Universidade do Minho
Escola de Psicologia

Ana Filipa Cunha Mendes

The Impact of Adverse Childhood Experiences on Emotional and Behavioral Problems in Institutionalized Children: The Contributions of Epigenetic Mechanisms

outubro de 2023

The Impact of Adverse Childhood Experiences on Emotional and Behavioral Problems in Institutionalized Children: The Contributions of Epigenetic Mechanisms
Ana Filipa Cunha Mendes
UMinho | 2023

Universidade do Minho
Escola de Psicologia

Ana Filipa Cunha Mendes

The Impact of Adverse Childhood Experiences on Emotional and Behavioral Problems in Institutionalized Children: The Contributions of Epigenetic Mechanisms

Dissertação de Mestrado
Mestrado em Psicologia Clínica na Infância e Adolescência

Trabalho efetuado sob a orientação da
Professora Doutora Isabel Soares e da
Professora Doutora Ana Mesquita

outubro de 2023

Agradecimentos

Com o fim de mais um capítulo, agradeço àqueles que fizeram parte dele e que tornaram possível a conclusão de um percurso que temos vindo a construir nestes últimos cinco anos.

À Professora Isabel Soares e à Professora Ana Mesquita, pelo conhecimento transmitido, o exemplo de profissionalismo e excelência e o apoio infalível nesta etapa. Acima de tudo, agradeço por me mostrarem o caminho, por serem inspiração para o meu percurso académico e profissional.

Ao meu pai, às minhas irmãs e aos meus tios. Esta conquista é vossa também, o vosso apoio incondicional permitiu-me crescer e chegar até onde estou. Obrigada por serem por serem a minha rede de segurança e não me deixarem cair, por serem abrigo nos momentos difíceis e por acreditarem em mim, especialmente quando eu própria não acreditei.

Às minhas amigas, que realizaram este percurso ao meu lado. À Eli, por seres conforto e carinho, pelo apoio emocional, por estares ao meu lado de igual forma na alegria e no desespero. À Xanna, pela confiança em mim, pela honestidade e clareza, por me manteres focada e não me deixares desistir. À Laura, por seres um exemplo a seguir, pela tua calma e sabedoria, por me tirares da confusão quando estou perdida.

Ao Nuno, por me teres dado a mão naquele dia e nunca mais me teres deixado. Por todas as conquistas que alcançamos juntos e todos os obstáculos superados. Foi um prazer ver-te crescer e um privilégio crescer contigo.

À Cátia, a outra metade do meu cérebro, por seres a melhor amiga que podia pedir, por me compreenderes melhor do que ninguém, por seres a primeira a estar ao meu lado quando preciso, por seres um apoio constante e inabalável.

Ao Cobain, por acreditares em mim e por me mostrares que não há nada que me impeça de alcançar o que quero, por seres a minha motivação e alegria quando estas faltaram, por sorrisos que iluminam os dias escuros.

Obrigada a todos.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Univesidade do Minho, 16 de outubro de 2023

Ana Filipa Cunha Mendes

O Impacto de Experiências Adversas na Infância e Problemas Emocionais e Comportamentais em Crianças Institucionalizadas: Contribuição de Mecanismos Epigenéticos

Resumo

Introdução: Experiências Adversas na Infância (EAI) consistem em acontecimentos negativos na infância com um impacto profundo a nível social e de saúde ao longo da vida, classificadas em dimensões de ameaça (ex., abuso físico) e de privação (ex., negligência), com impactos distintos a desenvolvimental. A adversidade pode ser biologicamente integrada através de processos epigenéticos, como a metilação do ADN. A metilação do gene NR3C1 compromete a sua expressão, alterando a reatividade ao stress através do eixo hipotálamo-pituitária-adrenal, resultando em problemas emocionais e comportamentais.

Objetivo: identificar o impacto diferencial e cumulativo das EAI na metilação do gene NR3C1 e subsequente impacto a nível emocional e comportamental, em crianças institucionalizadas.

Método: foram avaliadas 136 crianças de instituições portuguesas, considerando os motivos de admissão a cuidado institucional como EAI. Problemas emocionais e comportamentais foram avaliados através da CBCL 1.5-5 e o nível de metilação do gene NR3C1 foi avaliado através de amostras de saliva.

Resultados: exposição a um número mais elevado de EAI corresponde a níveis mais elevados de metilação do gene NR3C1. Adicionalmente, este estudo gera questões sobre a fiabilidade do instrumento CBCL 1.5-5 para medir problemas emocionais e comportamentais em contexto institucional.

Palavras-Chave: experiências adversas na infância; ameaça/privação; metilação do ADN; gene NR3C1; psicopatologia

The Impact of Adverse Childhood Experiences on Emotional and Behavioral Problems in Institutionalized Children: The Contribution of Epigenetic Mechanisms

Abstract

Introduction: Adverse Childhood Experiences (ACEs) consist of negative childhood events with a profound impact on social and health outcomes throughout life, classified into two dimensions, threat (e.g. physical abuse) and deprivation (e.g. neglect), with distinct developmental impacts. Adversity can be biologically embedded through epigenetic processes, such as DNA methylation. Methylation of the NR3C1 gene compromises its expression, altering stress reactivity through the hypothalamic-pituitary-adrenal axis, resulting in emotional and behavioral problems.

Objective: to identify the differential and cumulative impact of EAI on NR3C1 gene methylation and subsequent impact on emotional and behavioral levels in institutionalized children.

Method: 136 children from Portuguese institutions were evaluated, considering the reasons for admission to institutional care as ACEs. Emotional and behavioral problems were assessed using the CBCL 1.5-5 and the methylation level of the NR3C1 gene was assessed using saliva samples.

Results: children exposed to a higher number of ACEs display higher levels of NR3C1 gene methylation. Additionally, this study raises questions about the reliability of the CBCL 1.5-5 instrument for measuring emotional and behavioral problems in an institutional setting.

Keywords: adverse childhood experiences; threat/deprivation; DNA methylation; NR3C1 gene; psychopathology

Índice

Índice de Tabelas

The Impact of Adverse Childhood Experiences on Emotional and Behavioral Problems in Institutionalized Children: The Contribution of Epigenetic Mechanisms

Adverse Childhood Experiences (ACEs) can be conceptualized as negative childhood experiences associated with increased risk for adverse health and social outcomes throughout life. Adverse childhood experiences may include physical, sexual, and emotional abuse; physical and emotional neglect; exposure to violence, crime, and discrimination; parental death; caregiver disability attributed to psychopathology, substance abuse, and criminal behavior; unstable or inadequate care environment, such as low-quality institutional care, or other causes of psychological stress or trauma (Berens et al., 2017; Felitti et al., 1998).

