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Multi-Omics analysis of stress-related diseases

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List of terms and abbreviations

AA: Alpha-amylase

ACE: Adverse Childhood Experience

AUCg: Area Under the Curve with respect to ground

AUCi: Area Under the Curve with respect to increase

CBCL: Child Behavioral Checklist

CA: Childhood Adversity

CM: Childhood Maltreatment

CpGs: Cytosine-Guanine Dinucleotides

CRP: C-reactive Protein

CT: Childhood Trauma

CTCF: CCCTC-binding factor

Cort: Cortisol

DiffMeth: Differential Methylation between groups

DNAm: DNA Methylation

EA: Educational Attainment

EWAS: Epigenome-Wide Association Study

FAS: Fetal Alcohol Syndrome

***FKBP5*:** FK506 Binding Protein 5

GC: Glucocorticoids

GEWIS: Genome-Environment-Wide Interaction Study

GR: Glucocorticoid Receptor

GRE: Glucocorticoid Response Element

GWAS: Genome-Wide Association Study

GxE: Gene-Environment Interaction

HPA axis: Hypothalamic-Pituitary-Adrenal axis

HWE: Hardy-Weinberg-Equilibrium

LD: Linkage Disequilibrium

LES: Life Event Survey

lincRNA: Long intervening noncoding RNAs

MAD: Median Absolute Deviation

MAF: Minor Allele Frequency

MCS: Maltreatment Classification System

MDD: Major Depressive Disorder

MEL: Munich Event List

mQTL: methylation Quantitative Trait Locus

mRNA: Messenger RNA

PAPA: Preschool Age Psychiatric Assessment

PC: Principal Component

PCR: Polymerase Chain Reaction

PRS: Polygenic Risk Score

PTSD: Post Traumatic Stress Disorder

QC: Quality Control

SCZ: Schizophrenia

SES: Socioeconomic Status

SLE: Severe Life Event

snoRNA: small nucleolar RNA

SNP: Single Nucleotide Polymorphism

SON: Snijders-Oomen Non-verbal intelligence test

TAD: Topologically Associated Domain

TBS: Targeted-Bisulfite Sequencing

TF: Transcription Factor

WPPSI: Wechsler Preschool and Primary Scale of Intelligence

WGCNA: Weighted gene co-expression network analysis

Abstract

Exposure to childhood maltreatment (CM) has consistently been linked to an increased risk for developing psychiatric disorders later in life. Due to its reactivity to the environment, DNA methylation (DNAm), an epigenetic process regulating gene expression, has been proposed as a mechanism for the biological embedding of environmental exposures in early life, including CM. Although DNAm is among the best-studied epigenetic mechanisms in the context of CM, the understanding of the timing and trajectory of embedding remains limited and it is unclear if additional environmental exposures converge on similar DNAm alterations. This thesis extends our understanding of the temporal dynamics of biological embedding of CM as well as of the effects of concurrent exposures and genetic contributions. In this work, a broader adversity score, which, additionally to CM, includes socioeconomic status and other contextual stressors based on the life of adverse childhood experiences, and epigenetic biomarkers of prenatal exposure to tobacco and alcohol were examined as concurrent environmental exposures. The effects of CM alone compared to the effects of a broader adversity score on longitudinal DNAm trajectories were examined in the Berlin Longitudinal Child Study (Berlin LCS). The Berlin LCS cohort consists of 3 to 5-year-old children (n=173 at baseline) of whom 86 experienced CM. These children were monitored for up to 24 months with extensive psychometric and biological assessments as well as saliva collection at baseline ad 4 follow-up time points providing epigenome-wide DNAm levels using EPIC arrays and salivary biomarkers of stress. In general, there were only a few DNAm patterns associated with CM or adversity in general that were stable over this timeframe, but regions mapping to the genes *GRAREML*, *P3H3*, *ZNF562* and *GSTT1* showed significant changes, with CM and the adversity score significantly moderated DNAm trajectories over time. Children exposed to CM also showed epigenetic signatures of increased prenatal exposure to tobacco and alcohol, with the prenatal exposure score correlating with some of the differentially methylated regions associated with CM. Lastly, Weighted gene correlation network analysis (WGCNA) identified a module of 268 correlated CpGs which were exclusively associated with CM.

The findings from the Berlin LCS cohort were extended beyond childhood by replicating identified DNAm changes in an independent adolescent sample. This cohort (LMU cohort) included 640 adolescents with or without a diagnosis of major depressive disorder (MDD) assessed with the Kinder-DIPs as well as information on childhood abuse based on a self-report. There was only a small overlap of CpGs in CM-associated WGCNA modules, most likely due to the heterogeneity of cohorts. The prenatal exposure scores, however, could be replicated across tissues and age ranges highlighting the importance of including prenatal exposures in the investigation of early life adversities.

The last part of this work focused on *FKBP5,* a key modulator of the stress system, with known alterations of its epigenetic regulation following CM. Targeted-bisulfite-sequencing, a fine-mapping approach on the DNAm level, allowed the systematic study of DNAm changes at the *FKBP5* locus following CM, which validated previous findings and yielded new insights into the epigenetic regulation of *FKBP5* dynamics. The majority of the CpGs covered (n=41) were significantly differentially methylated between maltreated and non-maltreated children at baseline, of which 25 CpGs showed the same direction of effects after one year. Additionally, some of the methylation changes, especially within the 3' topologically associated domain (TAD), between baseline and the follow-up time point correlated with salivary biomarkers of acute and chronic stress. Genotype effects for the SNP rs1360780 could be detected for CpGs within intron 5 and the 3'TAD with some of the positions showing additive and interactive effects with CM. The stability of the findings after one year supports the notion that long-term effects of CM may be mediated by DNAm changes. More studies in large, deeply phenotyped, longitudinal cohorts, and across different developmental stages and exposures will be necessary to replicate these findings and to validate them with molecular experiments.

Zusammenfassung

Traumatische Erlebnisse im Kindesalter, wie z.B. Kindesmisshandlung wurden konsequent mit einem erhöhten Risiko für die Entstehung psychiatrischer Erkrankungen zu einem späteren Zeitpunkt im Leben in Verbindung gebracht. Aufgrund ihrer Anpassungsfähigkeit als Reaktion auf Umweltfaktoren, wurden epigenetische Mechanismen wie die DNA Methylierung (DNAm) als molekulare Grundlage für die biologische Einbettung („embedding“) von Umwelteinflüssen in den Mittelpunkt gerückt. Obwohl DNAm einer der meist untersuchten epigenetischen Modifikationen ist, sind Zeitpunkt und Stabilität der Einbettung von Umweltfaktoren weitestgehend unbekannt. Zusätzlich ist unklar, ob verschiedene Umwelteinflüsse miteinander interagieren und ähnliche epigenetische Modifikationen herbeiführen, da Faktoren wie Kindesmisshandlung oft mit anderen negativen Umwelteinflüssen wie niedrigem sozioökonomischem Status und pränatalem Alkoholbelastung auftreten.

Ziel dieser Arbeit ist es die Effekte von Kindesmissbrauch sowie Stressbelastung im Allgemeinen auf epigenetischer Ebene zu untersuchen und deren Stabilität im zeitlichen Verlauf zu ermitteln. Diese Effekte wurden in der Berlin Longitudinal Child Study, einer Longitudinal Studie mit 173 Kindern im Alter von 3-5 Jahren, von denen 86 Misshandlung ausgesetzt wurden, analysiert. Diese Kinder wurden über den Zeitraum von zwei Jahren begleitet und ihre Entwicklung (körperliche und psychische Gesundheit, kognitive und sprachliche Entwicklung) dokumentiert. Zusätzlich wurden umfangreiche biologische Parameter bestimmt und die DNA Methylierung zu jedem Zeitpunkt gemessen. Während die DNA Methylierung für die meisten Regionen über den gemessenen Zeitraum stabil blieben, gab es einige Regionen bei denen Misshandlung oder Stress im Allgemeinen die den longitudinalen Verlauf moderierten. Zusätzlichen zeigten Kinder die Misshandlung erfahren haben, signifikant mehr epigenetische Signaturen pränataler Alkohol- und Tabakbelastung, wobei diese Signaturen auch mit den Effekten von Kindesmisshandlung korrelierten. Weiterhin wurde ein modul-basierter Ansatz, „weighted gene co-expression analysis“ verwendet um ähnlich verändernde DNAm Positionen zu identifizieren, die ausschließlich mit Kindesmisshandlung assoziiert waren.

Im zweiten Teil dieser Arbeit, wurden die Ergebnisse aus der Kinderstudie in einer unabhängigen Kohorte, der LMU Kohorte validiert. Hier wurden 640 Jugendliche mit und ohne Diagnose einer Depression sowie der Information zu Misshandlungserlebnissen untersucht, um zu prüfen um die epigenetischen Effekte über die Kindheit hinaus stabil bleiben. Nur wenige DNAm Positionen aus dem modul-basiertem Ansatz konnten in einem modul-basierten Ansatz in der LMU Kohorte repliziert werden. Von den DNAm Regionen aus der Kinderstudie überlappten keine, mit denen die in der Jugendkohorte identifiziert wurden. Diese Ergebnisse kann mit großer Wahrscheinlichkeit der Heterogenität (Gewebe, Zeitraum, Maß für Misshandlung, etc.) zwischen den Kohorten zugeschrieben werden. Interessanterweise,

konnten die pränatalen Signaturen repliziert werden, was zeigt, dass diese Faktoren in zukünftigen Studien mit einbezogen werden sollten.

Der finale Teil dieser Arbeit bezieht sich auf das Gen *FBKP5,* das als ein wichtiger Modulator des Glucocortikoidrezeptors, mit für die Regulation der Stresshormonachse verantwortlich ist. Frühere Studien haben bereits gezeigt, dass Kindesmissbrauch zu epigenetischen Veränderungen in regulatorischen Regionen dieses Gens führt. Die Mehrheit aller DNAm Positionen (n=41) zeigten signifikante Unterschiede zwischen Kindern mit und ohne Misshandlung, wobei 25 dieser Unterscheide nach einem Jahr noch immer Effekte in gleicher Richtung aufwiesen. Zusätzlich korrelierten einige der Änderungen, besonders in der 3‘ topologisch assoziierten Domäne (TAD), über ein Jahr mit Änderungen in Biomarkern von akutem und chronischem Stress. Weiterhin konnten Genotypeffekte für den Polymophismus rs1360780 beobachtet werden, wobei manche Positionen in Intron 5 und der 3‘ TAD additive Effekte und Interaktionen mit Kindesmissbrauch zeigten. Mit dieser Arbeit konnten frühere Befunde zu dem FKBP5 gen repliziert werden und die wiederholten Messungen erlaubten es die Stabilität dieser Befunde nach einem Jahr zu zeigen. Die Ergebnisse dieser Studie unterstützen die Hypothese, dass epigenetische Marker zu den Langzeiteffekten von Kindesmisshandlungen beitragen. Dennoch werden diese Befunde in einer größeren Langzeitstudie mit gut dokumentierten Umwelteinflüssen validiert werden müssen.

1. Introduction

1.1. Genetic studies in psychiatric disorders

A large body of evidence suggests a substantial genetic component to many psychiatric conditions (Buxbaum et al., 2010). The genetic influence on all major psychiatric disorders has been consistently demonstrated by twin and adoption studies and the estimated broad-sense heritability (h^2), namely the proportion of phenotypic variation explained by genetic variation, ranges from 0.3 for depression to over 0.8 for schizophrenia (Agrawal, 2018; Baselmans et al., 2021; Kendall et al., 2021). A meta-analysis across five primary studies on the heritability of major depressive disorder (MDD) estimated an odds ratio (OR) of 2.84 (95% CI = 2.31-3.49) for an increased risk for first-degree relatives of MDD patients (Sullivan et al., 2000). The genetic contribution to the risk of many psychiatric disorders has been well established by family studies. While some loci with larger effect sizes have been detected in autism spectrum disorder (ASD) and schizophrenia (SCZ), few or no robust large effect size loci have been identified for the majority of psychiatric disorders (Jacquemont et al., 2006; Sebat et al., 2007). Genome-wide associations studies (GWAS) successfully identified numerous genetic loci with small effect sizes involved in many psychiatric disorders (Gratten et al., 2014). While some diseases are regulated by a single gene and show classical Mendelian patterns of inheritance, psychiatric disorders are best classified as 'complex traits', where the variability is a result of a large number of factors and can be dissected into sources of variation resulting from genetic factors, non-genetic factors and their interplay (Grotzinger et al., 2019; Visscher et al., 2012). The single nucleotide polymorphisms (SNPs) identified from GWAS using a case-control setting explain only a very small amount of the variance and cumulatively they only explain a fraction of the known genetic variance (Maier et al., 2018). This discrepancy between total heritability (estimated from family studies) and the proportion of phenotypic variation explained by all detected SNPs from GWAS was termed ‘missing heritability’ (N. Wray et al., 2014). Several explanations have been offered to address this problem: i) complex diseases are highly polygenic and GWAS do not capture variants with small effects that are rare, ii) family studies have overestimated heritability, genetic effects are generally non-additive but a result of complex interactions, and finally iii) that complex diseases mostly stem from an interaction of genetic predisposition with the environment (Owen & Williams, 2021; Sleeswijk et al., 2019; Woo et al., 2017). Especially, the last explanation emphasizes the need to more explicitly model the joint effect of genes and environment as a source of unaccounted variation (Kaprio, 2012).

