2021

The impact of social determinants of health on placental CpG methylation and severity of neurodevelopmental burden in children born extremely preterm

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

THE IMPACT OF SOCIAL DETERMINANTS OF HEALTH ON PLACENTAL CPG METHYLATION AND SEVERITY OF NEURODEVELOPMENTAL BURDEN IN CHILDREN BORN EXTREMELY PRETERM

by

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B.A., Providence College, 2018

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2021

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ACKNOWLEDGMENTS

I would sincerely like to thank my collaborators within the ELGAN project: my primary mentor for this project, Dr. Laurie Douglass, for her continued guidance and encouragement; Dr. Elizabeth Wilson for creating a collaborative space for the development of our vision for this study; and Dr. Karl Kuban for his insightful input on the cultivation of our approach to this research.

I would also like to thank one of my greatest literary assets, Brigid Lavelle, for her contribution to refining my work. Her efforts are always welcomed and invaluable.

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ABSTRACT

Background: It has long been accepted that the environment we experience can impact our well-being; throughout recorded history, the greatest prevalence and severity of disease has been experienced by marginalized and underserved populations. However, the translation of such nontangible influences into biological changes in our health has been elusive until the recent advent of epigenetic studies. Modifications outside of the genome play a critical role in regulating transcription as well as subsequent gene expression without altering DNA sequencing by controlling the accessibility of the DNA for interaction with key initiation proteins and enzymes. These modifications, which include DNA methylation, histone acetylation, and small noncoding microRNA regulation, have increasingly been found to have a fluid, adaptive response to experiences throughout life. Based on the literature supporting societal stressors negatively impacting neurologic outcome, as well as elucidating an association between epigenetic changes and adverse neurologic outcome, we hypothesize that alterations in CpG methylation sites associated with socioeconomic adversity will also be correlated with the incidence of Neurodevelopmental Disorders.

Methods: 889 of the 1,506 neonates initially recruited from 14 medical centers throughout the United States at their time of birth qualified to participate in this study. Placental samples were taken immediately following delivery and neonatal blood samples were taken within the first month of life. Children that survived were followed at 2 years old and 10 years old to evaluate for the presence of four possible Neurodevelopmental Disorders: cognitive impairment, Cerebral Palsy, Autism Spectrum Disorder, and epilepsy. Taking this data as well as demographic information into consideration, the entire cohort included in this study was first evaluated for aberrant methylation levels at 33 CpG sites previously associated with socioeconomic adversity to analyze the degree of significant correlation between altered methylation status and Neurodevelopmental Disorder prevalence. A secondary Epigenome-Wide Association Study was conducted for each of our 889 participants to pinpoint significant changes in CpG methylation in order to evaluate the relationship between altered methylation of particular genes and incidence of Neurodevelopmental Disorders. Taking the previous finding that cognitive impairment imposes a greater burden on both the individual and society than non-cognitive impairment into consideration, both analyses were categorized based on this measure of impairment severity: No Impairment, Non-Cognitive Impairment (diagnosed with Cerebral Palsy, Autism Spectrum Disorder, and/or epilepsy without cognitive deficit), and Cognitive Impairment (cognitive deficit with or without other neurodevelopmental disorders present).

Results: Primary analysis of the 33 CpG sites previously associated with socioeconomic adversity did not reveal any significant associations with Non-Cognitive or Cognitive Impairment. However, cg15519318 and cg10613063 (located in the *PCCB* gene) were marginally associated with Non-Cognitive Impairment while cg02765535 (located in the *NTN4* gene) was marginally associated with Cognitive Impairment. Secondary analysis of the entire epigenome found 4 CpG sites significantly associated with Non-Cognitive Impairment (cg07322235, cg13592565, cg13723879, and cg24387818) as well as 4 CpG sites significantly associated with Cognitive Impairment (cg23081580, cg14134658, cg00762003, and cg08546514).

Discussion: We were not able to define a significant relationship between the CpG methylation sites related to socioeconomic adversity and adverse neurodevelopmental outcomes. This could stem from several causes, including insufficient power as well as limiting our evaluation of the extensive list of environmental influences to the four measures of societal stress focused on in this study (low educational attainment, single relationship status, public health insurance, and receiving supplemental nutrition assistance). Investigating the epigenome for differential methylation that was significantly associated with the incidence of Neurodevelopmental Disorders identified CpGs associated with several important genes, including genes coding for Neuregulin-3 (*NRG3*) and Premature Ovarian Failure Actin Binding Protein 1B (*POF1B*) region with Non-Cognitive Impairment as well as genes coding for Six-Transmembrane Epithelial Antigen of Prostate 2 Metalloreductase (*STEAP2*), Ly1 Antibody Reactive (*LYAR*), 1-

Acylglycerol-3-Phosphate O-Acyltransferase 3 (*AGPAT3*), and Ninein-like protein (*NINL*) with Cognitive Impairment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ASD..... Autism Spectrum Disorder

CFC.....Cognitive Functional Class

CI.....Cognitive Impairment

CP.....Cerebral Palsy

DMR .....Differentially Methylated Region

DNM ..... De Novo Mutation

EA ..... Educational Attainment

ELGAN .....Extremely Low Gestational Age Newborn

EP..... Extremely Preterm

EWAS .....Epigenome-Wide Association Study

ID .....Intellectual Disability

IQ .....Intelligence Quotient

LPA..... Latent Profile Analysis

NCI.....Non-Cognitive Impairment

NDD.....Neurodevelopmental Disorder

NI .....No Impairment

OR..... Odds Ratio

SES.....Socioeconomic Status

INTRODUCTION

Neurodevelopmental Disorders (NDDs) negatively impact the quality of life of a significant portion of the global population, with nearly 1 in 6 children diagnosed in the United States alone.[1] This term is broadly used to describe conditions with atypical maturation and subsequent function of the central nervous system that can manifest in a myriad of phenotypic expressions.[2,3] These conditions are characterized by delayed and/or disturbed skill development in domains such as motor, social, language, and cognition.[4] Common diagnoses include Intellectual Disability, Autism Spectrum Disorder, Cerebral Palsy, and Epilepsy.[5] Intellectual Disability (ID) is a common neurodevelopmental disorder of cognitive impairment that impacts approximately 1-3% of the global population. This diagnosis is characterized by limited intellectual function and adaptive behavior and is classified by having an Intelligence Quotient (IQ) < 70.[6] Autism Spectrum Disorder (ASD) is a group of disorders affecting approximately 2% of the population characterized by dysfunctional social ability, restrictive and repetitive behavior, and impaired communication skills.[7] Cerebral Palsy (CP) encompasses a range of disordered movement and posture that afflicts 2-3 per 1000 live births.[8] Epilepsy is a neurological disease involving recurrent, unprovoked seizures that impacts 1.2% of the US population.[9] Given the vast collection of influences, both intrinsic and extrinsic to fetal development, that contribute to the etiology of these heterogenous disorders, NDDs have a spectrum of presentation and comorbidity that sheds light on the intricacies of neurodevelopment.

Genetics

Many diseases or disorders stem from heritable genetic mutations. For example, tuberous sclerosis complex (TSC) can arise from the autosomal inheritance of mutations in either *TSC1*, a gene located on chromosome 9 and encodes for the protein hamartin, or *TSC2*, a gene located on chromosome 16 and directs production of the protein tuberin. Both hamartin and tuberin play roles in the regulation of protein synthesis, and in the setting of tuberous sclerosis complex they cause hyperactivation of the mTOR signaling pathway. This upregulation of vital cell processes, such as cellular proliferation and metabolism, manifests as multisystem benign tumor growth, seizures, cognitive impairment, and ASD.[10] Familial transmission of X-linked mutations has also been identified, such as variants of the Chloride Voltage-Gated Channel 4 (*CLCN4*) gene responsible for a chloride/hydrogen ion exchanger in the brain that regulates ion homeostasis and intracellular trafficking. These mutations have been found to cause global developmental delays, ID, behavior disorders, and epilepsy.[11]

In addition to these heritable variants, numerous de novo mutations (DNMs) involved in crucial processes throughout fetal development have been associated with atypical neurodevelopment. Sporadic changes to Tubulin Alpha 1A (*TUBA1A*), which codes for the structural protein of the same name, Ras Homolog Family Member B (*RHOB*), which produces Rho-GTPase for regulation of cell signaling, and F-Box Only Protein 31 (*FBXO31*), which is associated with ubiquitination for proteasomal degradation, have been implicated in the development of spastic type Cerebral Palsy.[12] Some of the most common variants linked to epilepsy are within Sodium Voltage-Gated

Channel Alpha Subunit 1 (*SCN1A*), a gene that contributes to the neuronal voltage-gated sodium channel; these mutations give rise to a spectrum of epileptic syndromes, including generalized epilepsy with febrile seizures plus, myoclonic-atonic epilepsy, and Dravet Syndrome.[13,14] Notable mutations involved in ASD include Sodium Voltage-Gated Channel Alpha Subunit 2 (*SCN2A*), which codes for another component of the neuronal voltage-gated sodium channel that coordinates excitation, Chromodomain Helicase DNA Binding Protein 8 (*CHD8*), which is responsible for chromatin remodeling, and Pogo Transposable Element Derived with ZNF Domain (*POGZ*), which produces a vital mitotic protein.[15,16] DNMs in Catenin Beta-1 (*CTNNB1*), whose product beta-catenin plays a vital role in Wnt signaling, as well as Protein Phosphatase, Mg2+/Mn2+ Dependent 1D (*PPM1D*), which produces a phosphatase that regulates p53-mediated stress response, have been associated with intellectual disability.[17,18] Many genetic mutations have been linked to multiple disorders, such as the various missense mutations and polyalanine tract expansions within Aristaless Related Homeobox (*ARX*). This gene codes for a homeobox protein that coordinates neuronal migration, that have been associated with epilepsy, intellectual disability, autism, dystonia, and ataxia.[19] This varying phenotypic expression lends itself to the overlapping of symptoms experienced by NDD patients as well as frequent comorbidity.

