., 2016). Here, we searched for mQTLs based on results from the conditional Genome-wide Complex Trait Analysis (GCTA), which was used to identify mQTLs with the most representative, independent effect on each DNA methylation site in order to account for linkage disequilibrium (Gaunt et al., 2016). Based on mQTLdb search, we found that 2 out of 3 of Factor 1 probes (Probe 1 and Probe 10) were associated with known cis SNPs, suggesting that DNA methylation levels across these sites are likely to be under considerable genetic control. Interestingly, Probe 1 and Probe 10 are specific to conduct problems, while Probe 5 was significant in both hyperactivity and global problems. This suggests that these probes are likely to be influenced by genetic factors as well as environmental adversity and may suggest a specific GxE effect for conduct problems. See Table 3 for more details on SNP influences on Probe 1 and 10.

Discussion

Our goal in this study was to examine whether variability in *OXTR* DNA methylation profiles at birth predicted resilience as defined by psychopathological outcomes that were better than expected based on prenatal risks. Consistent with our hypothesis, Factor 1 methylation – as well as methylation of the individual probes (1, 5, 10) that make up the factor – was predictive of resilience to conduct problems in mid-childhood. In contrast, *OXTR* DNA methylation profiles did not predict resilience in domains of emotional, hyperactivity, and global symptomatology suggesting a potential role for *OXTR* in the development of conduct problems in particular. This is consistent with the fact that many social-cognitive processes such as empathy, attachment, bonding, and emotion recognition are disturbed in children with conduct problems. In addition, problems in social cognition associated with conduct-disordered behavior are typically marked by deficits in oxytocin levels.

Children who were resilient in the conduct problems domain in mid-childhood also had significantly fewer hyperactivity, emotional, and peer problems, higher levels of prosocial behavior, better social cognition, and lower scores on a measure of callous-unemotional traits compared with non-resilient youth. Thus, the group that was resilient to conduct problems was broadly resilient across multiple domains. However, this was probably not due to *OXTR* methylation profiles, which were not predictive of resilience as defined by emotional or hyperactivity problems. One possibility is that children who have fewer-than-expected conduct problems get along better with their peers, are both innately more prosocial and observe higher levels of prosocial behavior in their interactions with peers, and are thus buffered against the emergence of other forms of psychopathology relative to children with higher levels of conduct problems (Oland &

Shaw, 2005; Patterson et al., 1989). The role of *OXTR* DNA methylation in resilience beyond the conduct problems domain remains unclear.

It is important to note that there were no significant differences between resilient and non-resilient youths in levels of environmental risk in any of the developmental periods from prenatal to age 9. This rules out the possibility that resilient youth exhibited fewer conduct problems than non-resilient youth because they were exposed to less environmental risk after they were born. If epigenetic modifications in *OXTR* are consequences of exposure to stress, why would youth with similar levels of exposure to prenatal adversity vary in terms of *OXTR* methylation profiles? Recently, researchers have recognized that DNA methylation patterns may be allele-specific and the relationship between exposure to stress and DNA methylation may be moderated by gene variants. For example, one study found that adolescents that were homozygous for the l-allele of *5HTTLPR* and experienced more stressful life events had higher levels of *5HTTLPR* methylation. Stressful life events were not associated with methylation for s-allele carriers (van der Knaap et al., 2015). Another study found that decreased DNA methylation in the FK506 binding protein 5 *(FKBP5)* gene depended on early childhood abuse and the rs1360780 risk allele (Klengel et al., 2013). Although we could not examine direct SNP effects because of small sample size, our post-hoc analyses using the mQTLdb demonstrated that methylation of Probes 1 and 10 is significantly influenced by SNPs rs62243375 and rs237900 respectively. Interestingly, our results showed that Probes 1 and 10 were only related to conduct problems, while Probe 5 was related to global problems and hyperactivity. This provides indirect evidence for *OXTR* genotype moderating the relationship between adversity and DNA methylation in conduct problems. However, studies examining allele specific DNA methylation effects earlier in

child development, especially in the prenatal/neonatal period are lacking. More research is needed to examine the integrative effects of *OXTR* genotype and DNA methylation on the oxytocin pathway, especially during the critical prenatal period.

Increased methylation of *OXTR* is associated with decreased gene transcription and protein expression, which theoretically represents the molecular building blocks for behavioral phenotypes (Kumsta et al., 2013; Kusui et al., 2001; Mamrut et al., 2013). Interestingly, our results showed that *higher* levels of DNA methylation of *OXTR* at birth predicted resilience to conduct problems in mid-childhood. This pattern was unexpected in light of results showing that elevations in *OXTR* methylation are also associated with relatively high levels of callous-unemotional traits (Dadds et al., 2014; Cecil et al., 2014). However, this traditional view has been recently challenged with more and more studies finding an inverse relationship, highlighting the complexities in predicting behavioral phenotypes from DNA methylation (Jack, Conolley, & Morris, 2012; Reiner et al., 2015; Ziegler et al., 2015). In a human cohort, researchers found that only a minority of individual CpG sites had significant negative correlations with mRNA signaling across individuals and in a number of genes, higher DNA methylation was associated with higher gene expression (Lam et al., 2012). This can also be because the relationship between methylation, transcription, and expression can vary depending on the location of the CpG site. Of note, the three probes in our study mapped onto the 5' UTR region of gene, where an inverse correlation between DNA methylation and mRNA expression has previously been reported (Eckhardt et al., 2006). Thus, although we might theoretically predict that higher methylation would be associated with a lack of resilience to conduct problems, the mechanics of methylation are likely to be more complex than this.

Interestingly, our findings conflict with Cecil and colleagues (2014) work also

using data from the ALSPAC sample in which they found that higher *OXTR* methylation at birth was associated with higher callous-unemotional traits at age 13. Of note, Cecil et al (2014) found this relationship in *OXTR* probes that make up Factor 2, while Factor 1 probes were not associated with callous-unemotional traits in their study. Furthermore, the sample (N=39) was highly selected to include only youth who had early-onset and persistent conduct problems and the relationship between higher *OXTR* methylation at birth and callous-unemotional traits was only observed in the subgroup with low levels of internalizing profiles. Thus, although our analysis sample and Cecil et al's ostensibly come from the same cohort, they reflect very different groups of children.

The present findings should be interpreted in light of a number of limitations. This study focused specifically on DNA methylation of annotated probes located within the CpG island of *OXTR* and it is likely that differences across groups may be found in other genes (i.e. glucocorticoid or serotonergic pathways). Future studies may employ an epigenome-wide approach that would enable researchers to examine group differences in DNA methylation across the genome. In addition, we did not examine RNA expression and cannot explore the functional relevance of the probes in regards to gene expression and downstream biological mechanisms. However, we did select a region of *OXTR* that has previously demonstrated to be functional in utero. Although we provided indirect evidence for a potential GxE effect on DNA methylation via the mQTLbase data, we could not directly test it due to sample size. In general, the findings are based on a relatively small sample of youth, which limits statistical power to detect effects.

In summary, this is the first longitudinal study to examine the role of *OXTR* methylation in resilience across multiple domains. Our findings show that *OXTR* methylation at birth is exclusively related to resilience in the conduct problems domain in

middle childhood. This may be potentially reflective of a GxE effect where genotype moderates the relationship between environmental stressors and DNA methylation. These findings highlight the importance of the prenatal period for the development of childhood psychopathology and suggest a potential mechanism by which early experiences may be biologically embedded. Because of the important role of oxytocin in social impairment, understanding individual variations in *OXTR* methylation patterns might increase insight into risk and resilience factors that can bridge translational efforts in treatment and intervention approaches.

CHAPTER 5: A Longitudinal Epigenome-wide Analysis of Depression Trajectories in Adolescence (Part 1)

Introduction

Major Depressive Disorder (MDD) in children and adolescents represents a major worldwide public health burden. In the US, an estimated 3 million adolescents experienced an episode of depression in 2017, representing around 13% of the US population aged 12 to 17 (National Survey on Drug Use and Health, 2017). According to the Centers for Disease Control and Prevention, in 2014 rates of death by suicide surpassed deaths by traffic accidents among adolescents for the first time ever (Morbidity and Mortality Weekly Report-MMWR, 2016). The suicide rates among even younger youths (age 10 to 14) has doubled in the US between 2007-2014 (MMWR, 2016). Depression in adolescence predicts major depressive disorder in adulthood, other mental health disorders, substance abuse, suicide attempts, educational underachievement, unemployment, and early parenthood (Fergusson & Woodward, 2002).

The etiology of depression is still not fully understood. Genetic factors confer risk for the disorder with heritability of MDD estimated to be around 40%; but genetic

variants explain only a small proportion of heritability (for reviews of GWAS depression studies see Dunn et al., 2015; Tsang et al., 2017). Research has also shown that stressful life events also confer risk for depression above and beyond heritability (Kendler, Thornton, & Gardner, 2001; Kendler, Karkowski, & Prescott, 1999). Recent work utilizing the UK BioBank (N = 126, 522) found that the SNP-based heritability of MDD stratified by reported trauma exposure (24%) was much greater than MDD without reported trauma exposure (12%) suggesting a complex relationship between genetic risk, stressful life events, and MDD (Coleman et al., 2020).

Epigenetic research sits at the intersection between biological and environmental explanations for the development of depression. Although the human genome is static, the epigenome is dynamic and highly responsive to environmental input. The epigenome describes the chemical switches that sit on top of genes and modulate gene expression by either silencing or activating certain genes. Stress-induced epigenetic modifications are typically measured by examining DNA methylation, where methyl groups are added to cytosine-guanine-phosphate (CpG) sites (Jaenisch & Bird, 2003). Because the epigenome is highly responsive to stress signals from the environment, modifications that produce enduring changes in gene expression are a possible biological mechanism by which stressful life events increase risk for depression. Our study aims to examine the etiology of depression using epigenetic mechanisms through a developmental lens focusing on two sensitive periods: in utero and adolescence.

Heterogeneity in Depression

Major Depressive Disorder is a heterogenous phenotype across many domains. There is heterogeneity in presenting symptoms, both in terms of the type and combination. For example, researchers found over a 1,000 unique depression symptom

profiles in individuals diagnosed with MDD (N = 3707), representing only 3.6 patients per profile (Fried & Nesse, 2015). Depression is also heterogenous in severity and is better understood as a dimension ranging from normative response to life stress to a severe disorder (Beach & Amir, 2003; Ruscio & Ruscio, 2000). Finally, depression is also heterogeneous in clinical prognosis over time. Research has shown that depression can be both a time-limited, single episode phenomenon and a recurring and chronic lifetime disorder (Lorenzo-Luaces, 2015). It is clear that depression is better understood as a heterogenous symptom cluster rather than a discrete, underlying condition with demarcated boundaries and a homogeneous group of patients (Monroe & Anderson, 2015). However, in the search for biological vulnerabilities to MDD, research has largely relied on a categorical, disease model when operationalizing depression. As a result, despite decades of genetic, neurological, and biological research, slow progress has been made in identifying reliable biomarkers for MDD. In an effort to take this phenotypic heterogeneity into account, our study is the first to utilize a more dynamic measure of depression using trajectories that reflect severity and course over time.

Sensitive Periods of Development

Adolescence. Although depression in childhood is rare (less than 3% prevalence rate), there is a dramatic surge in depression rates during ages 13-17, with 17% of individuals experiencing a depressive episode before the age of 18 (Ge et al., 1994; Merikangas et al., 2010). There is a strong continuity of depression from adolescence into adulthood, while most depressed pre-pubertal children do not grow up to be depressed adults (Hankin et al., 2015a; Pine et al., 1999; Rutter et al., 2006). Therefore, adolescence represents a particularly significant developmental stage in which to study biological mechanisms underlying the development of depression.

In utero. Researchers have long posited that the risk for psychopathology can be embedded as early as the uterine period of development. With regards to depression research, a consistent finding is that maternal depression during pregnancy significantly increases risk for depression in offspring above and beyond postnatal maternal depression, contextual factors, and overall postnatal environment (Barker et al., 2011; Pawlby et al., 2009; Pearson et al., 2013; Plant et al., 2015) suggesting that the risk for depression could be traced back to prenatal development.

Our study is able to assess the biological programming of depression risk through a more developmentally relevant framework by examining epigenetic changes in sensitive periods of development (in utero and adolescence) that have the biggest potential to help us understand the causes of depression.

Literature Review of Epigenetics and Depression.

In the past ten years, there has been an explosion of DNA methylation studies examining the depression phenotype. In the nascent stages of behavioral epigenetics as a field, candidate gene approaches were most common. However, as the need for less biased and more comprehensive approaches grew, epigenome-wide studies have become more frequent, especially in the last five years. There are a number of reviews that describe this vast literature. For a comprehensive systematic review of approximately 70 depression EWAS and candidate gene studies published before 2018 see Li and colleagues (2019). For a systematic review more focused on work stress, burnout, and depression see Bakusic and colleagues (2017). For a narrative review focusing on seven specific candidate genes of depression see Chen and colleagues (2017). Finally, for a more focused systematic review of monozygotic twin DNA methylation studies see

Palma-Gudiel and colleagues (2020). Findings from some of these studies are broadly summarized below.

Candidate Gene Studies. For candidate-gene approaches, using case-control designs, the most frequently studied genes include brain derived neurotrophic factor (BDNF), the glucocorticoid receptor gene (NR3C1), and the serotonin transporter gene (SL6A4). The most robust candidate gene has been the BDNF with the majority of studies finding hypermethylation of BDNF to be associated with depression (e.g. Chagnon, Potvin, Hudon, & Préville, 2015; Choi et al., 2015; Fuchikami et al., 2011; Na et al., 2016; Roy et al., 2017).. The BDNF gene provides the instructions for making proteins often in the brain and spinal cord and are involved in promoting the growth, maturation, and maintenance of neurons as well as regulating synaptic plasticity. There have also been consistent associations of hypermethylation of SLC6A4 (e.g. Bayles et al., 2013; Philibert et al., 2008; Shi et al., 2017; Zhao, Goldberg, Bremner, & Vaccarino, 2013) and NR3C1 (e.g. Roy, Shelton, & Dwivedi, 2017; Bustamante et al., 2016; Na et al., 2014; Nantharat, Wanitchanon, Amesbutr, Tammachote, & Praphanphoj, 2015) and depression. SLC6A4 codes for a protein that is involved in the regulation of serotonergic signaling and *NR3C1* is the receptor to which cortisol and glucocorticoids bind; both have been implicated in mood and anxiety disorders. Other, less typically studied candidate genes have also shown differences in DNA methylation in depressed individuals including oxytocin receptor (OXTR; Chagnon et al., 2015; Reiner et al., 2015), monoamine oxidase A (MAOA; Melas & Forsell, 2015), tescalin (TESC; Han et al., 2017), and synapsin II (SYN2; Cruceanu et al., 2016).

Epigenome-wide Association Studies (EWAS). In the past few years, there has been a substantial increase in EWAS depression studies. The majority of studies are

cross-sectional, use the case-control design, and study populations in Western developed countries. Most studies use whole blood sampling (Byrne et al., 2013; Davies et al., 2014; Córdova-Palomera et al., 2015; Numata et al., 2015; Uddin et al., 2011), while a small handful examined brain tissue from deceased individuals (Kaut et al., 2015; Nagy et al., 2015; Sabunciyan et al., 2012). Sample sizes range from N = 12 to 473. The number of significant hits varies widely based on methodology from zero to 115; there are no specific genes that stand out as frequent replications. This lack of overlap may be due to the complexity of the depression phenotype as well as methodological differences in study design, technology platforms, type and timing of tissue sampling, assessment of depression symptoms including different measures at different stages in the lifespan, and small sample sizes.

Almost all epigenome-wide studies utilize convenience sampling of adults, most often in middle to late adulthood, making it difficult to disentangle DNA methylation changes as causes or consequences of depression. Only two cross-sectional studies conducted an EWAS in an adolescent sample (Boström et al., 2017; Dempster et al., 2014). Dempster and colleagues (2014) utilized 18 pairs of monozygotic twins discordant for depression and did not find any differentially methylated regions (DMR) that survived corrections for multiple testing. However, in a recent study, Bostrom and colleagues (2017) found that hypomethylation of a CpG site located on the promoter region of micro RNA 4646 (MIR4646) was related to an increased risk of depression. This finding was replicated in a validation sample and in a sample of post-mortem frontal cortex tissue in deceased subjects with a history of major depression. Genes related to MIR4646 play a major role in the conversion of omega-3 fatty acids, which have been previously associated with MDD (for a meta-analytic review see Lin & Su, 2007).

There has been one small longitudinal analysis examining DNA methylation in adolescence (N=23 cases and N=36 controls, Mage = 18.6) that attempted to identify epigenome-wide associations between changes in depression risk and changes in DNA methylation levels from baseline to 1 year follow up (Ciuculete et al., 2019). After FDR correction, no significant CpG probes were identified; however, there were 9 nominally significant probes. The largest methylation difference was detected at cg24627299 within the hepatocyte growth factor receptor (*MET*) gene, a gene involved in sending signals within cells and in cell growth and survival.

Fetal Programming Studies. There are a handful of longitudinal studies of DNA methylation assessed at birth predicting outcomes later in childhood that typically focus on externalizing disorders including ADHD (Neumann et al., 2019; van Mil et al., 2014), ODD (E. D. Barker et al., 2018), conduct problems (Cecil et al., 2018) and substance use (Cecil et al., 2016); these studies typically show robust DNA methylation differences. Although there is evidence that prenatal stress predicts internalizing outcomes later in childhood, less is known about the role of DNA methylation in this pathway. A small handful of studies have examined more proximal infant behavioral phenotypes in the context of prenatal stress and DNA methylation including cortisol reactivity (Oberlander et al., 2008) and aspects of neurobehavior such as self-regulation, hypotonia, lethargy, habituation, and reflexes (Appleton et al., 2015; Conradt et al., 2013b; Monk et al., 2016) but none have assessed more specific depression symptomatology later in life. To our knowledge, this is the first study (candidate gene or EWAS) to examine DNA methylation at birth and a depression phenotype later in childhood.

Current Study

We build upon previous epigenome-wide studies of depression in the following ways: (1) Studies in behavioral epigenetics continue to operationalize depression as a categorical construct assessed at a single time point. Researchers typically create two comparison groups based on a clinically significant cut-off point of a sum score of symptoms. We were able to utilize repeated measures of depression symptoms from early to late adolescence to identify groups that are homogenous in terms of their initial levels of symptomatology at the beginning of adolescence and the slope of their symptoms over time. In addition to providing novel insights into the biological mechanisms of depression over time, the ability to reduce heterogeneity in the depression phenotype may boost power to detect epigenetic changes. (2) We examine depression in early to late adolescence at a time that is developmentally relevant in understanding the causative mechanisms in the onset of depression. Most studies examine depression in mid to late adulthood, where it is unclear whether DNA methylation changes reflect a causal mechanism or instead represent the "wear and tear" that chronic depression and its accompanying sequela including substance use and health problems has on the epigenome throughout the lifespan. (3) The longitudinal nature of this study allows us to also examine depression through the fetal programming framework where we are able to examine whether epigenetic changes present at birth can set developmental trajectories that confer risk for depression later in adolescence. Moreover, the design allows us to test whether the same genes or genetic networks are implicated in depression at different sensitive periods potentially pointing to distinct pathways by which epigenetic modifications at different points in development increase risk for depression. (4) We utilize the largest sample size in an EWAS of depression to date.