1.2. Environment as a risk factor for psychiatric disorders

Previous research of environmental exposures as risk factors in psychiatry has yielded robust findings and several factors have consistently been associated with mental illness (Krapohl et al., 2017; Uher & Zwicker, 2017). These factors include general exposures such as low socioeconomic status (SES) (Lupien et al., 2009) and urbanicity (Guloksuz et al., 2015; Radhakrishnan et al., 2019) but also exposures specific to childhood, e.g., childhood maltreatment (CM) and household dysfunction (Teicher & Khan, 2019; Ujhelyine Nagy & Kuritarne Szabo, 2020) and range back to exposures during pregnancy such as perinatal stress (Molenaar et al., 2019; Rice et al., 2010) or prenatal substance abuse. While these effects are genetically controlled to a certain degree, findings from studies show a substantial contribution from environmental factors alone (G. D. Smith & Ebrahim, 2003; Van Os et al., 2008).

1.2.1. Early-life adversity induces long-lasting consequences

Childhood adversity (CA), also referred to as early-life stress (ELS), is among the most studied environmental risk factors for the development of psychiatric disorders later on in life (Gerke et al., 2018; Heim et al., 2008; Merrick et al., 2017). While the definition of the term CA varies across studies, it almost always encompasses a category of CM that comprises well-defined subtypes such as abuse and neglect (Burgermeister, 2007; Humphreys & Zeanah, 2015). Robust evidence for CA (including childhood abuse and neglect) being strongly associated with the onset and persistence of mental disorders has been presented by several large-scale and population-based mental health surveys (Green et al., 2010; Kessler et al., 2010; McLaughlin et al., 2010). For instance, a meta-analysis on the effects of childhood trauma (CT) across 26 studies showed an increased risk of developing MDD in adulthood (OR = 2.80, Z = 7.70, P < 0.001), with the strongest effect for the specific childhood stressors emotional abuse (OR = 2.78) and neglect (OR = 2.75) (Mandelli et al., 2015). Additionally, another meta-analysis across 184 studies on adult MDD reported that individuals with a history of CM were 2.66 (95% CI 2.38–2.98) to 3.73 (95% CI 2.88–4.83) times more likely to develop MDD in adulthood, presented with significantly earlier onset and were twice as likely to develop chronic or treatment-resistant depression (J. Nelson et al., 2017). These studies show that the effects of CA are not limited to childhood and adolescence, but that CA can have long-lasting effects accounting for disorders with the first onset in adulthood. The proportion of

mental disorders that can be attributed to CA has been estimated to be 40% in childhood to 19% in adulthood (Kessler et al., 2010).

Children experiencing maltreatment are more likely to be exposed to multiple adversities across developmental stages including exposure to caregiver mental illness, substance use, or domestic violence. Studies investigating the effects of CM have mostly focused on specific types of CM but did consider other adversities present in the environment (Brown et al., 2019). Various CA subtypes have as well been demonstrated to be associated with several psychiatric disorders (Carr et al., 2013; Guinosso et al., 2016; Lupien et al., 2009), however, CAs often occur in the context of multiple other adversities and are highly correlated making the separation of subtype-specific effects difficult (Dong et al., 2004). Exposures that occur alongside CM within a suboptimal caregiving environment include prenatal exposures such as maternal substance use, which need to be accounted for.

1.2.2. Prenatal exposures

Extending the timeframe for environmental effects to before birth, previous work has linked the occurrence of prenatal substance exposure (such as alcohol, smoking, or cocaine) and maternal psychopathology (maternal stress, perinatal depression, or anxiety) to a suboptimal caregiving environment, which is conducive to CM (Flannigan et al., 2021; Margolis et al., 2021; Min et al., 2017; Price et al., 2017). A study characterizing the co-occurrence of prenatal and postnatal stressors found that children with high prenatal adverse exposures were more likely to experience postnatal adversities (Lebel et al., 2019).

Findings from both animal and human studies have reported that exposure to prenatal adversity affects the brain and behavior of the offspring (Thomason et al., 2021; Zeng et al., 2015). The causal role of prenatal exposures on the etiology of neurodevelopmental disorders is supported by large population cohorts, which have accounted for a wide range of potential confounders, including postnatal stressors. Prenatal exposure to certain substances or exposure to maternal conditions via endogenous pathways, such as glucocorticoid (GC) signaling, lead to altered neurodevelopment (Antonelli et al., 2017; Franks et al., 2020). For instance, a prospective study on the long-term effects of prenatal synthetic GC exposure reported a significant association with general psychiatric disturbance (B=8.34 [95% CI: .23-16.45]) and inattention (B= .97 [95% CI: .16-1.80]) at 8 years, with the same direction of effect at 16 years (Khalife et al., 2013).

Another common prenatal exposure is alcohol use in pregnancy. Alcohol exposure in utero is associated with a well-described syndrome and collection of physical and psychological complications defined as a fetal alcohol spectrum disorder (FASD). Neurobehavioral consequences among individuals with FASDs include developmental delay, intellectual disability, hyperactivity, and hypersensitivity to stress. Children and adolescents with prenatal alcohol exposure were reported to experience high rates of early adversity (Flannigan et al., 2021). A study investigating the effects of prenatal alcohol exposure on brain structure in children with and without adverse childhood experiences found that prenatal and postnatal exposures interact with brain development differently and that the common and divergent effects on developmental trajectories require the consideration of multiple exposures (Andre et al., 2020).

While prenatal and postnatal exposures were individually found to be associated with the development of psychiatric disorders later on in life, it is likely they have converging effects on the overall risk and resilience trajectories. These correlates in offspring exposed to prenatal stress include altered neurodevelopment, neurocognitive processing as well as aberrant structural and functional connectivity (Roos et al., 2021). The role of biological alterations on various different levels (including epigenetic modifications, circuit-level alterations, changes on pathway levels) in mediating alterations in outcome following prenatal stress is expected to be crucial for embedding and as such is gaining attention. Additionally, prenatal and postnatal exposures may have cumulative effects or interact with each other, leading to worse outcomes than single exposures alone. Future research is needed to disentangle common and distinct effects of exposures during different stages of development and their potential interaction with genetic variants (Provençal & Binder, 2015).

1.3. The paradigm of gene-environment interactions in psychiatry

The investigation of Gene-by-Environment (GxE) interactions is based on the fact that, so far, neither genetic (G) nor environmental (E) factors alone are sufficient to explain the development of psychiatric disorders. The concept of GxE interaction is already well-established within the diathesis-stress or vulnerability-stress model in psychiatry, which describes that mental disorders are caused by the interactions of dispositional (diathesis) and the environmental (stress) factors (Broerman, 2020).

Within the framework of the diathesis-stress model, there have been various attempts to study the environmental effects on a phenotype that differ between individuals based on their genetic background or, worded differently, the differences

of genotypes in the susceptibility to environmental exposure (Karg & Sen, 2012; Rutter, 2008).

Effects of genetic variants and environmental exposures can co-occur in different manners and a key challenge is to distinguish “real” interactions from spurious ones. GxE correlations, in particular, can produce false-positive results in GxE research. These correlations can, for example, occur when an individual’s genotype influences the probability of environmental exposure and actually might represent a GxG interaction i.e., impulsivity (Bevilacqua & Goldman, 2013) and exposure to negative life events. The potential violation of the independence assumption for G and E needs to be considered when evaluating the findings of GxE studies (Ding et al., 2021; Perlstein & Waller, 2020).

1.3.1. Statistical modeling of GxE interactions

Two models that are commonly used to model GxE interactions are the additive and the multiplicative model. Within the additive model, a GxE interaction is given if the risk for the phenotype differs from the sum of risks when exposed to only G or only E. The multiplicative model constitutes an interaction if the phenotype risk when exposed to both, G and E, differs from the product of risks. Thus, a statistically significant GxE marks the joint occurrence of two factors (G and E) that produce synergistic (greater) or antagonistic (lesser) effects on an outcome that goes beyond the addition or multiplication of the single main effects. Based on this definition it is possible that the effect of the same gene on a certain outcome can go in different directions depending on the environment (Karg & Sen, 2012; Moffitt et al., 2006).

The biological interpretation of a statistically significant result from the multiplicative model is often difficult. Additionally, in some studies there might be a deviation from the additive model but not from the multiplicative model or vice versa, and that the model-dependency renders positive statistical interactions arbitrary. In conclusion, the model to be tested should be considered a priori and should ideally be biologically informed (Moffitt et al., 2006).

One of the first studies investigating GxE interactions in psychiatry was performed by Caspi et al., and looked at the moderation of childhood maltreatment (CM) by a polymorphism in the serotonin transporter gene (*SLC6A4*). They found that with exposure to CM, the polymorphism increased the risk of depression but the SNP had no effect without CM exposure (Caspi et al., 2003). However, while most of the later studies examining the altered depression risk in the presence of stress exposure found

effects in the same direction, some of them yielded inconsistent results or reported no significant effects (Culverhouse et al., 2018).

A study by Border et al. attempted to validate previous findings on 18 empirically identified candidate genes for depression and tested the main effects of the SNPs, SNP x E interactions, and gene-level interactions of E across multiple SNPs. Although the sample sizes ranged between 62,000 and 443,000 individuals, there was no clear evidence for interaction effects of the polymorphisms tested and traumatic events on depression phenotypes for any of the studied candidate genes. In conclusion, they suggested moving away from historic candidate gene approaches. Given the highly polygenic nature of psychiatric disorders in general, it is not clear why strong GxE with single candidate genes would be expected (Border et al., 2019).

This lack of reproducibility constitutes a major concern in the field of GxE studies, prompting the question of “true” interactions even exist. Conflicting results have been attributed to the heterogeneity in both outcome measures and measures of environmental exposures, but also differences in the modeling approaches and cohort characteristics across studies (Border et al., 2019; Karg & Sen, 2012).

1.3.2. From candidate genes to aggregated approaches

Taking into consideration the polygenic nature of complex diseases motivates the question of the combination of disease-relevant risk variants detected in GWAS might improve the assessment of disease risk as compared to single variants (Hyman, 2018). Polygenic risk scores (PRS) aggregate the estimated effect of many genetic variants on an individual's phenotype (Cano-Gamez & Trynka, 2020). PRS typically use the results of GWAS to predict quantitative phenotypes or disease risk at an individual level and combine markers into a score based on variation at multiple genetic loci and their associated weights. These scores aim to capture the additive effects explained by multiple markers. A PRS reflects an individual’s risk compared to others with a different genetic constitution but does not give a baseline or time frame for disease progression. While PRS does have a potential predictive ability, its current clinical use remains limited due to "insufficient" discriminative power at the individual level.

Employing a polygenic approach, approximately 1-2% of the variation in depression (Howard et al., 2019) and anxiety (Levey et al., 2020) could be explained. Following the advent of PRS, the joint analysis of environmental exposures and PRS (PRS x E) emerged. While in theory, GxE effects should be more likely to be detected if polygenic information is used, a meta-analysis of 5,765 individuals with depression did not show interaction effects of PRS and CT (Mullins et al., 2016). They reported

significant main effects of PRS, explaining 1.1% of the variance in phenotype ($p = 1.9 \times 10^{-6}$) and severe life events (SLE) and CT on MDD status ($p = 2.19 \times 10^{-4}$ and $p = 5.12 \times 10^{-20}$, respectively). The PRS x SLE interaction was not significant, PRS x CT interaction ($p = 0.002$) showed an inverse association with MDD status. This could be explained by the GWAS-derived PRS being based on a case-control comparison for a specific disorder and the additive nature of PRS, which does not model well the interaction with the environment of every variant included (Mullins et al., 2016).

While previous findings from candidate genes as well as polygenic and genome-wide approaches indicate GxE interactions, uncertainty regarding the question of whether and if GxE effects truly exist remains. The incorporation of biological data and the replication of a significant phenotypic GxE finding at an intermediate level, molecular or biological, could provide supporting evidence for true GxE interactions. A particular molecular process that has been proposed as a possible mediator for the embedding of environmental exposure in the genome is epigenetic mechanisms (Van Winkel et al., 2010).