Environment

In addition to the adverse effects genetic variance can have on neurologic outcomes, growing interest has been focused on the substantial impact the environment

can have on one's health. This study of external influences has its roots in investigating the tragic outcomes following exposure to chemicals such as aluminum, lead, and mercury. Exposure to these metals can occur through contaminated soil, water sources, and even household paint in the case of lead,[20] leading to the onset and/or exacerbation of various neurological deficits, including intellectual disability, autism spectrum disorder, epilepsy, cerebral palsy, and behavioral problems.[21–23] While all humans are susceptible to the toxic effects of these metals, the fetal period and the first three years of life have a markedly higher risk for adverse outcomes via perturbation of vital neurodevelopmental processes, such as cortical functional differentiation, synaptogenesis, and myelination.[20] Although initially assumed to be protected by the placental barrier, these chemicals are capable of passing directly to the fetus during gestation at varying rates.[24–26] In fact, the placenta has been found to enhance the transfer of mercury, with concentrations in umbilical cord red blood cells being as high as 100% greater than that of maternal red blood cells, resulting in graver effects at smaller maternal doses than previously anticipated.[27,28]

Another component of the environmental impact on health is exposure to harmful pathogens. Cyanobacteria, an alga that can bloom in fresh or brackish water, releases the toxic amino acid β-Methylamino-L-alanine (BMAA) that can cause cognitive impairment as well as future neurodegenerative disease upon exposure. Due to this toxins' ability to cross the placenta, pass to the infant via lactation, and traverse the rudimentary blood-brain barrier (BBB) with a notably higher rate of uptake than in adults, BMAA's effects are experienced most severely by younger children still undergoing neurodevelopment.[29]

Other maternal viral and bacterial infections have been found to have detrimental effects on fetal neurodevelopment, including influenza increasing fetal risk for ASD and low verbal Intelligence Quotient (IQ) scores.[30] Direct infection of the child can also result in detrimental outcomes, such as neonatal Herpes Simplex Virus (HSV) causing severe cognitive impairment and seizure disorder.[31]

While it is clear that these negative maternal exposures increase the risk of fetal anomalies, elements that are normally considered protective factors for a mother's health can also have detrimental effects on the fetus. Medications, in particular, that are prescribed to treat a vast array of maternal conditions can have teratogenic effects on the fetus. Valproate, an antiepileptic drug (AED) used in the treatment of seizures, bipolar disorder, and migraines, can cause neural tube defects, cognitive impairment with speech and psychomotor delay, ASD, and dyspraxia.[32] Isotretinoin, a retinoid used to treat severe cystic acne, has severe and diffuse impacts on the fetus, including microcephaly[33] with subsequent cerebral palsy, intellectual disability, ASD, and epielpsy.[34]

Additional complications can occur during pregnancy, birth, and early infancy that impose environmental strain on the developing child. Complications during the perinatal period such as hypoxic-ischemic events and stroke have a longstanding association with significant risk for causing and/or exacerbating NDDs, particularly CP.[12,35] Common maternal conditions such as gestational diabetes,[36] gestational hypertension,[37] and preeclampsia[38] have also been shown to increase the incidence of NDDs including ASD, cognitive impairment, and epilepsy. Intrauterine growth restriction has been associated with varying severity of ID, while excessive intrauterine growth,

typically associated with gestational diabetes, shows greater association with ID comorbid with ASD.[39] Intrauterine and placental infections with bacterial species such as *Ureaplasma urealyticum*, alpha-hemolytic *Streptococcus*, *Corynebacterium* sp. *Gardnerella vaginalis*, and *Staphylococcus* sp. have also been found to cause various neurological impairments including cognitive and motor delays. These occurrences have been connected via the presence of neonatal systemic inflammation, which is a byproduct of bacterial infection and has been associated with neurodevelopmental impairment.[40] Given the advancement of neonatal care within the last several decades, the increasing rate of extremely preterm (EP) infant survival has expanded our knowledge of the various consequences of immaturity and deficient organ growth.[41] The risk and severity of each of the outcomes described increases with prematurity; of particular relevance, children who are born extremely premature are at greater risk for cognitive impairment with significantly lower IQ,[42] CP, ASD, and epilepsy, as well as additional comorbidities among these NDDs.[43,44]

Social Factors

In addition to these well-established aspects of our tangible environment, recent studies have shifted focus toward the impact our social interactions have on neurodevelopment. It has long been understood that underserved and marginalized populations experience a diminished quality of life, and an archetypal measure used to explain this relationship has been race/ethnicity.[45,46] The persistence of racism in the fabric of our society has cultivated diffuse disparity among minorities, giving rise to

social, economic, and health inequity.[47] Race and ethnicity have classically been used as a generalized measure of socioeconomic status (SES) to explain differences in various health outcomes, however, recent studies have broken down this blanket term to analyze specific experiences of adversity and indicators of deprivation that stem from this discrimination. Examples include fewer opportunities for higher-level education, use of public insurance due to inability to afford private options, single-parent families, and food insecurity. The extensive list of these societal elements found to influence overall health has come to be collectively referred to as social determinants of health (SDOH).[47] Due to the importance of external stimulation in promoting maturation and the heightened susceptibility of the nervous system to environmental influence during early childhood, NDDs are experienced with greater frequency and severity in low and moderate-income countries where malnourishment, insufficient access to structured education, and inadequate healthcare fail to provide the environment needed to ensure optimal development.[48]

Cerebral Palsy and SES

A recent US study found an increased risk for CP among children born to mothers with lower educational attainment (EA) as well as non-Hispanic black ethnicity, both of which are individual-level measures of socioeconomic status (SES).[49] One study chose to consider not only individual-level SES through maternal ethnicity and maternal age, but also a composite neighborhood deprivation index of EA, income, and employment to evaluate the cumulative effects of deprivation on adverse neurologic outcomes. Maternal

minority status, low maternal age, and high levels of neighborhood deprivation were associated with increased rates of children with CP who were non-ambulant, had moderate to severe cognitive impairment, and severe comorbidities.[50] A similar project evaluated both levels of SES and additionally considered the possibility of low area-level SES negatively impacting neurologic outcome without the presence of personal deprivation. Despite having an individual measure of high maternal EA, children living in low SES neighborhoods had an increased risk for non-ambulatory status CP, highlighting the role both direct and indirect deprivation can play in mediating development.[51]

Epilepsy and SES

A recent Swedish study chose to evaluate the impact of neighborhood-level deprivation with and without the presence of personal deprivation, detecting a significant increase in outpatient registrations (healthcare use) in epilepsy patients that had recently moved from moderate to high levels of deprivation.[52] Within the scope of individual SES indicators, parental EA was associated with the highest risk of epilepsy, which was not affected by the level of neighborhood deprivation.[52] In furthering the study of area-level deprivation, a German project defined a positive correlation between neighborhood deprivation and antiepileptic drug (AED) adherence, bringing attention to the medical and social inequities faced daily through difficulty obtaining and paying for medication.[53] These social inequities were explored in a Nigerian study that sought to determine causal factors behind noncompliance. The most common reason for missed appointments was financial constraints that limited transportation and was seen disproportionately among

rural families with lower EA and overall SES. Similarly, the most frequent cause of poor drug compliance was cost, having the greatest impact on patients from lower social class. Following this socioeconomic trend, a positive correlation was found for both maternal EA and income with compliance.[54]

An American study chose to expand these measures of SES to assess the impact of food insecurity on epilepsy, finding those experiencing this particular hardship were at greater risk for more acute healthcare use, via hospitalizations and emergency department encounters, more frequent healthcare use, lower quality of life, and greater experience of adverse effects from AEDs. These effects were significantly greater within the non-Hispanic black population as well as among participants with public insurance.[55]

Cognitive Impairment and SES

The use of individual and residence-based SES measures has also been used in the context of ascertaining ID risk. A French study found a positively stratified risk among unemployment, low EA, and immigration status, with the greatest risk for severe ID among children living in highly deprived neighborhoods as well as having a single parent family.[56] These trends were replicated in a Taiwanese study, which used the degree of neighborhood urbanization as an area-level SES indicator and correlated increased urbanization with decreased incidence of ID.[57] Additionally, an Australian study associated increased risk of mild-moderate ID with increasing neighborhood-level social disadvantage.[58]

With regards to cognitive impairment, SES has proven to have a strong association with future outcomes. A recent UK study evaluating verbal and non-verbal IQ periodically from the age of 2 to 16 years old found not only a positive correlation between SES (quantified via parental education and occupation) and IQ but also a marked amplification of this gap in cognitive ability with progression through adolescence.[59] The development of IQ facilitated by higher SES, and conversely the decline of intelligence scores seen in children with low SES, highlights the accumulating effect such social components can have when experienced throughout childhood.[60] This is corroborated by a US study that found children born into the lower class, determined by parental education and occupation, had significantly lower Full-Scale IQ scores at 3 years old than children born into the middle and upper class.[61]

ASD and SES

Autism Spectrum Disorder presents an interesting divide in trends between US and international studies. American studies reveal a positive correlation between elements of SES, such as EA, and ASD prevalence.[62] These differences are seen between ethnicities as well; a recent temporal study of metropolitan Atlanta spanning 1991-2010 showed the greatest total number of ASD diagnoses among the non-Hispanic white population.[63] In contrast, several studies based in Sweden[64] and France[56] have uncovered an association similar to those previously described among other NDDs. Parental income and lower occupation class were both negatively correlated with ASD prevalence.[64] Additionally, neighborhood deprivation, unemployment, immigration status, and single-

parent families were associated with increased risk of ASD, with severe ID comorbidities most likely to occur with the highest composite score of deprivation.[56] Comparing these outcomes with the US findings highlights the pitfalls of the American healthcare system. Unlike the standardized access to diagnostics and treatment experienced with Swedish healthcare, the stratified opportunities available in the US can lead to case ascertainment bias, which is proposed as a likely contributor to the inverse stratification of ASD prevalence.[56] Despite these flaws, the metropolitan Atlanta study also revealed a greater rate of increase in ASD prevalence was found among the Hispanic and non-Hispanic Black populations, indicating an improvement in diagnostic and treatment availability.[63]

Epigenetics

Despite the well-established association between societal influences and adverse health outcomes, the mechanism of this biological manifestation of intangible factors had been elusive to the scientific community until very recently. The discovery of genetic mutations in the Methyl-CpG Binding Protein 2 (*MECP2*) gene and its causal role in adverse neurologic outcomes at the turn of the 21st century has brought about a new wave of research pointing to epigenetics as the malleable bridge between these two phenomena.[65]

X-linked DNMs of *MECP2*, a gene vital for transcriptional regulation via epigenetic modification, has been extensively studied as a causal factor in the development of intellectual disability, ASD, and Rett Syndrome, a progressive developmental disorder affecting females that involves loss of motor control and speech,

cognitive impairment, and seizures.[66] Investigating the disorders that result from dysregulation of epigenetic mechanisms has highlighted the vital role epigenetic mechanisms play in modulating our phenotypic expression and the fluid nature of our selective application of the genome throughout life.