It is not easy to determine the prevalence of ACE, as definitions, measures and sample features vary so much across studies. For example, a meta-analysis of 217 publications that studied the worldwide prevalence of sexual abuse during childhood found a combined prevalence of 17.7%, but it ranged from 0.1% to 71%, according to the definition of sexual abuse, geographic region, the source of the data, namely self-report or informant measures, among others (Stoltenborgh et al., 2011). Besides, youth studies that rely on self-report measures are at risk that this age group, particularly younger adolescents, may not feel comfortable reporting these experiences, even in confidential questionnaires. It is also possible that adolescents do not recognize these experiences as adversities, which could also underestimate the prevalence (Bellis et al., 2014; Polanczyk et al., 2003).

Epidemiological studies show that ACEs, namely exposure to neglect, abuse, caregiver psychopathology, and family or community violence, predict worse long-term outcomes regarding social and health domains, affecting immune, cardiovascular, and mental responses (Berens et al., 2017; Szyf, 2013). Concerning mental health, epidemiological and clinical studies show that children exposed to ACEs are at increased risk of developing internalizing and externalizing problems, including depression, anxiety, disruptive behavior, and substance use disorders (McLaughlin et al., 2012; Kessler et al., 2001).

Conceptual Models of Adverse Childhood Experiences

Considering the tremendous impact of ACEs together with the need to identify youth at greater risk for developing internalizing and externalizing symptoms, three theoretical models that organize ACEs by their underlying dimensions were proposed: individual risk (Henry et al., 2021), the Dimensional Model of Adversity and Psychopathology (DMAP; McLaughlin et al., 2014; Sheridan & McLaughlin, 2014), and cumulative risk (Evans et al., 2013; Felitti et al., 1998).

The individual risk approach examines ACEs individually as single indicators of risk for psychopathology (Henry et al., 2021). The DMAP approach (McLaughlin et al., 2014; Sheridan & McLaughlin, 2014) classifies ACEs onto dimensions of threat and deprivation since grouping ACEs by their underlying features could reveal patterns in the ways ACEs influence psychopathology. Finally, the cumulative risk approach sums exposure to individual risk factors to generate a total risk score (Evans et al., 2013; Felitti et al., 1998).

The DMAP approach is a theoretical model that predicts neurodevelopmental variability following adversity exposure. According to this model, adverse experiences can be sorted into two orthogonal dimensions: deprivation (low to high) and threat (low to high). Threat experiences refer to exposure to interpersonal violence that includes harm or threat of harm to the child, such as physical and sexual abuse or direct exposure to community violence. Deprivation experiences are considered exposures marked by the absence of expected environmental inputs, including the absence of age-typical complexity and stimulation, such as neglect, poverty, or institutionalization (McLaughlin et al., (2014); Sheridan & McLaughlin, 2014). This way, a map of adverse experiences includes quadrants of low threat and low deprivation, meaning safe, stimulating environments; low threat and high deprivation, such as neglect and institutionalization; high threat and low deprivation, in cases of violence exposure and abuse; and high threat and high deprivation, which represents an environment marked by multiple forms of adversity.

Particularly, children placed in low-quality institutional care are at greater risk for neurodevelopmental compromise, due to the absence of normative psychosocial stimuli, including age-adequate language exposure and responsive caregiver interactions (Berens & Nelson, 2015; Knudsen, 2004). Thus, the institutionalization of children has well documented neurobiological impact, namely a higher level of synaptic pruning (i.e., synapsis elimination process) and a globally decreased cortical thickness (McLaughlin et al., 2014).

Adding to the DMAP approach, the authors proposed that exposure to threat and deprivation experiences influences development in ways at least partly distinct, with threat affecting emotion reactivity and regulation, and deprivation affecting cognition (McLaughlin et al., 2014). A systematic review of literature by McLaughlin and colleagues (2019) showed that most studies point to reduced amygdala, medial prefrontal cortex, and hippocampus volume as well a heightened activation in the amygdala. On the other hand, the authors found evidence that youth exposed to deprivation events show reduced volume and altered function in frontoparietal regions.

Considering the cumulative risk approach, another relevant factor to take into consideration is the number of adverse experiences a child is exposed to and whether they occur simultaneously,

accounting for the interaction between them. The Adverse Childhood Experiences (ACE) Study (Felitti et al., 1998) provides evidence that the risk of health consequences increases with the number of adversity categories adult individuals were exposed to as children. This research revealed that people who had experienced four or more ACEs were more likely to report engaging in smoking, having poor self-rated health, contracting sexually transmitted infections, being physically inactive, and suffering from severe obesity. Additionally, they were at an increased risk for health issues like alcoholism, drug abuse, depression, and suicide attempts when compared to those who hadn't experienced any ACEs during their childhood.

When it comes to cases of severe adversity, most children are exposed to more than one form of adversity simultaneously. Thus, several forms of adversity can interact in complex ways over time, impacting development (Nelson et al., 2020). A score of four or more ACEs has been considered a cut-point, used clinically to define a high-risk status for several outcomes (Briggs et al., 2021). Specifically, a meta-analysis of thirty-seven ACE studies found considerable strength in the relationship between a score of four or more ACEs and the risk for twenty-three frequently screened adult health outcomes (Hughes et al., 2017).

Biological Embedding of Adversity: NR3C1 Gene Methylation

The pervasive and long-term impact of adversity upon the developing child have been shown to be biologically integrated through a process called biological embedding (Nelson et al., 2020). This process consists in the way initially transient homeostatic responses alter physiological systems in lasting ways (Hertzman, 2012).

According to Hertzman (2012), biological embedding occurs when four conditions are met: (a) experience, metaphorically, “gets under the skin” in a way that alters biological and developmental processes; (b) systematic differences in experience in different environments lead to systematic differences in biological and developmental states; (c) these differences are durable and stable; (d) These differences are capable of influencing several domains throughout life, such as health, wellbeing, learning, or behavior.

Epigenetic processes represent a central group of mechanisms through which biological embedding occurs and involve the stable alteration of a gene’s expression without changing the underlying nucleotide sequence. This happens, instead, through mechanisms that include, among others, the attachment of chemical residues to DNA molecules, as seen in DNA methylation (Essex et al., 2013).

DNA methylation is an epigenetic change that influences transcription through the addition of a methyl group to cytosine, usually in the context of a 5′-Cytosine-Phosphate-Guanine-3′ dinucleotide pair, known as a CpG site (Jaenisch & Bird, 2003; Bird, 2011). This process renders the portions of a gene that encode proteins less accessible to molecular transcription mechanisms that decode DNA sequences into messenger RNA and then specific protein products (Essex et al., 2013).

Palma-Gudiel and colleagues (2015) concluded, through their literature review, that ACEs were repeatedly associated with hypermethylation of the NR3C1 gene, which encodes the human glucocorticoid receptor, an important regulator of the hypothalamus-pituitary-adrenal (HPA) axis. Furthermore, Tyrka and colleagues (2015) reported association between NR3C1 gene hypermethylation and early child maltreatment in preschool age children from low socioeconomic backgrounds. Additionally, Perroud and colleagues (2011) found a significant association between physical neglect an NR3C1 methylation levels. Particularly, physical neglect victims presented higher methylation levels than participants that were not exposed to physical neglect. On the other hand, Hossack and colleagues (2020) found that subjects exposed to combat and traumatic childhood experiences showed lower levels of NR3C1 gene methylation, associated with a Posttraumatic Stress Disorder (PTSD) diagnosis.