1.4. Epigenetics as an underlying mechanism for gene-environment interactions

Epigenetic mechanisms refer to processes altering gene expression and translation that do not involve changes of the underlying DNA sequence. The term epigenome refers to the collection of chemical changes to the DNA and histones, such as methylation or acetylation. Epigenetic processes among others encompass DNA methylation (DNAm), non-coding RNAs (such as micro RNAs, long non-coding RNAs, or small interfering RNAs), and histone modifications. The effect of epigenetic modifications is exerted via direct or indirect changes to a chromatin structure (Venkatesh & Workman, 2015). Modifications of the chromatin structure are critical for the regulation of gene expression because they determine the accessibility and the sequential recruitment of regulatory factors to the underlying DNA (Quina et al., 2006). Although epigenetic changes are integral to some regular biological processes such as cellular differentiation and development, some alterations have been implicated in disease states (Portela & Esteller, 2010). Epigenetic marks are tissue-specific, each cell type has a distinct epigenetic pattern governing the timing and magnitude of gene expression by restricting areas of the genome available for transcription. Epigenetic changes can be introduced by several factors including aging, the environment/lifestyle, and disease state. After being introduced, epigenetic patterns are generally maintained by mitosis.

A large body of evidence suggests that some parts of the epigenome are responsive to external environmental factors and conditions including the social environment (Szyf et al., 2008). Therefore, it has been suggested that epigenetic modifications and mechanisms including DNAm, histone modifications, non-coding RNA, and chromatin conformation changes are involved in mediating GxE interactions by changing the expression of genes implicated in stress-related psychiatric diseases. As a result, epigenetic regulation is considered an additional layer of alteration that fine-tunes gene expression levels (Parade et al., 2021).

1.4.1. DNA methylation

The best investigated epigenetic mechanism is DNAm, which can take place at around 28 million CpG sites distributed across the human genome (Stirzaker et al., 2014). DNAm occurs by the addition of a methyl group (CH_3) to the DNA, specifically at the 5-carbon ring of cytosines in cytosine-phosphate-guanine dinucleotides (CpGs), resulting in 5-methylcytosine (5mC) (Figure 1) (Lister et al., 2009). DNAm can influence gene expression by affecting the interactions of the DNA with specific transcription factors and chromatin proteins. Methylation at CpGs has generally been associated with transcriptional repression (Khavari et al., 2010). DNAm patterns, however, are not constant across a gene but vary depending on the genetic architecture of a region. In actively transcribed genes, DNAm patterns relate to specific regulatory functions. For instance, CpGs are overrepresented in promotors of many genes (Weber et al., 2007). DNAm marks can be mitotically relayed during cell division, which can lead to stable alterations in gene activity and downstream biological processes. Across the genome, DNAm holds an essential role in genomic imprinting, a process in which gene expression occurs in a parent-specific manner (Peters, 2014). Areas not protected by imprinting undergo both active and passive demethylation. DNAm patterns proceed to differentiate by developmental stage and by tissue (Messerschmidt, 2012). DNAm patterns are introduced by at least three DNA methyltransferases, DNMT1, DNMT3a, and DNMT3b, which catalyze the transfer of a methyl group from *S*-adenosyl-L-methionine to cytosine-phosphate-guanine dinucleotides. These methyltransferases can be generalized into ones that maintain or copy methylation marks after DNA replication, and those that initiate new (de novo) methylation marks on DNA (Damelin & Bestor, 2007).

In order to understand the involvement of DNAm in disease, is essential to grasp the distribution of DNAm patterns and that these patterns can vary over time (e.g., development) and space (e.g., tissue specificity). A large amount of studies has

investigated the association of DNAm with different forms of psychopathology (Barbu et al., 2020; Rijlaarsdam et al., 2021; Wiegand et al., 2021), with the prospect that if DNAm is the causal link then reversing epigenetic marks may alleviate the disease burden. There is however also the possibility that DNAm marks represent a non-causal biomarker of stress-related disorders or environmental risk exposure. In this case, differences in DNAm may be a consequence of a condition instead of a causal mechanism (Barker et al., 2018). Even in this scenario, DNAm can have clinical utility and serve as a biomarker of disease. The majority of studies investigating DNAm in stress-related disorders such as MDD were performed in peripheral tissues (Penner-Goeke & Binder, 2019). Due to the tissue specificity of DNAm, studies in peripheral tissues may not detect brain-specific DNAm differences that may provide greater insight as neurobiological indicators of the disease (A. K. Smith et al., 2015). Most of the studies using postmortem brain tissue, however, are generally limited to smaller cohort sizes and are not suitable as biomarkers. DNAm measures from peripheral samples such as blood or saliva may be of particular relevance for psychiatric disorders because they are easily accessible in living patients (Barker et al., 2018). Overall, DNAm holds potential as a biomarker indexing both environmental exposure, such as childhood adversity, and vulnerability for psychopathology DNAm marks have been considered to be well suited as biomarkers as they are chemically robust and preserved in a range of sample sources. DNAm marks identified from large epigenome-wide association studies (EWAS) have been widely adopted to identify disease-specific biomarkers (Ahsan et al., 2017). More recently, DNAm marks have been aggregated to composite DNAm-based risk scores that may provide a molecular-level prediction of risk groups before disease onset or treatment response of subgroups (Zhang et al., 2017). For example, Barbu et al. developed a DNAm-based risk score that significantly discriminated MDD cases from controls in an independent cohort (Barbu et al., 2020). However, the extent to which the DNAm marks identified may represent a causal mediator remains unclear. In the case of the DNAm-based risk score, it is unclear what exactly is reflected by the score, as multiple environmental factors influence DNAm. The authors additionally developed a score based on samples from smokers, which reduced the discriminatory power of the score.

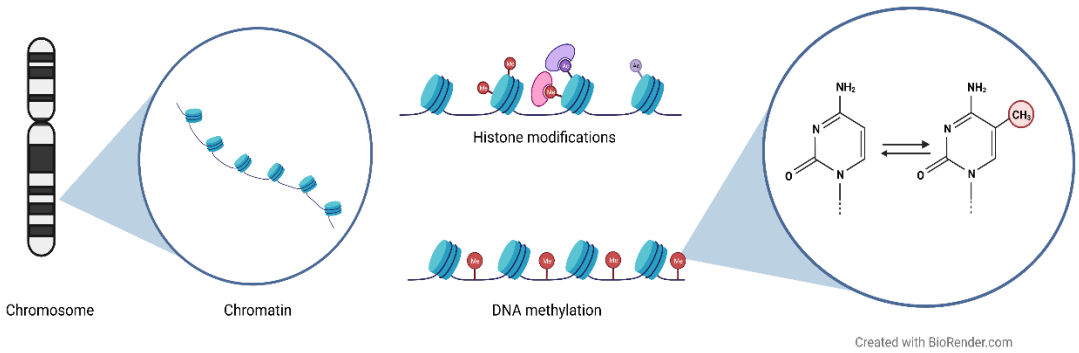


Figure 1: DNA methylation. Among other epigenetic mechanisms, DNAm regulates gene expression and may represent a means of biologically embedding early life stress including childhood maltreatment.

So far, over 70 studies have examined associations between CA and DNAm changes, however, findings between these studies remain inconsistent, which is partially due to differing sample characteristics and methodologies (Cecil et al., 2020a). The majority of studies investigating the effects of CA with EWAS or targeted candidate gene approaches focused on adults and used retrospective assessment of CA and measured DNAm in peripheral tissues (Parade et al., 2021). Some findings, especially for *NR3C1*, could be replicated across multiple studies in different tissues, while findings for other candidate genes were conflicting (Romens et al., 2015). One EWAS investigating the effects of CA on DNAm in two adult cohorts sampling peripheral blood and buccal cells, respectively, did not find any replicated associations on the level of individual CpGs but on the level of differentially methylated regions (DMRs). DMRs encompass several neighboring CpGs, which show the same direction of effect (Houtepen et al., 2018). Only a limited number of studies have examined the effects of CA in form of CM in children immediately following exposure and the temporal dynamics of these effects are unclear. Most of these studies employed a candidate gene approach, that extended some of the findings from adult samples to children. Even fewer studies applied EWAS to child cohorts and reported significant effects after correction. So far, longitudinal findings were presented for two candidate genes in children, *NR3C1* and *FKBP5*. The first study by Parent *et al.* looked at DNAm changes in preschoolers aged 2–5 years (n=260) within 6 months of documentation of CM and one year later, showing that the temporal dynamics of *NR3C1* methylation and its interaction with CM is complex (Parent et al., 2017). A study investigating DNAm changes in preschoolers (n=231) with moderate to severe CM in the previous 6 months found that CM is associated with change in *FKBP5* methylation over time in a six-month period, but only in children that were also exposed to high levels of other contextual stressors (Parade et al., 2017). Although all of these studies report significant

epigenetic associations, it is not reported whether the same loci are affected across studies. Additionally, the alterations in DNAm cannot be specifically attributed to CM as it is not clear if there is potential confounding by other life events, current symptoms, SES and other factors. Another point that needs to be considered is that previous studies varied age, tissues, measures of exposure and how candidate genes were targeted, further limiting the conclusions that can be drawn. The lack of longitudinal assessments often prohibits inferences about the dynamics over time in childhood. This work aims to address this data gap and is the first longitudinal study to analyze the genome-wide effects of CM on DNAm in children (n = 173) within a narrow age range (from 3 to 5 years of age at baseline) over the course of 2 years. Using a broader composite adversity score and correcting for prenatal exposures enabled the analysis of CM-specific DNAm changes and the assessment of other stressors' contribution to the methylation levels at similar sites.

1.4.2. Measuring DNA methylation

Several methods for the quantification of DNAm are available, varying in costs, coverage (genome-wide vs candidate gene approaches), and resolution. The most comprehensive method is whole-genome bisulfite sequencing (WGBS), which provides true genome-wide coverage by sequencing the whole genome including all 28 million CpGs after bisulfite treatment of DNA, however, this method is limited in application to larger samples because of the associated costs and bioinformatics resources required (Crary-Dooley et al., 2017).

1.4.2.1. Array-based DNA methylation measures

To date, various methods to measure DNAm at different resolutions and in genomic regions exist, ranging from genome-wide assays to targeted approaches. Methylation arrays allow the investigation of several selected methylation sites across the genome while providing high-throughput capabilities that minimize the cost per sample. The Infinium Methylation Epic Beadchip represents a more affordable platform for measuring DNAm for up to 850,000 single CpG sites across the genome. This array-based method is a suitable tool to identify associations between single (or clusters of) CpG sites and phenotypes of interest. While the Epic Beadchip is a cost-efficient tool to screen the genome for differentially methylated positions (DMP) and regions (DMR), it only assays ~3% of all genomic CpGs and might not cover the specific gene of interest well (Mansell et al., 2019). While the EPIC array has been designed to address the lack of coverage of regulatory regions of its predecessor, the 450k array, including 58% of FANTOM5 enhancers, only 7% distal and 27% proximal ENCODE regulatory elements

are represented. Additionally, the proportion of distal regulatory elements interrogated is still limited and the methylation level of one CpG probe per element is not always reflective of the neighboring sites (Pidsley et al., 2016). As the EPIC array does not necessarily cover the environmentally sensitive CpGs of interest, a sequencing-based approach might be more suitable to study these effects. Lasting effects of environmental risk factors may be reflected by very small changes in DNAm and a method to measure DNAm with high accuracy and sensitivity in candidate genes for large cohorts is needed to replicate previous findings. While whole-genome bisulphite sequencing is regarded as the current 'gold standard' for the fine mapping of methylated CpGs, the high costs of this approach make it unfeasible for DNAm measurements in large cohorts (Vargas-Landin et al., 2018).

1.4.2.2. Targeted-bisulfite sequencing as fine-mapping approach

Since changes related to environmental exposure might be cell-type specific and most studies rely on more complex tissues, assessing effects in mixed tissues requires high accuracy in order to detect small changes emerging from a small number of cells. While Illumina DNAm arrays represent accurate and cost-effective methods to measure DNAm, the currently available arrays lack coverage in key enhancer regions that are environmentally sensitive as they only include a small number of probes covering each site. Targeted-bisulfite sequencing (TBS) is a fine-mapping approach to measure DNAm levels which is based on bisulfite conversion coupled with targeted enrichment via PCR, followed by sequencing and subsequent quantification. TBS provides a candidate approach to perform high-resolution studies by increasing the depth of read coverage per CpG in regions of interest. Measuring DNAm changes with high accuracy and sensitivity in candidate loci increases the power to detect and replicate the embedding of GxE interactions on epigenetic layers and allows time-course experiments in larger numbers of samples to understand the stability of the alterations following environmental exposures. Roeh et al., developed and optimized an assay for the *FKBP5* locus, an important gene in the regulation of the stress system and previously linked to stress-related disorders (Roeh et al., 2018).