Epigenetic modifications occur to promote or downregulate the production of a given protein without altering the genetic sequence; this silencing or amplification results in varied phenotypic expression.[67] One major contributor to this process is the post-replication methylation of cytosine residues located 5' to guanosine residues, better known as CpG sites. CpG methylation is catalyzed by DNA methyltransferase and prevents the binding of key proteins involved in transcription, leaving this portion of DNA in a repressed state.[68] The presence of these methylations attracts additional binding proteins, such as the aforementioned *MECP2*, which in turn recruit enzymes that deacetylate histones; these modifications tighten chromatin structure, which further prevents gene expression.[65]

Methylation and SES

A recent study of this cohort identified 33 CpG sites within 21 genes associated with summative SES score, marital status, food insecurity, EA, and health insurance status.[69] These methylation patterns were mostly influenced by maternal single status, which affected the FRY Microtubule Binding Protein (FRY) gene responsible for mitotic structural integrity essential for cortex development and the Apoptosis Inducing Factor Mitochondria Associated 1 (*AIFM1*) gene that induces programmed cell death.[69] The

downregulation of these genes results in congenital NDDs, muscle atrophy, neurodegeneration, motor neuron disease, and severe encephalopathy.[69] Overall, these methylation patterns associated with SES enriched pathways involved in cellular development, growth and proliferation, muscular and hematological system development, and immune cell trafficking.[69] An additional study evaluated the impact of SES on imprinted genes, another form of epigenetic regulation that selectively represses genes in a parent-of-origin-specific manner, allowing for only one allele to be expressed for each gene.[70] A separate study identified 32 differentially methylated regions (DMRs) in response to SES, ethnicity, EA, and income in genes such as Insulin-like Growth Factor 2 (*IGF2*), which encodes the vital promoter of fetal growth, and Neuronatin (*NNAT*), which produces a protein that coordinates energy homeostasis, as well as the genes Maternally Expressed 3 (*MEG3*) and H19 Imprinted Maternally Expressed Transcript (*H19*) which are responsible for non-coding RNAs.[71] *IGF2* expression has also been found to modulate via differential DNA methylation in response to maternal depression and anxiety.[72] These responses to maternal stress, which can be caused by social stressors and adversity, can be interpreted as fetal reprogramming in preparation for the hardships experienced by their mother with the assumption that the child will also experience these hardships in their lifetime. This could be beneficial in theory, as seen in the hypomethylation of Hydroxysteroid 11-β Dehydrogenase 2 (*HSD11B2*), who's gene product is responsible for inactivating maternal cortisol, in children born into deprivation that results in lower cortisol levels and effective stress management in the child.[73] However, this modification can also be maladaptive, as proven by the hypermethylation

of the glucocorticoid receptor gene Nuclear Receptor Subfamily 3 Group C Member 1 (*NR3C1*) in response to maternal material deprivation, resulting in low birth weight and increased risk for development of future disease in the child.[74]

Methylation and NDDs

Considerable research has also been conducted to determine the extent to which epigenetic changes can predict long-term neurologic outcomes. A recent study identified 10 genes involved in the Hypothalamic-Pituitary-Adrenal (HPA) axis, a key component of both neurodevelopment as well as placental physiology, to be significantly associated with cognitive ability at 10 years old. In particular, *NR3C1* and Brain-Derived Neurotrophic Factor (*BDNF*), which promotes differentiation and plasticity, had the strongest association with moderate to severe cognitive impairment at age 10.[75] A deficiency of maternal leptin, the hormone responsible for satiety and heightened activity, has been found to increase methylation of the Leptin (*LEP*) gene in the fetus with subsequent decreased locomotion and hypotonicity.[70] Within a monozygotic twin study discordant for CP, differential methylation was noted in 25 genes. Most notably, Lymphotoxin-α (*LTA*), Lck Interacting Transmembrane Adaptor 1 (*LIME1*), immune signaling pathways genes (Heterogenous Nuclear Ribonucleoprotein L [*HNRNPL*], Ras Association Domain Family Member 5 [*RASSF5*], CD3-delta Molecule [*CD3D*], and Kalirin RhoGEF Kinase [*KALRN*]), and promoters of epileptic encephalopathy-associated genes (TBC1 Domain Family Member 24 [*TBC1D24*], F-Box Only Protein 9 [*FBXO9*], and Vasoactive Intestinal Peptide Receptor 2 [*VIPR2*]).[76] The highest-ranked

DMRs occurred within the *LTA* gene, which encodes for the brain inflammatory regulator TNF-β, and *LIME1*, which regulates MAPK signaling, while other prevalent DMRs enriched cytokine secretion and regulation of leukocyte-mediated immunity. This significant alteration of expression among key neuroinflammatory factors and signaling pathways highlights their role in adverse fetal neurodevelopment.[76] With regards to epilepsy, altered methylation levels in the promoter regions of genes associated with neuronal hyperactivity, including *BDNF* and the glutamate receptor subunits Glutamate Ionotropic Receptor AMPA Type Subunit 2 (*GRIA2*) and Glutamate Ionotropic Receptor NMDA Type Subunit 2B (*GRIN2B*), has been associated with its pathogenesis.[77] Additionally, aberrant methylation of the promoter region of the Reelin gene (*RELN*), which encodes for the neuronal migration protein Reelin, has been implicated in the development of hippocampal granule cell dispersion in Temporal Lobe Epilepsy.[77] Altered methylation of *RELN* has also been associated with the development of ASD, as well as the altered methylation of the Oxytocin Receptor (*OXTR*) gene and SH3 And Multiple Ankyrin Repeat Domains 3 (*SHANK3*), a gene coding for scaffolding protein that connects neurotransmitter receptors and ion channels to the cytoskeleton.[78]

SPECIFIC AIMS

Given the extensive findings supporting the positive correlation between socioeconomic adversity and adverse neurologic outcome, as well as the recent studies that associated increased CpG methylation with both SES and NDD, our research was conducted to determine if methylation patterns associated with SES could accurately predict neurologic outcome. We hypothesized that epigenetic changes contribute to an increased risk of poorer neurological outcomes, and we tested this hypothesis by evaluating whether placental tissue methylation alterations of 33 CpG sites previously associated with socioeconomic adversity[69] were also associated with poorer neurologic outcomes. In light of the significant impact social stressors can have on inducing preterm birth and, in turn, increased risk for NDDs,[42–44,69,71] this study was conducted within the Extremely Low Gestational Age Newborn (ELGAN) cohort. This participant pool is comprised of children born at less than 28 weeks' gestation that have been followed prospectively since birth.

The primary aim of this study was to determine if the aberrant methylation at 33 CpG sites associated with socioeconomic adversity (categorized as cumulative SES score, maternal education, marital status, and food insecurity) was also associated with adverse neurological outcomes at 10 years old (categorized as Non-Cognitive Impairment [CP, ASD, and epilepsy] and Cognitive Impairment [LPA]).

The secondary aim of this study was to evaluate the relationship between neurodevelopmental impairment and altered methylation status at all CpG sites throughout the epigenome. This approach was also categorized by type of impairment.

METHODS

Participants

This study was conducted using participant data from the Extremely Low Gestational Age Newborn (ELGAN) cohort. This is a multicenter study that enrolled newborns during 2002-2004 born at 23-28 weeks' gestation in data collection sites spanning 14 hospitals within 5 states.[44] Informed consent was given by mothers either upon arrival at the hospital for delivery or shortly after giving birth. The Institutional Review Board of each participating site approved of this study. Of the 1,506 infants born to 1,249 mothers that originally consented to participate in this longitudinal panel study, 1,198 children survived to ten years old.[43] Children from whom blood spots were collected during the first postnatal month to assess levels of inflammatory biomarkers (n=966) were invited to take part in the 10-year follow-up, and from that group 889 children agreed to participate.

Placental Tissue Collection

As previously described in prior research utilizing this database,[75,79] a total of 1,365 placentas were transported via a sterile exam basin following delivery to a separate room where sampling took place. Beneath the intact amnion at the midpoint of the longest distance between cord insertion and the placental disk was chosen as the location to remove a portion of the chorion and underlying trophoblast for each tissue sample. If this designated area of the amnion was not intact and chorionic tissue was already exposed, another area of the placental disk with preserved amnion was utilized. Sterile

forceps and scissors were used to gently pull back and cut the amnion in order to expose the chorion, and a piece of the underlying tissue was extracted with a second set of sterile forceps and scissors. Following removal, the tissue was placed in a sterile 2 mL cryogenic vial, treated with liquid nitrogen, and frozen at -80℃. Following collection at the 14 separate sites, frozen samples were sent to a microbiology laboratory in Boston, MA for processing and analysis.

DNA Extraction and Epigenome-Wide Association Study (EWAS)

A previous study[69] outlined the removal, preparation, and analysis of a fraction of the placental tissue biopsy. Following a rinse with 1x phosphate-buffered saline to remove any residual blood, a 0.2g portion of the frozen sample was homogenized in the lysis Buffer RLT with β-mercaptoethanol, and both DNA and RNA strands longer than 18 nucleotides were extracted by an AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Valencia CA). Unmethylated cytosines in the extracted DNA fragments were then bisulfite-converted to uracil by the EZ DNA kit (Zymo Research, Irvine, CA) and subsequently read as thymine residues, leaving the remaining cytosines to be quantified by the Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA). This chip contains probes that measure the methylation level of over 850,000 CpG sites at single nucleotide resolution.[80] Samples were randomized among plates and chips to minimize the batch effect. In accordance with previous recommendations,[81] this data was then imported into R for preprocessing using the *minfi* package.[82] Following quality control for failed samples and duplicates, 426 samples were retained for analysis. A normal-

exponential out-of-band correction method was implemented for background subtraction and dye normalization, followed by functional normalization that used the first two principal components of the control matrix.[83] After additional quality control excluded 806 probes with a p-value > 0.01 for 5% or more of our samples, 856,832 CpG sites were utilized for assessment. Calculated methylation levels were expressed as β values (β= methylated allele intensity (M)/[unmethylated allele intensity (U) + methylated allele intensity (M) + 100]) and were logit transformed to M values for statistical analyses.[84]

Measures of Socioeconomic Adversity

This study chose to replicate the SES variables previously utilized to associate 33 CpG sites with socioeconomic adversity: maternal educational attainment, marital status, type of health insurance, and food insecurity (Table 1).[69] SES adversity indicators for these measures include attaining a high school diploma or less, single relationship status, public health insurance, and receiving supplemental nutrition assistance (food stamps), respectively. This information was collected through a structured interview of the mother at enrollment for the ELGAN study and at 10-year data collection.

As many of these hardships are not experienced in isolation, a summative risk score of each SES indicator was created to evaluate the cumulative effect of experiencing multiple forms of socioeconomic adversity simultaneously. This is a sum of the previously mentioned hardships reported by each family with scores ranging from 0-4. The summative risk score was dichotomized as less than or equal to 1 (0 and 1) and greater than or equal to 2 (2, 3, 4), with scores of 2+ SES categories indicating greater

prenatal socioeconomic adversity based on previous studies of SES and placental CpG methylation that highlight the significance of cumulative effects.[73,85]

Measures of Neurodevelopment

All measures of neurodevelopment were previously outlined in a study of the comorbidity and severity of neurodevelopmental burden within the ELGAN cohort.[43]

Latent Profile Analysis Using Intellectual Quotient and Executive Function

Given its stronger predictive ability of academic achievement[44] as well as individual and societal long-term burden[86] when compared to IQ alone, Latent Profile Analysis (LPA) was used to classify participants into "Cognitive Functional Classes" based on their Intelligence Quotient and Executive Function.

Intelligence Quotient (IQ) was measured via the School-Age Differential Ability Scales II (DAS-II)[87] Verbal and Nonverbal Reasoning Scales.[88] Due to the strong correlation of DAS-II Verbal and Nonverbal IQ scores within this sample, overall IQ was determined as the mean of their scores. An overall IQ score of less than 70, or more than 2 standard deviations below the normative mean (100), was classified as intellectually disabled. All cognitive exams were administered by certified child psychologists. Executive Function (EF) and attention were assessed using the DAS-II along with the Developmental Neuropsychological Assessment-II (NEPSY-II)[89] to measure auditory attention, set switching and inhibition, verbal working memory, concept generation, and mental flexibility.