These epigenetic changes appear to impair HPA axis function and increase the predisposition of individuals exposed to early stress to disorders such as Major Depressive Disorder (MDD) or Borderline Personality Disorder (BPD) (Palma-Gudiel et al., 2015).

Therefore, ACEs have an influence on stress reactivity, controlled by the HPA axis. In response to stress, the release of corticotropin-releasing factor (CRF) at the paraventricular nucleus of the hypothalamus stimulates the production of adrenocorticotropic hormones (ACTH) by the anterior pituitary gland, which in turn promotes the secretion of glucocorticoids by the adrenal glands. Glucocorticoid hormones, mainly corticosterone in animals and cortisol in humans, are the final product of the HPA axis and participate in the control of homeostasis and the response of the organism to stressors (Habib et al., 2001). In humans, cortisol performs numerous functions at the level of the immune, digestive, and endocrine systems, including autoregulation of the HPA axis by negative feedback (Berens, 2017; Palma-Gudiel et al., 2015).

In both human and animal studies, ACEs predict HPA axis dysregulation that persists into adulthood, including patterns of hyperreactivity, suggesting a potential acquired resistance to glucocorticoid negative feedback (Danese & McEwen, 2012), or hyporeactivity, which suggests a blunted stress sensitivity or exaggerated suppression of the HPA axis (Lovallo, 2013). Despite one of the roles of the HPA axis being to prevent over-response to stress, prolonged exposure to elevated levels of

glucocorticoids has damaging effects on the developing brain, which may result in behavioral problems (GA, 2003; Teicher et al., 1997). Considering changes in HPA axis functioning, animal models of early stress have demonstrated altered expression of the glucocorticoid receptor (GR) and receptors for CRF, ACTH, among others (McEwen, 2012), particularly in rats receiving unfavorable maternal care, in which NR3C1 gene hypermethylation was found (Meaney & Szyf, 2005).

Concerning maternal care, rat pups may receive high or low levels of licking, grooming, and arch-backed nursing (LG-ABN) (Hetzman, 2012). The rat pups that get high levels of LG-ABN display differences in the function of their HPA axis: a low basal corticosterone level with an abrupt response to stressful circumstances and an abrupt decline back to baseline. Contrarily, rat pups that received low levels of LG-ABN show higher corticosterone baseline levels and a more blunted response to stressful situations. High LG-ABN pups have reduced total lifetime secretion of corticosterone compared with the low LG-ABN pups, showing less cognitive deterioration typical of aging. Prolonged exposure to high corticosterone levels, in turn, resulted in low LG-ABN rats showing a progressive deterioration in their memory, cognitive processing, and learning performance with age (Meaney, 2001). McGowan and colleagues (2009) attempted to replicate these findings in humans by examining NR3C1 gene expression and methylation in suicide victims, with or without childhood abuse, and control subjects who died suddenly by other causes. They found that childhood abuse victims showed reduced NR3C1 gene expression, suggesting that altered glucocorticoid expression is associated with a developmental history of adversity in humans. Furthermore, the same study found that persistent mother-infant interaction disruptions, as seen in childhood abuse experiences are associated with an increase in CRF expression and increased HPA response to stress, consistent with findings from animal models of early stress.

Emotional and Behavioral Problems

Adverse caregiving environments can promote probabilistic developmental pathways characterized by an increased risk for atypical brain development, relationship difficulties, maladaptive behavior, and psychopathology across the life span. Child maltreatment sensitizes neural function and neuroendocrine responses to stress exposure, thereby resulting in vulnerability to psychopathology, namely emotional and behavioral problems (Cicchetti & Handley,2017).

Several studies have shown differences in the associations between threat and deprivation and internalizing and externalizing problems. For example, Busso and colleagues (2017) found that experiences of threat (in this case, exposure to interpersonal violence) was associated with higher levels of internalizing and externalizing symptoms. Deprivation (in this case, poverty) was associated with

externalizing but not internalizing symptoms. In addition, Miller and colleagues (2018) found that threat (in this case, physical abuse, and severe discipline) was related to increased levels of internalizing and externalizing symptoms, and deprivation (in this case, lack of environmental enrichment) was related to increased externalizing symptoms. Deprivation was not a significant predictor of internalizing symptoms.

Study Aim and Hypothesis

The aim of this study consists in: 1) assessing the impact of different types of ACEs (threat, deprivation or both) and the number of ACEs a child has been exposed to on NR3C1 gene methylation in institutionalized children; 2) assessing the impact of ACEs type and number on the development of internalizing and externalizing problems in institutionalized children and 3) assessing the relationship between methylation level of the NR3C1 gene and the development of emotional and behavioral problems.

Considering the literature review presented above, hypothesis were made for each objective. For the first objective, it was proposed that 1) higher NR3C1 methylation levels are associated with deprivation experiences, and hypomethylation levels associated with threat experiences, and 2) children exposed to a higher number of ACEs display higher level of NR3C1 gene methylation, considering the cumulative effect of deprivation experiences, more frequently observed in this study's sample. For the second objective, it was proposed that 1) threat ACEs are associated with the development of internalization and externalization problems, and deprivation with externalization problems, and 2) children exposed to a higher number of ACEs display more emotional and behavioral problems. Finally, for the third objective, it was proposed that NR3C1 gene methylation is associated with emotional and behavioral problems.

Method

Sample

This study is part of a broader research project (PTDC/PSI-PCL/101506/2008) started in January 2010, conducted at Universidade do Minho and coordinated by Professor Isabel Soares. The original study selected 159 children from 28 Portuguese institutions from Braga, Porto and Lisbon, aged 30 to 78 months old by the time of assessment. Exclusion criteria included having severe mental or physical impairments, genetic diseases, and a diagnose of Autism Spectrum Disorder (ASD).

Concerning the ACEs that led children to be placed in institutional care, twelve were chosen to be considered in this study and divided into two categories, threat experiences and deprivation experiences (see Table 1). Taking this into account, children whose admission motive didn't belong in one of these groups were excluded, as were children without data concerning NR3C1 gene methylation levels and

CBCL scores, as these were essential measures for this study. Accordingly, the resulting sample consists in 136 children (N_{Male} = 80; N_{Female} = 56) from 28 Portuguese institutions from Braga (*n* = 14), Porto (*n* = 59) and Lisbon (*n* = 63), aged 36-78 months old (*m* = 54.88; *sd* = 11.135) by the time of assessment. Admission age to institutional care varies between 3 and 69 months old (*m* = 37.10; *sd* = 15.480), and institutionalization time between 2 and 59 months (*m* = 17.54; *sd* = 11.107).