1.4.3. *FKBP5*: a key modulator of the stress system

Studies investigating the biological correlates and mediators of the effects of early life stress in the form of CM on mental health, suggest that a dysregulation of hypothalamic pituitary adrenal (HPA) axis following CM leads to vulnerability to the effects of stress later in life (Agorastos et al., 2019; Dunn et al., 2019). The most consistent finding on the epigenetic programming of neuropsychiatric systems by exposure to adversity in rodent models and human cohorts relate to the genes of the

HPA axis by CM (Kular & Kular, 2018). A pioneer study reported an association of the epigenetic status of the GC receptor (GR) gene *Nr3c1* in the hippocampus with the quality of parental care of the pups, the levels of GR, and vulnerability to stress in rats (Weaver et al., 2004). This finding was extended to humans by the detection of DNAm changes in the *NR3C1* gene in the hippocampus of suicide victims with a history of childhood abuse, compared to suicide victims or controls with no reported childhood abuse (McGowan et al., 2009). Additionally, more recent studies have identified epigenetic alterations following adversity in other key player of the HPA-axis including the intracellular GR regulator FKBP5 as well as the hypothalamic arginine vasopressin (AVP), corticotropin-releasing hormone (CRH), and pituitary proopiomelanocortin (POMC) genes (Weaver, 2007). Upon stress activation, the release of CRH from the paraventricular nucleus of the hypothalamus is triggered, in turn stimulating the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. This causes the adrenals to secrete GCs, which have various physiological stress-coping effects and are responsible for terminating the axis activation. The most abundant circulating GC in humans is cortisol, which regulates various physiological processes (Tsigos & Chrousos, 2002). Within cells, GCs bind to two nuclear hormone receptors, the mineralocorticoid receptor (MR) and the GR. MRs have a 10-fold higher affinity for GCs than GRs, which indicates different roles for each of the receptors in the regulation of HPA axis activity (J. M.H.M. Reul & De Kloet, 1985; Johannes M.H.M. Reul et al., 2015). While MRs are almost saturated under basal GC levels, GR occupancy increases at elevated GC levels during the circadian peak or following stress exposure (Hartmann et al., 2021). Conversely, MRs are involved in basal activity and onset of stress-induced HPA axis activity, GRs primarily drive its termination.

The Hsp90-associated co-chaperone FK506-binding protein 51 (FKBP5), encoded by the *FKBP5* gene, is a negative regulator of GR activity and plays a key role in the termination of the stress response by GRs (Binder, 2009). FKBP51 binds to the receptor complex and impedes the nuclear translocation of the GR. In return, GR activation induces *FKBP5* mRNA and protein expression, providing an ultra-short feedback loop for GR activation. FKBP5 induction can vary across individuals and has been proposed as a marker of GR sensitivity. FKBP5 transcription is steroid-regulated and mediated by binding of the GR to GC responsive elements (GRE). GREs are located throughout the locus ranging from upstream of the FKBP5 promoter to introns 2, 5, and 7 of the gene (Anthony S Zannas et al., 2015). *FKBP5* includes several polymorphisms and the best-characterized haplotype spans the entire gene containing up to 18 SNPs in strong linkage disequilibrium (LD) in Caucasians ($r^2 > 40.8$, distance

4500 kb, 1,000 genomes next generation sequencing project). This haplotype is commonly tagged by the SNPs rs3800373, rs9296158, or rs1360780.

Klengel et al. reported that *FKBP5* expression level differences in response to GR signaling that were linked to the epigenetic regulation of the *FKBP5* locus. Additionally, *FKBP5* was shown to confer genetic risk for stress-related disorders, specifically in the presence of CM (Klengel et al., 2013). Based on their findings, the authors propose the following model of GxE interactions with rs1360780: The risk allele causes differential interactions between the enhancer and the transcription start site (TSS) upon GR activation. Chromatin conformation capture experiments have shown that the "risk allele" of rs1360780 enables the structural interaction of TSS with downstream enhancers located in GRE of intron 2, which does not occur in carriers of the "protective allele" (Figure 2). This structural interaction leads to an increased FKBP5 expression by GR. This interaction causes an altered in transcriptional induction of *FKBP5* and persistent overexpression of *FKBP5* leads to a prolonged GR activation. The allele-dependent changes in chromatin structure together with the prolonged GR activity introduce DNA demethylation at CpGs located within and proximal to GREs. During specific developmental periods, the demethylation is stable, and thus results in long-term transcriptional changes of *FKBP5* upon GR activation (Klengel et al., 2013).

Differential methylation within the *FKBP5* locus has been reported for various mental disorders including MDD and Post Traumatic Stress Disorder (PTSD) and could partially be connected to CA (Klinger-König et al., 2019; Xie et al., 2010). Demethylation following CA has been shown in regulatory elements in intron 2 and 7 of *FKBP5* (Klengel et al., 2013; Parade et al., 2017; Tyrka et al., 2015; Wiechmann et al., 2019), consequently increasing gene expression following GR signaling and impeding the negative feedback mechanism of the HPA axis.

Interestingly, the interaction with the genotype and adversity seems to be restricted to early life, as no relationship between *FKBP5* DNAm and adversity has been reported in adults (N. Alexander et al., 2020; Binder et al., 2008). This implies the existence of a vulnerable period during childhood in which adverse events can influence the stress response in adulthood, affecting risk for stress-related phenotypes (Dunn et al., 2019). Dunn et al. investigated several conceptual models that have been proposed to account for the effects of adverse experiences on neurodevelopment. The authors concluded that a sensitive period model best fits most DNAm sites associated with adversity, with experiences occurring before 3 years of age having disproportionate influences on methylation. As a conclusion, epigenetic changes as a result of GxE interaction effects have been proposed to be established during sensitive

periods (e.g., development) and to remain stable over time. Nevertheless, only few studies have investigated DNAm changes within *FKBP5* following CA directly in children. Apart from the study described above by Parade et al., a study by Tyrka et al. reported the demethylation of two CpGs within intron 7 of *FKBP5* in salivary DNA in children exposed to CM.

The work presented here is the first to systematically investigate *FKBP5* methylation with a fine-mapping approach directly in a child cohort. While the EPIC array includes 29 CpGs within the FKBP5 locus, only few CpGs are located in/near the regulatory regions (GREs, CTCF) which have been shown to regulate transcription. The fine mapping approach is necessary to assess methylation of CpGs relevant for the epigenetic regulation of this gene and to investigate the effects of adversity, especially CM, on these sites. Additionally, the repeated measures in the cohort allow the assessment of the stability of association between CM and salivary DNAm within *FKBP5*. DNAm can exhibit different temporal dynamics, varying between the nearly absolute stability of the DNA sequence and short-term variations typical of mRNA levels. Previous studies have identified sequence and other marker characteristics that are associated with DNAm stability including GC content, CpG density proximity to repeat elements. The latter has been suggested to be a result from interplays involving CCCTC-binding factor (CTCF), specificity Protein 1 (SP1) and DNA methyltransferases (Byun et al., 2012). Understanding the stability of the environmentally induced epigenetic changes during development would help elucidate the association between CM and the development of psychopathology later on in life. Information on the stability of DNAm marks following exposure would be important for the design of studies, as well as for the statistical analysis of methylation data.

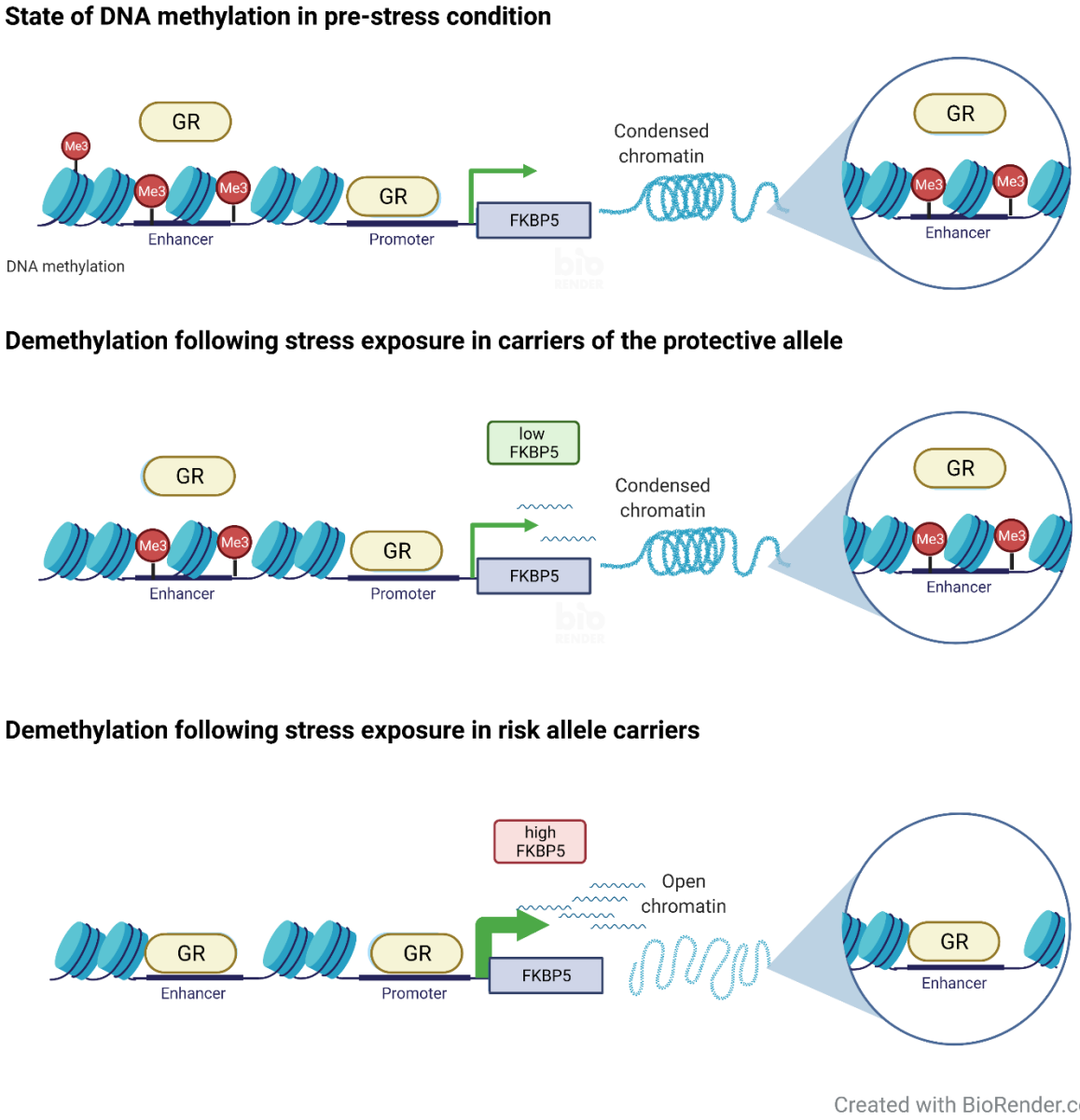


Figure 2: Genotype-dependent epigenetic regulation of the FKBP5 locus. *FKBP5* shows robust induction by GR across different tissues, however there is significant variation in expression patterns. Epigenetic modifications, such as DNAm, constitute an additional layer of regulation of gene expression and the relationship between genotype and environmental factors**.** The SNP rs1360780 with the risk allele T is located within an enhancer region of intron 2 of the *FKBP5* gene and was repeatedly shown to be the functional variant conferring risk in the haplotype. Mechanistically, the tagged haplotype alters the ultrashort feedback loop between the glucocorticoid receptor (GR, shown in red) and FKBP5. rs1360780 is located close to a functional glucocorticoid responsive element (GRE, shown in green) and its T-allele facilitates the binding of the intron 2 GRE to the promoter in response to GR activation, which leads to an increased transcriptional response of *FKBP5.* This results in increased GR resistance in T allele carriers, which affects the negative feedback of the stress response. GxE studies have shown an interaction effect of the *FKBP5* haplotype and early-life stress with risk allele carriers being exposed to prolonged and higher cortisol levels.

1.5. Aim of the research

The main objectives of this thesis were to first determine if CM specific variation in DNAm exists and if it is more accentuated in the context of additional adversity, i.e., if different adversities contribute to DNAm changes at the same CpGs or genes. The latter is based on the fact that CM is embedded in an environment with multiple adversities. Additionally, this work aimed to characterize how CM-associated variation in DNAm changes over a short timeframe of two years during childhood. This thesis specifically addressed the lack of longitudinal data in the research of the effects of CM on DNAm. Longitudinal data allows the examination of the implied temporal order of associations (i.e., clarify the direction of effects) and account for other exposures. The timing of the effects of CM on epigenetic regulation could also be used to identify specific windows of biological embedding. Additionally, the genetic contribution on related outcomes was investigated using PRSs.

Second, this thesis focused on *FKBP5,* a key modulator of the stress system, with known alterations of its epigenetic regulation following CM. The aim here was to validate the findings previously reported in a child cohort and investigate the stability of the changes of DNAm in regulatory regions of *FKBP5*. This work systematically examined the effects of CM on DNAm employing a fine-mapping approach, addressing the lack of coverage of array-based technologies. In order to replicate previously reported genotype effects, the interaction between rs1360780 and CM on *FKBP5* methylation was tested. Additionally, the correlation between DNAm changes, genotype, and biodata (including cortisol) was investigated.

The last part of this thesis aimed to validate the longitudinal array-based findings in an independent cohort with a different age range. Here, I tested if the maltreatment-specific DNAm patterns could be replicated in an adolescent cohort, showing that effects of CM on DNAm remain stable beyond childhood and impact the risk of developing mental disorders later in life.