LPA subgrouping corresponded with levels of Cognitive Functional Class (CFC): CFC 1 (normal) with mean IQ and EF scores within normal range on all measures, CFC 2 (low-normal) with mean IQ and EF scores 0.5-1 standard deviation (SD) below the norm, CFC 3 (moderate impairment) with mean IQ and EF scores 1.5-2.5 SD below the norm, and CFC 4 (severe impairment) with mean IQ and EF scores 3-4 SD below the norm. Participants were identified as having cognitive impairment if they fell under the classification of CFC 3 or CFC 4.[43]

Cerebral Palsy

Neurological examiners assessed participants for CP by utilizing a standardized-manual and data collection form, along with viewing an instructional CD to minimize examiner variability.[90]

Autism Spectrum Disorder

After participants completed the Social Communication Questionnaire,[91] the parents of children deemed to be at risk for ASD were asked to take part in the Autism Diagnostic Interview-Revised (ADI-R).[92] Those whose ADI-R score met the criteria for ASD were subsequently given the Autism Diagnostic Observation Schedule-2 (ADOS-2).[93] Official classification with ASD in this study required participants to meet the standardized criteria outlined in both ADI-R and ADOS-2. All evaluators were given research-level training in the administration and scoring of these assessments.

Epilepsy

Study coordinators aided the parents of participants in completing part one of a validated seizure screen for their child.[94] The parents of children with a positive part one screen completed a structured interview with a pediatric epilepsy specialist who rated the events as seizure or not-seizure. Interview responses were reviewed and rated by a second epilepsy specialist to classify the described events as seizures or not-seizures. A third epilepsy specialist was introduced for the 3% of participants for which the first two epilepsy specialists were in disagreement. Epileptologists were unable to interview 43 of the 273 participants with a positive initial screening, however inverse probability weighting[95] based on gestational age and initial seizure screen result was used to account for this missing data. For the purposes of this study, a diagnosis of epilepsy required two or more unprovoked seizures.[96]

Quantifying/Categorizing Severity of Impairment

Children with non-cognitive neurodevelopmental disorders have been found to have less impact on their family as well as a higher likelihood of living independent lives in comparison to children with neurodevelopmental disorders that include cognitive impairment.[43] Meanwhile, school failure secondary to cognitive impairment is the most costly childhood condition, both individually and societally, outside of complex chronic disease.[86] Given these distinctions, a three-level categorization was devised to group participants based on their overall level of impairment severity. Children were placed in the No Impairment group (Category I) if they were free of any significant

neurodevelopmental impairment. The Non-Cognitive Impairment group (Category II) consisted of participants who were without cognitive deficit (IQ > 70, CFC 1 or 2) but were diagnosed with at least one of the other NDDs within this study (CP, ASD, and/or epilepsy). Finally, the Cognitive Impairment group (Category III) included all children with cognitive impairment regardless of the presence of comorbid NDDs (CP, ASD, and/or epilepsy). Visual and hearing impairments (N=7) were not included in this categorization.[97]

Statistical Analyses

The primary analysis evaluated the relationship between socioeconomic adversity and neurologic outcome through robust logistic regression models. These tests were implemented to determine whether aberrant methylation levels at 33 CpG sites associated with SES (Table 1) are correlated with prevalence and severity of neurodevelopmental impairment. These CpG sites were selected based on a previous study[69] that determined their strong association with experiencing various forms of socioeconomic adversity by conducting an Epigenome-Wide Association Study. Methylation level served as the independent variable for each regression model while the dependent variable was the presence or absence of adverse neurodevelopment, which was categorized based on type of neurological burden. Participants were divided into groups of No Impairment (Category I), Non-Cognitive Impairment (Category II), and Cognitive Impairment (Category III), which correlated with their severity of their impairment. Participants included in the NCI category were diagnosed with ASD, CP, and/or epilepsy but had

normal cognitive ability (CFC 1 or CFC 2). The CI category consisted of all participants classified as CFC 3 or CFC 4 via LPA, regardless of comorbidity with other NDDs. Each regression model was adjusted for socioeconomic and clinical covariates, which were selected based on established associations with both methylation and NDDs including infant sex, gestational age, maternal age, and race. The range of β values was transformed from 0-1 to 0-100 to better visualize the changes in percent methylation reflected in the Odds Ratio (OR) outcomes. The criterion for a CpG site to be significantly associated with neurodevelopmental impairment was a p-value < 0.05. OR, logit OR estimate, SD, z-score, and p-value are reported for each regression.

A secondary analysis investigated the possible correlation of altered CpG methylation with adverse neurologic outcomes by conducting an epigenome-wide analysis of methylation at the level of individual CpG sites. Similar to the first portion of this study, participants were categorized into No Impairment (NI), Non-Cognitive Impairment (NCI), and Cognitive Impairment (CI) and the models were adjusted for infant sex, gestational age, maternal age, and race. Given the increased probability for false positives created by conducting multiple comparisons, a False Discovery Rate (FDR) < 0.10 was used in order to maintain a low false-positive rate while limiting missed significant findings. The log-fold change of average M value (log_2FC), average rate of gene expression (AveExpr), t-statistic, unadjusted and adjusted p-value, log-odds of differential methylation (B), standard error, and associated gene name are reported for each CpG site.

Table 1: Epigenome-wide analysis of the association between DNA methylation and socioeconomic factors.[69]

Socioeconomic factors	CpG	Chromosome	Gene	Genomic location	Relation to CpG island
Summative score					
	cg09625398	8	-	-	-
	cg26546864	15	C15orf26	TSS1500	North Shore
	cg03873518	22	GRAMD4	Body	North Shore
	cg11230111	20	CDH4	Body	-
	cg12217222	1	DR1	5'UTR	Island
	cg15154763	18	C18orf63	TSS1500	North Shore
	cg24032568	10	PPP2R2D	Body	-
	cg09236163	15	C15orf26	1stExon	Island
	cg11173636	10	-	-	-
	cg08707025	8	DLGAP2	Body	Island
	cg21574864	15	FAM103A1	Body	South Shelf
	cg10830144	11	-	-	-
	cg15519318	12	-	-	-
	cg01134296	17	MMP28	TSS1500	South Shore
Marital Status					
	cg21075783	19	-	-	-
	cg14194715	13	FRY	Body	-
	cg15555622	19	ZNF833P	5'UTR	South Shelf
	cg07980716	X	AIFM1	Body	-
	cg10613063	3	PCCB	Body	-
	cg22167148	1	-	-	North Shelf
	cg00606069	X	PHEX	3'UTR	-
	cg24715563	1	RHOU	3'UTR	-
	cg15090198	1	USP48	3'UTR	-
	cg00323466	4	KCNIP4	Body	-
	cg27037498	7	COA1	Body	-
	cg10904858	10	PLCE1	5'UTR	South Shelf
	cg05963700	8	-	-	-
	cg02765535	12	NTN4	Body	-
	cg05186250	15	ZNF609	Body	-
Food Stamps					
	cg00121303	20	MACROD2	ExonBnd	-
	cg01314054	6	-	-	-
Education					
	cg01412404	3	-	-	-
Insurance Status					
	cg19839372	6	-	-	-

KEY: TSS1500=0–1500 bases upstream of Transcriptional Start Site; Body=between ATG and stop codon; 5'UTR= Untranslated Region upstream from start codon; ExonBnd=within 20 bases of an exon boundary

RESULTS

Demographics

Demographic characteristics, including infant sex, gestational age, birthweight, maternal age, and race, among the 889 participants that survived to age 10 were recorded and separated into the three previously designated categories of neurodevelopmental outcome (Table 2). The majority of this cohort had overall normal neurodevelopment (NI N=601, 67.6%), however approximately 1 in 4 children experienced moderate to severe cognitive impairment (CI N=214, 24.1%) and nearly 1 in 10 children were diagnosed with non-cognitive neurological disorders, including epilepsy, autism spectrum disorder, and cerebral palsy, without cognitive impairment (NCI N=74, 8.32%). Infant sex was uniformly stratified among the No Impairment and Non-Cognitive Impairment categories (NI female=310 [51.6%] vs male=291 [48.4%]; NCI female=37 [50.0%] vs male=37 [50.0%]), however there was a slightly higher prevalence of cognitive impairment seen in males (CI: female=87 [40.7%] vs male=127 [59.3%]). While the percentage of participants with a gestational age of 25-26 weeks was comparable across all groups (NI=45.1%, NCI=41.9%, CI=45.8%), the proportion of participants born at 23-24 weeks gestation notably increases within the Cognitive Impairment category (CI=31.8%) and even more so within the Non-Cognitive Impairment category (NCI=39.2%). A similar trend is seen regarding birthweight: the percentage of participants with a birthweight z-score >1 remains fairly constant for all types of impairment (NI=11.8%, NCI=10.8%, CI=11.2%), while the proportion of infants with a birthweight more than 2 SD below the mean (NI=4.66%) increases in those diagnosed with both cognitive (CI=8.41%) and non-

cognitive (NCI=9.46%) impairments. Interestingly, there was a larger presence of children with a birthweight z-score <-1 in the cognitively impaired grouping (CI=16.8%) in comparison to those without impairment (NI=12.6%), yet a somewhat smaller population among those with NDDs but normal cognition (NCI=10.8%). Mean maternal age was comparable across the NI (29.7, SD=6.56) and NCI (29.4, SD= 6.70) groups but was lower for CI (27.97 SD=6.83). In evaluating participant race, the stratification of White, Black, and "Other" subjects was relatively similar among participants with solely non-cognitive impairment (NCI: W=75.7%, B=17.6%, O=6.76%) and those without impairment (NI: W=68.2%, B=20.9%, O=10.9%). In contrast to these categories, cognitive impairment was more notably prevalent among Black and "Other" children (W=44.1%, B=42.7%, O=13.3%).

With regards to factors of socioeconomic adversity, among the 889 participants 21.7% had a single parent, 14.6% had a parent with a high school diploma or less, 35.1% qualified for public insurance, and 22.6% qualified for supplemental nutrition assistance via food stamps.[69]

Neurodevelopmental Impairment

Among the 889 families that agreed to participate in the 10-year follow-up, five children were present but unable to complete the assessments and eleven did not accompany their caregiver to the update visit, resulting in 873 children that fulfilled the measures of neurodevelopment at age 10.[44] Forty of these children were assigned the

lowest score on some if not all tests due to severe motor, visual, and cognitive disability.[43]

Assessment of the cognitive ability of 873 children (100% of the recruited cohort at age 10 years) via Latent Profile Analysis found 214 participants to have moderate to severe cognitive impairment (CFC 3 or 4, respectively), equating to almost 1 in 4 subjects receiving this diagnosis. Of the 849 participants assessed for CP (97% of the cohort), 93 children, or approximately 1 in 10 subjects, met the diagnostic criteria assessed for this study. Within the 857 participants assessed for ASD (98% of the cohort), 61, or nearly 1 in 15 subjects, possessed clinically diagnosable features. Among the 845 participants evaluated for epilepsy (97% of the cohort), 66 participants, which is nearly 1 in 14 children within the cohort, screened positive for this disorder.[43] Based on these findings, the CI category included 214 participants, while the NCI category included 74 participants (Table 2).