Methods

Participants

The ARIES subsample of ALSPAC was used. Final analytic samples for analyses included only participants who had both depression and methylation data available (N = 830 for prenatal analyses and N= 893 for adolescent analyses). Analyses at birth and adolescence largely included the same participants (90% match across data sets, N = 801 participants present in both sets of analyses). DNA methylation in adolescence was measured either at age 15 (n = 222, 25% of the participants) or at age 17 (n = 671, 75% of the participants). See Figure 5 for descriptive flowchart of sample.

Measures

Depression Trajectories. Depression was assessed using The Short Moods and Feelings Questionnaire (SMFQ; Angold, Costello, Messer, & Pickles, 1995), a 13-item child self-report questionnaire that enquired about the occurrence of depressive symptoms over the past 2 weeks. Scores range from 0-26. A cut-off of 11 and above has been used to describe clinically significant symptoms (Joinson et al., 2012). The SMFQ has been validated as a tool for assessing depressive symptoms in adolescence (Turner et al., 2014) and distinguishes children with depression from those who are not depressed in general population samples (Sharp et al., 2006). Previous studies done with ALSPAC data have shown that the mean SMFQ score and variability increase from childhood to adolescence (Niarchou, Zammit, & Lewis, 2015; Sequeira, Lewis, Bonilla, Smith, & Joinson, 2017). By the age of 18, 8% of the sample meets ICD-10 criteria for depression (Niarchou et al., 2015). This study uses SMFQ data collected at mean ages 12.5, 13.5, 16, and 17.5.

DNA Methylation. DNA methylation was assayed according to standard protocol described in the Introduction to the thesis (p. 19-22). In an attempt to reduce non-

biological differences between probes, samples were functional normalized using 10 principal components derived from control probes using the "meffil" package in R.

Covariates for DNA Methylation at Birth. Research has shown that a number of prenatal environmental factors impact differential DNA methylation in newborns including infant birthweight (Filiberto et al., 2011), infant gestational age or pre-term birth (H. Lee et al., 2012; Schroeder et al., 2011), and maternal age (Adkins et al., 2011). In addition to these covariates, we also controlled for maternal substance use including smoking cigarettes, marijuana use, and alcohol use. Mothers were asked about substance use at different time points throughout pregnancy.

Cigarette Use. Smoking is considered one of the most powerful environmental modifiers of DNA methylation across the lifespan. Research has shown that mother's prenatal smoking has large effects on infant DNA methylation in cord blood (Küpers et al., 2015; Lee et al., 2015). Mothers were asked how many times per day did they smoke cigarettes at three time points (first, second, and third trimester). Data from each time point was entered into the model as a separate continuous covariate.

Alcohol Use. Chronic alcohol use in pregnancy has a number of deleterious developmental effects on the fetus, often culminating in fetal alcohol syndrome (FAS). Some research has shown differential methylation profiles in infants who were born with FAS (Laufer et al., 2015; Portales-Casamar et al., 2016). However, research has shown that there are no consistent adverse developmental consequences of low to moderate alcohol use during pregnancy (see for review Henderson, Gray, & Brocklehurst, 2007). Mothers were asked to rate on a 6-point scale, how often they used alcohol ranging from "none" to "greater than 9 glasses daily" during the first and third trimesesters of

pregnancy. Data from each time point was entered into the model as a separate continuous covariate.

Marijuana Use. To our knowledge there is no research examining the effects of marijuana use on epigenetic changes in human offspring. However, numerous animal models have shown that chronic prenatal exposure to cannabinoids triggers epigenetic changes that have suppressive immunological effects on offspring (see Zumbrun, Sido, Nagarkatti, & Nagarkatti, 2015 for a review). Therefore, as a precaution, we included marijuana use as a covariate in our analyses due to its potential influence on development. Mothers were asked to rate on a 6-point scale, how often they used marijuana or any cannabis products ranging from "none" to "every day" at the same time points they were asked about cigarette smoking. Data from each time point was entered into the model as a separate continuous covariate.

Data were available regarding illicit drug use during pregnancy (e.g. cocaine, heroin). However, none of the mothers in our analytic sample indicated any illicit drug use during pregnancy. Tables 4 and 5 show descriptive statistics for EWAS analyses at birth and in adolescence, respectively broken down by depression trajectories.

Covariates for DNA Methylation in Adolescence

Substance Use. Research has shown a robust link between depression and substance use in adolescence (Armstrong & Costello, 2002; Kandel et al., 1999). Research has also shown that chronic substance use is related to individual differences in DNA methylation patterns (Parira, Laverde, & Agudelo, 2017; Rotter et al., 2013). Therefore, we included cigarette smoking and marijuana use as covariates. Covariate time points were matched with available DNA methylation time points. For example, adolescents with methylation data collected at age 15 had corresponding covariates

measured at age 14.5 years, while adolescents with DNA methylation data collected at age 17 had corresponding covariates measured at 16.5 years. At age 14.5 and 16.5, adolescents were asked about the frequency of their cigarette smoking ranging from "I have only ever tried smoking cigarettes once or twice" to "I usually smoke one or more cigarettes every day." At the same time points, adolescents were asked about the frequency of their marijuana use similarly ranging from "I have only ever tried cannabis once or twice" to "I usually use or take cannabis every day."

Data regarding illicit drug use was available but was focused on whether or not an adolescent had experimented with drugs and did not reflect problematic or chronic use. Data regarding alcohol use was only measured at age 14.5 years and not at 16.5 years. Because the majority of our sample had DNA methylation data at age 17, alcohol use was not included as a covariate.

Data Analysis Plan

The subsequent three chapters will describe the methods, results, and discussion points for (1) latent class growth curve modeling of depression trajecotries in MPLUS (2) epigenome-wide analyses of depression trajectories and gene annotation, and (3) follow-up regional and gene network analyses.

CHAPTER 6: A Longitudinal Epigenome-wide Analysis of Depression Trajectories in Adolescence (Part 2)

Latent Class Growth Curve Modeling of Depression Trajectories

Methods

Due to the heterogeneous nature of the course of depression over time, we utilized a latent class growth curve model in MPLUS to extract homogenous subgroups of adolescents with distinct developmental trajectories of depressive symptoms. We

utilized the full ALSPAC sample (N = 15,445) to retain power and controlled for sex. It is well-known that females are at twice the risk for developing depression compared to males. Therefore, it is likely that sex has significant effects on the growth factors of the model including the intercept and slope as well as trajectory classification (i.e. classification into one trajectory class over another may be due to sex and not depression score). Only participants who had depression data available from at least one time point were included in the analysis (N = 8,360). Mplus handles missing data by the standard approach of Missing At Random (MAR) under Maximum Likelihood (ML). This means that it uses all the data that is available to estimate the model using full information maximum likelihood where each parameter is estimated directly without filling in missing data values for each individual (Muthén & Muthén, 1998). Previous research deriving depression trajectories from the ALSPAC cohort has demonstrated little difference on the shape of trajectories, distribution of trajectory membership, or associations of trajectories with outcomes when comparing individuals with at least 1 measurement of depression symptoms with participants with at least 3 or more measures (Kwong et al., 2019).

Following guidelines in the field based on simulation studies (Nylund et al., 2007), number of classes were determined by the following fit indices: Lo-Mendell-Rubin adjusted likelihood ratio test (LMR-LRT) and the bootstrap likelihood ratio test (BLRT), where significant p-values prompt the rejection of the k-1 model in favor of the K-class model. Other considerations also included model convergence, lower Bayesian Information Criterion values, higher entropy values (near 1.0), no less than 1-2% of participants in a class, and higher posterior probabilities (values > 70% indicating good model fit) (Jung & Wickrama, 2008).

Extracting Depression Class Trajectories

Class solutions were examined in a sequential order starting from a two-class structure. The two-class model indicated a "low" (*N* = 7087; 85%) and a "high" (*N* = 1273; 15%) depression group (LMR-LRT, *p* = 0.000; BLRT, *p* = 0.000; Entropy = 0.83; BIC=296434.645). Because significant LMR-LRT and BLRT values prompt the rejection of the k-1 model in favor of the k model, this meant that a two-class solution was a better model fit than a one-class solution. The three-class model indicated a "low" (*N* = 6732; 77%), "increasing" (*N* = 949; 14%), and "moderate/decreasing" (*N* = 679; 9%) group (LMR-LRT, *p* = 0.000; BLRT, *p* = 0.000; Entropy = 0.80; BIC = 248937.648). Again, significant p-values indicated that a three-class solution was a better model fit that a two-class solution. The four-class model yielded a "low" (*N* = 6350; 76%), "increasing" (*N* = 775; 9%), "moderate/decreasing" (*N* = 978; 12%), and always high (*N* = "257"; 3%) group (LMR-LRT, *p* = 0.000; BLRT, *p* = 0.000; Entropy = 0.79; BIC = 294141.890). Although the four-class model converged and p-values were significant, the results were unreliable due to solutions being local maxima that did not resolve with increased random starts. In MPLUS, the estimation algorithm attempts to converge on the globally best solution with the largest loglikelihood – one set of parameter values. However, sometimes it converges on a local maximum solution, which is the best solution around the parameter, but not the best one. This coupled with increased BIC value, decreased entropy, only 3% of participants in one class, and posterior probabilities dropping to 70%, the three-class solution was chosen as the most reliable model fit for the analyses. Depression trajectories were classified on the entire ALSPAC sample. See Figure 6 for visual representation of depression trajectories. When applied to the ARIES DNA methylation subset, the final analytic sample retained similar class proportions. In the

birth EWAS, 80% of participants were classified in the low trajectory (n = 662), 11% in the increasing trajectory (n = 94), and 9% in the moderate/decreasing trajectory (n = 74). In the adolescent EWAS, 80% of participants were classified in the low trajectory (n = 720), 11% in the increasing trajectory (n = 77), and 9% in the moderate/decreasing trajectory (n = 64).

There is a vast literature examining longitudinal depression trajectories in adolescence. Using various trajectory modeling techniques, the number of unique class trajectories range from 3 to 6 with usually a consistent "low" group and variations of "increasing", "high", "moderate", and "decreasing" groups (for a review see Schubert et al., 2017). In a recent meta-analysis examining 20 longitudinal studies published in the past 20 years, a random pooled effect estimate identified a consistent "no or low" group (56% of the sampled study populations), a consistent "moderate" group (26%) and a variations of "high", "increasing" or "decreasing" groups (12%) (Shore et al., 2017). Regarding the ALSPAC cohort specifically, the number and types of depression class trajectories has also varied. Using 7543 adolescents with data between 10.5 years and 18.5 years of age, 3 trajectory classes were identified using a dichotomous depression score (i.e. using the SMFQ clinical cut-point of 11): "persistently low" (74%), "later-adolescent onset" or "increasing" group (17%) and an "early-adolescence onset" or "stably moderate/high" group (9%) (Frances Rice et al., 2002). These trajectory groups closely match our classifications. On the other hand, using a sample of 3525 ALSPAC individuals with measurements extending into adulthood (i.e. mean age 24), 5 trajectories were identified: "low" (71%), "early-adult-onset" (11%), individuals who started with low depression symptoms that increased during adolescence and young adulthood, "adolescent-limited" (9%), individuals who experienced elevated levels of depression

symptoms only during adolescence, “childhood-limited” (6%), individuals who started with elevated levels of depression symptoms in childhood that decreased. and “childhood-persistent” (3%) individuals with moderate levels of depression symptoms that continued to increase and stay high during adolescence and into young adulthood (Kwong et al., 2019). Although there are differences likely due to the result of extending measurements well into young adulthood, Kwong and colleagues’ results are still broadly comparable to the three-class solutions.

Describing the Depression Class Trajectories.

Descriptive statistics for the depression trajectories for the analytic sample for the birth EWAS and for the adolescence EWAS sample were near identical and are discussed here more broadly. See Tables 4 and 5 for more specific descriptive statistics of depression trajectories separated by EWAS analytic sample. The majority of the increasing trajectory (70%) and the moderate/decreasing trajectory (around 65%) was made up of females. Females made up less than half of the low trajectory group (around 48%). For the low depression trajectory, mean depression scores (i.e. SMFQ; total 26, clinical cut-off 11) stayed in the 2-6 range across all 4 times points. For the increasing trajectory, mean depression scores started low (M = ~ 5), increased by age 13 (M = ~10), and continued to increase into late adolescence (M= ~15). For the moderate trajectory, mean depression scores were clinically significant at age 12 (M= ~13) and slightly decreased in later adolescence to be below the clinical cut-point (M= ~9).

An ANOVA with Tukey HSD was used to assess any potential mean differences in covariates across the three trajectory groups. For the birth analytic sample, the three trajectories did not significantly differ on most covariates including gestation length, maternal age at birth, birth weight, mother’s alcohol use and mother’s smoking during

pregnancy. Individuals in the increasing trajectory had mothers with increased use of marijuana, though this was only a significant difference compared to the low group and only in the 3rd trimester.

For the adolescent analytic sample, individuals in the increasing and moderate trajectories had significantly higher levels of adolescent cigarette smoking compared to the low group. Individuals in the increasing and moderate trajectories had higher levels of adolescent marijuana use compared with the low group, though differences were only significant in the moderate vs. low group. Results regarding cigarette smoking are consistent with a robust literature describing increased smoking by depressed adolescents and adults (see reviews e.g. Chaiton et al., 2009; Fluharty et al., 2017; Lee & Pausova, 2013; Weinberger et al., 2017) with less robust results describing increased marijuana use (Brook et al., 2011; Passarotti et al., 2015)

CHAPTER 7: A Longitudinal Epigenome-wide Analysis of Depression Trajectories in Adolescence (Part 3)

Epigenome-Wide Analyses (EWAS) of Depression Trajectories

EWAS Methods

For our EWAS analyses, we were particularly interested in the increasing trajectory as those individuals show a dramatic increase in depression symptoms from very low during pre-adolescence to more severe levels by age 18. In order to test whether youth whose symptoms of depression increased across adolescence were biologically distinct from those with stable low or moderate symptoms, we compared patterns of DNA methylation (1) at birth and (2) in adolescence for the increasing versus low trajectories and for the increasing versus moderate trajectories. In order to increase power in our EWAS analyses, we also combined the increasing and moderate/decreasing trajectories to form a

high group and compared patterns of DNA methylation to the low group. Thus, we conducted 6 EWAS in total. In addition, as supplementary analyses, we also ran a more "traditional" case-control design EWAS using depression at a fixed time point (age 17.5). We used the clinically significant SMFQ cut point of 11 to categorize participants into depressed (N = 173) and non-depressed groups (N = 567) and compared patterns of DNA methylation at birth and in adolescence.

Due to the large size and subsequent processing burden of the data (i.e. 485,000 data points per individual for 1,000 individuals across 2 time points), initial data cleaning and variable derivation was conducted using the University of Pennsylvania School of Arts and Sciences high performance computing cluster in which "jobs" were submitted using Linux code to run on multiple computers simultaneously. Once data were cleaned and processed, EWAS analyses were able to run on a personal computer. EWAS analyses were performed using a general linear model using the 'CpG assoc' package implemented in R. All analyses controlled for sex, cell type proportion (as described in the Introduction), and sample ID for additional batch effect controls. As described in the Introduction, probes known to be polymorphic or cross-reactive were removed prior to analysis. In addition, participants with non-Caucasian or missing ethnicity (based on self-reports, $n = 28$) were removed prior to the analysis to control for race/ethnicity effects on DNA methylation. Analyses using DNA methylation at birth included the following covariates that potentially have effects on DNA methylation levels in cord blood: infant birthweight, mother's age, infant gestation length, mother's alcohol, marijuana, and cigarette use during pregnancy. Analyses using DNA methylation in adolescence included the following covariates: adolescent marijuana and cigarette use.

An EWAS essentially entails conducting hundreds of thousands of linear regressions at one time. If 485,000 CpG sites are being interrogated, around 1/20 or 20,000 can be false positives (i.e. significant hits that are significant by chance and not due to underlying biological differences) due to Type 1 error if using traditional $\alpha = 0.05$ thresholds. The multiple comparison problem in epigenome-wide analyses is most often addressed through Benjamini & Hochberg's (1995) false discovery rate (FDR) correction, which estimates and controls for the proportion of false positives in an analysis. We utilized the FDR correction in all our analyses ($q < .05$). Because DNA methylation analyses yield very small effect sizes, statistical power to detect those small effects is always a concern. It is likely that low statistical power results in some number of relevant CpG hits that do not cross the threshold of significance after FDR correction. Therefore, many researchers report hits that are approaching significance. We used the most liberal genome-wide threshold proposed in the literature ($\alpha = 10^{-6}$) to identify hits we label as nominal (Rakyan et al., 2011). Hits below this liberal threshold are less likely to be true hits not detected due to power concerns. Once FDR-corrected differentially methylated CpG sites or "hits" are identified, individual linear regressions are performed comparing mean methylation levels on that CpG site between trajectory groups to extract more specific regression statistics including standardized betas, standard error, and adjusted R values. CpG hits are then mapped to their respective gene sites through extensive probe annotation available from Illumina that includes probe location within genes (annotated by University of California, Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu; UCSC Genome Bio- informatics, Santa Cruz, CA, USA), CpG islands and shores, and regulatory features.

As with genome-wide association studies, epigenome-wide statistical inflation resulting in increased rates of false positive is also of concern. High genomic inflation is thought to be caused by population stratification, technical batch effects, sample quality, and unknown relatedness between samples (Devlin & Roeder, 1999). Usually, the inflation is quantified using the genomic inflation factor (λ), which is defined as the ratio of the median of the empirically observed distribution of the test statistic to the expected median. Therefore, the lambda is used to calculate the deviation of a distribution of residuals from a null distribution. A genomic inflation factor of 1.0 or lower reflects no evidence of inflation while increasing values reflect inflation. Researchers also often visually inspect quantile-quantile (QQ) plots that are able to graph the deviations of the observed distribution from the expected null distribution.

Results: DNA Methylation at Birth.

High Versus Low Trajectories. At birth, no probes were differentially methylated

between the high (increasing + moderate) versus low trajectories after FDR correction, nor were any probes approaching significance.

Increasing Versus Low Trajectories. At birth, no probes were differentially methylated between the increasing and low trajectories after FDR correction when controlling for smoking, alcohol, and marijuana use during pregnancy as well as birth characteristics, sex, cell type, and plate number. One probe, cg08214693 was approaching significance but still did not meet nominal significance cut-offs. Cg08214693 was hypomethylated in the increasing trajectory and was annotated to SCRIB (scribbled planar cell polarity protein). See Table 6 for more details. Absolute mean percentage methylation between the increasing and low trajectory groups was

1.3%. Inspection of the QQ plot (Supplementary Figure 4) and a lambda statistic of 0.79 provided little evidence of inflation of test statistics. See Figure 7 for manhattan plot of EWAS.

Increasing Versus Moderate Trajectories. At birth, no probes were differentially methylated between the increasing versus moderate trajectories after FDR correction, nor were any probes approaching significance.

Supplementary Analyses. When examining depression at a single time point at age 17.5, there were no probes differentially methylated between the depressed and non-depressed groups after FDR correction, nor were any probes approaching significance.

Results: DNA Methylation at Adolescence

High Versus Low Trajectories. In adolescence, one probe was differentially methylated between the high (increasing + moderate) and low trajectories after FDR correction when controlling for covariates (See Table 7 for more details). Cg06758781 was hypomethylated in the high group (q = 0.02) and was annotated to AACS (Activates acetoacetate to acetoacetyl-CoA). Inspection of the QQ plot (Supplementary Figure 5) and a lambda statistic of 0.856 provided little evidence of inflation of test statistics. See Figure 8 for Manhattan graph of results.