2. Material & Methods

2.1. Study populations

2.1.1. Berlin Longitudinal Child Study Cohort

Within the scope of the Berlin Longitudinal Child Study (Berlin LCS), 173 children and their caregivers were recruited, of which 86 presented with documented CM exposure, i.e., emotional and physical abuse and/or neglect within 6 months, and 87 non-maltreated matched controls (Entringer et al., 2020; Martins et al., 2021; Winter et al., in review). The maltreatment and control groups were frequency-matched for age, sex, and SES. Children were aged between 3 and 5 years at study entry and underwent detailed assessments of psychiatric and behavioral symptoms, development, and physical health at study entry and at 4 follow-up appointments at 6 month-intervals within the time frame of 2 years. Additionally, children provided saliva samples for biological measurements, genotyping and measurement of DNAm (Entringer et al., 2020; Martins et al., 2021; Winter et al., in review). Demographics and the study design are summarized in Figure 3 and Table 1. Findings in the Berlin LCS cohort have already partially been published in: Martins, J., Czamara, D., Sauer, S., Rex-Haffner, M., Dittrich, K., Dörr, P., ... & Binder, E. B. (2021). Childhood adversity correlates with stable changes in DNA methylation trajectories in children and converges with epigenetic signatures of prenatal stress. *Neurobiology of stress*, *15*, 100336.

Exclusion criteria from the study included parents under the age of 18 years, neurodevelopmental disorders, serious medical disease as well as serious medical disease of the parents. Inclusion into the maltreatment group was based on the Maltreatment Classification System (see chapter 2.2 measures of CA below) (English et al., 2002), according to which children needed to have experienced maltreatment in the form of physical or emotional abuse or neglect within the past 6 months. For an inclusion into the control group, children were screened to not have been exposed to any severe critical or traumatic life event. Due to the timing of the initial study entry and to dropout not all families reached T4. The rate of non-completion was higher among maltreated children (62.45%) compared to non-maltreated children (40.22%). The effect of dropouts on the findings was assessed using a sensitivity analysis (as described in section 2.8.3 on validation).

Approval for the study was obtained from the ethics committee of Charité – Universitätsmedizin Berlin. All procedures were conducted in accordance with the Ethical Principles for Medical Research as established by the Medical Association

Declaration of Helsinki. Written informed consent was obtained from all participants after the procedures were fully explained. Children gave consent by painting or signing a form that was appropriate for the children's age range. Caregivers received monetary compensation for participation and children received a small gift. All caregivers received diagnostic results and referral for psychosocial or medical follow-up.

While no maltreatment-specific intervention took place, maltreated children did receive “care as usual”. The children for whom treatment was assessed as necessary were referred to the Social Pediatric Center of the Charité or other appropriate facilities. At each visit, families received feedback about the child's health and developmental status and recommendations for follow-up, where necessary (e.g., psychological consultation, dentist visit, etc.). Between 44.7% and 62.2% of the families adhered to the recommendations across the time points.

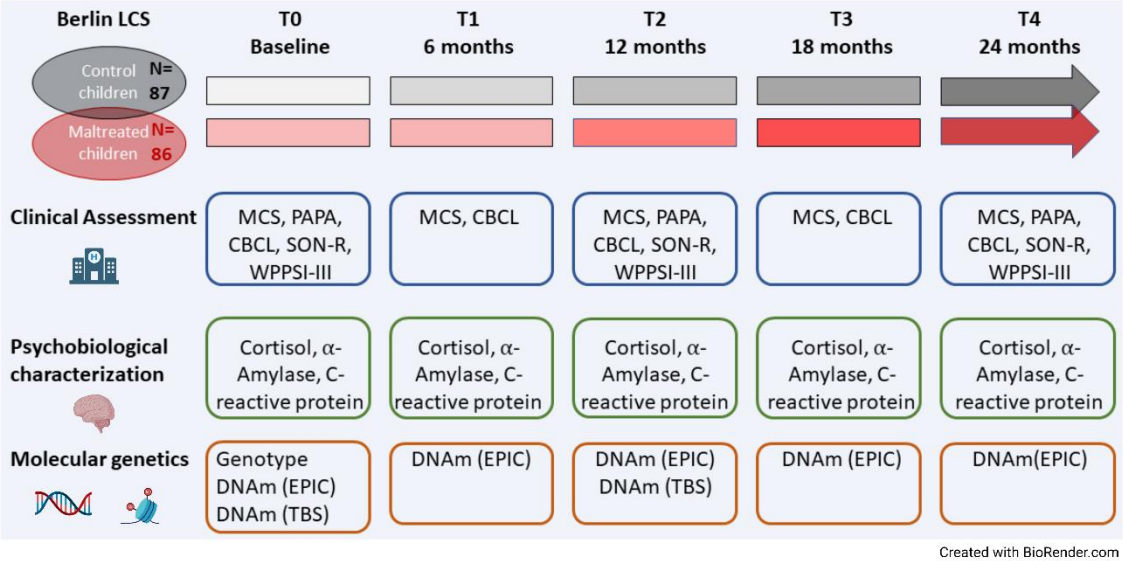


Figure 3: Study Design of the Berlin LCS cohort. Clinical assessments, Biodata samples and array-based DNA methylation (DNAm) measurements were performed every 6 months. Additionally, the fine-mapping approach for a targeted DNAm measurement of the *FKBP5* locus was applied to the baseline and T2 samples. Clinical assessments include the documentation of maltreatment events with the maltreatment classification system (MCS), psychometric measurements such as the preschool age psychiatric assessment (PAPA) and the Child Behavioural Check List (CBC) as well as developmental measures such as the Snijder-Oomens Intelligence test (SON-IQ) and the Wechlser Preschool and Primary Scale of Intelligence (WPPSI). Biodata samples included Cortisol (Cort), Alpha-amylase (AA) and C-reactive protein (CRP). Figure modified from Martins et al., (2021).

Table 1: Demographics of the Berlin LCS cohort. Demographic data, developmental and behavioral diagnostics, stratified by time point and case-control status (children exposed to maltreatment and non-maltreated children). Mean and standard deviation is reported for the quantitative measures.

	T0	**T1**	**T2**	**T3**	**T4**
Total sample size	173	146	130	111	90
Sex, CM[1] Sex, no CM	m=45, f=41 m=46, f=41	m=36, f=30 m=42, f=38	m=30, f=30 m=36, f=34	m=25, f=22 m=34, f=30	m=21, f=17 m=27, f=25
Age, maltreated[1] Age, no CM	4.36±0.83 4.11±0.73	4.84±0.85 4.60±0.73	5.33± 0.81 5.03±0.74	5.85±0.83 5.54±0.72	6.39±0.82 4.04±0.71
Number CM[1] Number no CM	86 (49.7%) 87 (50.3%)	66 (45.2%) 80 (54.8%)	60 (46.2%) 70 (43.8%)	47 (42.3 %) 64 (57.7 %)	38 (42.2 %) 52 (57.8 %)
Adversity score CM[1] Adversity score no CM	4.05±1.84 1.04±1.05	4.17±1.71 0.96±0.97	4.56±1.72 1.39±1.09	4.70±1.69 1.34±0.96	5.00±1.67 1.57±1.02
SES[2] CM[1] SES no CM	9.38±4.46 16.10±3.60	9.88±4.70 16.01±3.50	9.75±4.85 16.41±3.55	9.76±4.63 16.40±3.72	10.18±4.45 15.94±3.83
SON_IQ[3] CM[1] SON_IQ no CM	90.09±17.33 106.32±12.22	-	93.45±17.40 109.14±13.00	-	95.5±16.74 113.73±13.3
WPPSI[4] CM[1] WPPSI no CM	90.42±13.33 105.09±11.96	-	92.46±14.24 106.19±9.36	-	93.37±15.29 107.69±8.24
CBCL[5] CM[1] CBCL no CM	54.82±11.39 41.37±8.97	53.31±10.82 41.51±9.59	50.53±11.24 39.1±7.55	51.66±11.18 41.30±7.76	50.70±12.48 40.37±8.17
PAPA[6] CM[1] PAPA[2] no CM	49 (28.3%) 18 (10.4%)	-	36 (20.5%) 12 (8.2%)	-	17 (18.9%) 10 (11.1%)

[1] CM includes any subject which experienced at least one maltreatment event of sufficient severity and refers to the maltreated group

[2] Socioeconomic status (SES)

[3] SON_IQ is a non-verbal intelligence test, which was used as developmental measure

[4] WPPSI Wechsler preschool and primary scale of intelligence, developmental measure with spoken component

[5] Child behavioral check list, a caregiver report form for problematic behavior

[6] Preschool age psychiatric assessment (PAPA), at least one symptom in the PAPA questionnaire

2.1.2. LMU Cohort

The LMU cohort (Halldorsdottir et al., 2019) comprised 385 youths aged 8 to 18 years with MDD (mean age=15.13 years [SD=2.07], 68% female) and 255 healthy control subjects (mean age=15.02 years [SD=2.24], 64% female). Cases in this cohort were recruited from two child and adolescent clinics in Munich, with inclusion criteria for the cases being a MDD diagnosis based on ICD-10 using a standardized semi-structured clinical interview at study entry and intellectual capacity to complete clinical measures. Diagnostic criteria according to the ICD-10 are met if the patient experienced persistent depressed mood, reduction of energy, and decreased activity. Additional symptoms

may occur include decreased enjoyment, interest, concentration and self-esteem and increased feelings of guilt and worthlessness. The depressed mood may also be accompanied by somatic symptoms such as disturbed sleep, psychomotor retardation, agitation, loss of appetite, weight loss and loss of libido. Patients are diagnosed with mild, moderate or severe depressive episodes based on the number and severity of the symptoms. The control group was recruited via the clinic's website, flyers and local advertisement. Inclusion criteria for the control group comprised no past or current mental disorder based on the Kinder-DIPS (Schneider et al., 2017) and intellectual capacity to complete clinical measures. Participants received a 20 Euro voucher as compensation for participating in the study. Exposure to environmental stressors including childhood abuse was evaluated using four items on a self-report questionnaire which were adapted from the Life Event Survey (Adams & Adams, 1991) and the Munich Event List (Wittchen et al., 1989) (see chapter on measures of CA below). Study design and demographic data are summarized in Figure 4 and Table 2.

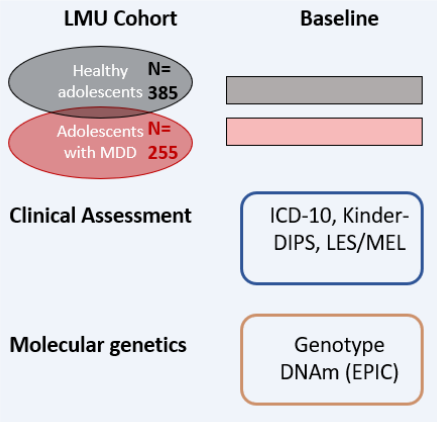


Figure 4: Study Design of the LMU cohort. Clinical assessments for MDD and exclusion of comorbidities were assessed using the Kinder-DIPS, exposures were measured using questions adapted from the LES (Life Event Survey) and the MEL (Munich Event List). Genotyping and array-based DNAm measurements are available for this cohort as well.

Table 2: Demographics of the LMU cohort. Demographic data, psychobiological diagnostics and exposure measures, and the standard deviation are reported for the quantitative measures.

	baseline
Total sample size	640
Sex, MDD cases[1] Sex, controls	m=124, f=261 m=91, f=164
Age, MDD cases[1] Age, controls	15.13±2.07 15.02±2.24
Exposed to CA[2] Not exposed to CA	392 (61.25%) 248 (38.75%)
Age exposed to CA Age not exposed to CA	14.69±2.19 15.20±2.13

[1] Participants with MDD episode at study begin
[2] Participants with history of childhood abuse

2.2. Measures of childhood adversity

2.2.1. Childhood maltreatment

The occurrence and features of CM in the Berlin LCS cohort (i.e., subtype, duration, severity of the event) were assessed at baseline and all follow-up timepoints (T0-T4). At the follow-up visits, information on ongoing maltreatment for each 6-month interval were collected. The properties of CM were assessed using the Maternal Interview for the Classification of Maltreatment (MCS). The MCS provides specific criteria for classifying and quantifying the occurrence and features of subtypes of maltreatment and responses were coded accordingly by trained clinicians. A range of maltreatment subtypes are covered by the interview including emotional maltreatment (i.e., emotional abuse and/or emotional neglect), physical neglect (i.e., failure to provide and/or lack of supervision), physical abuse, and moral, legal and/or educational maltreatment (English et al., 2002). For each maltreatment incidence, severity is rated on a 5-point scale ranging from mild (1) to severe or life-threatening maltreatment (5). Additionally, age of onset was specified for each incidence. Severity cutoff scores were used to include children in the maltreatment group (emotional maltreatment ≥ 2, physical abuse ≥ 1, physical neglect ≥ 2).