When considering the entire cohort, 601 (67.6.%) children were without impairment, while 288 (32.4%) were found to have at least one neurodevelopmental disorder. Within the pool of participants diagnosed with neurodevelopmental impairment, the majority had a single diagnosis (N=171, 59.4%) in comparison to those with comorbid NDDs (two diagnoses N=90, 31.3%; three diagnoses N=25, 8.68%; four diagnoses N=2, 0.69%).[43] Epilepsy had the highest rate of comorbidity, with 71% of participants having other NDDs (N=47): 41 subjects were comorbid with LPA 3 or 4, while 6 subjects were comorbid with ASD or CP (Table 2). Among the children that possessed more than one neurodevelopmental diagnosis, moderate to severe cognitive

impairment (CFC 3 or 4) ran the highest risk for comorbid NDDs. Participants with cognitive impairment were 5.3 times more at risk for epilepsy, 5.5 times more at risk for CP, and 7.1 times more at risk for ASD in comparison to those with normal cognition.[43]

Table 2: Demographics and NDD Distribution of ELGAN Cohort at Age 10 Years

	No Impairment	Non-Cognitive Impairment	Cognitive Impairment
	N=601 (67.6%)	N=74 (8.32%)	N=214 (24.1%)
Sex:			
Female	310 (51.6%)	37 (50.0%)	87 (40.7%)
Male	291 (48.4%)	37 (50.0%)	127 (59.3%)
Gestational Age:			
23-24	90 (15.0%)	29 (39.2%)	68 (31.8%)
25-26	271 (45.1%)	31 (41.9%)	98 (45.8%)
27	240 (39.9%)	14 (18.9%)	48 (22.4%)
Birthweight Z-Score:			
< -2	28 (4.66%)	7 (9.46%)	18 (8.41%)
< -1	76 (12.6%)	8 (10.8%)	36 (16.8%)
<= 1	426 (70.9%)	51 (68.9%)	136 (63.6%)
> 1	71 (11.8%)	8 (10.8%)	24 (11.2%)
Maternal Age (SD):	29.7 (6.56)	29.4 (6.70)	27.7 (6.83)
Race:			
White	405 (68.2%)	56 (75.7%)	93 (44.1%)
Black	124 (20.9%)	13 (17.6%)	90 (42.7%)
Other	65 (10.9%)	5 (6.76%)	28 (13.3%)
CP by limb distribution:			
Quad/Double Hemiplegia	0 (0.00%)	12 (16.4%)	36 (17.8%)
Hemiplegia	0 (0.00%)	6 (8.22%)	10 (4.95%)
Diplegia	0 (0.00%)	17 (23.3%)	12 (5.94%)
None	574 (100%)	38 (52.1%)	144 (71.3%)
ASD:			
0	601 (100%)	53 (71.6%)	174 (81.3%)
1	0 (0.00%)	21 (28.4%)	40 (18.7%)
Epilepsy:			
0	601 (100%)	49 (66.2%)	173 (80.8%)
1	0 (0.00%)	25 (33.8%)	41 (19.2%)

SES CpG Sites and Neurodevelopment

Multivariable logistic regression analysis of the 33 SES-associated CpG sites did not reveal statistically significant associations between altered methylation patterns and neurologic impairment (Table 3, Table 4). Odds Ratio plots were also constructed for each of the 66 regression models (Figure 1, Figure 2), which did not illustrate significant findings. To maintain a suitable scale, cg12217222 was excluded from both OR plots due to the values falling exceptionally outside of the range for both NCI (0.26084497) and CI (52.9347837). None of the P(>|z|) values calculated from these models surpassed the 95% confidence interval utilized for this experiment (characterized by a two-tailed z-score range of $Z < -1.96$ and $Z > 1.96$ with $P(>|z|) < 0.05$). However, three of these tests produced trend-level significance with outcomes surpassing the 90% confidence interval.

CpG sites cg15519318 and cg10613063 were found to have a notable correlation with incidence of Non-Cognitive Impairment, as indicated by p-values of 0.052 and 0.081, respectively. CpG site cg15519318 was pinpointed to chromosome 12, however the Infinium MethylationEPIC BeadChip array was unable to reveal additional information about cg15519318's associated gene, genomic location, or its relation to the CpG island.[69] cg10613063, a CpG site found on chromosome 3, is located within the body of the Propionyl-CoA Carboxylase Subunit β (*PCCB*) gene that codes for the β subunit of the mitochondrial enzyme of the same name.[69] The final notable SES-related CpG, cg02765535, was found to be associated with Cognitive Impairment with a p-value of 0.073. This site is positioned on chromosome 12 within the body of the Netrin 4 (*NTN4*) gene,[69] which produces a laminin-related protein from the netrin family.

With regards to the epigenetic manifestation of SES, altered methylation at cg15519318 is associated with the summative risk score comprised of marital status, insurance status, educational attainment, and food insecurity that illustrates the cumulative effect of socioeconomic hardships. Aberrant methylation at both cg10613063 and cg02765535 is more specifically associated with the parent having a single relationship status.[69]

Table 1: Logistic Regression Estimates and Odds Ratio Probabilities for SES-Related CpG Sites and Non-Cognitive Impairment

CpG Site	Estimate	OR	Std. Error	z value	Pr(>|z|)
cg09625398	-0.0114541	0.98861123	0.0201631	-0.5680734	0.56998513
cg26546864	-0.0758443	0.92696049	0.07441051	-1.0192691	0.30807524
cg03873518	-0.0061452	0.9938736	0.00619156	-0.9925184	0.32094472
cg11230111	-0.0114385	0.98862668	0.01122796	-1.0187506	0.30832137
cg12217222	-1.343829	0.26084497	4.79719883	-0.2801279	0.77937941
cg15154763	0.01749919	1.0176532	0.01239001	1.41236299	0.15784311
cg24032568	0.00804108	1.00807349	0.06335256	0.1269258	0.89899912
cg09236163	-0.0197012	0.98049158	0.04147699	-0.4749917	0.6347929
cg11173636	0.00604161	1.0060599	0.00789665	0.76508499	0.44422094
cg08707025	-0.0580185	0.94363245	0.07954021	-0.729424	0.46574234
cg21574864	-0.0009984	0.99900213	0.02606983	-0.038296	0.96945165
cg10830144	-0.0060004	0.99401759	0.01965995	-0.3052083	0.76020748
cg15519318	-0.018247	0.98191852	0.00940406	-1.9403265	0.05234002
cg01134296	-0.2613615	0.77000252	0.1724451	-1.5156215	0.12961509
cg21075783	-0.0025212	0.99748196	0.00771744	-0.3266905	0.74390198
cg14194715	0.00427985	1.00428902	0.02326301	0.18397659	0.85403182
cg15555622	0.04249417	1.04340997	0.07717056	0.55065258	0.58187186
cg07980716	-0.0154469	0.98467177	0.0198983	-0.7762936	0.43757565
cg10613063	-0.1263885	0.88127244	0.07250637	-1.7431358	0.08130989
cg22167148	-0.0200577	0.98014209	0.01588347	-1.2628049	0.20665931
cg00606069	-0.0164043	0.98372947	0.02038624	-0.8046774	0.42100585
cg24715563	-0.2353931	0.79026015	0.31340284	-0.7510879	0.45259975
cg15090198	-0.0356111	0.96501551	0.38250892	-0.0930988	0.92582511
cg00323466	-0.0021631	0.99783927	0.0093597	-0.2311042	0.81723383
cg27037498	0.19504621	1.21536715	0.36530871	0.5339216	0.59339578
cg10904858	-0.0094207	0.99062354	0.12879202	-0.0731466	0.94168948
cg05963700	-0.0196846	0.98050791	0.05965205	-0.3299897	0.74140773
cg02765535	-0.0040517	0.99595649	0.02597853	-0.1559637	0.87606166
cg05186250	-0.0883383	0.9154511	0.21639826	-0.4082211	0.68311138
cg00121303	0.01129231	1.01135631	0.02553851	0.44216807	0.65836759
cg01314054	-0.0071354	0.99288998	0.01919559	-0.3717218	0.71009999
cg01412404	-0.004384	0.99562558	0.00753147	-0.5820926	0.56050434
cg19839372	-0.0398375	0.96094557	0.02638541	-1.5098307	0.13108664