Table 1

Threat and Deprivation Adverse Childhood Experiences

	n	%
Threat		
Physical Abuse	12	8.8
Sexual Abuse	2	1.5
Psychological maltreatment	15	11.0
Exposure to criminality	6	4.4
Dysfunctional relationships	18	13.2
Family violence	47	34.6
Deprivation		
Neglect	108	79.4
Abandonment	33	24.3
Low Socioeconomic Status	41	30.1
Poor housing conditions or homelessness	31	22.8
Lack of parenting skills	65	47.8
Parental Physical impairment	4	2.9

Note. N = 136

Measures

A sociodemographic questionnaire was used to collected data such as reason for admission to institutional care, information concerning the child's filiation, socioeconomic status and health and developmental status.

Emotional and Behavioral Problems

To assess the development of emotional and behavioral problems, the Portuguese version of the Child Behavior Checklist for children with 1.5-5 years of age (CBCL/1.5-5) (Achenbach et al., 2014) was

used. The CBCL is a component of the Achenbach System of Empirically Based Assessment (ASEBA) and a reliable and valid measure with strong test–retest reliability (α = .68 - .92). This measure is composed of 99 items coded on a 3-point scale, from 0 "*not true*" to 2 "*very true* or *often true*", considering the child's behavior in the past two months (Achenbach & Rescorla, 2000). While scoring, these items are organized into seven syndrome scales: Emotionally Reactive, Anxious/Depressed, Somatic Complaints, Withdrawn, Attention Problems, Aggressive Behavior, and Sleep Problems. The CBCL also provides three composite scales, Internalizing, Externalizing and Total Problems, that result from the sum of scores of the syndrome scales. Specifically, the Internalizing Problems scale is a product of the sum of the Emotionally Reactive, Anxious/Depressed, Somatic Complaints and Withdrawn scales' scores and the Externalizing Problems scale is a product of the sum of the Attention Problems and Aggressive Behavior scales' scores. The Total Problems scale results of the sum of the scores for Internalizing and Externalizing Problems. These three composite scales were used to assess the development of emotional and behavioral problems. A higher score translates to a higher level of internalizing, externalizing and total problems.

Epigenetic analysis – NR3C1 methylation level

For genetic analysis and DNA methylation profiling, saliva samples were collected using OraGene 500 devices (DNA Genotek). DNA was extracted according to the manufacturer and quantitative DNA methylation analysis of the glucocorticoid receptor gene (NR3C1) was performed through EpiTyper technology and MassARRAY system (Agena Bioscience Inc). Following van der Knaap and colleagues (2014) and McGowan and colleagues (2009), three amplicons (i.e. genomic regions) within the NR3C1 CpG site were selected for analyses, namely two regions covering the edges of the NR3C1 CpG site, henceforth called NR3C1_1 and NR3C1_3, and the region that encompasses exon 1F, called NR3C1_2.

Procedure

Approval by the Portuguese Social Services and the National Commission for Data Protection was obtained. The Portuguese Social Services are responsible for managing institutions and are the legal guardian of institutionalized children. The National Commission for Data Protection is responsible for ensuring that ethical requirements in relation to human research are carried out by Portuguese entities. Written informed consent was gathered from biological parents, institution directors, and caregivers. After selecting the participants, institutional staff was consulted to identify the assigned caregiver to each child. Children's sociodemographic questionnaires were completed with help from institutional staff and with access to the child's individual file, used to gather information on the child's prior experiences to

institutionalization, such as the reason for admission to institutional care. Children's files were filled by social workers based on the information that was available to them. To assess the occurrence of emotional and behavioral problems, each child's assigned caregiver completed the Portuguese version of the CBCL/1.5-5. Finally, for genetic analysis, children's saliva samples were collected, a process during which children were instructed to place a cotton swab in the mouth and chew for a minute.

Data Analysis

Data analysis was run with IBM® SPSS®-27 software. Statistical significance was considered with $p < .05$.

To allow comparison between types of ACE, children were divided into groups according to the type of ACE that led to admission in institutional care. As there are only 4 children that went through threat experiences and no deprivation experiences, and such a small sample wouldn't allow for statistical testing, it was not possible to create groups of exclusively threat ACEs, exclusively deprivation ACEs and both types of ACEs, as planned. Instead, children were divided according to having been exposed or not exposed to threat experiences and being exposed to a high (three or more) or low (up to two) number of deprivation experiences. Accordingly, 4 groups were created as follows:

- No Threat/ Low Deprivation (NoT-LowD): children who experienced no threat ACEs and up to 2 deprivation ACEs (n = 45).
- No Threat/ High Deprivation (NoT-HighD): children who experienced no threat ACEs and 3 or more deprivation ACEs (n = 26).
- Threat/ Low Deprivation (T-LowD): children who experienced any number of threat ACEs and up to 2 deprivation ACEs (n = 44).
- Threat/ High Deprivation (NoT-HighD): children who experienced any number threat ACEs and 3 or more deprivation ACEs (n = 21).

Due to the non-normality of the study variables, nonparametric statistical tests had to be used. Thus, to assess differences in the NR3C1 gene methylation according to the type of ACE (deprivation, threat, or both), Kruskal-Wallis (H) analysis of variance were performed for each of the three studied segments of the NR3C1 gene.

Additionally, with the purpose of assessing the impact of threat ACEs, children were divided into two groups, according to having or not experienced threat ACEs (Threat - T and No Threat - NoT), regardless of the number of deprivation ACEs. This way, these two groups allowed for comparison between children who experienced threat ACEs, and children who didn't. As such, Mann-Whitney (U) tests

were performed with these two groups to assess the difference in the NR3C1 gene methylation levels in these 2 groups of children.

To assess the impact of the number of ACEs a child was exposed to on NR3C1 gene methylation levels, the sample was divided into 2 groups according to the number of ACEs. Therefore, and according to literature, the sample was divided into children exposed to 1-3 ACEs (n = 97) and 4+ ACEs (n = 39). To explore the differences in methylation levels between these groups, Mann-Whitney (U) tests were performed for each NR3C1 gene segment.

To evaluate the impact of the type of ACE on the development of internalizing and externalizing problems, Kruskal-Wallis (H) tests were performed to compare the Internalizing, Externalizing and Total Problems scores between the four ACE type groups (NoT-LowD, NoT-HighD, T-LowD and T-HighD). Also, to assess the impact of threat experiences on the development of internalization and externalization problems, Mann-Whitney (U) tests were performed to compare the scores from the groups T and NoT.

To assess the impact of the number of ACEs a child was exposed to on the development of internalizing and externalizing problems, Mann-Whitney (U) tests were performed to compare the scores from the groups 1-3 and 4+. Finally, to evaluate the relationship between NR3C1 gene methylation levels and the development of emotional and behavioral problems, a Linear Regression was performed between the methylation level of the three NR3C1 gene segments and the Total Problems scale score.