2.2.2. Childhood abuse

Environmental stressors, including childhood abuse, in the LMU cohort were assessed with a comprehensive questionnaire adapted from the Life Event Survey (Adams & Adams, 1991) and the Munich Event List (Wittchen et al., 1989). Each item was rated

dichotomously (“yes” or “no”). Participants with the positive endorsement of any item were rated as having a history of childhood abuse.

2.2.3. Composite adversity score

In this work, a broader measure was used to separately analyze the burden of stress in general in order to identify alterations that were specific to CM. This composite adversity score (ranged: 0–10) is based on Adverse Childhood Experience (ACE) categories and sums up: low SES (0 or 1, Winkler & Stolzenberg Index (Winkler & Stolzenberg, 1999)), exposure to contextual stressors, and critical life events (0 or 1, list included in the Preschool Age Psychiatric Assessment), and exposure to different maltreatment categories (range 0–7). Children were included in a high or low adversity group by using a median split of the adversity score (high: score ≥ 3, low: score ≤ 2).

2.3. Outcome measures

2.3.1. Mental health outcomes

Psychopathology was assessed at baseline, T2, and T4. To assess psychiatric disorders by administering the electronic version of the Preschool Age Psychiatric Assessment (PAPA). The PAPA implements a developmentally sensitive and fully structured assessment based on caregiver reports (Egger & Angold, 2004). In this interview, the presence, frequency, duration, and onset of symptoms for a 3-month period and diagnoses are generated according to DSM-IV, including depressive disorders (i.e. MDD, dysthymia), anxiety disorders (i.e., social phobia, specific phobia, separation anxiety disorder, generalized anxiety disorder, posttraumatic stress disorder), attention deficit hyperactivity disorder, conduct disorder, oppositional defiant disorder, and mutism. All interviews were conducted by specifically trained clinicians. In this work, the subscales for internalizing symptoms (PAPA_int) and externalizing symptoms (PAPA_ext) were used in addition. Maternal MDD and anxiety were assessed at baseline using the Beck-Depression-Inventory (BDI) and Beck-Anxiety-Inventory (BAI) (A.T. Beck et al., 1996; Aaron T. Beck et al., 1988).

2.3.2. Developmental measures

Children completed standardized neuropsychological testing for cognitive, verbal, and motor developmental domains at T0, T2, and T4. Nonverbal cognitive development was evaluated with the Snijders Oomen Nonverbal Test (SON) for the appropriate age

range of 2½ to 7 years. This well-validated test provides standardized intelligence quotient (IQ) scores (Tellegen & Laros, 1993). Verbal development was assessed with the German Wechsler Preschool and Primary Scale of Intelligence - Fourth Edition (Wechsler, 2002). These verbal subtests yield two verbal IQ scores, reflecting vocabulary and verbal comprehension. The mean of both scores was used in downstream analyses. Percentiles were converted into the metric of standardized IQ scores for our analyses.

2.3.3. Behavioral outcomes

Behavioral and emotional problems were assessed at each time point (T0-T4) using an age-appropriate caregiver-report version of the Child Behavior Checklist (CBCL) depending on the child's age (1.5-5 years) (Achenbach, 1999).

2.4. Saliva samples & Biodata assays

Saliva samples were collected at each time point (T0-T4) at three times during the clinical visits at 9, 10, and 11 a.m. using oral swabs specially designed for small children and were immediately stored at −80 °C. Salivary α-amylase (AA) and cortisol (Cort) were measured at three-time points on the day of assessment (9, 10, and 11 a.m.). Salivary C-reactive protein levels (CRP) was measured at the 11 a.m. time point. Saliva for extraction was collected using ORAgene DNA kits (OG500) at 9 a.m. during the clinical visit. DNA was extracted together from the samples collected at all time points (T0-T4). An automated and standardized procedure based on magnetic beads for 2 × 400 µl saliva samples with the PerkinElmer Chemagic360 system was used for DNA extraction.

CRP concentration was measured using a commercial kit (Salimetrics) with a sensitivity of 10 pg/ml. Intra-assay and inter-assay coefficients of variability were 6% and 13%, respectively. Cort concentration was measured using a commercial ELISA kit (Salimetrics) with a sensitivity of 0.007 µg/dL. Intra-assay and inter-assay coefficients of variability were 7% and 11%, respectively. AA was analyzed using a commercially available assay kit (Salimetrics) according to the manufacturer's instructions. Intra-assay and inter-assay coefficients of variability were 4% and 10%, respectively.

As a measure for AA and Cort, the area under the curve (AUC) was used for each of the markers with respect to ground (AUC_g) which was computed on flow-rate corrected levels (FR) as measured for AA and Cort. For downstream analyses, a log-

transformation of the CRP values was utilized. A number of different readouts could be extracted from the measures at three-time points reflecting different aspects of the cortisol dynamics over time: Cortisol levels at baseline (9 a.m.), peak levels (10 a.m.), increase (peak-baseline), AUC with respect to ground (AUC_g), AUC with respect to increase (AUC_i). The readout best reflecting HPA´-axis activity in this sample was the AUC_g measure, as it was significantly correlated with all measures and best correlated with baseline ($r = 0.59$, $p = 6.66*10^{-16}$) and peak levels ($r = 0.76$, $p < 2.2*10^{-26}$). In addition, some of the assessments of the children could not always be performed in the same order but the AUCg measure is robust against this kind of difference.

2.5. Genotype data

2.5.1. Genotyping

Samples were genotyped using the Illumina GSA-24 v2.0 BeadChip. After filtering by SNP call rate (exclusion at < 95%), sample call rate (exclusion at < 98%) as well as for Hardy-Weinberg Equilibrium (HWE; p-value for HWE $< 10^{-5}$) and minor (MAF, MAF < 0.01), 469,592 SNPs and 167 IDs (83 cases, 84 controls) remained.

As no genotyping method is 100% accurate and given that genotype mistakes can lead to increased random error and bias in gene-disease associations, various checks were employed to detect genotyping errors, including testing for deviations from the HWE. According to the HWE in absence of other evolutionary influences allele and genotype frequencies in a population will remain constant from generation to generation. Certain departures from the HWE suggests genotyping errors (Chen et al., 2017; Wigginton et al., 2005). One specific SNP within the *FKBP5* locus (rs1360780, located at chr6:35639794) was extracted to compute interaction effects with CM using the R-package SNPstats (Clayton & Clayton, 2012).

2.5.2. Imputation

Pre-phasing was performed with shapeit v2 (Delaneau et al., 2008) and imputed with impute 2 (Howie et al., 2011) with the 1,000 genomes phase 3 reference panel. SNPs were filtered by imputation quality (info-score < 0.6), minor allele frequency (MAF; > 0.01), and HWE ($< 1*10^{-5}$) with qctool v2 (Wigginton et al., 2005). For SNPs with low MAF the power of detection is extremely low even for large effects. Therefore, only SNPs passing a MAF threshold were considered for downstream analysis to yield a reasonable statistical power. SNPs that are associated with the trait of interest but also present with highly significant deviations from the HWE were excluded from downstream analysis. Frequently HWE deviations toward an excess of heterozygotes reflect a technical problem in the assay, for example, non-specific amplification of the

target region. After quality control, 9,522,926 SNPs and 173 IDs remained for downstream analysis. SNPs passing the QC were pruned for linkage(window size = 100, step size = 5, $r^2 = 0.2$) with plink v1.9 (Chang et al., 2015) and used to compute principal components. The first three principal components (PCs) explaining 35% of the genotypic variance. In all models, the genotype PCs were used as covariates to correct for population structure and relatedness.

2.5.3. Polygenic Risk scores

Polygenic risk scores for educational attainment (EA) (J. Lee et al., 2018), MDD (Howard et al., 2019), and SCZ (Ripke et al., 2014) were computed for different P-value thresholds (0.001, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 1) using the PRSice 2 software (Choi & O'Reilly, 2019). For each PRS the P-value threshold for which the correlation with the phenotype (PAPA for MDD and SCZ, and SON_IQ or WPPSI for EA) was highest was selected for downstream analysis. Additive and interaction effects of the PRS and the environment (either CM or the adversity score) on the outcome were tested using linear models and comparing the improvement of the model compared to a null model using ANOVA.

2.6. DNA methylation

2.6.1. Array-based measurements

2.6.1.1. Berlin LCS sample

The Methylation EPIC BeadChip (Illumina Inc, San Diego, CA, USA) was used to measure DNAm in the Berlin LCS cohort. Samples from all timepoints were extracted, plated, and run together. Randomization of the samples was performed with regards to maltreatment, age, and sex to avoid confounding batch effects and CM. Hybridization and array processing were performed according to manufacturers' instructions. Data were normalized using functional normalization (with npc=4) implemented by the minfi package (Aryee et al., 2014). Artifacts in the beta-value distribution were identified by visual inspection and thirty-four samples were excluded. Additionally, three samples were removed due to large amounts of missing values (>5% of CpGs missing). Batch effects were identified and removed with the Empirical Bayes' method *ComBat* implemented in the R package *sva* (Leek et al., 2012). Two iterations of batch corrections were performed for Chip barcode and position (row/column), which were the most significant batches. Known cross-reactive and polymorphic probes were excluded from downstream analyses (Y.-A. Chen et al., 2013). Further, probes with detection p-value > 0.01 in more than 25% of the samples were also removed. CpG-

sites located on the X or Y were excluded (McCartney et al., 2016). After quality control (QC), a total 830,206 CpGs and 634 samples (by timepoint (case; control) T0: 167 (84,83), T1: 128 (63,65), T2: 125 (57,68), T3: 104 (44,60), T4: 110 (46,64)) were used for downstream analysis. Cell composition of the samples was estimated using the deconvolution method described by Smith et al. and was corrected for in all statistical models (A. K. Smith et al., 2015).

2.6.1.2. LMU sample

From the blood samples collected, DNA was extracted according to standard procedures. Methylation analyses were performed at the Max Planck Institute of Psychiatry in Munich, Germany. DNAm from blood samples from the LMU cohort was also measured with the Infinium Methylation EPIC BeadChip (Illumina Inc, San Diego, CA, USA). Samples were randomized with respect to case-control status, childhood abuse, age, and sex. Hybridization and array processing were performed according to manufacturers' instructions. Data were normalized using quantile normalization as a first step followed by a beta-mixture quantile (BMIQ) normalization step implemented by the wateRmelon package. Artifacts in the beta-value distribution were identified by visual inspection and four samples were excluded. Batch effects were identified and removed with the Empirical Bayes' method *ComBat* implemented in the R package *sva* (Leek et al., 2012). Filtering of the probes (polymorphic, cross-reactive probes, detection-pvalue, location on X or Y chromosome) was performed for the Berlin LCS sample. After QC, 636 samples and 728,868 probes remained for downstream analysis. The cell composition of the samples was estimated using the Houseman method.

2.6.2. Targeted bisulfite sequencing of the Berlin LCS sample

2.6.2.1. Amplicon selection

Targeted bisulfite sequencing of the *FKBP5* locus in the Berlin LCS samples was performed according to the protocol described by *Roeh* et al., who optimized amplifications of 28 regions within the *FKBP5* locus covering 302 CpGs within GR and CTCF binding sites as well as the transcription start site of the gene (Roeh et al., 2018). Regulatory regions within the *FKBP5* locus include upstream, downstream, and intronic enhancer regions with GREs and CTCF sites. CTCF sites create boundaries between so called topological associating domains (TADs) in chromosomes. TADs partition the genome into blocks (at a scale of 100 to 1,000 kb), in which loci show a higher contact frequency compared to the rest of the genome. This means that sequences within TADs are known to physically interact with higher probability than with sequences outside of

the TAD. Stimulating with a synthetic GR agonist, dexamethasone, Wiechmann et al. (2019) examined the effects of GR activation on DNAm in the different categories of regulatory regions (Wiechmann et al., 2019).

Based on this study, 11 of the amplicons covering regulatory regions of interest (*FKBP5* enhancers, intron 5, intron 7 as well as CTCF binding sites) plus one control amplicon H19 were selected. Two PCR targets were not successful and were excluded from further analysis (amplification failed for *FKBP5* PCR 13.1 and the pooling *FKBP5* PCR 18 failed). Amplicons were selected to cover previously described GREs from (Klengel et al., 2013)] and the *GR* ChIP-Seq from the ENCODE project (Bernstein et al., 2012). Additionally, Amplicons covering *CTCF* binding sites were selected using HI-C peaks (Rao et al., 2015), *CTCF*-ChIA-Pet interactions from a lymphoblastoid cell line (GM12878, (Tang et al., 2015)) and *CTCF* ChIP-Seq information from the ENCODE project (Bernstein et al., 2012). Amplicons selected and the number of CpGs covered are summarized in the Table 3.

Table 3: PCR targets selected for amplification. PCR targets are named according to the HAM-TBS method developed by Wiechmann and colleagues (2019). Targets were selected if they were located in functionally relevant regions of the FKB5P locus.