Figure 1: Non-Cognitive Impairment Odds Ratio Plots for SES-Related CpG sites

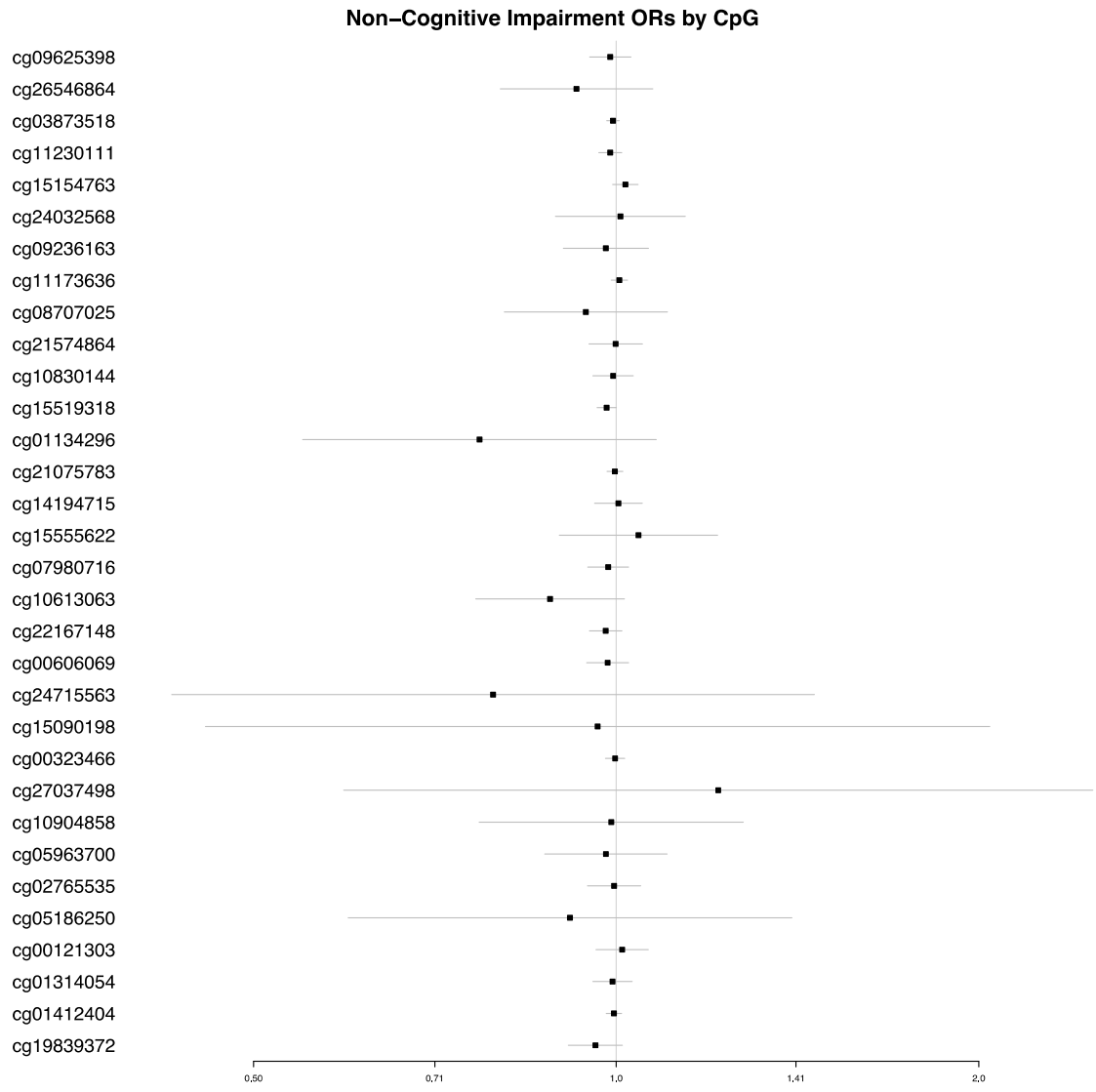
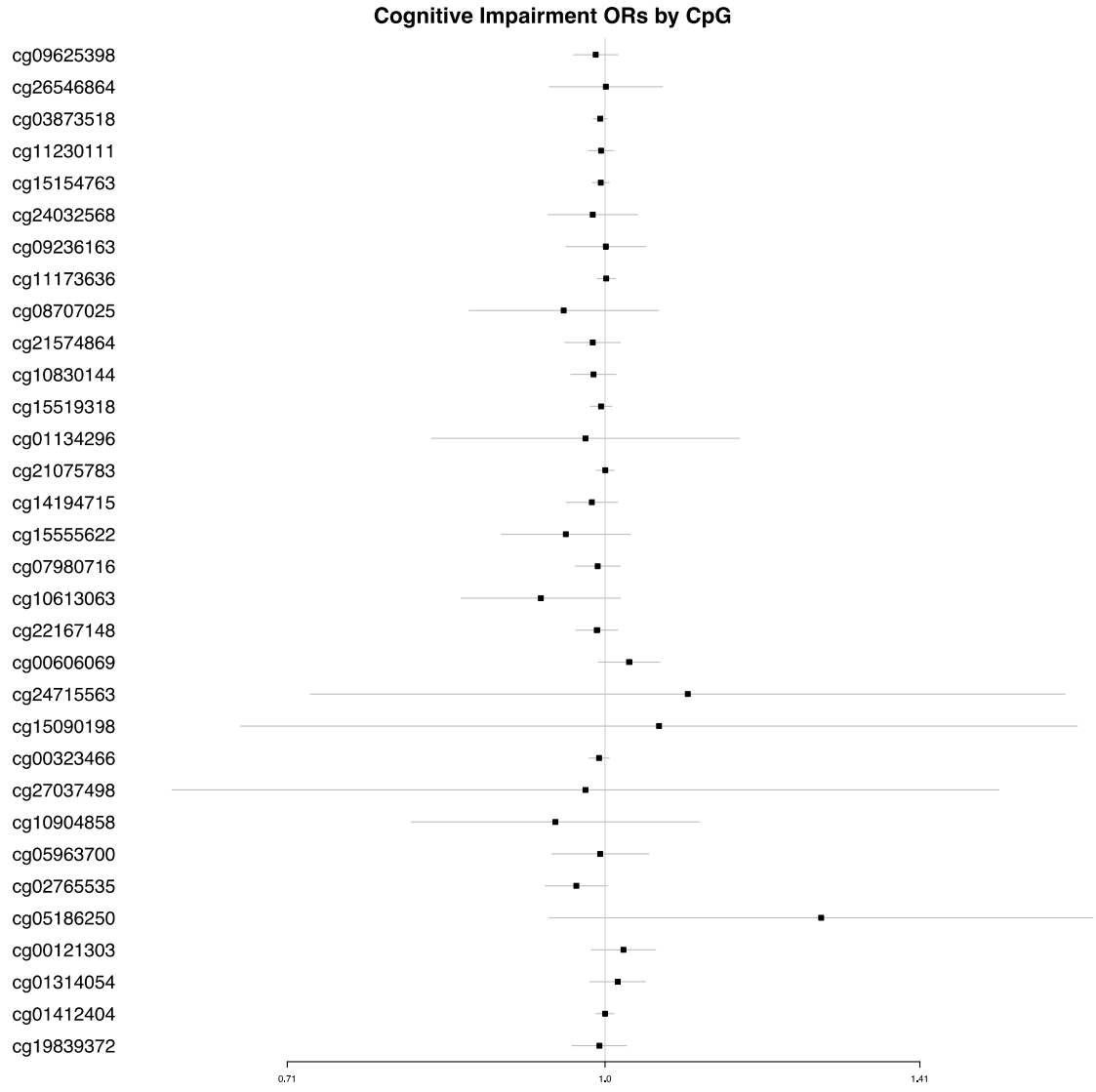


Table 2: Logistic Regression Estimates and Odds Ratio Probabilities for SES-Related CpG Sites and Cognitive Impairment

CpG Site	Estimate	OR	Std. Error	z value	Pr(>|z|)
cg09625398	-0.0101648	0.98988666	0.01235071	-0.8230152	0.41049932
cg26546864	0.00094546	1.00094591	0.03160877	0.02991127	0.97613782
cg03873518	-0.0053134	0.99470068	0.00398954	-1.3318374	0.18291361
cg11230111	-0.0042937	0.9957155	0.00698656	-0.6145655	0.53884169
cg12217222	3.96906066	52.9347837	3.02853813	1.31055331	0.19000872
cg15154763	-0.0045575	0.99545283	0.00478684	-0.9520974	0.34104759
cg24032568	-0.0133296	0.98675886	0.02497538	-0.5337089	0.59354293
cg09236163	0.00093665	1.00093709	0.02218648	0.04221725	0.96632551
cg11173636	0.00126858	1.00126939	0.0050488	0.25126443	0.80160967
cg08707025	-0.0450376	0.95596151	0.05272695	-0.854167	0.39301247
cg21574864	-0.0134076	0.9866819	0.01546322	-0.8670631	0.38590745
cg10830144	-0.0125791	0.98749965	0.0125388	-1.0032163	0.3157565
cg15519318	-0.0042289	0.99578006	0.00603063	-0.7012311	0.48315882
cg01134296	-0.0212822	0.97894267	0.0858582	-0.2478761	0.80423027
cg21075783	0.00031116	1.00031121	0.00507513	0.0613112	0.95111137
cg14194715	-0.014393	0.98571008	0.01438899	-1.0002789	0.31717554
cg15555622	-0.0427148	0.95818462	0.03583491	-1.1919885	0.23326577
cg07980716	-0.008001	0.99203095	0.01257384	-0.6363192	0.52456842
cg10613063	-0.0700369	0.93235942	0.0444778	-1.5746483	0.11533766
cg22167148	-0.0088997	0.99113975	0.01160252	-0.7670519	0.44305067
cg00606069	0.02635417	1.02670451	0.01728365	1.52480328	0.12730817
cg24715563	0.09034357	1.09455028	0.21014396	0.42991278	0.66725909
cg15090198	0.05902881	1.0608058	0.23309184	0.2532427	0.80008067
cg00323466	-0.0064495	0.99357129	0.00561317	-1.148989	0.25056053
cg27037498	-0.0213664	0.97886028	0.23033758	-0.0927611	0.92609336
cg10904858	-0.0541995	0.9472431	0.08017647	-0.6760028	0.4990389
cg05963700	-0.0052185	0.99479511	0.02712678	-0.1923738	0.84744942
cg02765535	-0.0312261	0.96925639	0.01742017	-1.7925262	0.07304872
cg05186250	0.23592301	1.26607683	0.15145857	1.55767356	0.11931065
cg00121303	0.0202311	1.02043714	0.01794338	1.12749655	0.2595326
cg01314054	0.01394231	1.01403996	0.01547489	0.90096306	0.36760796
cg01412404	5.41E-05	1.00E+00	0.00489181	0.01105989	0.99117566
cg19839372	-0.0061268	0.99389194	0.01524029	-0.4020129	0.68767453

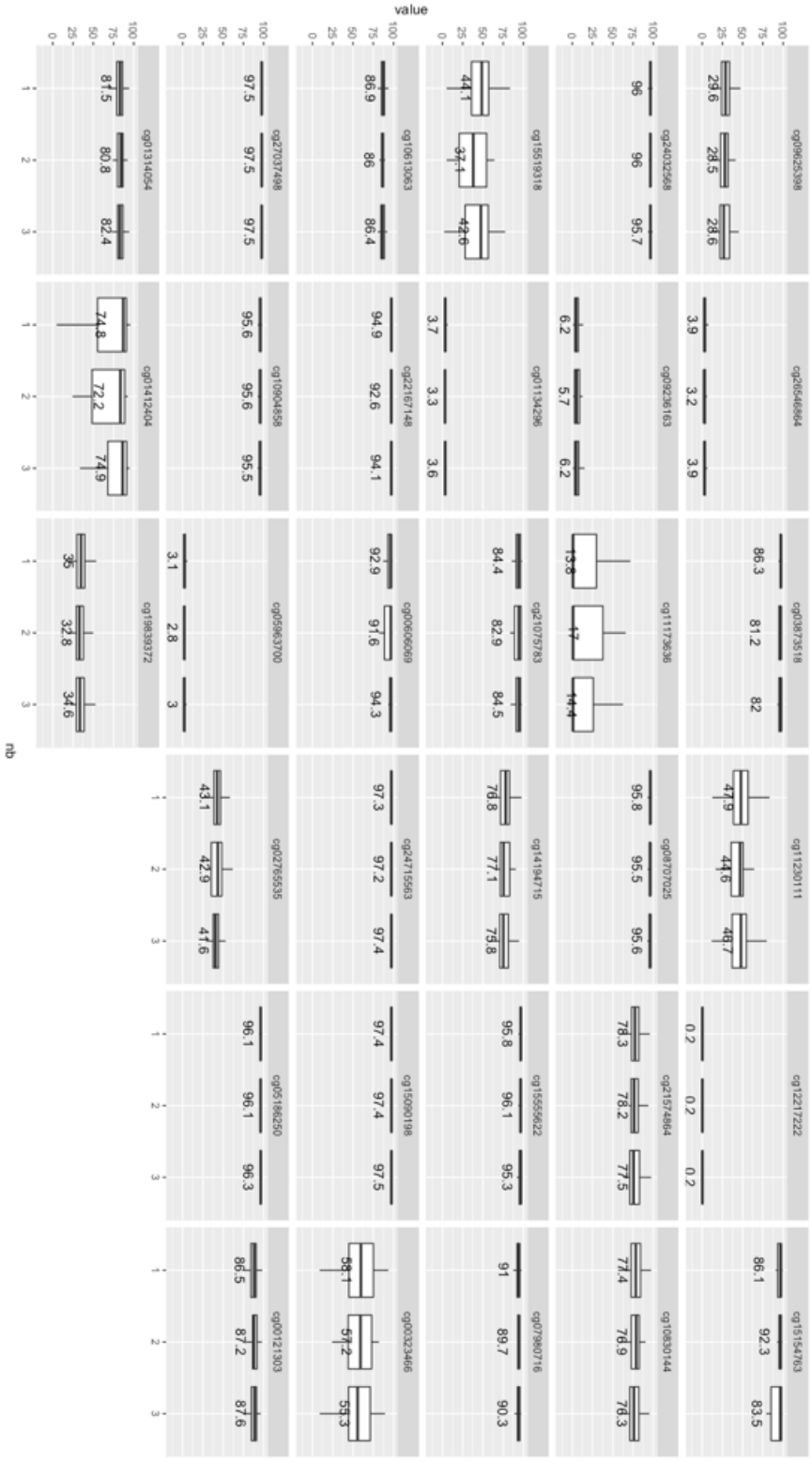
Figure 2: Cognitive Impairment Odds Ratio Plots for SES-Related CpG Sites



The box plots for these comparisons illustrate minimal differences in methylation level as percentages for all 33 CpG sites across all three categories (Figure 3). For the cg15519318 site, participants with no impairments had a median methylation level of 44.1%, while those with non-cognitive impairments had a median methylation of 37.1%. Variance between these groups was also seen at cg10613063, where the median level of methylation for the NI group was 86.9% but for the NCI group was 86%. For the third notable difference, the median methylation of cg02765535 for the NI category was 43.1% while for the CI population the median methylation level was 41.6%.