Increasing Versus Low Trajectories. In adolescence, one probe was differentially methylated between the increasing and low trajectories after FDR correction when controlling for smoking, marijuana use, cell type, sex, and plate number while 6 probes were approaching significance (See Table 8 for more details). Cg06460328 was hypermethylated in the increasing group (q = 0.030) and was annotated to CBFA2T3 (CBFA2/RUNX1 translocation partner 3). Absolute mean percentage methylation between the increasing and low trajectory groups was 2%. All 6 nominal hits

were also hypermethylated in the increasing group with methylation differences ranging from 1% - 2.5%. See Table 8 for further details. Inspection of the QQ plots (Supplementary Figure 6) and lambda statistic of 1.09 provided evidence of some mild inflation of test statistics. See Figure 9 for Manhattan graph of results.

Increasing Versus Moderate Trajectories. In adolescence, no probes were differentially methylated between the increasing versus moderate trajectories after FDR correction.

Supplementary Analyses. When examining depression at a single time point at age 17.5, there were no probes differentially methylated between the depressed and non-depressed groups after FDR correction, nor were any probes approaching significance.

Follow-up Regional Analyses

Data Analysis Plan

EWAS studies typically interrogate DNA methylation at the individual CpG level. Although this is very informative, it does not take into account the broader context of the DNA methylation status of its neighboring CpG sites, as sometimes a gene is not turned on and off by the action of a single CpG site but rather a cluster of CpG sites in close proximity to one another through co-methylation. Results have shown moderate levels of correlations (0.25-0.40) in proximal CpG sites up to a distance of 1kb apart and no significant correlations once inter-pair distances reach around 2kb (Saffari et al., 2018).

Therefore, researchers have started to additionally interrogate differentially methylated regions (DMRs) as complementary analyses to EWAS. This region-based approach is statistically more powerful with a lower rate of false positive findings and has the potential to be more biologically informative than individual CpGs. Many bioinformatic packages to interrogate DMRs are available. We utilized the DMRcate

package in R (Peters et al., 2015), which identifies and ranks the most differentially methylated regions across the genome. It is a data-driven agnostic approach that does not favor known annotated genomic regions (i.e. known CpG islands) and has the ability to assess all 450k probes.

First, estimates of differential methylation at individual CpG sites are derived using the limma package in R (this package is similar to CpGassoc used for the single CpG site EWAS). Identical covariates to the EWAS were used in all regional analyses. The corresponding t-statistic obtained with each probe's beta value is utilized in the DMR-finding function to which a Gaussian kernel smoothing method is applied. A kernel smoother is a statistical technique used to estimate the real value of a function as the weighted average of neighboring observed data. The weight is defined by the kernel, meaning that closer points are given higher weights. A Gaussian kernel is a kernel with the shape of a Gaussian or normal distribution curve. The length of the nucleotide region can be user specific, though the authors of the package suggest a bandwidth of 1000 nucleotides (lambda = 1000) and a scaling factor of 2 ($C = 2$; at least 2 CpG sites in a region). Significant p-values were again FDR-corrected for multiple testing. Data output consists of significant regions ranked by their corresponding p-values as well as genomic coordinates and gene associations.

Results: Regional Analyses with DNA Methylation at Birth

Using DNA methylation analysed at birth, no regions were differentially methylated in either high (moderate + high) versus low, increasing versus low, or increasing versus moderate analyses.

Results: Regional Analyses with DNA Methylation in Adolescence

High Versus Low Trajectories. In adolescence, regional analyses identified 1 differentially methylated region using the fully adjusted model (q <0.05). See Table 9. This region was mapped to the Small Nucleolar RNA (SnoRNA) family of non-coding RNAs.

Increasing Versus Low trajectories. In adolescence, regional analyses identified 3 differentially methylated regions using the fully adjusted model (q <0.05). See Table 10. These regions were mapped to Zinc Finger and BTB Domain Containing 44 (ZBTB44), Bladder Cancer-Associate Protein (BLCAP), and the Small Nucleolar RNA (SnoRNA) family of non-coding RNAs.

Increasing Versus Moderate Trajectories. No regions were differentially methylated between the increasing and moderate trajectories.

Discussion of Genes Implicated in EWAS and Regional Analyses

Utilizing the ALSPAC longitudinal cohort to extract latent class trajectories of depression symptoms, we conducted EWAS analyses using DNA methylation obtained at birth and during mid-adolescence. To examine potential fetal programming effects, we assessed whether DNA methylation patterns present at birth differentiated the increasing trajectory class compared to the low and moderate/decreasing trajectory classes. In our birth EWAS analyses, the closest hit to significance (q = .074) was annotated to the SCRIB gene and hypomethylated in the increasing group compared to the low depression group. The SCRIB gene is a scaffold protein that is part of a pathway of genes called the Scribble complex that is involved in cell migration, cell polarity (i.e. spatial differences in shape, structure, and function of a cell), and cell proliferation in epithelial cells (Anastas et al., 2012). Loss of cell polarity is a hallmark of epithelial cancers and therefore regulators of polarity are hypothesized to play a major role in suppression of

tumorigenesis. Recent studies have shown that increased expression on SCRIB is related to adverse clinical outcomes in breast cancer and that reducing its expression reduced the growth of human breast cancer cells (Anastas et al., 2012). A recent EWAS of depression symptomatology measured in an elderly cohort of 742 monozygotic Danish twins, also identified 3 CpG sites mapped to the SCRIB gene in regional analyses that were differentially methylated in the depressed group (Starnawska et al., 2019). Interestingly, the SCRIB gene was not in our top 50 hits in our EWAS using DNA methylation in adolescence. Results of this hit are presented for informational purposes. However, this CpG site did not reach even more liberal nominal level of significance and therefore should be interpreted with caution.

Although research on externalizing disorders such as ADHD, conduct problems, and ODD (Barker et al., 2018; Cecil et al., 2018; Neumann et al., 2019; van Mil et al., 2014) have shown robust association with DNA methylation at birth, much less is known about fetal programming effects of internalizing disorders To our knowledge, this was the first study to assess whether DNA methylation profiles at birth can predict depression outcomes later in development. Interestingly, our analyses did not show any potential fetal programming effects. It is possible that prenatal risks may be embedded more broadly as traits and predispositions (i.e. emotional reactivity) that may be exacerbated by the psychological, behavioral, and physiological consequences of the trait including emotion dysregulation, difficulty in social relationships, and unhealthy coping behaviors throughout childhood. This can culminate in development of depression in adolescence triggered by more specific developmental demands during this sensitive period, which includes a multitude of neural and hormonal changes. This is supported by the fact DNA methylation studies examining prenatal stress and more trait-like features in infancy find

robust results (Appleton et al., 2015; Conradt et al., 2013b; Monk et al., 2016), and the fact that the prevalence rates of depression in childhood is rare and increases dramatically in adolescence (Hankin et al., 2015b). In addition, disorders like ADHD and ODD manifest much earlier in childhood, while the incubation period for depression is much longer, and therefore may include more downstream cascading effects that are unmeasured. More research is needed to elucidate the potential fetal programming effects that underlie internalizing disorders via DNA methylation mechanisms.

Given the dearth of EWAS studies of depression occurring in adolescence, we also examined whether DNA methylation in mid-adolescence predicted depression trajectory groups. When collapsing the two clinically significant depression groups (increasing and moderate/decreasing) into one "high" category and comparing it to the low group for a more powerful analysis, one CpG hit annotated to the AACS gene was significant even after correction for multiple testing and hypermethylated in the "high" group. No other depression EWAS has identified this gene. AACS is hypothesized to be involved in utilizing ketone body (i.e. energy source that is mainly produced in the liver) for fatty acid-synthesis during adipose tissue development (Hasegawa et al., 2012). High-fat diet obesity has been shown to induce unusual metabolism of ketone bodies through inflammatory mechanisms (Puchalska & Crawford, 2017). Research on the expression of AACS is scant and exclusively has been assessed in animal models. Interestingly, one study has shown increased expression of AACS as a result of inflammatory mechanisms due to a high fat diet (Yamasaki et al., 2016)

Our results did not show DNA methylation differences between the increasing and moderate trajectory. There was one CpG site that was nominally significant but not

annotated to a specific gene and therefore the function of this differential methylation in largely unknown.

When comparing the increasing trajectory group to the low group, one CpG hit annotated to the CBFA2T3 gene was significant even after correction for multiple testing and was hypermethylated in the increasing group. No other depression EWAS has identified this gene. The CBFA2T3 gene is responsible for transcription repression. Research has shown that the expression of CBFA2T3 was significantly reduced in breast cancer cell lines and breast tumors and CBFA2T3 has emerged as a candidate gene of breast cancer tumor suppression (Kochetkova et al., 2002; Kumar et al., 2006).

Additionally, there were 6 CpG hits that were nominally significant ($p < 1 \times 10^{6}$) that were annotated to 6 genes: LRTOMT (Leucine Rich Transmembrane And O-Methyltransferase Domain Containing), NUMA1 (Nuclear Mitotic Apparatus Protein 1), LDB1 (LIM Domain Binding 1), USF2 (Upstream Transcription Factor 2, C-Fos Interacting), HEY2 (Hes Related Family BHLH Transcription Factor With YRPW Motif 2), and BLCAP (BLCAP Apoptosis Inducing Factor). Not much is known about the specific biological mechanism of LRTOMT except that it is an O - methyl transferase heavily implicated in the morphology and physiology of ear development and its mutation has been consistently studied as leading to autosomal recessive non-syndromic hearing loss (i.e. non-specific genetic hearing loss) (Charif et al., 2012; Taghizadeh et al., 2013; Vanwesemael et al., 2011). NUMA1 is also an understudied gene that codes for the spindle protein NuMA; spindle fibers form a protein structure that divides the genetic material in a cell (Quintyne et al., 2005). Only one study has assessed expression of NUMA1 directly and linked increased expression to epithelial ovarian cancer due to its role in aneuploidy (i.e. presence of an abnormal number of chromosomes in a cell, as

often seen in cancer cells) (Brüning-Richardson et al., 2012). However, more research needs to be done to elucidate whether NUMA1 is consistently involved in carcinogenesis.

LDB1 is a transcription cofactor (i.e. modulates the effects of transcription factors by recruiting other proteins for binding). It is part of a complex that maintains the function of erythroid cells (i.e. most common blood cell and principal means of delivering oxygen to the body tissues via blood flow through the circulatory system) through transcription activation (Matthews & Visvader, 2003). Due to its role in modulation of transcription factors it has been studied in the context of cancer progression. Studies have shown that overexpression of LDB1 is associated with negative prognosis factors in colorectal, head, and neck cancer (García et al., 2016; Simonik et al., 2016).

USF2 (and its counterpart USF1) are transcription factors (i.e. proteins that bind to the DNA sequences of their target genes and participate in the regulation of a large number of genes) and part of the basic helix-loop-helix (bHLH) class. USF2 in particular appears to be crucial for embryonic development, brain function, metabolism, iron homeostasis and fertility while USF1 has more specific roles in metabolism and immune system functioning (Horbach et al., 2015). Recent research suggests that deregulation of transcription factors can cause tissue damage and suggests a major role for transcription factors in the inappropriate growth of cancer cells. In fact, the USF genes seem to exhibit a tissue protective and tumor suppressive function in several cancer types (Horbach et al., 2015). Most consistently, downregulation of USF2 has been linked to the proliferation of breast and prostate cancer (Chen et al., 2006; Ismail et al., 1999; Kivinen et al., 2004; Tan et al., 2019). Similarly to USF2, HEY2 is a bHLH transcription factor, involved primarily in the regulation of cell differentiation of the cardiovascular system and the heart itself

(Iso et al., 2002). Increased expression of HEY2 has been linked to the progression of prostate, liver, and pancreatic cancer (Cavard et al., 2009; Tradonsky et al., 2012; Wu et al., 2016). One

EWAS comparing 20 medication free patients with MDD and 19 control subjects (Mage = 44) found 363 differentially methylated CpG sites after FDR correction for multiple testing, one of which was annotated to HEY2. However, it is important to note that the study did not correct for cigarette smoking and most importantly did not control for cell-type heterogeneity which greatly limits our ability to identify this as a true replication.

Finally, the BLCAP gene encodes a protein that reduces cell growth by stimulating apoptosis (i.e. cell death) and is hypothesized to play a major role in the regulation of tumor cell proliferation and survival. Studies have shown that decreased expression of the BLCAP gene was associated with the progression of cervical, renal, bladder, and tongue cancer tissue, and conversely increased expression in breast cancer tissues (Gromova et al., 2012). This gene was also implicated in regional analyses (6 CpG sites) comparing the increasing to low trajectory group suggesting that it may be the most consistent finding in our EWAS though it only reached nominal significance in the single CpG site analysis.

In addition to BLCAP, two other differentially methylated regions were identified in regional analyses. Two CpG hits were identified on the ZBTB44 gene. Not much is known about the ZBTB44 gene, except that it is primarily involved in transcription regulation and nucleic acid binding (www.genecards.org). Four CpG sites were identified in a region composed of several small nucleolar RNAs (snoRNAs). snoRNAs are a type of non-coding RNA (i.e. RNA transcripts that never get translated to protein and consequently are never expressed). For a long time, non-coding RNA have been

considered as cellular housekeeping maintenance molecules or “junk DNA” due to their non-coding nature. However, recent research has demonstrated that in addition to DNA methylation and histone modification, non-coding RNAs are essential mechanisms of epigenetic changes and function as post-transcriptional modulators of gene expression, especially during development and disease progression (Peschansky & Wahlestedt, 2014; Watson et al., 2019; Wei et al., 2016). snoRNAs are crucial for ribosomal RNA (rRNA) maturation and functionality (Gaviraghi et al., 2019). Because hyperactive ribosomal biogenesis is widely observed in cancer, an increasing body of work has linked increased expression of snoRNAs to renal, colorectal, lung, prostate, and breast cancer (Baral et al., 2018; Gao et al., 2015; Mannoor et al., 2014; Martens-Uzunova et al., 2015; Okugawa et al., 2017; su et al., 2013).

CHAPTER 8: A Longitudinal Epigenome-wide Analysis of Depression Trajectories in Adolescence (Part 4)

Gene Networks and Functional Enrichment Analyses

Methods

After decades of genomic research, it is now best understood that genes often act in concert with each other rather than in isolation. It has been increasingly apparent that the function of a single gene cannot explain genetic liability for phenotypically complex biomedical and psychological disorders that are most likely the result of polygenic interactions. A list of differentially methylated genes or regions, although informative, does not describe how genes may be acting in unison with each other and other unidentified genes to confer risk. To assess whether the genes identified in our analyses are related to one another, we inputted them in a bioinformatic webserver called GeneMANIA (http://www. genemania.org). GeneMANIA mines all publicly available

biological datasets of all mapped genes in the human genome to create genetic networks. Researchers can input a list of genes into the server to examine any possible connections between genes using this comprehensive literature review. Gene networks are compiled based on several categories including: (1) co-expression; genes are linked if their expression levels are similar across conditions in gene expression studies, (2) physical interaction; genes are linked if their proteins are known to physically interact, (3) gene interaction; genes are linked if the expression of one gene depends on the presence/absence of another, (4) shared protein domains; genes are linked if they code for the same protein domain, (5) co-localization; genes are linked if they are both expressed in the same tissue or if their gene products are both identified in the same cellular location, (6) pathways; genes are linked if they participate in the same reaction within a pathway, and finally (7) predicted; genes are linked if they have similar functional mechanisms. Once a list of genes is inputted into the server, a network is created based on those seven categories. The weighting of connections between genes is chosen automatically using linear regression to make genes on the inputted list interact as much as possible with each other, and as little as possible with genes not in the inputted list. The network weighting prioritizes gene-ontology, where genes are connected based on similar biological functions. For more detailed description of network bioinformatics see Mostafavi et al., 2008 and Warde-Farley et al., 2010.

In order to better understand the underlying biological processes of the gene network, GeneMANIA also provides functional enrichment analyses using Gene Ontology (GO) categories. Similarly, GeneMANIA mines the GO database, which is a bioinformatics initiative to categorize a vocabulary of known genes and their products into an organized graph structure describing what is known about the biological function

of known genes. GeneMANIA compares the functional profile (i.e. biological pathway describing the cellular or physiological role) of the inputted gene set to the functional profiles described by GO to examine whether the network is significantly enriched for particular functions. Functional enrichment analyses also use the FDR ($q < .05$) correction for multiple testing. GO analyses provide the number of genes in the network that are implicated in a biological process out of the total number of genes identified in that process in the literature (i.e. coverage).

The comparison of DNA methylation values in adolescence in the increasing versus low trajectories produced multiple hits that were annotated to multiple genes (1 FDR corrected significant, 6 nominally significant). We ran gene network and functional enrichment analyses using genes obtained from these comparisons. snoRNAs are not genes and therefore were not inputted in the analysis. In exploratory analyses, we wanted to examine the addition of AACS, a significant hit in the combined "high" versus low trajectory groups to assess whether it is biologically connected to those genes annotated in the increasing versus low trajectory group analyses.

Results: Description of the Gene Network

Results of inputting annotated genes into the network analysis showed that rather than acting in isolation, these genes form a compact cluster network based on known genetic and physical interactions, shared pathways and protein domains as well as protein co-expression data. See Figure 10 for visual representation of the gene network. In gene ontology analyses, the most enriched gene ontology biological functions were related to sequencing specific DNA binding, bhlh transcription factor binding, and cardiac development, which corresponds to our literature review of annotated genes. See Table 11 for comprehensive gene ontology descriptions.

Interestingly, ZBTB47, a gene identified in regional analysis but not the EWAS, emerged as part of this network though a physical reaction with CBFA2T3. LRTOMT and NUMA1 were only tangentially related to the network while CBFA2T3, HEY2, USF2, BLCAP, and LDB1 formed a tighter "cancer gene" cluster. This makes sense as the biological function of LRTOMT and NUMA1 is still largely unknown. Because network analyses use existing data to create connections between genes, this does not necessarily mean that LRTOMT and NUMA1 are unrelated to this cancer network, but rather more research is necessary to elucidate their function. LRTOMT and NUMA1 were related through co-expression which is consistent with our data as a single CpG hit was annotated to both genes suggesting they are in close proximity. They are connected to the network through NUMA1 and LDB1 co-expression, which suggests that they are potentially biologically relevant to the cancer network. Interestingly CBFA2T3, the only FDR corrected significant hit, appeared to be central to the 5-gene cancer network and was connected to USF2, BLCAP, LDB1 through predicted connections. HEY2 is connected to the network through genetic interactions with BLCAP and USF2, which makes sense as HEY2 and USF2 both regulate transcription factors.

In exploratory analysis, we assessed the role of AACS, the FDR corrected significant hit from analyses comparing the combined "high" group to the low trajectory group, in the gene network. AACS did not appear to be relevant to the cancer gene network and was only related to CBFA2T3 through a small genetic interaction (See Figure 11). The fact that AACS was not part of the cancer gene network associated with the increasing trajectory could suggest that epigenetic correlates of depression that increases throughout adolescence are distinct from epigenetic correlates of symptoms of depression that are moderately high throughout adolescence (as seen in the combined

increasing and moderate/decreasing groups). On the other hand, there is much less research on the biological function of AACS compared to other genes in the network and AACS may be more centrally implicated than these analyses show.