Results

ACE Type and NR3C1 Gene Methylation Level

The Kruskal-Wallis (H) test was performed to compare the NR3C1 gene methylation levels of the four ACE type groups. According to the Kruskal-Wallis (H) test, there's a marginally significant difference on the methylation level of segment 1 of the NR3C1 gene between the four groups of ACE type, H (3, 136) = 7.673; p = .053 (see table 2).

Table 2

Kruskal-Wallis test for the difference of NR3C1 gene methylation level between ACE type groups

	M	SD	H	*p*
NR3C1_1	.024	.004	7.673	.053

NR3C1_2	.022	.004	2.106	.551
NR3C1_3	.034	.004	.128	.998

Note. N = 136

Although pairwise comparisons showed a significant difference between groups NoT-LowD and NoT-HighD, $p = .015$ (see table 3), when applying the Bonferroni adjustment to account for multiple comparisons, the significance level increased to $p > .05$, indicating that this result did not remain statistically significant after correcting for multiple comparisons.

Table 3

Pairwise Comparisons of ACE Type Groups

ACE Type Groups	Test Statistic	Std. Error	*p*	*Adj. Sig.*
NoT-LowD – T-LowD	-5.056	8.315	.543	1.000
NoT-LowD – T-HighD	-18.890	10.365	.068	.410
NoT-LowD – NoT-HighD	-23.437	9.662	.015	.092
T-LowD – T-HighD	-13.834	10.403	.184	1.000
T-LowD – NoT-HighD	18.381	9.702	.058	.349
T-HighD – NoT-HighD	4.547	11.507	.693	1.000

Note. N = 136

No other results were statistically significant, namely results concerning segment 2 ($H = 2.106$, $p = .551$) and segment 3 ($H = .128$, $p = .988$).

Additionally, to compare the NR3C1 gene methylation levels of children that were or not exposed to threat ACEs, Mann-Whitney (*U*) tests were performed with these two groups. No results were statistically significant, considering methylation levels of segment 1 ($U = 2{,}275.500$, $p = .889$), segment 2 ($U = 2{,}174.500$, $p = .561$) and segment 3 ($U = 2{,}283.500$, $p = .916$).

NR3C1 Gene Methylation Level and Number of ACEs

The Mann-Whitney (*U*) test was performed to compare the NR3C1 gene methylation levels of Group 1-3 ACEs and Group 4+ ACEs. The Mann-Whitney (*U*) showed statistically significant difference between the two groups for the segment 1 of the NR3C1 gene, $U = 1{,}467.500$, $p = .040$ (see table 4). Group 4+ (mean rank = 79.37) had significantly higher methylation levels than Group 1-3 (mean rank = 64.13).

Table 4

Mann-Whitney Test for the difference of NR3C1 gene methylation level between ACE Number Groups

Variable	1-3	4+	U	*p*
	Mean Rank	Mean Rank		
NR3C1_1	64.13	79.37	1,467.500	.040
NR3C1_2	69.24	66.65	1,819.500	.728
NR3C1_3	67.66	70.58	1,810.500	.695

Note. N = 136

No other results were statistically significant, namely results concerning segment 2 ($U = 1{,}819.500$, $p = .728$) and segment 3 ($U = 1{,}810.500$, $p = .695$).

ACE Type and Internalizing, Externalizing and Total Problems Scores

A Kruskal-Wallis test was performed to compare Internalizing, Externalizing and Total Problems scores between the four ACE type groups, whose descriptive statistics are presented in table 5.

Table 5

Descriptive Statistics for Internalizing, Externalizing and Total Problems Scores

	M	SD	Range
Internalizing Problems			
NoT-LowD	12.689	1.372	0-39
NoT-HighD	15.269	1.907	2-44
T-LowD	14.068	1.345	1-41
T-HighD	7.714	1.386	1-23

Externalizing Problems			
NoT-LowD	12.889	1.150	2-32
NoT-HighD	16.538	1.404	3-29
T-LowD	14.773	1.340	3-43
T-HighD	10.952	1.663	1-26
Total Problems			
NoT-LowD	37.644	3.373	6-89
NoT-HighD	46.154	4.145	9-90
T-LowD	42.500	3.817	8-120
T-HighD	27.524	4.155	5-76

According to the Kruskal-Wallis test, there's a statistically significant difference on both Total Problems score (H (3, 136) = 9.326; p = .025) and Internalizing Problems score (H (3, 136) = 10.669; p = .014) between the four groups of ACE type (see table 6).

Table 6

Kruskal-Wallis test for the difference of Internalizing, Externalizing and Total Problems Scores in the total sample

	M	SD	H	*p*
Total Problems	39.279	23.288	9.326	.025
Internalizing Problems	12.860	9.063	10.669	.014
Externalizing Problems	13.897	8.118	7.300	.063

Note. N = 136

Considering the results for Total Problems, pairwise comparisons showed a significant difference between groups T-HighD and T-LowD (p = .017) and between groups T-HighD and NoT-HighD (p = .004) (see table 7). However, when applying the Bonferroni adjustment to account for multiple comparisons, only the difference between groups T-HighD and NoT-HighD remained statistically significant (p = .021). Specifically, the group NoT-HighD (M = 46.154) shows significantly higher scores for total problems than the group T-HighD (M = 27.524).

Table 7

Pairwise Comparisons of ACE Type Groups – Total Problems Scores

ACE Type Groups	Test Statistic	Std. Error	*p*	Adj. Sig.
T-HighD – NoT-LowD	18.157	10.411	.081	.487
T-HighD – T-LowD	24.956	10.449	.017	.102
T-HighD – NoT-HighD	33.697	11.558	.004	.021
NoT-LowD – T-LowD	-6.798	8.352	.416	1.000
NoT-LowD – NoT-HighD	-15.540	9.705	.109	.656
T-LowD – NoT-HighD	8.741	9.745	.370	1.000

Note. N = 136

Considering the results for Internalizing Problems, pairwise comparisons showed a significant difference between groups T-HighD and NoT-LowD ($p = .026$), between groups T-HighD and T-LowD ($p = .004$) and between groups T-HighD and NoT-HighD ($p = .003$) (see table 8). However, when applying the Bonferroni adjustment to account for multiple comparisons, only the difference between groups T-HighD and T-LowD ($p = .023$) and between groups T-HighD and NoT-HighD ($p = .018$) remained statistically significant the significance. Specifically, the groups NoT-HighD ($M = 15.269$) and T-LowD ($M = 14.068$) both show significantly higher scores for internalizing problems than the group T-HighD ($M = 7.714$).

Table 8

Pairwise Comparisons of ACE Type Groups – Internalizing Problems Scores

ACE Type Groups	Test Statistic	Std. Error	*p*	Adj. Sig.
T-HighD – NoT-LowD	23.119	10.402	.026	.258
T-HighD – T-LowD	30.146	10.440	.004	.023
T-HighD – NoT-HighD	34.260	11.549	.003	.018
NoT-LowD – T-LowD	-7.027	8.345	.400	1.000

NoT-LowD – NoT-HighD	-11.141	9.696	.251	1.000
T-LowD – NoT-HighD	4.115	9.737	.673	1.000

Note. N = 136

No other results were statistically significant, namely results concerning Externalizing Problems ($H = 7.300$, $p = .063$).