There are several CpG sites with expansive interquartile ranges across all impairment categories, such as cg00323466 and cg01412404, but there are also numerous sites with limited ranges not visible on the graph, including cg12217222 and cg27037498. Locations with minimal variation between participants are at the extremes of methylation, as characterized by the 0.2% median methylation across categories at cg12217222 and 97.5% median methylation across categories at cg27037498. Conversely, variation both within groups and between groups increases as methylation levels fall farther away from the extremes, as seen in the interquartile ranges of approximately 35%, 40%, and 25% for the NI, NCI, and CI groups at cg01412404.

Figure 3: Box Plots of Methylation Levels for each SES-Related CpG Site in NI (1), NCI (2), and CI (3)



EWAS: Associating CpG Methylation and Neurodevelopmental Outcome

Although the logistic regression analysis of the SES-related CpG sites produced statistically insignificant results, a secondary EWAS found several associations between altered CpG methylation and poor neurodevelopmental outcome. Non-Cognitive Impairment was found to be significantly correlated with four aberrant CpG sites: cg07322235 (log_2FC=-0.55, q=3.33E-03), cg13592565 (log_2FC=-1.17, q=3.53E-02), cg13723879 (log_2FC =0.93, q=3.53E-02), and cg24387818 (log_2FC=0.71, q=4.17E-02) (Table 5). Cognitive Impairment was also found to be associated with four abnormally methylated CpG sites: cg23081580 (log_2FC=-0.36, q=4.01E-02), cg14134658 (log_2FC=0.26, q=7.83E-02), cg00762003 (log_2FC=-1.05, q=7.83E-02), and cg08546514 (log_2FC=0.40, q=7.83E-02) (Table 5). As indicated by negative log_2FC values, four of the eight significant sites were hypomethylated in comparison to the accepted methylation level, while the other four remaining sites exhibited hypermethylation. This equal stratification of increased and decreased methylation levels was also seen within groups, with both CI and NCI categories having two hypomethylated CpG sites and two hypermethylated CpG sites.

With regards to the location of these CpG sites, six of the eight significantly altered methylation patterns were positioned within known protein-coding genes (Table 5). Of the four CpG sites associated with Non-Cognitive Impairment, cg13723879 was found within the Neuregulin-3 (*NRG3*) gene and cg24387818 was discovered within the Premature Ovarian Failure Actin Binding Protein 1B (*POF1B*) gene. All four altered methylation patterns significantly correlated with Cognitive Impairment were situated

within the coding region of known genes: cg23081580 was found within the Six-Transmembrane Epithelial Antigen of Prostate 2 Metalloreductase (*STEAP2*) gene, cg14134658 was located in the Ly1 Antibody Reactive (*LYAR*) gene, cg00762003 was detected in the 1-Acylglycerol-3-Phosphate O-Acyltransferase 3 (*AGPAT3*) gene, and cg08546514 was observed within the Ninein-like protein (*NINL*) gene.

Table 3: EWAS CpG Sites Significantly Correlated with NCI and CI

ID	logFC	AveExpr	t	P Value	Adj. P Value	B	SE	UCSC RefGene Name
Non-Cognitive CpG sites:								
cg07322235	-0.5531412	4.92291803	-6.025147	3.85E-09	0.00332926	3.33142572	0.09180543	
cg13592565	-1.172266	4.92872096	-5.4108503	1.09E-07	0.03529501	2.48665419	0.21665097	
cg13723879	0.93007798	-5.1916186	5.3876075	1.23E-07	0.03529501	2.44852911	0.17263284	NRG3; NRG3; NRG3
cg24387818	0.70643436	-4.1984521	5.29951487	1.93E-07	0.0416581	2.26716037	0.1333017	POF1B
Cognitive CpG sites:								
cg23081580	-0.3609776	-6.1329647	-5.5725535	4.64E-08	0.04006052	6.92101831	0.06477777	STEAP2; STEAP2; STEAP2
cg14134658	0.25847994	2.1669353	5.23815371	2.64E-07	0.07826763	5.57945301	0.04934562	LYAR; LYAR
cg00762003	-1.0485589	1.94139886	-5.1866412	3.42E-07	0.07826763	5.14148867	0.20216531	AGPAT3; AGPAT3
cg08546514	0.40284331	-0.1579744	5.17503489	3.62E-07	0.07826763	5.27514757	0.07784359	NINL

KEY (Tables 5 and 6): $\log_2$FC= $\log_2$ fold change (FC) of average M values between two levels of a given phenotype; AveExpr= average rate of gene expression; t= moderated t-statistic for test of association; Adj. P Value= adjusted p value for FDR<0.1; B= log-odds that the CpG site is differentially methylated; SE= Standard Error.

DISCUSSION

The purpose of this study was to evaluate the relationship between exposure to socioeconomic adversity during gestation and the development of neurological disorders through epigenetic modification of DNA methylation levels. Given the previously well-defined impact of socioeconomic status on neurologic outcome[45,46,49–64] as well as epigenetic alterations,[69–74] we hypothesized that the manifestation of societal hardships in epigenetic changes would also correlate with the incidence of Neurodevelopmental Disorders (NDDs). Our primary findings revealed marginal associations of the SES-related CpG sites cg15519318 and cg10613063 with Non-Cognitive Impairment, as well as cg02765535 with Cognitive Impairment, however no significant correlations between these two categories of variables. Our secondary findings from evaluating the relationship between altered methylation status and neurodevelopment uncovered 4 CpG sites associated with Non-Cognitive Impairment and 4 CpG sites associated with Cognitive Impairment. While these findings shed light on the interplay of epigenetics and neurological phenotypic expression, this study was unable to prove, or disprove, our original hypothesis.

Demographics and Neurodevelopmental Disorder Prevalence

An increase in both categories of NDD incidence in neonates born at 23-24 weeks gestation as well as those with below-average birthweight is consistent with previous findings that gestational age is inversely correlated with risk for neurodevelopmental impairment.[42–44,69,71] The unequal sex-stratification seen in the CI group (male N=127,

female N=87) is consistent with previous findings that rates of intellectual disability are significantly higher among males.[63] This trend has been identified in many other NDDs including ASD[63] and CP,[98] while sex differences within epilepsy are more evenly split between subtypes.[99] Given the greater number of participants with ASD or CP diagnoses in comparison to those with epilepsy, the equal distribution of the NCI group between females (N=37) and males (N=37) is an interesting deviation from the expected stratification towards males. Due to our exclusion of probes for CpGs on sex chromosomes from our analysis, we are unable to determine whether these findings were a result of methylation alterations in genes located on the sex chromosomes.

The notably higher level of white children diagnosed with Non-Cognitive Impairments (ASD, CP, and epilepsy) in comparison to Black and "Other" children in this study was an intriguing and unexpected outcome. Our findings mirror the racial differences extensively documented in the US population for Autism Spectrum Disorder prevalence, which has been majorly attributed to the under-ascertainment of cases due to insufficient access to healthcare and diagnostic evaluation.[63] Given the strict adherence of our providers and researchers to implementing protocol uniformly for each participant, this bias should not exist among the participants included in this study. However, a possible explanation of these racial differences in NCI prevalence could be that expecting mothers with low SES, which is a population disproportionately comprised of non-white communities,[49] had greater difficulty accessing healthcare at one of the study's fourteen institutions due to other financial constraints, such as transportation access and costs,[54] thus preventing them from having the opportunity to partake in this study. In light of the

majority of participants being white (63%) and experiencing zero of the four measures of socioeconomic adversity (62%), future projects should strive to make participation in these studies more accessible for a spectrum of populations in order to increase the statistical power of these societal influences that predominantly impact low SES populations as well as the external validity of their findings.

Interestingly, ASD was the least prevalent NDD within our cohort (N=61, 7.12%), while Cerebral Palsy, a disorder with an established trend of higher prevalence in Black populations,[49] was the most prevalent Non-Cognitive Impairment (N=93, 11.0%). The absence of this trend in our cohort could coincide with recent developments in CP incidence beyond the scope of perinatal complications. Classically, the development of Cerebral Palsy has been largely attributed to complications during delivery (hypoxic-ischemia, stroke, bradycardia, etc.)[35]; due to the inadequate access to quality healthcare for Black populations, this has resulted in a higher incidence of this disease within the Black community.[49] The improvement of delivery practices, such as fetal heart monitoring and cesarean section procedures, in the last several decades without significant decrease in CP prevalence has revealed that these perinatal events in the setting of CP are a by-product of previously established abnormalities and genetic predispositions, rather than a causal factor of the disease.[35] Given that all of the participants within this cohort were delivered in one of fourteen medical centers and therefore exposed to the same conditions, the quality of care between patients in each medical center should be of the same level. Further research of this cohort could be done

to determine whether the quality of care offered at each institution impacted CP prevalence.

SES-Associated CpG Methylation & Neurodevelopmental Disorders

Contrary to our initial hypothesis that the biological manifestation of environmental stress displayed in 33 CpG sites previously associated with SES would also be associated with neurodevelopment, our findings did not indicate a significant correlation between altered methylation patterns at these sites and worsened neurologic outcome. We chose to explore this topic because finding a correlation between these particular methylation sites and neurodevelopment could have served as a social predictive measure for neurologic deficit. This, in turn, could have contributed evidence to support legislation that improves the quality of and accessibility to various public resources concerning health and overall quality of life. While the four socioeconomic factors evaluated in this study represent considerable sources of adversity, there are a substantial amount of other societal factors that contribute to adverse health outcomes that should be explored in the future. This includes disparate educational opportunities,[100] healthcare quality and access,[101] housing conditions,[102] employment exclusions,[102] transportation limitations,[103] and many others. Given our lack of significant findings, it is possible that the CpG alterations related to the four SES indicators evaluated in this study have a weaker correlation with neurologic outcome than the epigenetic changes associated with other forms of social adversity. Thus, these four measures may not play

as important of a role in neurodevelopment in comparison to other types of societal stress.