Broader Discussion of EWAS, Regional, and Gene Network Analyses

Overall, our results demonstrated a link between genes implicated in cancer genesis and progression and individuals who demonstrated increasing levels of depression symptoms as they progressed through adolescence. The prevalence of depression in patients with various types of cancer exceeds that observed in the general population and is associated with a poorer prognosis and higher mortality rate Pasquini & Biondi, 2007). This of course makes intuitive sense; individuals faced with a life-threatening illness and painful treatment would be more likely to develop depression as a result. However, there is a body of research that suggests that increased depression prevalence is not solely a reaction to the socioemotional and physical stress after a cancer diagnosis and posits that there may be a bi-directional relationship between depression and cancer with common underlying pathophysiology.

Whether depression earlier in life can be conceptualized as a risk factor for developing cancer later on has long been debated. Several large-scale longitudinal epidemiologic studies have reported significant associations between depression symptoms and subsequent development of cancer (Dalton et al., 2002; Penninx et al., 1998), and some have not (Kaplan & Reynolds, 1988; Zonderman et al., 1989). A meta-analysis using eight longitudinal, population-based studies found a small but significant increased risk for cancer among depressed individuals (relative risk = 1.19) (Oerlemans et al., 2007). The most dominant theory of shared underlying pathophysiology is chronic inflammation, followed by lesser studied hypotheses of malfunctions in DNA repair.

Chronic inflammation propagates increased wear and tear on several biological systems in the body impairing their functions. In terms of cancer, impaired functioning of immune cells, most specifically, natural killer (NK) cells creates a immunosuppressive environment that promotes tumor growth; it has now been evident that an inflammatory microenvironment is an essential component of all types of tumors (Baniyash et al., 2014; Grivennikov et al., 2010). Because pro-inflammatory responses over-activate the HPA, the main mechanism through which cortisol shuts down and inflammatory response, chronic inflammation has been widely studied as both a result of and precursor to depression (Miller et al., 2009; Moriarity et al., 2020; Raison & Miller, 2011, 2013; Su, 2012). For example, one meta-analysis found that chronic inflammation preceded the development of depression even after controlling for a wide range of factors associated with risk for depression (Valkanova et al., 2013).

It has also been hypothesized that psychosocial stress may have a negative impact on DNA repair and cell apoptosis which leads to the initiation and production of abnormal cells, a primary drive of induction of tumor growth and spread (Kiecolt-Glaser et al., 2002). Stress may decrease the ability of DNA repair enzymes, like methyltransferase, in carrying out maintenance tasks of tumor suppression.

Our pattern of results potentially supports both of these two theorized biological mechanisms in different ways. The implication of the AACS gene (with a theorized critical function in adipose tissue development and consequently obesity) when comparing individuals with any depression in adolescence compared to individuals with none suggests that underlying inflammatory mechanisms may differentiate the two groups broadly. This is supported by a vast literature demonstrating the link between obesity and depression (for several reviews and meta-analyses see: (Atlantis & Baker,

2008; Blaine, 2008; de Wit et al., 2010; Luppino et al., 2010). This pattern of results may be due to the addition of the moderate/decreasing group whose sustained high levels of depression even pre-adolescence may better predict or reflect more chronic activation of the immune system. However, it is important to note that no other genes directly related to the immune system were implicated suggesting more research needs to be done on the function of AACS in the context of depression.

When examining the increasing group specifically, no genes directly implicated in inflammatory processes were identified and instead a cluster of genes heavily implicated in cancer genesis emerged. Many of the biological function of those genes were DNA repair and apoptosis and several have been identified as tumor suppressing genes. However, these results should not serve as a definite evidence for the implication of cancer related processes underlying the development of depression. The relationships between inflammation, DNA repair processes, depression, and cancer are likely to be infinitely complex as both disorders involve the maladaptive disruption of multiple biological systems and can reflect a wide range of risk factors. In addition, our analyses used peripheral blood to obtain DNA methylation levels and most studies assessing expression of our identified genes used target tissues (colon, pancreas, breast). DNA methylation patterns are tissue-specific (see broader discussion on page 78) and therefore we cannot make definitive conclusions on the concordance of DNA methylation and expression of these genes in the blood versus in target tissue during tumor growth. However, based on review of gene databases (e.g. genecards), all genes annotated in our analyses are as expressed in the blood as in other tissues and are hypothesized to have immune system functioning roles. Most importantly, gene annotations are only as comprehensive as the research that informs them. Cancer research dominates the research

on the functional role of thousands of genes including the ones identified in our analyses. It is possible that annotated genes have other functions that many be related to psychological phenotypes in different ways. More research on the functional role of genes beyond the context of cancer is necessary for more comprehensive understanding of novel hits in EWAS.

Limitations

There are several additional limitations of the EWAS design that should be noted. EWAS is frequently underpowered due to a combination of very small effect sizes (see more in-depth discussion on page 80), punitive multiple test corrections, and small sample sizes due to data availability and the cost of microarray assays. No formal power analyses for EWAS exist, however, some studies have tried to estimate sufficient sample size for adequate power using data stimulations. Tsai and Bell (2015) found that in a case-control design, N = 1,000 (or 500 pairs) was necessary to detect DNA methylation differences between 0-5% in terms of genome-wide significance at 80% power; N = 200 was necessary in monozygotic twin designs. Our sample size is one of the largest seen in EWAS (birth EWAS: low = 662, increasing = 94, moderate/decreasing = 74; adolescent EWAS: low = 720, increasing = 77, moderate = 64). However, group membership was heavily skewed towards the low depression trajectory and therefore, our analyses might not have been adequately powered. Issues of power likely contribute to EWAS replication difficulties similar to those seen in GWAS.

There are also several limitations of our specific study in particular. First, we were unable to control for antidepressant use in our study. Research has shown that antidepressants may affect DNA methylation of certain sites in candidate genes such as BDNF, SLC6A4, HTR1A and HTR1B (Serotonin receptor 5HT1A subtype variants),

IL11 (interleuken-11), as well as more global whole genome changes (for recent systematic review see Webb et al., 2020). It is possible that individuals in the increasing group trajectory were more likely to use antidepressants throughout adolescence. Therefore, DNA methylation differences found in the increasing symptom trajectory may be confounded by anti-depressant effects on DNA methylation. Second, we measured DNA methylation at age 15.5 and did not have DNA methylation data available at baseline at age 12.5. This may be especially important for the increasing trajectory group, where individuals were not experiencing clinical symptoms of depression at ages 12.5 and 13.5. Although it can be argued that we may be tapping into an epigenetic pathway that has already been calibrated earlier on, we could not completely rule out issue of reverse-causation. Third, depression is a heterogenous disorder that is often co-morbid with other psychological disorders. Because we did not assess for co-morbidity, it is unclear if our trajectory groups differed on prevalence of related psychopathology further obscuring DNA methylation results. Future epigenetic research should focus less on specific psychiatric diagnoses and instead emphasize broad trait-level vulnerabilities, such as emotion dysregulation or impulsivity. Further discussion on clinical phenotypes in behavioral epigenetic research is on page 81. Fourth, we did not control for the presence of other biomedical disorders especially those related to inflammatory processes including cancer, Type II diabetes, or cardiovascular disorder, though prevalence rates of diseases associated with aging in adolescence is very low. Finally, due to issues of power, we were not able to stratify our sample by gender though it was controlled for at every level of analysis. Given the 2:1 depression gender ratio that emerges in adolescence and the differential hormonal changes related to puberty for teenage girls, it is highly likely that underlying epigenetic mechanisms for depression may be gender-specific, especially

for those girls with increasing depression symptoms over time. The need for higher-powered gender specific EWAS for depression is compounded by the fact that our most significant hit, that was also central to the cancer gene network, has been consistently identified as a breast cancer tumor suppressor.

CHAPTER 9: Thesis Discussion

The Tissue Issue

One of the most salient debates in the viability of using DNA methylation to understand complex psychological phenotypes is the "tissue issue.' DNA methylation profiles are tissue-specific, which means that each tissue (e.g. blood, brain, skin) has its own unique DNA methylation profile as part of normative tissue differentiation. Since most behavioral epigenetic studies utilize peripheral tissue, most commonly blood, a major debate is whether peripheral tissue samples have utility for the study of disorders that are thought to be primarily manifest in the brain. Simply put, does DNA methylation that we observe in the blood, have anything to do with what is going on in the brain? One study sought to characterize intra- and inter-individual methylome variation across whole blood and multiple regions of the brain (Davies et al., 2012). They found that DNA methylation at CpG island around promoter sites was largely conserved between blood and brain regions, while CpG shores and intragenic regions showed tissue-specific DNA methylation differences. Most strikingly, inter-individual DNA methylation differences found in the blood were correlated ($p < 0.001$) with inter-individual differences in the brain (correlation = 0.76 in the cerebellum and 0.66 in the cortex). This means that differential DNA methylation patterns between two individuals that are detected in peripheral blood are also present in the brain suggesting that peripheral tissues are still relevant despite tissue-specific DNA methylation patterns. Similarly, another study found

that interindividual variation in DNA methylation are highly correlated between whole blood and brain when probes are in CpG promoter regions (Hannon et al., 2015). However, they found that interindividual variation in DNA methylation between blood and brain exists in 1-3% of 450k probes and warned against using blood DNA methylation patterns as proxies for DNA methylation in the brain. It is important to note that DNA methylation differences as assessed by candidate gene studies are often substantiated with similar patterns in post-mortem brain samples, albeit not within the same individual (e.g. Keller et al., 2010; Labonte et al., 2012; McGowan et al., 2009; Stenz et al., 2015)

Although DNA methylation patterns in the blood may not always be aligned with DNA methylation patterns in the brain, we argue that peripheral tissues can still provide useful information about etiology of psychopathology. Peripheral tissue types may seem irrelevant under the assumption that psychological disorders are primarily disorders of the brain and are the result of dysfunction of neural circuitry. However, there has recently been an explosion of research exploring the connection between the immune system, chronic inflammation, and psychological disorders beyond depression (for various reviews see Mitchell & Goldstein, 2014; Renna et al., 2018, 2018; Su, 2012; Monica Uddin & Diwadkar, 2014). Similarly, a new research base focusing on the gastrointestinal system has linked altered gut microbiome functioning to psychological disorders as well (for reviews see Groen et al., 2018; Mayer & Hsiao, 2017; Nguyen et al., 2018). Perhaps psychological disorders are caused by disruptions in multiple body systems in addition to the brain and the traditional dichotomy between mind and body should be reconceptualized when thinking about disease etiology. Peripheral tissue may not be an exact proxy for brain processes but can instead be thought of as a window to

disrupted pathways in other body systems that interact with, are a consequence to, or a precursor to processes in the brain.

Furthermore, even if large scale access to post-mortem brain tissue was easier and sample size was not an issue, there are other considerations to take into account about the limitations of brain tissue sampling. First, one cannot rule out the profound effects that death can have on DNA methylation patterns in the brain, especially if death was traumatic or due to illness. Second, post-mortem samples will not tell us anything about the etiology of psychological disorders and how they unfold over time during sensitive periods of development. Third, if the hope is that DNA methylation will one day be a biomarker for either the onset of psychological disorders or as evidence of wear and tear, it must be easily and reliably accessed. It is likely that blood-based epigenetic studies will continue, and emerging evidence suggests that limitations to this approach can be surmountable, though confirmation in brain tissue remains important.

Effect Size

While epigenetic studies in cancer and other disorders typically manifest DNA methylation differences of ~20% when comparing cases and controls, studies in behavioral epigenetics examining psychological phenotypes often have effect sizes ranging from 1-10% and sometimes even smaller differences are reported. Similarly, our studies reported mean DNA methylation differences in the range of 1-2%. It has been posited that large changes in DNA methylation as a result of stress would hinder any possible social-emotional development in the same way that DNA methylation leading to cancer renders the tissue completely lost of its normative function and as a result, large effect sizes are not to be expected when assessing stress and psychopathology (Breton et al., 2017).

Furthermore, is important to note that there is usually a strong statistical significance reported with these small differences, suggesting that even though the effects are small, there is little variability in the measured values. An important example of this is the robust literature linking maternal smoking during pregnancy and DNA methylation in infant blood where effect sizes range from 1-13% (Breton et al., 2017). It is perhaps also useful to recategorize what we see as small effects. Very small changes in DNA methylation can have large effects on transcriptional activity. For example, one study analyzing the DNA methylation of the imprinted insulin-like growth factor II (IGF2) gene in umbilical cord blood, found that for every 1% difference observed in DNA methylation, there was a doubling or halving of IGF2 transcription (Murphy et al., 2012). Effect sizes are also isolated to DNA methylation differences in one CpG site and the collective effect of multiple CpG sites on one gene or across many genes is largely unmeasured (to our knowledge, there is no capacity to estimate regional effect sizes. Though it is always imperative to question the clinical significance of effect sizes, researchers must take into account the context in which they are being examined to truly determine if they are relevant. The best way to assess the implications of a difference in methylation is to further examine downstream processes such as level of gene expression; though this was not available in the ALSPAC cohort.

Psychological Phenotypes in Behavioral Epigenetics

Difficulties with EWAS replication have historically been discussed in terms of issues with power, technology, and statistical analysis. Less discussed is the likelihood that the lack of replication and consequently, any meaningful biological understanding of epigenetic pathways that underlie mental health disorders is due to the manner in which psychological phenotypes are conceptualized in behavioral epigenetic studies.

Psychological constructs are typically treated as categorical disease categories in the same vein as biomedical disorders like cancer, but there is little evidence to suggest that the actual underlying structure of psychological phenotypes matches that conceptualization. In fact, this has been an important debate in the field of clinical psychology where the current DSM-5 (Diagnostic and Statistical Manual of Mental Disorders – 5) classification of psychological disorders in terms of a categorical disease model has been vehemently criticized as ultimately failing to "carve nature at its joints". Extensive heterogeneity and comorbidity demonstrate that diagnoses are more like heterogeneous constellations of features in multidimensional space within the context of normative human experience and processes (Lilienfeld, 2014). Limiting samples to individuals who only meet criteria for one particular psychological disorder or other ways to methodologically obtain a "cleaner" experimental phenotype is not reflective of the true nature of psychopathology where individuals often meet criteria for multiple disorders at one time or have a lifetime history of multiple diagnoses. Our current classification of psychological phenotypes may have utility in terms of reliable identification and treatment, but it is severely limiting in its ability to study biological pathology and etiology and should not be used as the default operationalization of phenotypes in behavioral epigenetic studies. If psychological research in epigenetics is to be elucidating and fruitful, as much care is to be taken in understanding and conceptualizing the phenotype as has been taken to understand epigenetic methodology.

In these two studies we have attempted to remedy this somewhat by moving beyond a case-control design. In study 1, we conceptualized psychopathology using confirmatory factor analysis to extract conduct, hyperactivity, emotion problems factors as well as a global psychopathology score. In study 2, we conceptualized depression

using longitudinal trajectories that take into account change in symptoms over time. There are many ways in which the psychological phenotype can be refined in future studies for an increased possibility in finding underlying biological pathways. First, as we attempted in our first study, psychological phenotypes can be broadened to get at more meaningful underlying factors. Research in clinical psychology has found that the structure of mental disorders can be potentially summarized by three core psychopathological dimensions: internalizing (i.e. liability towards mood disorders like depression and anxiety), externalizing (i.e. liability towards impulse and behavioral control disorders like ADHD and substance use), and thought disorders (i.e. liability towards disordered and disorganized thinking and symptoms of psychosis like schizophrenia and bipolar disorder) (Caspi et al., 2014). In behavioral epigenetics, psychological phenotypes may be expanded upon into these dimensions with the additive bonus of increased sample size and statistical power.

Disorders can also be grouped in other meaningful ways, for example, neurodevelopmental disorders such as autism, ADHD, and schizophrenia may have similar underlying epigenetic mechanisms as research has already identified overlap in genetic risk among these disorders (Owen et al., 2011). It would also be interesting to examine DNA methylation variation on an even broader scale of those who have psychopathology and those who have less or not at all. Researchers have posited some evidence pointing to one general underlying dimension dubbed the “p factor” that described an individual’s propensity to develop psychopathology period, where individuals are classified on a low to high psychopathology dimension (Caspi et al., 2014). It is plausible that epigenetic changes confer risk for psychopathology on a

broader scale and lack of replicability may be due to the mistaken assumption that different psychological disorders emerge from different epigenetic underpinnings.

On the other hand, psychological phenotypes could also be narrowed into transdiagnostic endophenotypes that are "closer" to underlying epigenetic vulnerability in the lengthy pathway between DNA methylation and complex behavioral and emotional phenotypes. Endophenotypes can be described as constructs that provide the means for identifying the downstream trait or facets of more complex observable behaviors as well as the upstream consequences of genetic and epigenetic processes (Gottesman & Gould, 2003). Although endophenotypes have been more traditionally thought of as simpler underlying biological processes (e.g. cortisol reactivity, sensory motor gazing, eye-tracking, reward learning), that definition has been expanded to include transdiagnostic personality traits that underpin psychopathology (e.g. neuroticism, impulsivity). Endophenotypes are particularly useful due to the recurring nature of psychopathology in a lifespan perspective as they are not state dependent and are more stably manifested in the individual whether the psychological disorder is currently present or not (Gottesman & Gould, 2003). The endophenotype concept fits within the Research Domain Criteria (RDoC) framework, which was developed as an alternative to the DSM classification system as a way to organize psychological disorders on transdiagnostic dimensional domains (e.g. arousal and regulatory systems, cognitive processes) that focus on pathophysiology across several units of analysis (e.g. genetics, physiology, behavior) (Insel & Cuthbert, 2009). As discussed previously, studies examining prenatal stress, DNA methylation, and infant neurobehavioral outcomes have utilized endophenotypes such as cortisol reactivity (e.g. Houtepen et al., 2016; Oberlander et al., 2008; Tf et al., 2008). There are a few studies who have also examined DNA methylation and its relation

to cortisol reactivity later in development (Alexander et al., 2014, 2014; Ouellet-Morin et al., 2013). For example, in one EWAS, researchers found that the methylation of one locus in the Kit ligand gene (KITTLG) in adults mediated the relationship between childhood trauma and cortisol stress reactivity (Houtepen et al., 2016). Although endophenotype outcomes in behavioral epigenetic should also be expanded to include other measures of cognitive, physiological, and biological functioning, it would also be interesting for DNA methylation studies to examine transdiagnostic trait-like endophenotypes such as neuroticism (i.e. the tendency to exhibit frequent and intense negative emotions) or impulsivity instead of traditional disease model approaches.

Another consequence of overreliance on the disease model in studying psychological phenotypes, is the lack of research on epigenetic pathways that promote resilience as well as risk. It is likely that epigenetic mechanisms such as DNA methylation play a major role not only in elucidating why some individuals go on to develop psychopathology as a result of environmental stress but also why some do not. Study 1 of this thesis is an attempt to begin to understand the biological underpinnings of resilience to psychopathology in a sensitive period of development, but much more work needs to be done in this area. Contrast to the thousands of EWAS and candidate gene studies focusing on risk for negative outcomes, there is a lack of a substantial literature in understanding the epigenetics of protective factors and resilience to psychopathology. There have been a few studies examining the role of DNA methylation to resilience to acute stress in animal models (Elliott et al., 2010; Taff et al., 2019; Uchida et al., 2011; Wang et al., 2018), but very few exist in humans. There is some preliminary work examining resilience to PTSD in combat soldiers. For example, one study found that in soldiers with a diagnosis of PTSD, resilience, as measured by a range of coping

strategies, was associated with DNA methylation age acceleration suggesting that aspects of resilience may come at a biological cost (Mehta et al., 2018). To our knowledge, no studies have examined epigenetic mechanisms of resilience to psychopathology in childhood and much more work is needed to be done in this area. It would also be interesting to examine how protective factors such as social support and maternal warmth may alter epigenetic signaling pathways to promote resilience, especially early on in development.