PCR name	PCR location (hg19)			# CpGs covered	Functional region
	chr	start	stop		
FKBP5 PCR 1.2	chr6	35558361	35558652	5	Intron 7 Enhancer
FKBP5 PCR 1.4	chr6	35558459	35558774	5	Intron 7 Enhancer
FKBP5 PCR 2	chr6	35569680	35569946	5	Intron 5 Enhancer
FKBP5 PCR 3	chr6	35578686	35578916	3	Intron 5 Enhancer
FKBP5 PCR 12	chr6	35683267	35683538	4	Proximal Enhancer
FKBP5 PCR 13.1	chr6	35693391	35693722	7	Proximal Enhancer
FKBP5 PCR 17	chr6	35696695	35697046	5	Proximal Enhancer
FKBP5 PCR 18	chr6	35697684	35697842	2	Proximal Enhancer
FKBP5cg14284211	chr6	35570168	35570410	1	intronic Enhancer
CTCF PCR 5.1	chr6	35703966	35704310	3	5'TAD
CTCF PCR 17	chr6	35490554	35490990	17	3'TAD

2.6.2.2. Library preparation and sequencing

Bisulfite treatments were performed on each sample which were then pooled to run one PCR amplification per amplicon. In three experiments 3 x 50 ng (total of 150 ng) of DNA was used per sample using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA). Between 1-5 µl of bisulfite-converted DNA were used for each PCR amplification employing Takara EpiTaq HS Polymerase (Clontech, Saint-Germain-en-Laye, France) with 49 amplification cycles. Quantification of the PCR amplicons was measured with the Agilent 2200 TapeStation (Agilent Technologies, Waldbronn, Germany). Amplicons were then pooled in equimolar quantities for each sample. In order to remove primer dimers and high molecular DNA fragments, AMPure XP beads (Beckman Coulter, Krefeld, Germany) were used for a double size selection (200–500 bp).

Libraries were prepared using the TruSeq DNA PCR-Free HT Library Prep Kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Each library was quantified using the Qubit® 1.0 (Thermo Fisher Scientific Inc., Schwerte, Germany), then normalized to 4 nM and pooled. Library concentrations and fragment sizes were checked via Agilent's 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and quantitative PCR using the Kapa HIFI Library quantification kit (Kapa Biosystems, Wilmington, MA). Paired-end (PE) sequencing was performed on an Illumina MiSeq Instrument (Illumina, San Diego, CA) with their MiSeq Reagent Kit v3 (2× 300 cycles) with the addition of 30% of PhiX Library.

2.6.2.3. Preprocessing of the TBS data

The quality control of the raw sequencing data was performed using FastQC (Andrews et al., 2015). Adapter sequences were trimmed using Cupadapt (Martin, 2011) and reads were aligned against a bisulfite-converted reference restricted to the PCR targets. The alignment was run using Bismark (Krueger & Andrews, 2011), a specialized aligner that maps bisulfite-treated reads to a reference genome. Paired-end (PE) reads were subsequently stitched with an in-house Perl script, which also removed low-quality tails of overlapping PE reads. Methylation levels for all CpGs, CHGs, and CHHs were quantified using the R package methylKit (Akalin et al., 2012). QC of the DNAm levels included the detection of PCR artifacts, the removal of samples with insufficient bisulfite conversion rate (< 95%) as well as the exclusion of CpGs with coverage lower than 1,000 reads.

2.6.3. DNA methylation-based risk scores

DNAm based risk scores for prenatal smoking- and alcohol exposure were calculated based on previous epigenetic studies. Prenatal smoke exposure was estimated by using a DNAm score based on 15 CpG-sites identified by Richmond et al., maternal alcohol intake during pregnancy and 658 CpGs from a fetal alcohol syndrome (FAS) study by Portales-Casamar and colleagues (Portales-Casamar et al., 2016; Richmond et al., 2018). For the construction of the epigenetic scores for prenatal alcohol and smoking exposure, the CpGs identified in the previous studies were included. The beta values measured in the Berlin LCS cohort (using the EPIC array) were weighted by effect size reported by the authors and summed up for a score (Martins et al., 2021).

2.7. Statistical analysis

2.7.1. Linear mixed models

Differentially methylated CpGs between exposed children and non-exposed children at baseline were identified using general linear models (glm; function in R) (Hastie & Pregibon, 2017). Significant changes over time for single CpGs were analyzed using linear mixed models implemented in the lme4 R-package (D. Bates et al., 2007). All models included age, sex, and cell type composition (Buccal, CD14, CD34) as covariates as well as the first three PCs (PC1, PC2, PC3) from the genotypes in order to correct for different ethnicities and relatedness. The following models were tested: assessing significant DNAm changes occurring over time (Model 1), assessing DNAm changes over time with additive effect of the environment (maltreatment or adversity in general) (Model 2) and assessing interactive effects of DNAm over time and the environment (Model 3).

Model 1:

Model over time (methylation changes due to the aging of the children)

Beta ~ age + sex + PC1 + PC2 + PC3 + Buccal + CD14 + CD34 + Time + (1|Subject)

Model 2:

Additive model (changes over time where the environment adds to the effect):

Beta ~ age + sex + PC1 + PC2 + PC3 + Buccal + CD14 + CD34 + Time + E + (1|Subject)

Model 3:

Interactive model (the environment modulates the changes over time):

Beta ~ age + sex + PC1 + PC2 + PC3 + Buccal + CD14 + CD34 + Time x E + (1|Subject)

E represents the stress measure of the environment (either CM or adversity score). P-values were computed by comparing the models with a corresponding nested model using ANOVA (Model1, Model2) and ANOVA (Model2, Model3).

2.7.2. Differentially methylated regions

DMRs were identified by aggregating the results from the linear mixed models using comb-p (Pedersen et al., 2012). P-values for each model of all CpGs available (n = 830,206) were combined into regions by choosing a seed CpG (with $p < 1 \times 10^{-4}$) and extending the region by significant neighboring CpG within the range of 500 bp. All results reported were corrected for multiple testing at FDR at 10% over all identified regions using the Benjamini-Hochberg Method (Benjamini & Hochberg, 1995).

2.7.3. Weighted Gene Co-expression Network Analysis

The most variable 10% of all CpGs at baseline (n = 83,021) that passed QC were selected for downstream analyses by filtering by median absolute deviation (MAD) and taking the CpGs presenting with a MAD in the 90th percentile into further analyses (Rousseeuw & Croux, 1993). Weighted correlation network analysis (WGCNA) was conducted on the baseline DNAm levels (T0) to identify co-methylation structures. The best soft thresholding power was determined to be 5 and the tree-cut height was set to 0.25. The first soft thresholding power to reach an r^2 of 0.8 or better with mean connectivity in the hundreds is considered a good scale-free topology fit. As recommended by the authors, a signed network was constructed (Langfelder & Horvath, 2008).

Stability analysis of the results was performed using a bootstrap approach. Network construction was repeated 50 times by resampling 66% of the data in order to identify, which modules remained stable and should be considered for annotation. The module-trait relationship was then investigated for the stable modules. A summary of all analyses is depicted in Figure 5.

2.7.4. Functional Annotation

Enrichment for pathways was computed by mapping the CpGs to genes using the EPIC array annotation and then using the gene list as an input for FUMA, a web tool for functional mapping and annotation (Watanabe et al., 2017). CpGs within all stable modules were tested for enrichment in specific pathways and overlap with GWAS hits. Additionally, significant DMRs were investigated for overlap with pathways. All n=36,420 genes represented by CpGs on the EPIC array were used as background for the enrichment tests.

2.8. Validation

2.8.1. Power analysis

A power analysis tailored to longitudinal models using the R-package longpower (Donohue et al., 2013) was performed. Functions for computing power and sample size for linear models of longitudinal data included in this package are based on the formulas from Diggle et al., (1994) and Liu et al., (1997). These formulas are expressed in terms of marginal model or Generalized Estimating Equations parameters. Mixed effect model parameters (e.g., random intercept and/or slope) of a pilot model (the fitted model of the CpG with median effect size was used) are translated into marginal model parameters so that the formulas can be applied to investigate the power-sample size relationship for two sample longitudinal designs assuming known variance. Power for our given sample size of the smaller group (n = 81) was estimated for each of the models described above with the parameters of interest being: time, time + adversity and time x adversity and an effect size estimate (delta) for a 5% methylation change in the pilot model.

2.8.2. Sensitivity analysis

In order to test the sensitivity of the model to dropouts, the additive model (time + adversity) and the interactive model (time x adversity) were re-run using only the complete samples for T3 (n=102) and T4 (n=83). Additionally, the direction of effect remaining stable using the complete cases at time point 4 was compared to the results using the complete data. An overview of all validation steps is shown in Figure 5.

2.8.3. Validation in the LMU cohort

A linear model to test for the effects of CA on DNAm was run using the LMU samples. Similar to the models over time in the Berlin LCS cohort, this model was corrected for

age, sex, cell composition (CD8T, CD4T, NK, Bcell, Mono, Gran), and the first three PCs of the genotype.

Model (testing for effects of CA):

Beta ~ age + sex + PC1 + PC2 + PC3 + CD4T + CD8T + NK + Bcell + Mono + Gran + Time + (1|Subject).

Analogously to the procedure for the Berlin LCS sample, results from this model were aggregated to DMRs with the comb-p software using the same settings. These DMRs were then attempted to overlap with the DMRs identified with Model 2 (time + E) and Model 3 (time x E) in the Berlin LCS cohort.

Further, DNAm based scores for prenatal smoke and prenatal alcohol exposure were calculated as described above for the individuals of the LMU cohort. This was done in order to check if the effects of prenatal exposures remain stable past childhood and if it affects case-control status in adolescents.

Finally, the module-centric approach was repeated in the LMU cohort by running WGCNA. The top ten percent most variable CpGs were used as an input with the same settings applied on the Berlin LCS sample (softThresholdingPower and Networktype). Modules identified were overlapped with modules from the Berlin LCS cohort and were checked for association with CA, case-control status, and prenatal exposure scores. Modules that correlated with any type of adversity or case-control status were annotated using FUMA as previously described.

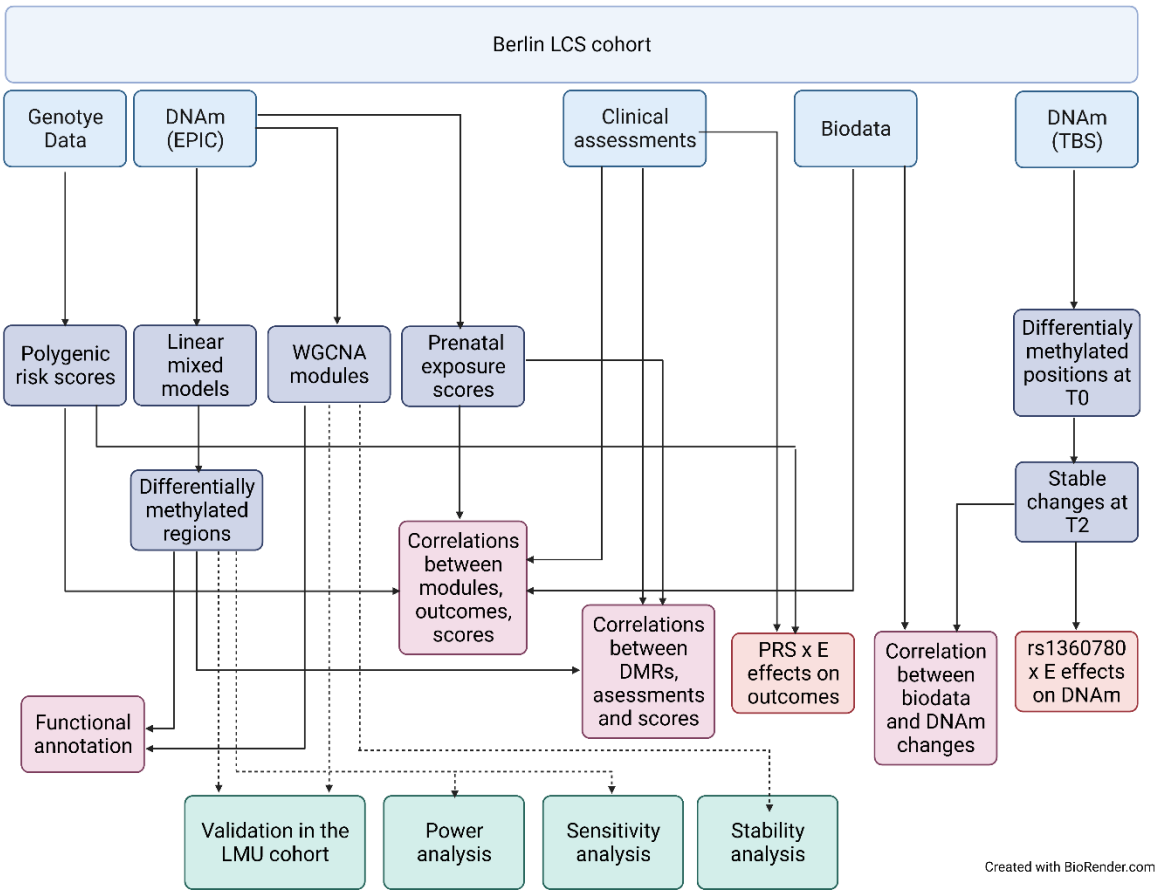


Figure 5: Analysis Flowchart. The top layer shows the available Data (light blue boxes) from the Berlin LCS cohort (genotype, DNAm, clinical assessments, and biodata). Primary analyses are shown in the second layer (dark blue boxes). The additional analyses such as correlations between primary findings or annotations and outcomes (shown in red) and interaction analyses (shown in orange) are located in the third layer. Validation steps are shown in green on the bottom layer. Arrows denote the input required for each step, dashed arrows show which validation steps were taken for which findings.