To compare Internalizing, Externalizing and Total Problems scores of children that were or not exposed to threat ACEs, a Mann-Whitney (U) test was performed with these two groups. No results were statistically significant, scores for Internalization Problems ($U = 2{,}077.000$, $p = .315$), scores for Externalization Problems ($U = 2{,}148.000$, $p = .487$) and Total Problems scores ($U = 2{,}148.500$, $p = .304$).

Number of ACEs and Internalizing, Externalizing and Total Problems Scores

Mann-Whitney (U) tests were performed to compare the Internalizing, Externalizing and Total Problems scores of Group 1-3 ACEs and Group 4+ ACEs. According to the Mann-Whitney (U) test, there's a marginally significant difference between the two groups for the Internalizing Problems score, U = 1,449.500, $p = .059$ (see table 9).

Table 9

Mann-Whitney Test for the difference of Internalizing, Externalizing and Total Problems Scores between ACE Number Groups

Variable	1-3	4+	U	*p*
	Mean Rank	Mean Rank		
Internalizing Problems	72.54	58.45	1,499.500	.059
Externalizing Problems	69.64	65.67	1,781.000	.595
Total Problems	71.30	61.53	1,619.500	.190

Note. N = 136

Group 1-3 (Mean Rank = 72.54) had significantly higher Internalizing Problems score than Group 4+ (Mean Rank = 58.45). These results suggest that there is a significant difference in Internalizing

Problems scores between Group 1-3 and Group 4+, with Group 1-3 exhibiting higher levels of internalizing problems.

No other results were statistically significant, namely results concerning Externalizing Problems scores (U = 1,781.000, p = .595) and Total Problem scores (U = 1,619.500, p = .190).

NR3C1 Gene Methylation Levels and Total Problems Scores

A Linear Regression was performed to assess the impact of NR3C1 gene methylation level on Internalizing, Externalizing and Total Problems scores. The methylation level of each NR3C1 gene segment did not predict the score for Total Problems (see table 10).

Table 10

Regression of NR3C1 Gene Methylation Level and Total Problem Scores

	B	SE	*t*	*p*	95% CI	
					Lower Bound	Upper Bound
NR3C1_1	440.538	518.318	.850	.397	-584.747	1465.823
NR3C1_2	-185.756	507.261	-.366	.715	-1189.170	817.657
NR3C1_3	568.555	559.428	1.016	.311	-538.050	1675.159
F	.615					
R^2	.014					

Note. N = 136

Discussion

The first years of life present a particularly important developmental period, with environmental cues programming the genome in anticipation of long-term environmental exposures. Such early life events produce changes that will alter immunity, cardiovascular and mental responses throughout life, which contributes to the knowledge that ACE alter DNA methylation levels in both the brain and peripheral systems (Szyf, 2013).

Taking this into account, ACEs alter the development of stress response regulation systems, such as glucocorticoid receptor expression, and thus enhance the effect of stress in adulthood and vulnerability for emotional and behavioral problems (Heim & Nemeroff, 2001).

With this newfound knowledge comes the potential to improve screening and intervention strategies to decrease exposure to childhood adversity, limiting the effects of such exposure, and helping those who are dealing with these effects (Berens et al., 2017). Taking this into account, the importance of understanding the mechanisms through which adverse childhood experiences produce durable physiological changes and how those changes translate into psychopathological conditions has become essential to the development of effective and evidence-based prevention and treatment strategies.

The aim of this study was to evaluate the differential impact of the threat and deprivation ACE and the number of such experiences a child was exposed to on the development of emotional and behavioral problems and the role of NR3C1 gene methylation levels in this relationship.

Considering the impact of the type of ACE on NR3C1 gene methylation levels, no conclusions can be made concerning the differential impact of threat and deprivation experiences. The lack of significant results could be explained by the way the groups of type of ACE were arranged. It was proposed that children exposed to deprivation experiences would display higher levels of NR3C1 gene methylation and that threat experiences would be associated with lower NR3C1 gene methylation levels. Given that there was not a group of children exposed exclusively to threat experiences, this was not the ideal setting to assess this hypothesis. Even if a less conservative approach was used, without applying the Bonferroni correction, there would only be a significant difference between groups NoT-LowD and Not-HighD. Although, seeing as neither of these groups were exposed to threat experiences, being the distinction between them the number of deprivation experiences, this result would support the hypothesis that a higher number of ACE is associated with an increased level of NR3C1 gene methylation instead.

Regarding the relationship between the type of ACE and the development of emotional and behavioral problems, it was hypothesized that exposure to threat experiences would lead to a higher score for internalizing and externalizing problems and that exposure to deprivation experiences would lead to a higher score for externalizing problems. Following this reasoning, it would be expected that children exposed to threat experiences showed higher scores for emotional and behavioral problems by means of a higher internalization problems score, given that exposure to both types of adversity are hypothesized to result in externalization problems. However, it was found that children exposed to more severe conditions (i.e., exposure to both types of ACE and a high number of deprivation experiences) scored lower for emotional and behavioral problems than children only to a higher number of deprivation experiences. Congruently, for internalizing problems, children exposed to both types of ACE and a higher number of deprivation experiences report lower internalizing symptoms than children exposed to threat

experiences and a lower number of deprivation experiences and children exposed only to a higher number of deprivation experiences.

This result is particularly intriguing since it goes against literature on the matter. One interpretation for such results is that institutional caregivers might not be a reliable informant to complete psychological assessment. A study by Gearing and colleagues (2015) examined the discrepancies between self-reported and staff caregiver reported psychopathology in adolescents, using the YSR and CBCL measures. The authors found that, even though caregivers report higher numbers of overall problems than adolescents, they report significantly lower severity of such problems, which shows that it is possible that caregivers might be minimizing the emotional and behavioral problems of the children they care for. Additionally, it was reported that congruency in these reports increases with the length of stay of the child in institutional care and the quality of the caregiver-child relationship. Since this study includes children that have been in institutional care for as little time as 2 months, the turnover of the caregivers might not allow them to know the child well enough to be able to report emotional and behavioral problems accurately. Taking this into account, this study provides evidence that the CBCL 1.5-5 may not be a reliable instrument to measure emotional and behavioral problems in institutional context.

As for the relationship between the number of ACEs and NR3C1 gene methylation, children who were exposed to four or more ACEs were found to have a higher methylation level compared to children who were exposed to 1-3 ACEs. Following the cumulative risk approach (Evans, et al., 2013; Felitti et al., 1998), this result supports de proposed hypothesis that children exposed to a higher number of ACEs will display higher level of NR3C1 gene methylation. Furthermore, it is consistent with results found by Parade and colleagues (2016), that observed higher NR3C1 gene methylation levels were associated with a higher ACE composite score in maltreated preschool-aged children, as well as with results found by Perroud and colleagues (2011), that reported a positive correlation between NR3C1 gene methylation and the number of abuse and neglect experiences.