Future Research Directions

In addition to more refined clinical phenotypes as outcomes measures and a greater focus of resilience and protective factors, more developmentally relevant longitudinal designs are needed to push the field forward. However, this is easier said than done as most longitudinal cohorts established decades ago could not have foreseen the need for more frequent extractions of blood samples to assess temporal timing of DNA methylation. Research utilizing longitudinal designs, including the ALSPAC cohort in this thesis, is greatly limited by needing to make do with what existing data is available. Future launches of longitudinal cohorts may have the benefit of establishing designs and timing of assessments that may be more conducive to hypotheses of underlying biological mechanisms. Studies with more frequent sampling of DNA methylation across a period of time are greatly needed, not only to better establish temporal order, but to gain better understanding the timing of epigenetic changes. How quickly after stressors can DNA methylation changes be identified? Are they temporary fluctuations or more permanent cellular reprogramming phenomena?

Additionally, innovated epigenetic designs beyond the traditional candidate gene and EWAS case-control samples are necessary. Epigenetic research will be most robust

when integrating multiple levels of analysis. The distance between DNA methylation and a complex behavioral phenotype is vast and in between lies a number of cascading processes. Future research should continue to include multi-omics measures including genomics, transcriptomics, metabolomics, proteomics, and imaging data (Lin & Tsai, 2019). Furthermore, the polygenic risk score approach that has been utilized in application of GWAS data should also be adapted to epigenetic research, given that multiple CpG sites of multiple genomic regions are likely acting and interacting in accordance with one another.

Finally, the interpretation of EWAS is often limited by how little is actually known about the biological functioning of newly identified genes as much of what is known about gene function is through cancer research. In theory, EWAS are hypothesis generating analyses where novel genes implicated in psychological phenotypes are discovered. However, there is very little if any follow-up (for example, candidate gene analyses) on novel hits in the field of behavioral epigenetics. There are a large number of novel genes in many EWAS that have not been more closesly examined. If the field is to continue to grow, researchers must conduct more in-depth follow-up analyses on how these genes are related to psychological phenotypes.

Clinical Implications

The seminal study conducted by Weaver and colleagues (2004), demonstrated in an animal model that early life experience became embedded through DNA methylation of the GR gene to propagate an anxious phenotype later in life. Perhaps the most striking result of this study was that the epigenetic changes were *reversible.* Central infusion of a histone deacetylase inhibitor into the brain effectively removed the methyl tags on the GR gene and removed group differences in DNA methylation, GR transcription and

expression, hypothalamic-pituitary adrenal (HPA) responses to stress, and most importantly, the actual anxious phenotype. Previously anxious mice were now not distinguishable from their non-anxious control counterparts. While epigenetic pharmacotherapy is an exciting concept in treatment of mental illness, these results demonstrate more of a proof of principle rather than possible reality in human psychopathology. Current epigenome-editing technology uses DNA methyltransferase (DNMT) inhibitors acting on DNA methylation and histone deacetylase (HDAC) inhibitors targeting histone post-translational modification (i.e. another mechanism of epigenetic mechanisms not discussed in this thesis) (Kular & Kular, 2018). However, in humans these modifiers affect DNA methylation globally, exerting broad effects on the epigenome, and current technology cannot target individual loci. And even if that technology comes to fruition, it would be unclear which loci to safely target and in what tissue without adverse pleiotropic side-effects. Unlike in cancers where malignant tumors can be localized and targeted with global methylation changes, it remains to be seen how this would be possible in complex psychological phenotypes that have social, emotional, cognitive, and behavioral components.

Although direct biological intervention may not be possible, epigenetic research has the potential to inform the classification and treatment of mental health disorders in other ways. The most obvious utility is the central aim of this thesis: to better understand the etiology and mechanisms of psychopathology. In addition, DNA methylation changes can also serve as potential biomarkers that can predict and track clinical outcomes as well as potentially classify particular subtypes of a disorder. For example, one longitudinal study assessing postpartum depression identified 116 transcripts, related to estrogen signaling, that were differentially expressed between cases and controls during the 3rd

trimester that then predicted with 88% accuracy who went on to develop postpartum depression in two separate sample cohorts (Mehta et al., 2014). Similarly, another study posited the predictive utility of DNA methylation of HP1BP3 and TTC9B, both of which are regulated by estrogen with 80% accuracy (Guintivano et al., 2014). Another study using an epigenome-wide approach examined DNA methylation in the context of treatment response to antidepressant medication. Results identified differential DNA methylation in two genes, CHN2 and JAK2, that distinguished responders from non-responders with CHN2 being replicated in an independent sample (Ju et al., 2019).

The future of behavioral epigenetics, aided by strides in technological advances, improved bioinformatic methods, more meaningful and developmentally relevant phenotypes, and innovative research designs, looks bright. However, the field is still in its infancy and researchers must use caution in overinterpreting new discoveries. The more that novel discoveries of the epigenome are uncovered, the more of our ignorance of the complexities in relationships between genes and environments is revealed. It is unlikely that epigenetics, like genetics before it, will be the final piece of the puzzle in solving the disease burden of mental illness. However, it continues to hold enormous potential for better understanding of the etiology of psychopathology and for better, more precise treatment of it.

APPENDIX: LIST OF TABLES

Table and Figures from Study 1: Variation in DNA Methylation of the Oxytocin Receptor Gene Predicts Children's Resilience to Prenatal Stress (2017)

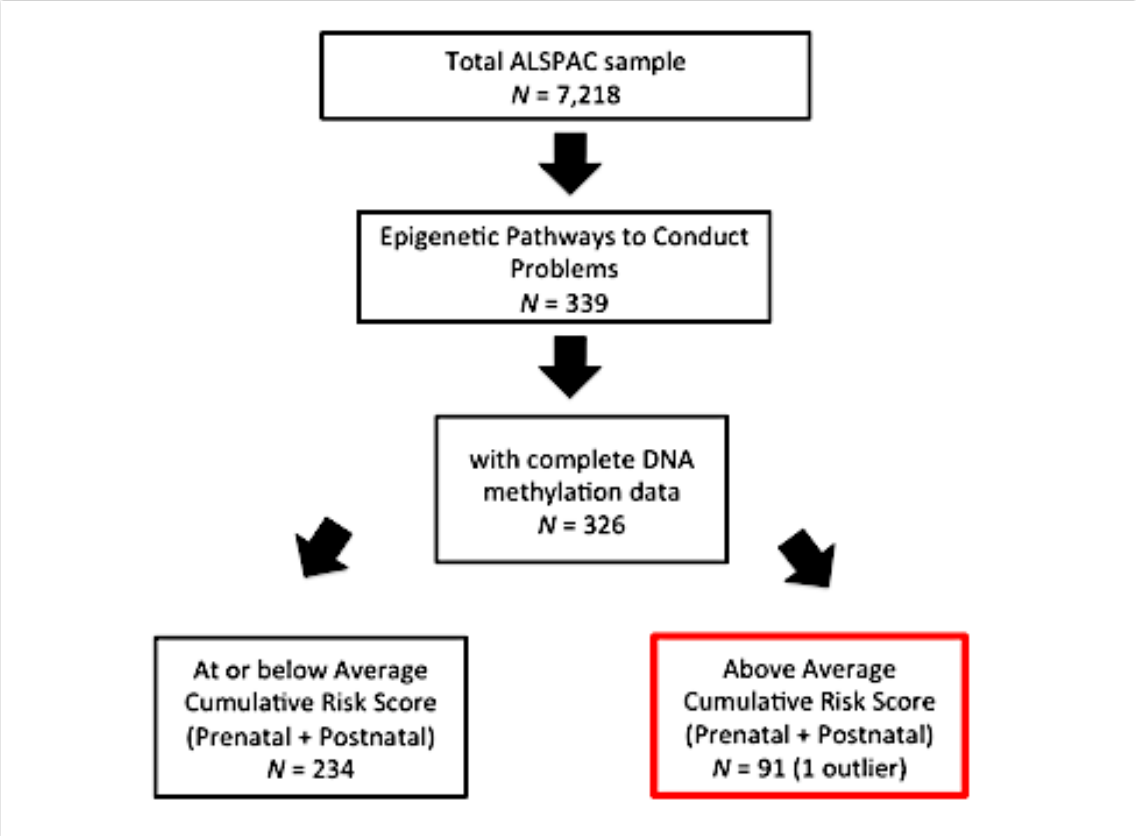
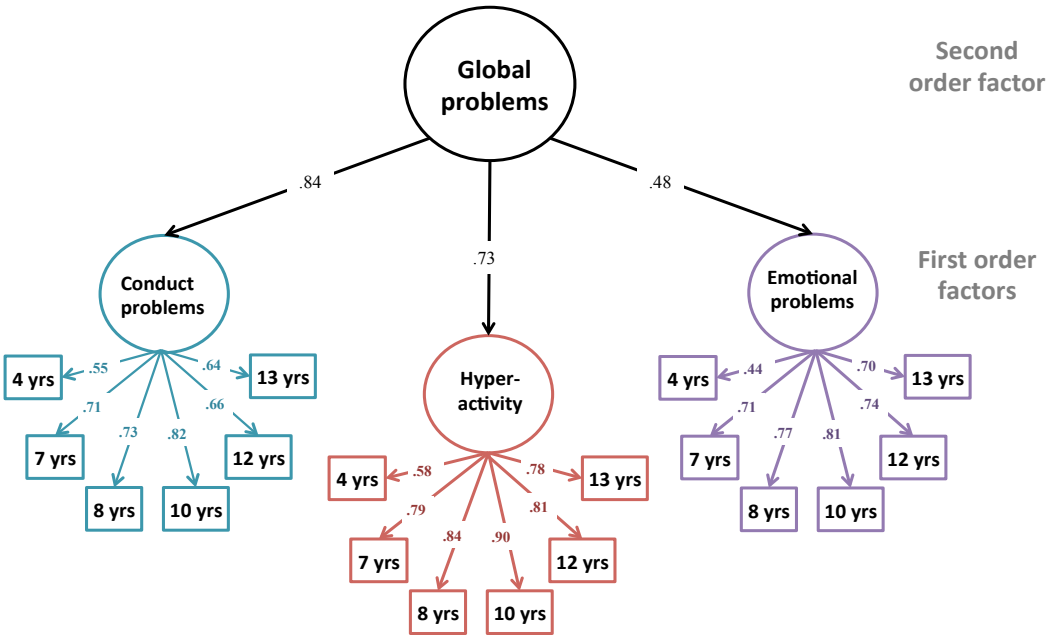
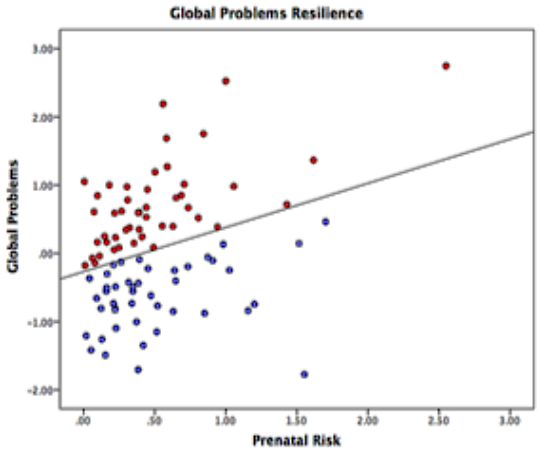


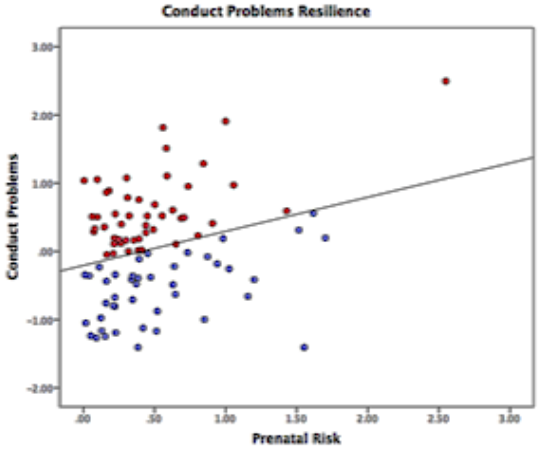
Figure 1. Flowchart of sample selection from ALSPAC cohort.

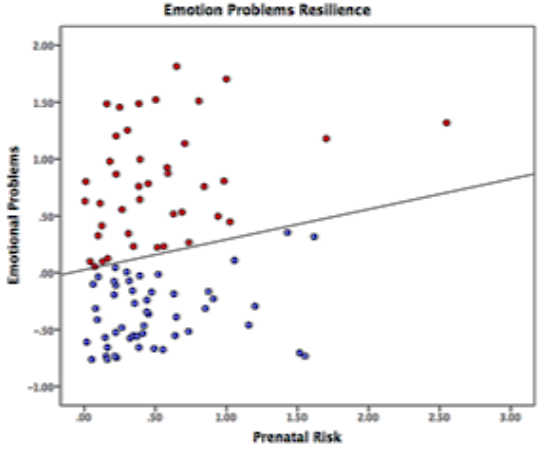


Model Fit: X^2 (132) = 462.16; p <.001; CFI = .88; TLI = .86; RMSEA = .08, 90% CIs = .08, .09

Figure 2. Confirmatory factor analysis of Strengths and Weaknesses Questionnaire (SDQ) subscales of conduct problems, hyperactivity, and emotional problems and global problems.


Global Problems Resilience
Global Problems
Prenatal Risk


Conduct Problems Resilience
Conduct Problems
Prenatal Risk


Emotion Problems Resilience
Emotional Problems
Prenatal Risk

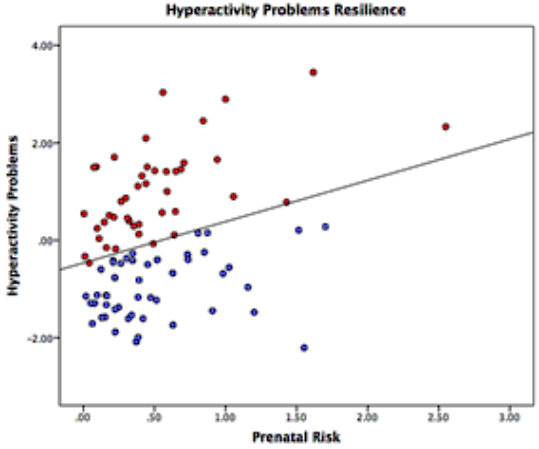

Hyperactivity Problems Resilience
Hyperactivity Problems
Prenatal Risk

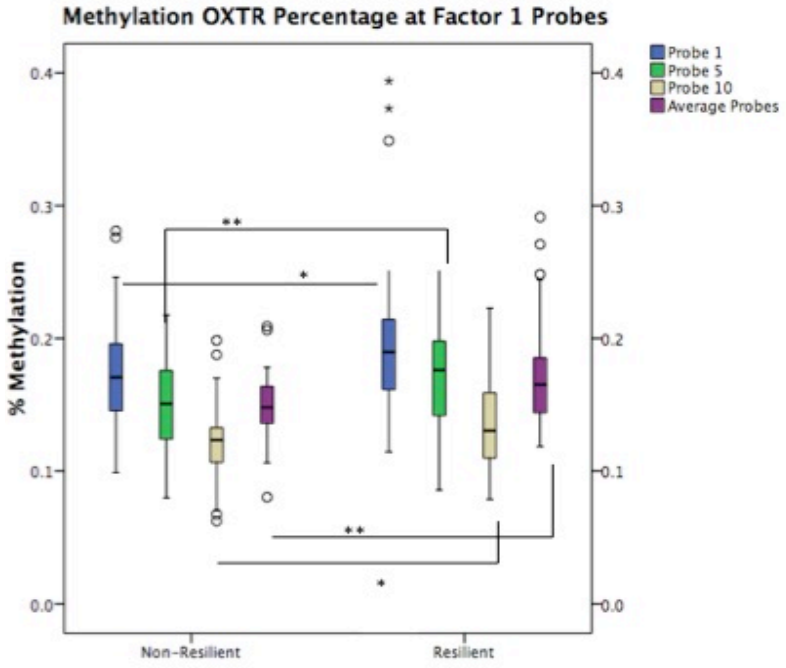
Figure 3. Linear Regression models used to classify resilient and non-resilient groups to global, conduct, hyperactivity, and

Table 1. *Multiple Linear Regression Predicting Factor 1 Methylation and Individual Probes at Birth by Types of Resilience*

	Global		**Conduct**		Hyperactivity		Emotional	
Parameter	β	95% CI	**β**	**95% CI**	β	95% CI	β	95% CI
Factor 1 Methylation	.220	.000-.025	**.323***	**.006-.031**	.154	-.006-.024	.015	-.013-.013
Probe 1	.153	-.009-.040	**.245***	**.003-.051**	.051	-.022-.033	.022	-.022-.025
Probe 5	.274	.004-.041	**.283***	**.006-.042**	.280*	.005-.042	.027	-.018-.020
Probe 10	.073	-.012-.021	**.244***	**.002-.035**	.055	-.015-.024	-.042	-.021-.014

Note: β = Beta Weights; CI = Bootstrapped Confidence Intervals; Analyses controlled for sex and cell type
*$p < .05$, **$p < .01$, ***$p < .001$.

emotional problems. Red (top half) dots represent the non-resilient group while the blue (bottom half) represent the resilient group.



		Resilient (N = 44)		Non-Resilient (N = 47)	
Probes	*%*	*M*	*SD*	*M*	*SD*
Average	2.2	.19	.04	.17	.06
Probe 1	2.5	.18	.05	.15	.04
Probe 5	2.3	.14	.04	.12	.03
Probe 10	1.7	.17	.04	.15	.02

Figure 4. *This figure shows mean methylation percentages at each individual probe that makes up Factor 1 controlling for sex and estimated cell–type composition.* $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

Table 2. *Descriptive Statistics of Resilient and Non-Resilient groups in Conduct Problems*

	Resilient (*N* = 44)	Non-Resilient (*N* = 47)	
	N (%)	*N* (%)	
Gender			
Male	20 (46.5)	23 (53.5)	
Female	24 (50.0)	24 (50.0)	
	M (SD)	*M (SD)*	*T-test*
Environmental Risk			
Prenatal	0.54 (0.46)	.047 (.429)	-0.750
Ages 0-7	5.96 (4.46)	6.58 (5.80)	0.564
Ages 8-9	0.85 (1.81)	0.99 (1.76)	0.387
	M (SD)	*M (SD)*	*F-test*
Psychopathology			
Hyperactivity	-0.55 (1.10)	0.45 (1.22)	16.56***
Emotional Problems	0.00 (0.67)	0.31 (0.70)	4.90*
Peer Problems	-0.10 (0.67)	0.17 (0.63)	3.72^{t}
Prosocial Behavior	0.61 (0.64)	-0.40 (1.03)	31.18***
Social Cognition (Age 7)	2.24 (2.33)	5.16 (3.65)	18.14***
Callous-Unemotional Traits (Age 13)	1.79 (0.54)	2.33 (0.61)	16.87***

, *Note: All psychopathology outcomes controlled for sex*
, $^{**}p < .01$, $^{***}p < .001$.