Although the first portion of this experiment did not culminate in significant results, several interesting trends emerged from its analysis. Two of the three SES-related CpG sites having the strongest trends with neurologic outcome are located within genes that have considerable impact on neurodevelopment. Propionyl-CoA Carboxylase Subunit β, the protein coding gene that includes CpG site cg10613063 associated with Non-Cognitive Impairment, is a mitochondrial enzyme highly expressed in the fetal brain during the embryonic stage. Although not directly involved in neurological processes beyond early gestation, inadequate function of this enzyme in the adrenal gland, liver, and kidney can result in movement disorders, intellectual disability, and epilepsy secondary to metabolic disorders such as Propionic Acidemia.[104] Similarly, the protein coding gene Netrin 4 contains the CpG site cg02765535, which was found to be marginally associated with Cognitive Impairment. This protein contributes to axon guidance, neurite growth and migration, and neuron remodeling,[105] and its malfunction has been linked to several neurodevelopmental disorders including Tourette Syndrome.[106]

With regards to the association of these three CpG sites with summative SES risk score (cg15519318) and single marital status (cg10613063 and cg02765535), this also reflects the original study's finding[69] that these two forms of adversity had the greatest number of associations with altered DNA methylation (summative score=14 sites, single=15 sites) in comparison to the other SES indicators (food insecurity=2 sites, high school diploma or less=1 site, and public health insurance=1 site). These outcomes

further highlight that every component of our external environment and societal experience does not have the same biological impact, particularly when they accumulate and interact with one another.[73] Breaking down the umbrella term of socioeconomic status into its multiple components allows us to determine the more prominent promoters of adverse health outcomes, such as parental marital status. By taking a finer lens to environmental and social factors, we hope to promote the development and implementation of health risk evaluations that not only incorporate but place weight on the role of societal factors in development and exacerbation of disease.

Another interesting trend can be seen in the pattern of variance among the SES-CpG box plots (Table 3). As previously noted, the CpG sites with methylation levels occurring close to either 0% or 100% were most uniform among all participants and variation increased as methylation levels travelled away from these extremes. The lack of variation at CpG sites such as cg12217222, which has a median of only 0.2% methylation with no visible range across all subjects and is thus unencumbered by methylation to proceed with transcription, could indicate that the proteins they code for benefit from adapting to the experience of socioeconomic adversity but play an essential role common to every human being regardless of neurodevelopmental status. Ironically, the gene containing cg12217222 codes for Down-Regulator of Transcription 1 (*DR1*), a TATA Box Binding Protein-associated phosphoprotein that controls the rate of RNA polymerase II transcription by inhibiting the assembly of the preinitiation complex.[107] This further supports the concept that adaptive genetic expression is a crucial and active component of daily life.

In similar theory, the sites with greater variation may play a role expressing genes involved in neurodevelopment. Potassium Voltage-Gated Channel Interacting Protein 4 (*KCNIP4*), the gene that contains the variably methylated cg00323466, encodes a protein that inhibits neuronal excitability via A-type currents in response to intracellular calcium.[108] Given the involvement of this protein in such a vital neurological process and the extensive implication of dysregulated neuronal excitability in epilepsy,[109] ASD,[110] and ID,[111] this spectrum of phenotypic expression across participants could lend itself to the intricacies of each NDD's pathogenesis as well as which degrees of physiological deviation correlate with each disorder. Given the lack of literature concerning this pattern, further research should be conducted regarding its validity and whether it could serve as a predictive marker of susceptibility to alteration.

EWAS CpG Methylation & Neurodevelopmental Disorders

Although our primary analysis of the epigenetic connection between socioeconomic adversity and neurodevelopment proved to be insignificant, evaluation of CpG alterations throughout the epigenome in the presence of neurodevelopmental impairment was very informative. The differentially methylated protein-coding genes uncovered in this study have a spectrum of influences on health outcomes. Several of these genes play integral roles in the development and function of the central nervous system. For example, *NRG3*, the gene that contains NCI-associated CpG site cg13723879, encodes the ligand for epidermal growth factor tyrosine kinase ERBB4 that activates intracellular signaling cascades for neuroblast proliferation, migration, and

differentiation.[112] Prior studies have linked diminished production and function of this protein to several neurological disorders, including Alzheimer's disease, ASD, and cognitive deficit in the setting of schizophrenia.[113] 1-Acylglycerol-3-Phosphate O-Acyltransferase 3 (*AGPAT3*), the gene containing the CI-associated CpG site cg00762003, produces an enzyme of the same that converts lysophosphatidic acid into phosphatidic acid within the phospholipid biosynthetic pathway and contributes to retrograde transport from the Golgi complex.[114] The AGPAT3 enzyme also acts on lysophosphatidylcholine, the major source of docosahexaenoic acid (DHA) that is critical in neurodevelopment.[115] Upregulation of this enzyme could cause a "nutritional toxicity" of excessive DHA accumulation in the brain that results in competitive displacement of other fatty acids, such as arachidonic acid and omega-6 fatty acids, that play vital roles in myelination, plasticity, and neuronal signaling.[116,117] Ninein-like protein, which is produced by the *NINL* gene containing CI-associated CpG site cg08546514, is involved in the microtubule organization of interphase cells and cilia for organelle development and cell growth.[118] Decreased expression of this gene has been implicated in the development of Joubert Syndrome, a complex neurodevelopmental disorder resulting from cerebellar malformation that is characterized by hypotonia and ataxia, delayed acquisition of motor and language skills, and intellectual disability.[119] Additional adverse neurologic outcomes include Autism Spectrum Disorder and forms of progressive vision and hearing loss, such as Usher Syndrome, Fundus Dystrophy, and Inherited Retinal Disorder.[118]

Other identified genes were not directly involved in neurodevelopment; however, their malfunction could lead to detrimental effects on the nervous system. The Six-Transmembrane Epithelial Antigen of Prostate 2 Metalloreductase (*STEAP2*) gene that contains the CI-associated CpG site cg23081580 produces an enzyme of the same name that functions as an integral membrane protein responsible for stimulating cellular uptake of iron and copper.[120] Diminished function of this transporter can cause aceruloplasminemia, a severe neurodegenerative disorder resulting from the accumulation of iron in the brain that can cause cognitive decline as well as various movement disorders including cerebellar ataxia, chorea, and dystonia.[121] The gene product of Premature Ovarian Failure Actin Binding Protein 1B (*POF1B*), which includes the NCI-associated CpG site cg24387818, plays a critical role in tissue organization and cytoskeleton regulation through binding actin filaments for cell adhesion. Loss of protein function due to translocation in the critical region of the X chromosome long arm can result in several abnormalities, including X-linked intellectual disability and deafness.[122]

One gene of particular interest has no obvious connection with the central nervous system. Ly1 Antibody Reactive (*LYAR*), which carries the CI-associated CpG site cg14134658, encodes for a cell growth-regulating nucleolar protein involved in rRNA production maintenance, transcriptional regulation, and promotion of cell proliferation in undifferentiated cells. This protein also negatively regulates the innate immune response by impairing the DNA-binding activity of transcription factor Interferon Regulatory Factor 3 and inhibiting Nuclear Factor kappa B-mediated expression of proinflammatory

cytokines. The upregulation of this protein has been implicated in pathologies outside of the scope of extrauterine neurodevelopment, including the lethal neural tube defect exencephaly, in which the fetal brain develops outside of the skull, and neuroblastoma tumorigenesis.[123,124] However, given *LYAR*'s roll in suppressing the immune response in order to preserve the host, upregulation of this gene could be a byproduct of placental or uterine infection during gestation. As previously mentioned, systemic inflammation in response to infection within the underdeveloped brain can induce chemokine production and recruit immune cells through the primitive blood brain barrier and into the parenchyma, resulting in white matter damage.[41]

The differentially methylated sites correlated with adverse neurodevelopment were uniformly stratified based on the type of neurodevelopmental impairment, however when the structure of these categories is taken into consideration, the same number of changes in methylation pattern attributed to one diagnosis (cognitive impairment) is associated with a group of several diagnoses (cerebral palsy, autism spectrum disorder, and epilepsy). This outcome reflects the greater prevalence of cognitive impairment, with 214 participants being diagnosed by an LPA of 3 or 4, in comparison to CP, ASD, and epilepsy, whose pooled population was 220 participants (Table 2). However, despite the possibility of similar origins and notable risk for comorbidity amongst them,[125] these are three vastly different diseases with varying pathologies and presentations. Although the four NCI sites are likely not associated with all three disorders, there is no way to assume the distribution of these associations based on this model. Additionally, while the NCI category strictly includes participants with CP, ASD, and/or epilepsy diagnoses, the CI

category includes all participants with cognitive impairment regardless of comorbidity with other NDDs. This is of particular interest when CpG sites such as cg23081580 and cg08546514, which are associated with the CI category, are components of genes implicated in the development of CP and ASD. Further stratifying this variable to analyze the correlation of these CpG sites with each diagnosis could provide greater insight into the similarities and nuances of each disease's pathogenesis with the hope of elucidating epigenetic risk level of comorbidity as well as specific targets for therapy.

Limitations

Several factors should be taken into account while interpreting the results of this study. We did not utilize the methylation status of participants at 10 years old in our analysis, but rather solely evaluated the presence of an NDD diagnosis in correlation with placental DNA methylation. Future study including CpG methylation at 10 years old could possibly reveal stronger associations with directionality. Additionally, the analysis of methylation status was restricted to placental DNA. Although it is well established that the placenta acts as a bridge connecting the fetus to its environment and thus embodies the physiological manifestation of fetal experience and exposure,[24–28] it is also known that certain types of methylation occur in tissue specific patterns.[126] Our approach to this study was to evaluate placental DNA methylation patterns as a "snapshot" of the signaling pathways occurring during fetal development in order to determine if this "snapshot" could serve as a predictive measure for neurologic outcome. As such, we do not make the assumption that these CpG alterations would be found in the brain of the

child during gestation or at 10 years old. In light of these restrictions, future studies should investigate DNA methylation in a cell type from the nervous system that could be evaluated at birth and throughout life to provide a consistent measure with greater tissue specificity.

With regards to the participant pool, conducting research with the multicenter ELGAN cohort helped increase magnitude and diversity of patient population; however, given the stratification of each category with numerous variables, the group sizes considerably downsize during analysis. This is notable with types of neurological impairment (24.5% LPA 3 or 4, 11.0% CP, 7.81% epilepsy, 7.12% ASD) as well as in types of socioeconomic adversity (21.7% single parent, 14.6% low parental EA, 35.1% public insurance, and 22.6% food insecurity), particularly when calculating the summative score. As previously discussed, expanding future cohorts by recruiting additional medical centers could increase the power behind potential findings. Additionally, despite the strict protocol that was developed and implemented for all forms of participant evaluation and data collection, the vastness of this program increases heterogeneity of study samples due to the possibility of slightly different approaches to the diagnostic methods by each provider/researcher.

Future Directions

Although the 33 SES-associated CpG sites in question were not significantly correlated with an increased risk for adverse neurologic outcomes, a considerable amount of previous research has illuminated this relationship between societal factors and

neurodevelopment.[45,46,49–64] Given that this well-established association exists, this avenue could be further explored by evaluating the correlation of the CpG sites identified by EWAS in this study, as well as other sites previously associated with neurodevelopmental impairment,[70,75–78] with socioeconomic adversity. As previously mentioned, in order to gain a more specific understanding of neurodevelopmental impairment, future models could account for specifics NDD diagnoses individually, severity, and comorbidities. Additionally, exploring the interactive effects of CpG methylation and socioeconomic adversity on NDDs as well as possible confounding variables could elucidate a stronger relationship between societal stress and adverse neurodevelopment.

A future direction we plan to explore is the possible modifying effect of changes in SES on neurologic outcomes by calculating the change in each participant's summative risk score of socioeconomic adversity between birth and 10 years old. By categorizing participants into those who experienced an increase, no change, or decrease in adversity, we hope to evaluate the impact of SES indicators on NDD prevalence beyond gestation.

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