Concerning the relationship between the number of ACE and the development of internalizing and externalizing problems, it was once again found an association that goes against those found in previous studies. Once more, these results could be explained by the fact that institutional caregivers might not be a reliable informant when assessing the development of emotional and behavioral problems in the children they care for.

Finally, no relationship was found between the type and number of ACE and the development of externalizing problems, as well as between NR3C1 gene methylation levels and the development of emotional and behavioral problems. Once again, these results, or lack of thereof, might be a product of

the fact that psychological assessment measures, in this case, the CBCL 1.5-5, were filled by institutional caregivers, as was explored above.

Limitations and Future Research

Concerning the sample, it is important to emphasize that a control group without a history of ACE is missing from this analysis, which would have been useful to compare NR3C1 gene methylation levels and the development of emotional and behavioral problems of children exposed to ACE with control subjects. On that note, it should also be noted that every child in this sample is institutionalized, which constitutes a deprivation experience itself. Taking this into account, there are two ways to deal with this fact in future research: adding children from the community to the study, as a way to compare these two settings, and take institutionalization into account as a deprivation experience for data analysis. Furthermore, to properly examine the difference in NR3C1 gene methylation levels and the development of emotional and behavioral problems associated with different ACE types, it would be ideal to study a sample whose reasons for admission to institutional care allow for the correct distribution of subjects across three groups, threat experiences, deprivation experiences, and both, instead of the four alternative groups that were created. This proved to be a particularly important topic, given that the way the groups were arranged, without a group exposed exclusively to threat experiences, ended up undermining data analysis. Additionally, several participants had to be excluded, because their reason for admission to institutional care was difficult to classify as either a threat or deprivation experience and only experiences with extensive literature on the subject were included.

As suggestions for future research, this study focuses only on two features of ACE, number and type, so future studies could include other characteristics of adversity, such as developmental timing and chronicity of exposure to ACE. Lastly, it would be interesting for future studies to assess the interaction between ACE and behavioral and emotional problems using other informants together with caregivers, such as kindergarten/school staff through the TRF, to increase the accuracy of psychological measures.

Conclusion

Nowadays, the importance of understanding the mechanisms through which adverse childhood experiences produce durable physiological changes and how those changes translate into psychopathological conditions has become essential to the development of effective and evidence-based prevention and treatment strategies. It is important to emphasize that, as stated by Finkerlhor and colleagues (2015), regardless of the lasting impact of ACE, authorities should strive to protect children exposed to adverse conditions, as they deserve protection and a safe environment. Addressing adversity

in childhood should not be downplayed even when it may not result in lasting harm. Promoting children's well-being and their right to be protected from adversity is a societal responsibility and should be guaranteed.

Acknowledgements

This study was conducted within the Psychology Research Centre, University of Minho, coordinated by Professor Isabel Soares, and partially supported by the Portuguese Foundation for Science and Technology (PTDC /PSI-PCL/101506/2008).

References

Achenbach, T. M., & Rescorla, L. A. (2000). *Manual for the ASEBA Preschool Forms & Profiles*. Burlington, VT: University of Vermont, Research Center for Children, Youth, & Families.

Achenbach, T. M., Rescorla, L. A., Dias, P., Ramalho, V., Lima, V. S., Machado, B. C., & Gonçalves, M. (2014). Manual do sistema de avaliação empiricamente validado (ASEBA) para o período pré-escolar e escolar: Um sistema integrado de avaliação com múltiplos informadores.

Bellis, M. A., Hughes, K., Leckenby, N., Perkins, C., & Lowey, H. (2014). National household survey of adverse childhood experiences and their relationshipwith resilience to health-harming behaviors in England. BMC Medicine, 12, 72. http://dx.doi.org/10.1186/1741-7015-12-72

Berens, A. E., Jensen, S. K., & Nelson, C. A. (2017). Biological embedding of childhood adversity: from physiological mechanisms to clinical implications. *BMC medicine*, 15(1), 1-12.

Berens, A. E., & Nelson, C. A. (2015). The science of early adversity: is there a role for large institutions in the care of vulnerable children?. *The Lancet*, *386*(9991), 388-398.

Bird, A. (2011). The dinucleotide CG as a genomic signalling module. *Journal of molecular biology*, 409(1), 47-53.

Briggs, E. C., Amaya-Jackson, L., Putnam, K. T., & Putnam, F. W. (2021). All adverse childhood experiences are not equal: The contribution of synergy to adverse childhood experience scores. *American Psychologist*, *76*(2), 243.

Busso, D. S., McLaughlin, K. A., & Sheridan, M. A. (2017). Dimensions of adversity, physiological reactivity, and externalizing psychopathology in adolescence: Deprivation and threat. *Psychosomatic medicine*, *79*(2), 162.

Cicchetti, D., & Handley, E. D. (2017). Methylation of the glucocorticoid receptor gene, nuclear receptor subfamily 3, group C, member 1 (NR3C1), in maltreated and nonmaltreated children: Associations with behavioral undercontrol, emotional lability/negativity, and externalizing and internalizing symptoms. *Development and psychopathology*, 29(5), 1795-1806.

Danese, A., & McEwen, B. S. (2012). Adverse childhood experiences, allostasis, allostatic load, and age-related disease. *Physiology & behavior*, 106(1), 29-39.

Essex, M. J., Thomas Boyce, W., Hertzman, C., Lam, L. L., Armstrong, J. M., Neumann, S. M., & Kobor, M. S. (2013). Epigenetic vestiges of early developmental adversity: childhood stress exposure and DNA methylation in adolescence. *Child development*, 84(1), 58-75.

Evans, G. W., Li, D., & Whipple, S. S. (2013). Cumulative risk and child development. *Psychological bulletin*, *139*(6), 1342.

Felitti, V. J., Anda, R. F., Nordenberg, D., Williamson, D. F., Spitz, A. M., Edwards, V., & Marks, J. S. (1998). Relationship of childhood abuse and household dysfunction to many of the leading causes of death in adults: The Adverse Childhood Experiences (ACE) Study. *American journal of preventive medicine*, 14(4), 245-258.

Finkelhor, D., Shattuck, A., Turner, H., & Hamby, S. (2015). A revised inventory of adverse childhood experiences. *Child abuse & neglect*, *48*, 13-21.

GA, C. (2003). Van de Kar LD. Neuroendocrine pharmacology of stress. *Eur J Pharmacol*, 463, 235-272.

Gearing, R. E., Schwalbe, C. S., MacKenzie, M. J., Brewer, K. B., & Ibrahim, R. W. (2015). Assessment of adolescent mental health and behavioral problems in institutional care: discrepancies between staff-reported CBCL scores and adolescent-reported YSR scores. *Administration and Policy in Mental Health and Mental Health Services Research*, *42*, 279-287.