Table 3. *OXTR Single Nucleotide Polymorphism (SNP) effects on Probe 1 and Probe 10*

Timepoint	SNP	SNP chr	SNP pos	A1	A2	CpG site	CpG chr	CpG pos	Beta	T-stat	Effect Size
Birth (Probe 1)	rs62243375	3	8810462	T	C	cg00078085	3	8810592	0.613	0.00	0.008
Birth (Probe 10)	rs237900	3	8808696	A	G	cg12695586	3	8810077	-0.328	0.00	0.004

Note: chr = chromosome, pos = position

Table and Figures from Study 2: A Longitudinal Epigenome-wide Analysis of Depression Trajectories in Adolescence

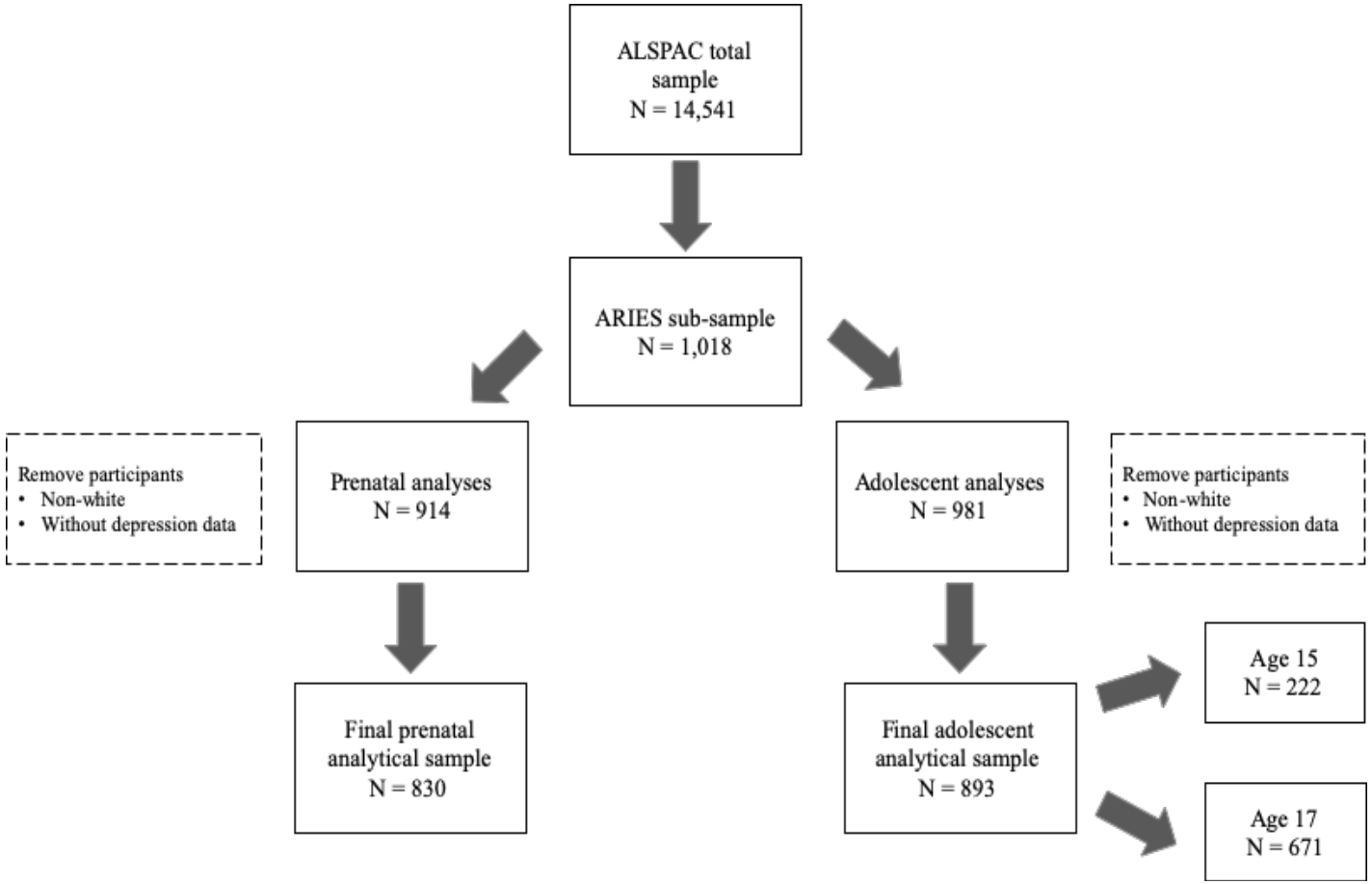


Figure 5. *Flowchart of sample selection from ALSPAC cohort*

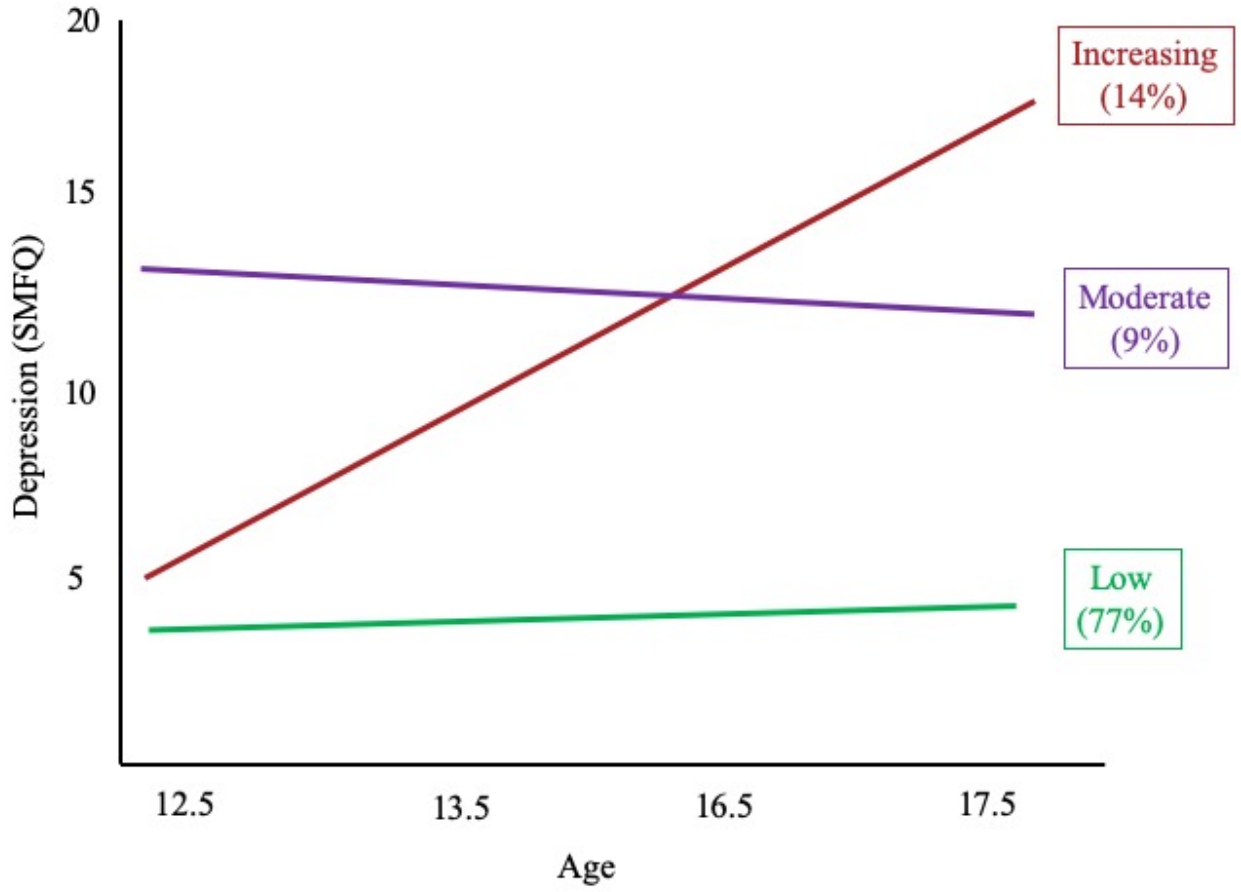


Figure 6. *Depression trajectories (measured with Short Moods and Feelings Questionnaire; SMFQ) obtained through latent class growth curve modeling using the full ALSPAC sample (N = 8,360).*

Table 4. *Descriptive Statistics for EWAS analyses at birth broken down by depression trajectories.*

	Low	**Increasing**	**Moderate/ Decreasing**
		Birth EWAS (*N* = 830)	
N *(%)*	662 *(80)*	94 *(11)*	74 *(9)*
N *(%)* female	311 *(47)*	66 *(70)*	48 *(65)*
Birth covariates	M *(SD)*	M *(SD)*	M *(SD)*
Maternal smoking freq			
1st trimester	0.93 *(3.46)*	1.71 *(5.04)*	1.32 *(4.49)*
2nd trimester	0.60 *(2.69)*	1.04 *(3.65)*	1.16 *(3.87)*
3rd trimester	0.70 *(2.98)*	1.55 *(4.75)*	1.28 *(4.69)*
Maternal marijuana freq			
1st trimester	0.02 *(0.19)*	0.05 *(0.43)*	0.01 *(0.12)*
2nd trimester	0.01 *(0.16)*	0.05 *(0.43)*	0.03 *(0.17)*
3rd trimester	0.02 *(0.22)*^{a}	0.10 *(0.62)*^{ab}	0.03 *(0.17)*^{b}
Maternal alcohol use freq			
1st trimester	0.77 *(0.81)*	0.84 *(0.86)*	0.64 *(0.69)*
3rd trimester	0.80 *(0.79)*	0.79 *(0.88)*	0.81 *(0.81)*
Child gestation length (weeks)	39.60 *(1.48)*	39.33 *(1.56)*	39.68 *(1.45)*
Maternal age at birth (years)	29.65 *(4.27)*	29.49 *(4.71)*	29.88 *(5.17)*
Child birthweight (grams)	3509.50 *(476.92)*	3372.17 *(480.59)*	3472.26 *(461.89)*
Adolescent Dep (age 12.5)	2.94 *(2.34)*	5.31 *(2.56)*	12.80 *(3.64)*
N *(%)* clin sig dep	2 *(.03)*	2 *(2)*	57 *(77)*
Adolescent Dep (age 13.5)	3.59 *(2.93)*	9.34 *(5.19)*	11.64 *(4.53)*
N *(%)* clin sig dep	20 *(3)*	34 *(36)*	39 *(53)*
Adolescent Dep (age 16)	5.40 *(3.22)*	15.49 *(5.78)*	10.15 *(5.72)*
N *(%)* clin sig dep	53 *(8)*	73 *(78)*	30 *(41)*
Adolescent Dep (age 17.5)	5.06 *(3.85)*	14.41 *(5.11)*	8.75 *(4.66)*
N *(%)* clin sig dep	73 *(11)*	73 *(78)*	25 *(34)*

Note*: M = Mean, SD = Standard Deviation; ANOVA with Tukey HSD was used to assess mean differences in covariates, columns with different superscripts are significantly different from each other; Dep = SMFQ Depression score; freq = frequency; clin sig dep = clinically significant depression; Range of values for smoking, marijuana, and alcohol use are 0 (none) to 6 (frequent daily use)*

Table 5. *Descriptive Statistics for EWAS analyses at adolescence broken down by depression trajectories.*

	Low	**Increasing**	**Moderate/ Decreasing**
			Adolescent EWAS (*N* = 893)
N *(%)*	720 *(80)*	77 *(11)*	96 *(9)*
N (*%)* female	346 *(48)*	54 *(70)*	61 *(64)*
	M *(SD)*	M *(SD)*	M *(SD)*
Adolescent Age	17.14 *(1.03)*	17.15 *(1.06)*	17.10 *(1.08)*
Adolescent Smoking	0.85 *(1.46)*[a]	1.68 *(1.99)*[b]	1.62 *(1.80)*[b]
Adolescent Marijuana	0.42 *(0.97)*[a]	0.75 *(1.37)*[ab]	0.93 *(1.48)*[b]
Adolescent Dep (age 12.5)	2.86 *(2.30)*	5.27 *(2.52)*	12.69 *(3.47)*
N *(%)* clin sig dep (SMFQ $\geq$ 11)	2 *(.03)*	2 *(2)*	56 *(77)*
Adolescent Dep (age 13.5)	3.53 *(2.89)*	9.49 *(5.20)*	11.73 *(4.56)*
N *(%)* clin sig dep	20 *(3)*	32 *(39)*	39 *(53)*
Adolescent Dep (age 16)	5.35 *(3.21)*	14.88 *(5.89)*	9.87 *(5.39)*
N *(%)* clin sig dep	43 *(8)*	53 *(73)*	21 *(39)*
Adolescent Dep (age 17.5)	4.99 *(3.77)*	14.83 *(5.22)*	8.93 *(4.79)*
N *(%)* clin sig dep	72 *(10)*	58 *(82)*	35 *(36)*

Note*: M = Mean, SD = Standard Deviation; ANOVA with Tukey HSD was used to assess mean differences in covariates, columns with different superscripts are significantly different from each other; Dep = SMFQ Depression score; freq = frequency; clin sig dep = clinically significant depression; Range of values for smoking and marijuana are 0 (none) to 6 (frequent daily use)*

Table 6. *EWAS comparing increasing* (*N* = 94) *to low* (*N* = 662) *groups using DNA methylation obtained at birth*

CpG	Gene	Chr	Position	Location details	b (SE)	p	q	Adj R	M (SD) low	M (SD) increasing	Meth diff
cg08214693	SCRIB	8	144885540	Island, 5' UTR, promoter	-2.12e-02 (3.80e-03)	7.44e-06	0.074	0.025	0.965 (0.018)	0.952 (0.049)	1.3%

Note: *Chr = chromosome; b = unstandardized beta; SE = standard error, q = adjusted FDR value; Adj = Adjusted; M = mean; SD = standard deviation.*

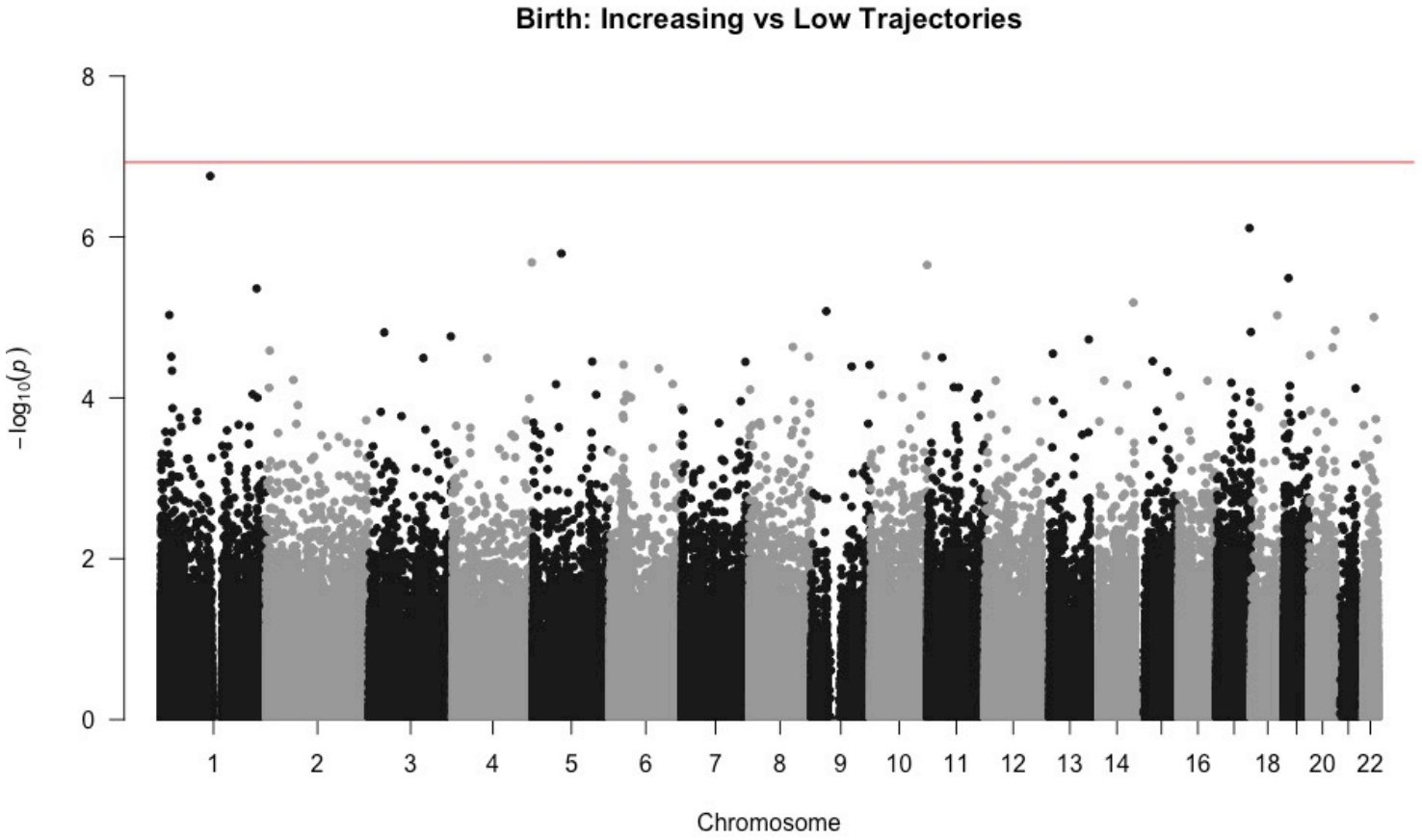


Figure 7. *Manhattan plot of EWAS comparing increasing (N = 94) to low (N = 662) groups using DNA methylation obtained at birth. Red line represents FDR significance.*

Table 7. *EWAS comparing combined high* (*N* = 173) *to low* (*N* = 720) *groups using DNA methylation obtained at adolescence*

CpG	Gene	Chr	Position	Location Details	b (SE)	p	q	Adj R	M (SD) low	M (SD) increasing	Meth diff
cg06758781	AACS	12	125570653	South Shore; Body	-1.23e-02 (2.21e-03)	3.53e-08	0.015	0.021	0.136 (0.048)	0.156 (0.042)	2%

Note: *Chr = chromosome; b = unstandardized beta; SE = standard error, q = adjusted FDR value; Adj = Adjusted; M = mean; SD = standard deviation; Location details = location in genomic space, location on the gene, and whether CpG is near the promoter region*