Habib, K. E. (2001). Gold PW, Chrousos GP. *Neuroendocrinology of stress. Endocrinol Metab Clin North Am*, *30*, 695-728.

Heim, C., & Nemeroff, C. B. (2001). The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. *Biological psychiatry*, *49*(12), 1023-1039.

Henry, L. M., Gracey, K., Shaffer, A., Ebert, J., Kuhn, T., Watson, K. H., ... & Compas, B. E. (2021). Comparison of three models of adverse childhood experiences: Associations with child and adolescent internalizing and externalizing symptoms. *Journal of abnormal psychology*, *130*(1), 9.

Hertzman, C. (2012). Putting the concept of biological embedding in historical perspective. *Proceedings of the National Academy of Sciences*, 109(Supplement 2), 17160-17167.

Hossack, M. R., Reid, M. W., Aden, J. K., Gibbons, T., Noe, J. C., & Willis, A. M. (2020). Adverse childhood experience, genes, and PTSD risk in soldiers: a methylation study. *Military medicine*, *185*(3-4), 377-384.

Hughes, K., Bellis, M. A., Hardcastle, K. A., Sethi, D., Butchart, A., Mikton, C., ... & Dunne, M. P. (2017). The effect of multiple adverse childhood experiences on health: a systematic review and meta-analysis. *The Lancet Public Health*, *2*(8), e356-e366.

Jaenisch, R., & Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature genetics*, 33(3), 245-254.

Kessler, R. C., Avenevoli, S., & Merikangas, K. R. (2001). Mood disorders in children and adolescents: an epidemiologic perspective. *Biological psychiatry*, *49*(12), 1002-1014.

Knudsen, E. I. (2004). Sensitive periods in the development of the brain and behavior. *Journal of cognitive neuroscience*, *16*(8), 1412-1425.

Lovallo, W. R. (2013). Early life adversity reduces stress reactivity and enhances impulsive behavior: Implications for health behaviors. *International journal of psychophysiology*, 90(1), 8-16.

McEwen, B. S. (2012). Brain on stress: how the social environment gets under the skin. *Proceedings of the National Academy of Sciences*, *109*(supplement_2), 17180-17185.

McGowan, P. O., Sasaki, A., D'alessio, A. C., Dymov, S., Labonté, B., Szyf, M., ... & Meaney, M. J. (2009). Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nature neuroscience*, *12*(3), 342-348.

McLaughlin, K. A., Green, J. G., Gruber, M. J., Sampson, N. A., Zaslavsky, A. M., & Kessler, R. C. (2012). Childhood adversities and first onset of psychiatric disorders in a national sample of US adolescents. *Archives of general psychiatry*, *69*(11), 1151-1160.

McLaughlin, K. A., Sheridan, M. A., & Lambert, H. K. (2014). Childhood adversity and neural development: deprivation and threat as distinct dimensions of early experience. *Neuroscience & Biobehavioral Reviews*, 47, 578-591.

McLaughlin, K. A., Sheridan, M. A., Winter, W., Fox, N. A., Zeanah, C. H., & Nelson, C. A. (2014). Widespread reductions in cortical thickness following severe early-life deprivation: a neurodevelopmental pathway to attention-deficit/hyperactivity disorder. *Biological psychiatry*, *76*(8), 629-638.

McLaughlin, K. A., Weissman, D., & Bitrán, D. (2019). Childhood adversity and neural development: A systematic review. *Annual review of developmental psychology*, *1*, 277.

Meaney, M. J. (2001). Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. *Annual review of neuroscience*, *24*(1), 1161-1192.

Meaney, M. J., & Szyf, M. (2005). Maternal care as a model for experience-dependent chromatin plasticity?. *Trends in neurosciences*, *28*(9), 456-463.

Miller, A. B., Sheridan, M. A., Hanson, J. L., McLaughlin, K. A., Bates, J. E., Lansford, J. E., ... & Dodge, K. A. (2018). Dimensions of deprivation and threat, psychopathology, and potential mediators: A multi-year longitudinal analysis. *Journal of abnormal psychology*, *127*(2), 160.

Nelson, C. A., Bhutta, Z. A., Harris, N. B., Danese, A., & Samara, M. (2020). Adversity in childhood is linked to mental and physical health throughout life. *bmj*, 371.

Palma-Gudiel, H., Córdova-Palomera, A., Leza, J. C., & Fañanás, L. (2015). Glucocorticoid receptor gene (NR3C1) methylation processes as mediators of early adversity in stress-related disorders causality: a critical review. *Neuroscience & Biobehavioral Reviews*, 55, 520-535.

Parade, S. H., Ridout, K. K., Seifer, R., Armstrong, D. A., Marsit, C. J., McWilliams, M. A., & Tyrka, A. R. (2016). Methylation of the glucocorticoid receptor gene promoter in preschoolers: Links with internalizing behavior problems. *Child Development*, *87*(1), 86-97.

Perroud, N., Paoloni-Giacobino, A., Prada, P., Olié, E., Salzmann, A., Nicastro, R., ... & Malafosse, A. (2011). Increased methylation of glucocorticoid receptor gene (NR3C1) in adults with a history of childhood maltreatment: a link with the severity and type of trauma. *Translational psychiatry*, *1*(12), e59-e59.

Polanczyk, G. V., Zavaschi, M. L., Benetti, S., Zenker, R., & Gammerman, P. W. (2003). Sexual violence and its prevalence among adolescents, Brazil. Revista de Saude Publica, 3(1), 8–14.

Sheridan, M. A., & McLaughlin, K. A. (2014). Dimensions of early experience and neural development: deprivation and threat. *Trends in cognitive sciences*, *18*(11), 580-585.

Stoltenborgh, M., van Ijzendoorn, M. H., Euser, E. M., & Bakermans-Kranenburg, M. J. (2011). A global perspective on child sexual abuse: Meta-analysis of prevalence around the world. *Child Maltreatment*, 1(2), 79–101. http://dx.doi.org/10.1177/1077559511403920

Szyf, M. (2013). DNA methylation, behavior and early life adversity. *Journal of Genetics and Genomics*, 40(7), 331-338.

Teicher, M. H., Ito, Y., Glod, C. A., & Andersen, S. L. (1997). Preliminary evidence for abnormal cortical development in physically and sexually abused children using EEG coherence and MRI.

Tyrka, A. R., Ridout, K. K., Parade, S. H., Paquette, A., Marsit, C. J., & Seifer, R. (2015). Childhood maltreatment and methylation of FK506 binding protein 5 gene (FKBP5). *Development and psychopathology*, *27*(4pt2), 1637-1645.

Van Der Knaap, L. J., Riese, H., Hudziak, J. J., Verbiest, M. M. P. J., Verhulst, F. C., Oldehinkel, A. J., & Van Oort, F. V. A. (2014). Glucocorticoid receptor gene (NR3C1) methylation following stressful events between birth and adolescence. The TRAILS study. *Translational psychiatry*, *4*(4), e381-e381.