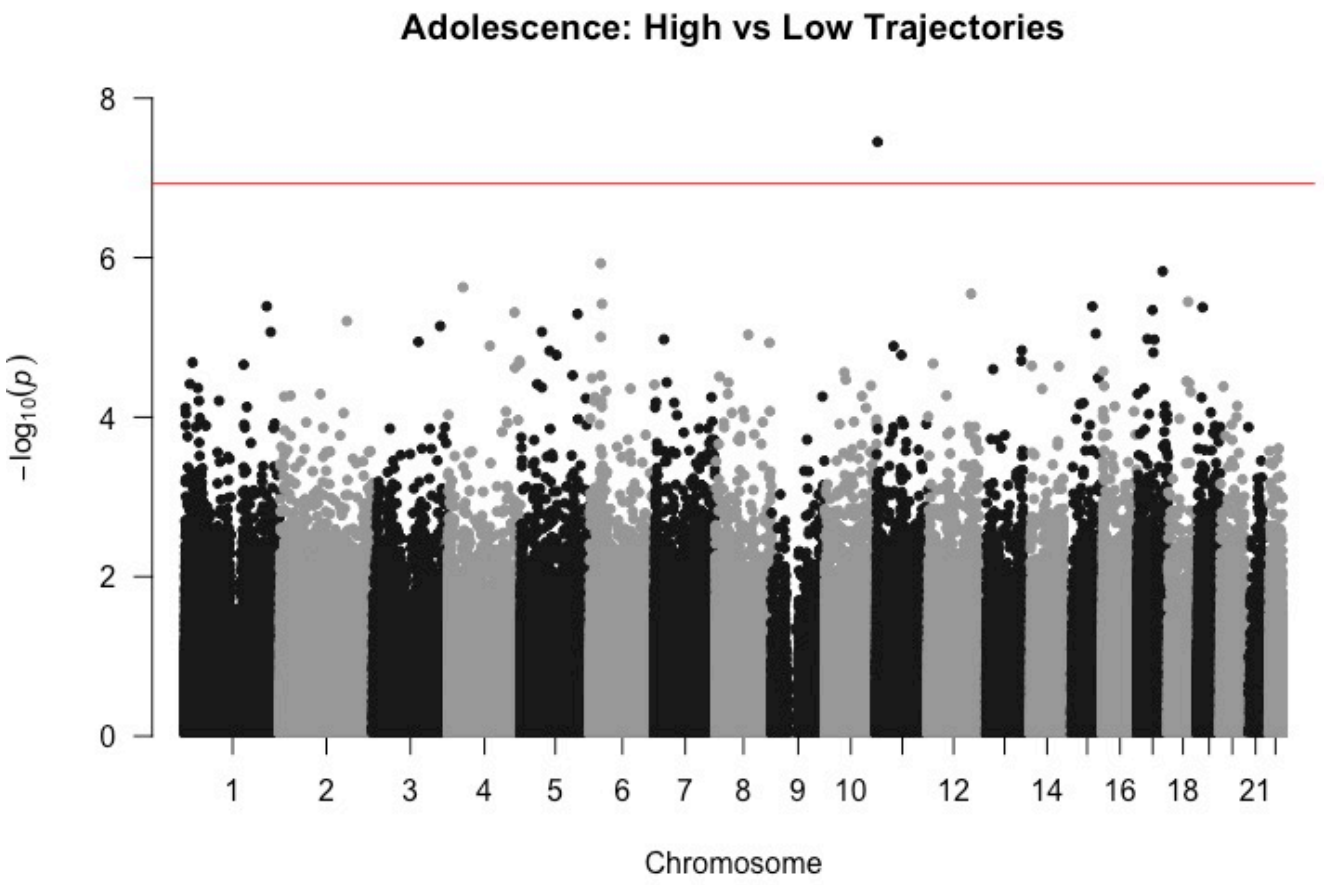


Figure 8. *Manhattan plot of EWAS comparing combined high* (*N* = 173) *to low* (*N* = 720) *groups using DNA methylation obtained at adolescence. Red line represents FDR significance.*

Table 8. *EWAS comparing increasing* (*N* = 96) *to low* (*N* = 720) *groups using DNA methylation obtained at adolescence*

CpG	Gene	Chr	Location Details	b (SE)	p	q	Adj R	M(SD) low	M(SD) inc	Meth dff
cg06460328	**CBFA2T3**	**chr16**	**North Shelf; 5'UTR; Body**	**-2.64e-02 (4.85e-03)**	**7.17e-08**	**0.030**	**0.296**	**0.136 (0.048)**	**0.156 (0.042)**	**2%**
cg15414828	LRTOMT/ NUMA1	chr11	Island; 5'UTR, 1st exon; promoter	-1.36e-02 (2.62e-03)	2.77e-07	0.059	0.061	0.080 (0.021)	0.090 (0.021)	1%
cg00624332	LDB1	chr10	Island; TSS200; promoter	-1.48e-02 (2.94e-03)	6.75e-07	0.060	0.046	0.062 (0.023)	0.074 (0.027)	1.2%
cg14558639	USF2	chr19	Island; Body; promoter	-2.58e-02 (5.16e-03)	7.18e-07	0.060	0.052	0.080 (0.040)	0.100 (0.044)	2%
cg05901451	HEY2	chr6	Island; 5'UTR, 1st exon	-2.97e-02 (5.95e-03)	7.79e-07	0.060	0.062	0.224 (0.048)	0.242 (0.049)	1.8%
cg18786593	--	chr2	South Shore	-0.020 (4.14e-03)	9.61e-07	0.060	0.078	0.096 (0.034)	0.106 (0.034)	1%

cg21772776	BLCAP	chr20	Island; 5'UTR, 1st exon; promoter	-2.87e-02 (5.80e-03)	9.81e-07	0.060	0.075	0.138 (0.047)	0.163 (0.042)	2.5%

Note: *Chr = chromosome; b = unstandardized beta; SE = standard error, q = adjusted FDR value; Adj = Adjusted; M = mean; SD = standard deviation; inc = increasing; Meth diff = percentage methylation difference. Location details = location in genomic space, location on the gene, and whether CpG is near the promoter region*

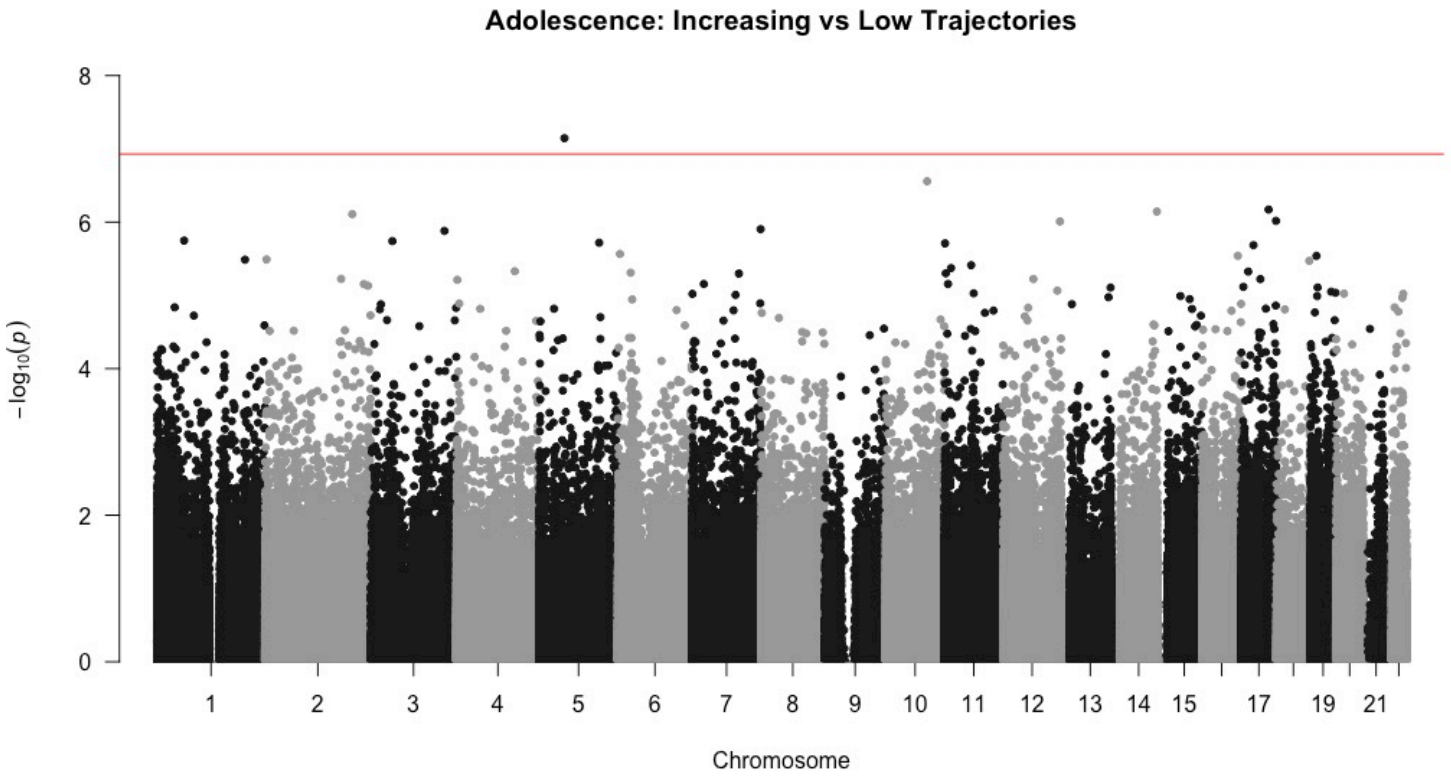


Figure 9. *Manhattan plot of EWAS comparing increasing* (*N* = 96) *to low* (*N* = 720) *groups using DNA methylation obtained at adolescence. Red line represents FDR significance.*

Table 9. *Regional analyses comparing combined high (N = 173) to low (N = 720) groups using DNA methylation obtained at adolescence*

DMR position (hg19)	Number of probes in the DMR	DMR p-value	Gene
Chr3:127347876-127347978	4	0.000	SNORA33, SNORA81, SNORD66, SNORD2, SNORD5, SNORD63, SNORD61, SNORA24, SNORA18

Note: DMR. = differentially methylated region

Table 10. *Regional analyses comparing increasing* (*N* = 96) *to low* (*N* = 720) *groups using DNA methylation obtained at adolescence*

DMR position (hg19)	Number of probes in the DMR	DMR p-value	Gene
Chr3:127347876-127347978	4	0.000	SNORA33, SNORA81, SNORD66, SNORD2, SNORD5, SNORD63, SNORD61, SNORA24, SNORA18
Chr11:130184046-130184122	2	0.000	ZBTB44
Chr20:36155925-36156146	6	0.000	BLCAP

Note: DMR. = differentially methylated region

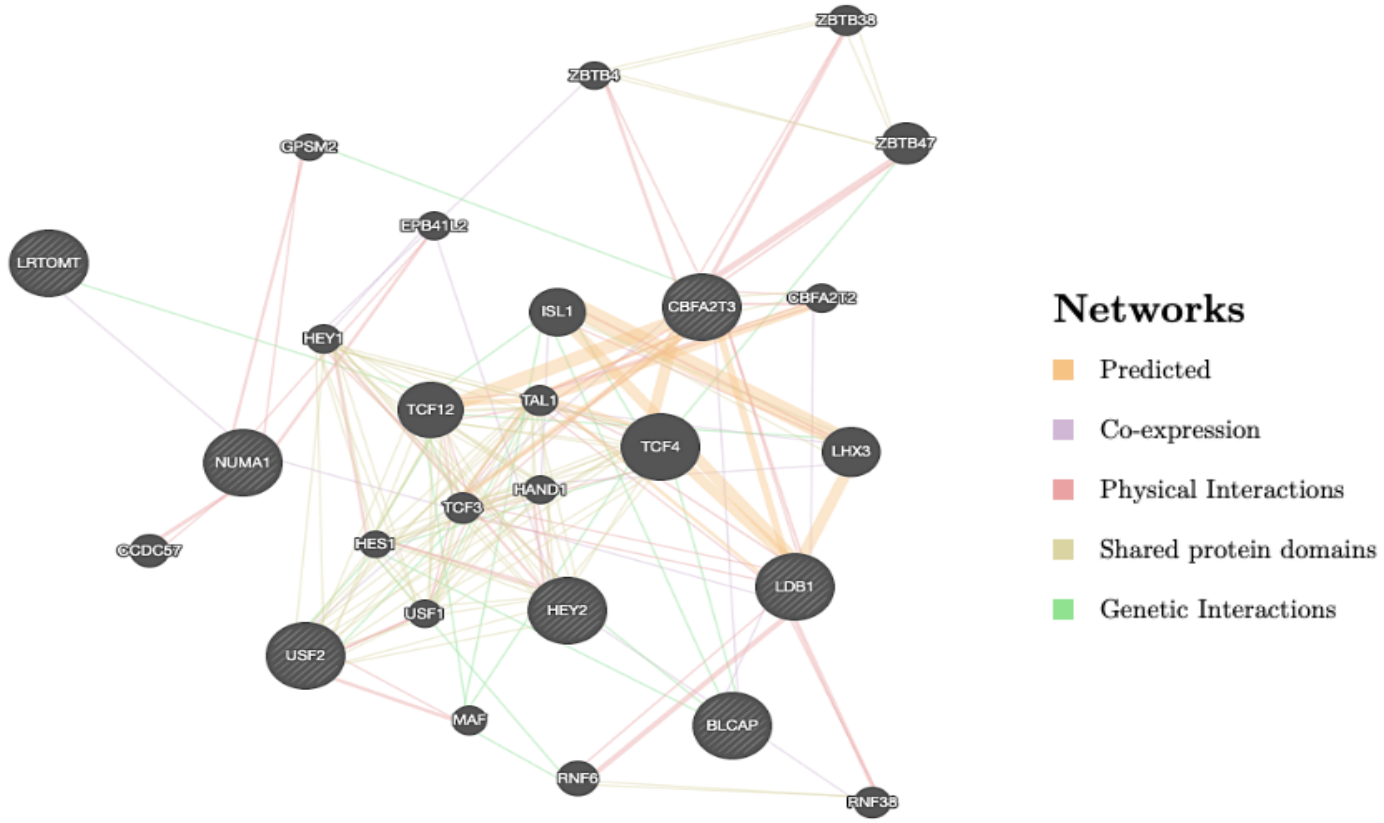


Figure 10. *GeneMANIA gene network analysis using significant and nominal hits from increasing versus low groups EWAS using DNA methylation in adolescence. Striped black circles represent genes associated with the probes found to be related to depression trajectories in the EWAS. Solid black circles represent additional genes predicted by GeneMANIA based on genetic and physical interactions, shared pathways and protein domains as well as protein co-expression data.*

Table 11. Gene ontology analyses from *GeneMANIA gene network analysis using significant and nominal hits from increasing versus low groups EWAS using DNA methylation in adolescence.*

Function	FDR	Coverage
sequence-specific DNA binding	1.60e-8	10/255
bHLH transcription factor binding	2.79e-7	5/18
cardiac septum morphogenesis	0.000002	5/27
cardiac septum development	0.000007	5/37
cardiac ventricle development	0.00005	5/56
cardiac chamber morphogenesis	0.00006	5/60
muscle structure development	0.00009	7/244
cardiac chamber development	0.00009	5/70
regulatory region DNA binding	0.0001	7/268
transcription regulatory region DNA binding	0.0001	7/267
regulatory region nucleic acid binding	0.0001	7/268
RNA polymerase II transcription factor binding	0.0001	5/74
sequence-specific DNA binding RNA polymerase II transcription factor activity	0.0004	6/200
cardiac right ventricle morphogenesis	0.0004	3/10
cardiac ventricle morphogenesis	0.0006	4/47
heart morphogenesis	0.0007	5/120
histone deacetylase binding	0.0007	4/51
chordate embryonic development	0.0007	5/125
embryo development ending in birth or egg hatching	0.0007	5/125
aorta morphogenesis	0.0008	3/14
ventricular septum morphogenesis	0.0008	3/14
aorta development	0.0009	3/15
embryonic organ development	0.001	5/144`
transcription factor complex	0.002	5/155
mesenchymal cell differentiation	0.002	4/68
smooth muscle cell differentiation	0.002	3/19
ventricular septum development	0.002	3/21
mesenchyme development	0.003	4/80
regulation of neuron differentiation	0.004	5/188
E-box binding	0.004	3/27
heart development	0.005	5/203
artery morphogenesis	0.006	3/30
RNA polymerase II activating transcription factor binding	0.007	3/32
outflow tract morphogenesis	0.007	3/33
artery development	0.007	3/33
regulation of neurogenesis	0.007	5/228
regulation of nervous system development	0.01	5/256

regulation of binding	0.01	4/127
regulation of DNA binding	0.02	3/46
in utero embryonic development	0.02	3/47
activating transcription factor binding	0.02	3/48
muscle cell differentiation	0.02	4/151
mesenchymal cell development	0.03	3/60
negative regulation of binding	0.04	3/61
blood vessel morphogenesis	0.04	4/170
regulation of vasculogenesis	0.04	2/10
stem cell differentiation	0.04	4/171
cardiocyte differentiation	0.04	3/64
cardiac epithelial to mesenchymal transition	0.05	2/12
endocardial cushion morphogenesis	0.05	2/12
cardiac left ventricle morphogenesis	0.05	2/12
protein heterodimerization activity	0.05	4/191
blood vessel development	0.05	4/193

Note*: Coverage = how many genes in this network/how many genes identified in this process overall in the literature*

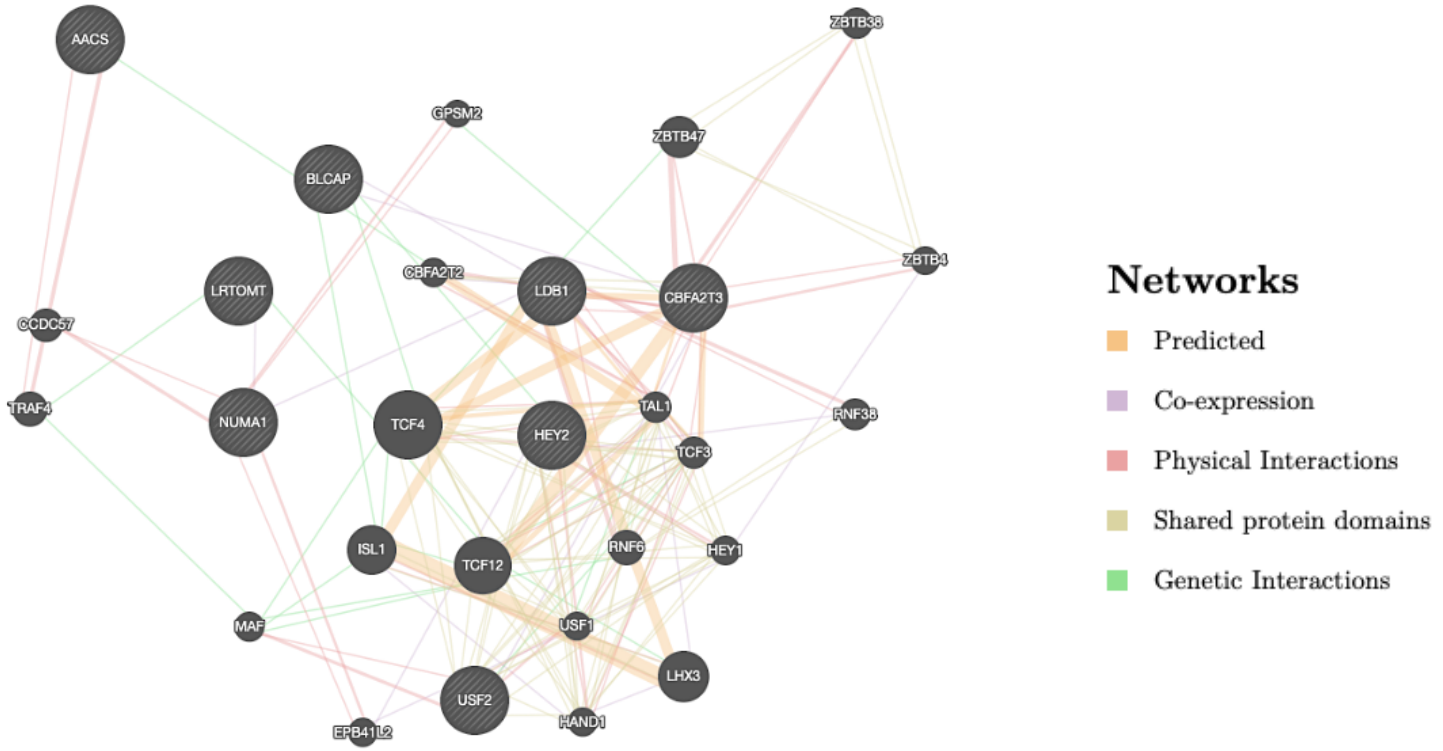


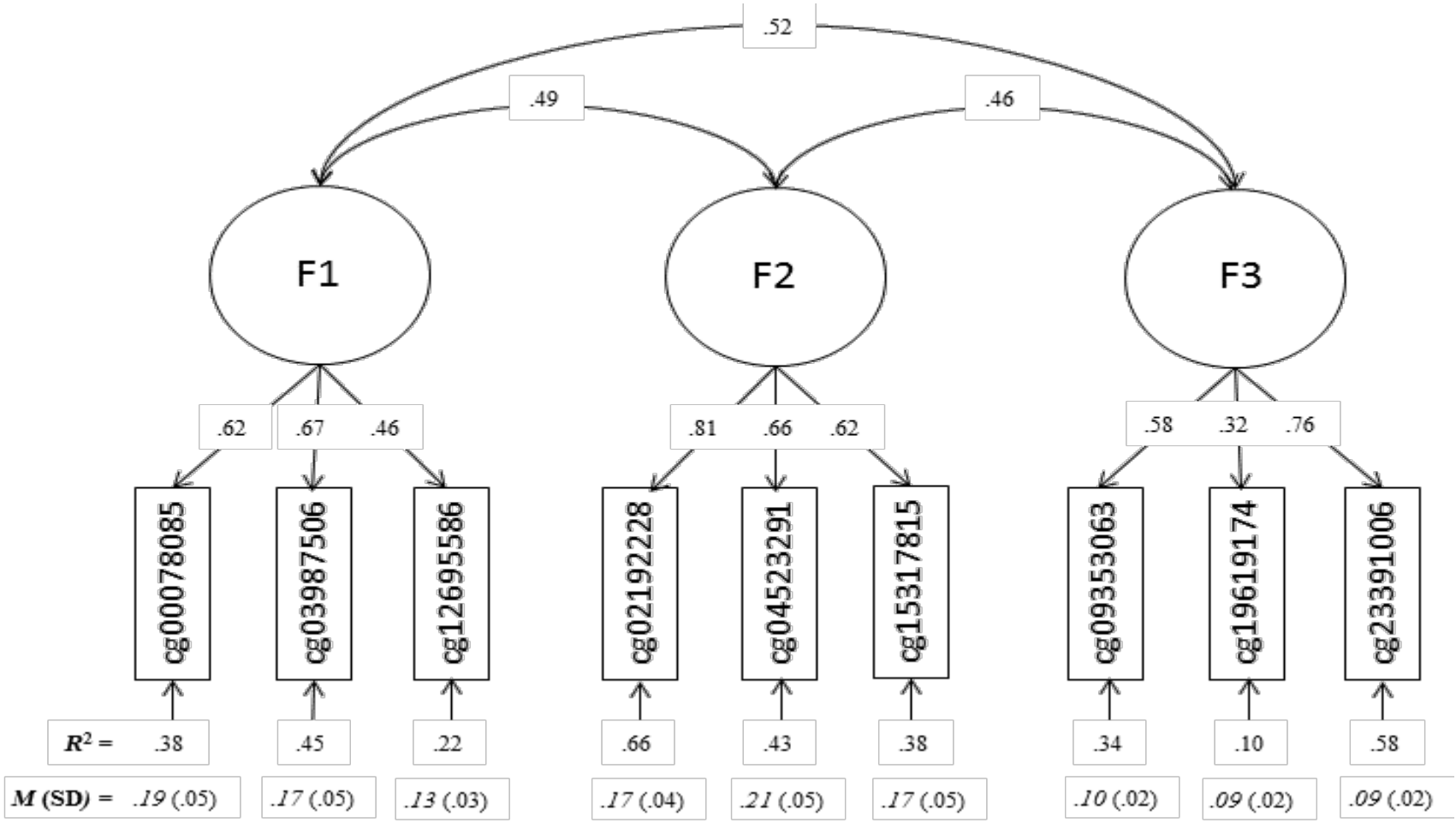
Figure 11. *GeneMANIA gene network analysis using significant and nominal hits from increasing versus low groups EWAS using DNA methylation in adolescence with the addition of AACS from high versus low groups EWAS. Striped black circles represent genes associated with the probes found to be related to depression trajectories in the EWAS. Solid black circles represent additional genes predicted by GeneMANIA based on genetic and physical interactions, shared pathways and protein domains as well as protein co-expression data.*

Supplementary Section

SI 1. *Factor analysis procedure for reducing OXTR methylation data and results obtained from Cecil and colleagues (2014) for study 1.*

Procedure:
We used exploratory factor analysis (EFA) to examine associations between the 12 OXTR probes at birth. EFA is a data reduction technique that groups correlated probes into a smaller set of factors which account for shared variance between them – an advantageous method when the pattern of relationships between variables (i.e. probes) is not known. The optimal number of factors was determined by comparing fit statistics between models estimating 1 to 5 factors. Model fit was first established using the chi-square statistic, which tests the difference between observed and expected covariance matrices, producing a non-significant value if this difference is close to zero . In the event of a significant chi-square value, we examined additional relative fit indices , including the mean square error of approximation (RMSEA; acceptable fit =< .08), the Comparative Fit Index and Tucker-Lewis Index (CFI & TLI; acceptable fit => .90). As a next step, confirmatory factor analysis (CFA) was run to validate the factor structure identified by the EFA. Once methylation factors at birth were confirmed, we tested whether they remained consistent from birth onwards (i.e. birth vs age 7; age 7 vs age 9), by examining correlations between probes in each factor, and mean levels of DNA methylation in probes within each factor.

Results:
Correlations between the 12 OXTR probes at birth can be found in SI3. Using EFA, we identified 3 methylation factors at birth (containing 3 probes each), which showed the best model fit: X2 (33) = 41.15, p = .16. We then used CFA to validate the 3-factor model and extract factor scores (i.e. containing shared variance between probes in each factor). Model fit was satisfactory (X2 (24) = 70.03 p <.01; CFI = .91; TLI = .86; RMSEA = .08, 90% CIs = .06, .10). See SI4 for probe descriptive statistics, standardized loadings and factor correlations. Probe correlations for each factor remained consistent between birth and age 7, as well as between age 7 and age 9, but mean levels varied across time (see SI5). We present findings relating to Factor 2 as it associated with both the environment and CU. Of note, all probes included in this factor were physically located on the same Exon (i.e., 2) of OXTR . Details pertaining to Factor 1 and Factor 3 are available upon request.



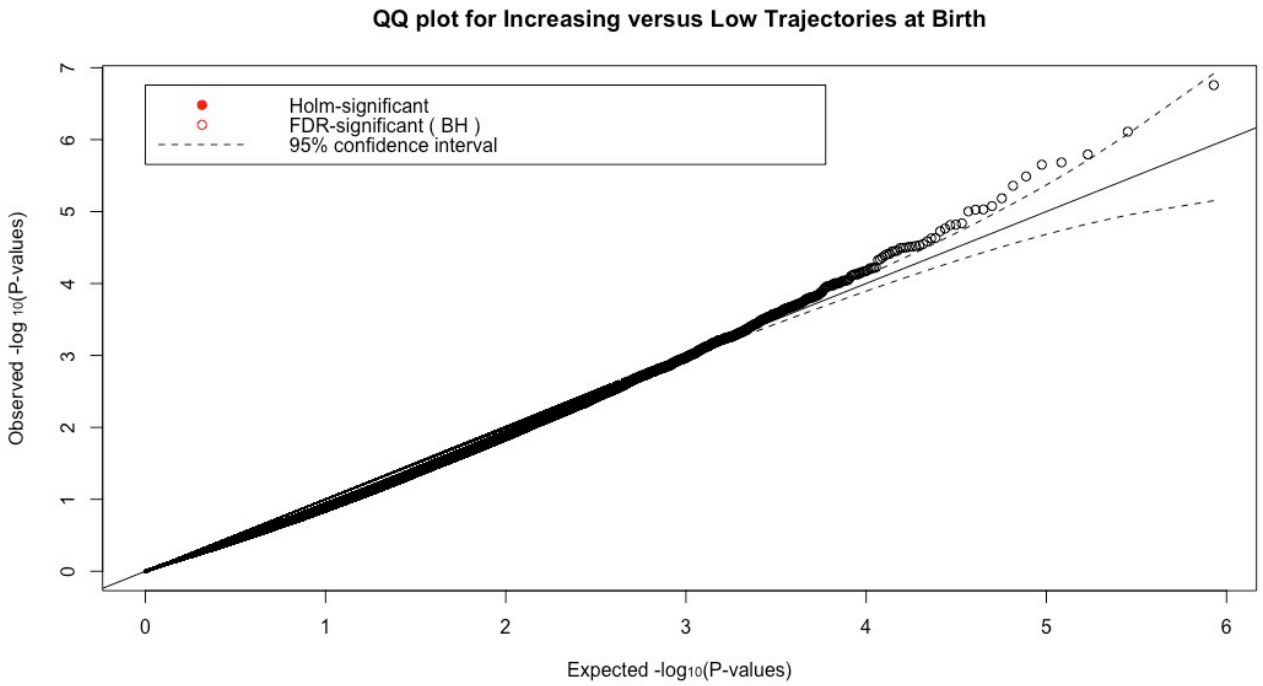
SI 2. *Confirmatory Factor Model of OXTR methylation patterns at birth obtained from Cecil and colleagues (2014) for Study 1*

	Prenatal Period				Early Childhood (birth-Age 7)					Mid-Childhood (Age 8-9)			
	Life Events	Contextual Risks	Parental Risks	Interpersonal Risks	Life Events	Contextual Risks	Parental Risks	Interpersonal Risks	Direct Victimization	Life Events	Contextual Risks	Parental Risks	Interpersonal Risks
Prenatal Period													
Life Events	-												
Contextual Risks	.21**	-											
Parental Risks	.21**	.28**	-										
Interpersonal Risks	.21**	.35**	.34**	-									
Early Childhood (birth - Age 7)													
Life Events	.39**	.01	.09	.13*	-								
Contextual Risks	.31**	.37**	.32**	.21**	.35**	-							
Parental Risks	.22**	.22**	.61**	.24**	.14**	.35***	-						
Interpersonal Risks	.34**	.21**	.29**	.53***	.43***	.42***	40***	-					
Direct Victimization	.19**	.11*	.19**	.25**	.26***	.22***	.20***	.32***	-				
Mid-Childhood (Age 8-9)													
Life Events	.32**	.12*	.11*	.17*	.41***	.24***	.11*	.28***	.29***	-			
Contextual Risks	.08	.11	.09	.14*	.08	.19***	.09	.13*	.13*	.35***	-		
Parental Risks	-.01	.13*	.17**	.07	.06	.13*	.23***	.17**	.06	.12*	.09	-	
Interpersonal Risks	.24**	.13*	.19**	.22**	.25***	.19***	.27***	.48***	.32***	.35***	.25***	.19***	-
Direct Victimization	.01	.04	.07	.15**	.08	.03	.02	.11*	.27***	.15**	.11*	.01	.11*

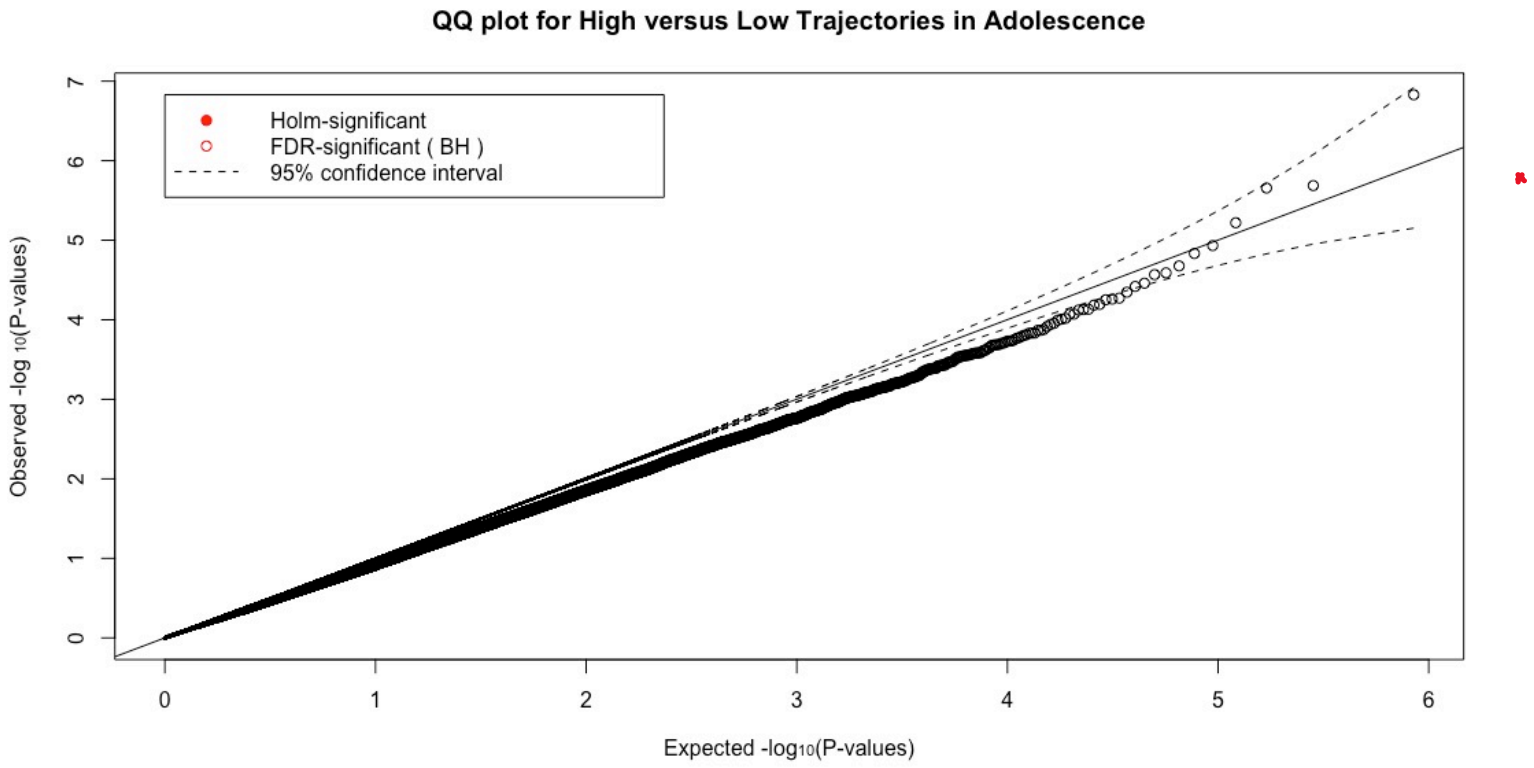
N.b. *** = $p < .001$; ** = $p < .01$; * = $p < .05$.

Confirmatory Factor Analysis model fit indices for each developmental era (full indices provided when chi-square value is significant): (i) *Cumulative prenatal risk score*: X^2 (2) = .60, p = .73; (ii) *Cumulative early childhood risk score*: X^2 (5) = 14.94, p = .01; CFI = .95; TLI = .90; RMSEA = .07, 90% CIs = .03, .12; (iii) *Cumulative mid-childhood risk score*: X^2 (5) = 4.04, p = .54.

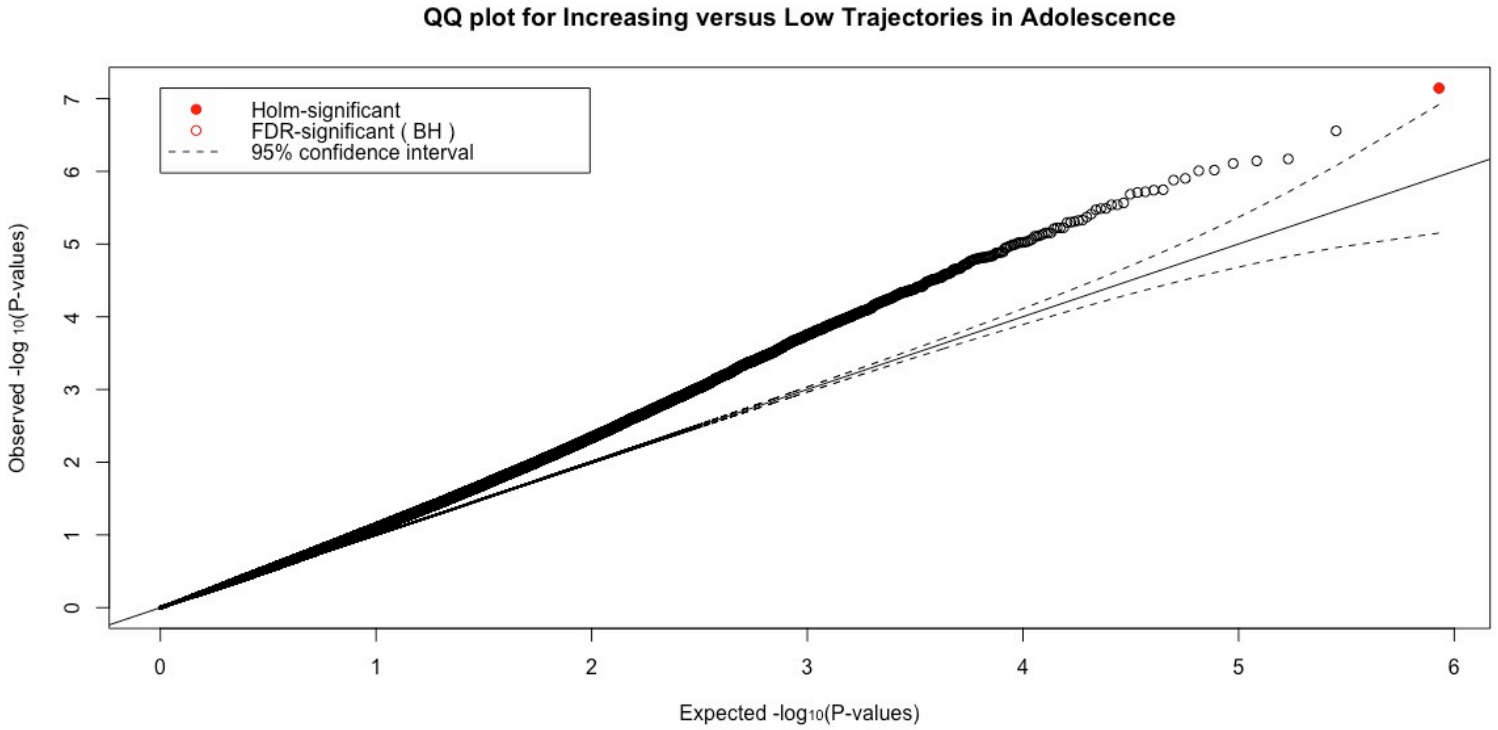
SI 3. Intercorrelations between environmental risk domains across developmental periods and confirmatory factor models obtained from Cecil and colleagues (2014) for Study 1



SI 4. QQ-plot for EWAS comparing increasing (*N* = 94) *to low* (*N* = 662) *groups using DNA methylation obtained at birth*



SI 5. Q-Q plot for EWAS comparing combined high (N = 173) to low (N = 720) groups using DNA methylation obtained at adolescence



SI 6. Q-Q plot for EWAS comparing increasing (*N* = 96) *to low* (*N* = 720) *groups using DNA methylation obtained at adolescence.*

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