3. Results

In this dissertation, I investigate DNAm changes following exposure to CM or early-life adversity in general, directly following the maltreatment exposure in a child cohort within a narrow time frame over the course of two years. I examine 1) whether CM specific DNAm changes, that can be isolated from the variety of exposures present in the environment, exist, 2) if these changes remain stable over time and 3) if these changes are associated with clinical or developmental outcomes and salivary biological measures such as AA, CRP, and cort. Further, PRS were computed and their interaction effect with CM and/or CA on developmental outcomes and clinical assessments were examined. Parts of these findings have already recently been published in a separate manuscript (Martins et al., 2021). Findings from the Berlin LCS cohort including 173 children were validated in an independent adolescent cohort (LMU sample, including 640 adolescents) showing that some of the identified DNAm changes remain stable well beyond childhood. Finally, I focused on the effects of exposures to CM and adversity on DNAm within regulatory regions of a candidate gene, namely *FKBP5*, using a fine-mapping approach. The stability of these DNAm changes was investigated as well as their interaction with a genetic risk variant (rs1360780) within *FKBP5*, exploring how the genotype might modify reactivity to exposures. A manuscript describing these findings is currently in preparation.

3.1. Childhood adversity correlates with stable changes in DNA methylation trajectories

Using the data from the baseline measurements, an association analysis comparing DNAm levels between the maltreated (n = 86) and the control group (n = 87) as well as the groups with high (n = 81) and low adversity scores (n = 92) was performed for each single CpG (n = 830,621). In the following, a longitudinal analysis across all time points was conducted by computing linear mixed models in order to test for associations of CM or adversity (adversity score) with epigenetic trajectories: (1) a model over time (Model 1) estimating the general effects over time occurring due to the aging of the children, (2) an additive model (Model 2) estimating the effects where maltreatment or adversity add to the effects over time and finally (3) an interactive model (Model 3) estimating the effects over time that are moderated by CM or adversity.

Moving away from the analysis on a single CpG level, the results of the linear mixed models were then aggregated to DMRs. In addition to the models using all 5-time points, Models 1 – 3 were also run using only the first three-time points, which capture short-term effects on DNAm trajectories (effects over one year). Due to the reduced sample sizes at time points 4 (n = 111) and 5 (n = 90), DMR analysis for Model 2 and Model 3 was only performed using the first three-time points.

Finally, a weighted correlation network analysis was performed to identify clusters of highly correlated CpGs that might be associated with prenatal or postnatal exposures. This network-based approach can be used to screen for relevant CpGs associated with a specific exposure or outcome, which could potentially be used as biomarkers.

3.1.1. Effects of CM and adversity on baseline DNAm

Association studies between DNAm levels and maltreatment or the adversity score were carried out using the baseline data. CpGs were beforehand limited to the top 10% of most variable CpGs over time according to the median absolute deviation. While the EWAS for adversity yielded no significant CpGs after correction for multiple testing, testing for CM resulted in 4 CpGs with significant differential methylation (diffMeth, denoting the mean % methylation difference between the groups) after correction for multiple testing: cg01221528 ($p = 9.35*10^{-8}$, diffMeth = -1.91%) mapping onto exon 9 of the *NT5C1B* gene, cg18047890 ($p = 3.01*10^{-8}$, diffMeth = 3.66%) mapping onto an open sea region on chromosome 12, cg09323083 ($p = 2.37*10^{-7}$, diffMeth = -0.90%) mapping onto intron 7 of the *TBCD* and cg17313621 ($p = 2.59*10^{-7}$, diffMeth = -2.01%) mapping to a different open sea region on chromosome 21.

3.1.2. Effects of early-life adversity on longitudinal epigenetic trajectories

Models (1)-(3) (Model over time, Additive model, and Interactive model) were run for each CpG using either CM or the adversity score as exposure. Since only a few CpGs passed the multiple testing correction and the significant CpGs showed only small methylation changes for the respective model (< 2% diffMeth) between exposed and non-exposed children, an approach aggregating single CpGs into regions was applied. For each model, neighboring CpGs were combined into DMRs showing the same direction of effect.

3.1.2.1. Effects of aging

Aggregating the results from Model 1 across all time points yielded 7 DMRs with significant DNAm changes over the total observation period of 24 months. These DMRs mapped onto six genes (*GSTM3/5, MCCC1, GSDMS, KCNQ1, AURKC, BLCAP*) and one long coding RNA (*LINC22001*) (see supplementary table S1). Model 1 was also applied to the first three-time points only. The analysis of short-term effects identified 9 DMRs mapping onto *C5orf63, RUFY1*, *HLA* response elements, *NPY, RP11-73B2.6, MESTIT1, PIWIL2, CIDEB* , and *AIRE* (shown in supplementary table S2).

3.1.2.2. Additive effects of time and exposure

Testing the additive effects of time and CM or the adversity score with Model 2 yielded 2 significant DMRs with additive effects of time and CM. The two DMRs mapped onto *HLA-B* (dmr1_m2) and the pseudogene *ZFP91* (dmr2_m2). The same two DMRs emerged for additive effects of time and the adversity score. Effects over time were observed for both DMRs in the non-exposed group but blunted in exposed individuals (see Figure 6). In addition, both DMRs showed associations with sex (Figure 8) with the lowest DNAm levels over time in exposed boys and highest in non-exposed girls.

3.1.2.3. Interaction effects of time and exposure

While Model 3, which investigated the changes over time moderated by CM (time x CM), showed no significant DMRs, the model for time x adversity score yielded 4 significant DMRs. These DMRs mapped onto the genes *GAREML* (dmr1_m3), *P3H2* (dmr2_m3), *ZNF562* (dmr3_m3) and *GSTT1* (dmr4_m3). Many of the DMRs described above mapped to genes previously reported to be associated with prenatal exposures including maternal smoking and FAS as shown in supplementary table 2. At baseline, DNAm levels within all these regions were negatively associated with adversity score. Two of the DMRs (dmr1_m3, dmr2_m3) showed reduced DNAm changes over time in the exposure group (3.0% and 1.2% difference in exposed vs 2.6% and 0.4% difference in non-exposed). Dmr3_m3 showed small changes over time but different directions for the high adversity group (hypermethylation of 0.2%) and low adversity group (demethylation of 0.1%). This DMR was significantly correlated with SES (Pearson correlation r = 0.17, p = 0.03). Dmr4_m3 was demethylated over time with larger changes over time in the high adversity group (0.09%) compared to the low adversity group (0.05%). At baseline dmr4_m3 was correlated with externalizing symptoms (Pearson correlation r = 0.14, nominal p = 0.03), cortisol (Pearson correlation r = 0.13, nominal p = 0.04), and CRP levels (Pearson correlation r = 0.14,

nominal p = 0.03). Changes over time by adversity are shown in the right panel of Figure 6 for all four DMRs.

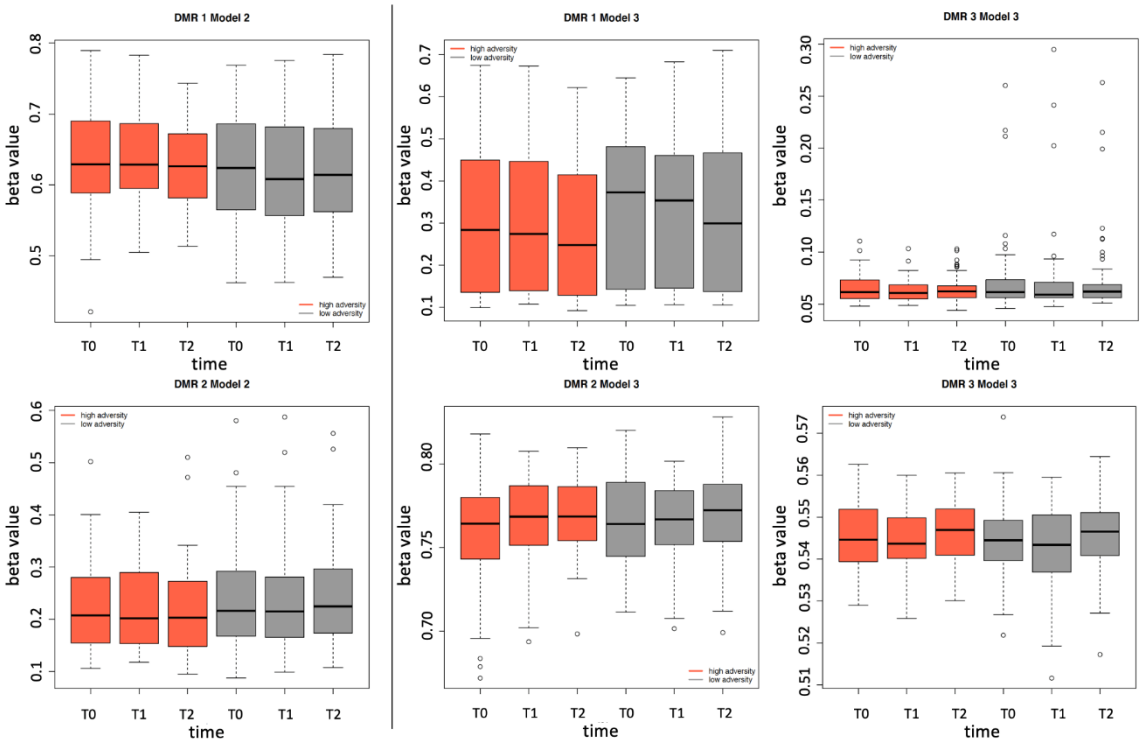


Figure 6: Differentially methylated regions (DMRs) over time. Mean DNAm changes over time are shown for all of the DMRs, split by adversity score (groups were obtained using a median split, the high scoring group is shown in red and the low scoring group in grey). DMRs which were yielded from the time + adversity model (Model 2) are shown in the left panel, and the DMRs from the time x adversity model (Model 3) are shown in the right panel.

Investigating the direction of effect of all DMRs from Model 2 (additive model) and Model 3 (interactive model) revealed that all DMRs except one (dmr1_m1) are hypomethylated in the exposed group compared to the non-exposed group. Differential methylation for each CpG for all DMRs is shown in Supplementary Figure S1.

3.1.3. Epigenetic signatures of prenatal exposure

Based on previous findings that exposure to post-natal adversities are preceded by prenatal exposures within the context of suboptimal caregiving, I next investigated the presence of established epigenetic markers of prenatal adversity such as alcohol and tobacco exposure. Based on differentially methylated CpGs following prenatal alcohol and prenatal smoke exposure, which were identified in previous studies, I calculated poly-epigenetic scores for the Berlin LCS sample. DNAm levels of these CpGs in our cohort were weighted by the effect sizes and then summed up to yield scores for both exposures (see chapter on DNAm-based risk scores above). While both epigenetic

scores were highly correlated (Pearson correlation r = 0.89, nominal p < $2.2*10^{-16}$), none of the CpGs used to compute the scores (prenatal alcohol exposure score: n = 658 CpGs, prenatal smoke exposure score: n = 15 CpGs) overlapped, and only one pair of CpGs were located within the same locus (*MYO1G*). This suggests that the exposures themselves are correlated.

On average, children with documented prenatal smoke exposure (n = 23) or with missing information (n = 36) had a higher epigenetic score for smoking exposure as compared to those without reported exposure (*t*-test, n = 108, mean (smoking/missing) = 0.143, mean (non-smoking) = 0.139, nominal p = 0.14; documented smoking yes vs no: nominal p = 0.07, Figure 7 left panel). This finding validates the prenatal smoke exposure score in the Berlin LCS cohort. Maltreated children had significantly higher baseline scores for prenatal smoke exposure (nominal p = 0.03) compared to non-maltreated children (Figure 7, middle panel). The prenatal smoke exposure was positively correlated with the adversity score (Pearson correlation r = 0.16, nominal p = $8.8*10^{-5}$). Similarly, maltreated children had a significantly higher epigenetic score for prenatal alcohol exposure at baseline than non-maltreated children (nominal p = 0.04) (Figure 7, right panel). Both epigenetic prenatal exposure scores correlated with the adversity score, however, these correlations were not significant (smoke exposure: r = 0.12, nominal p = 0.11 and alcohol exposure: r = 0.14, nominal p = 0.05).

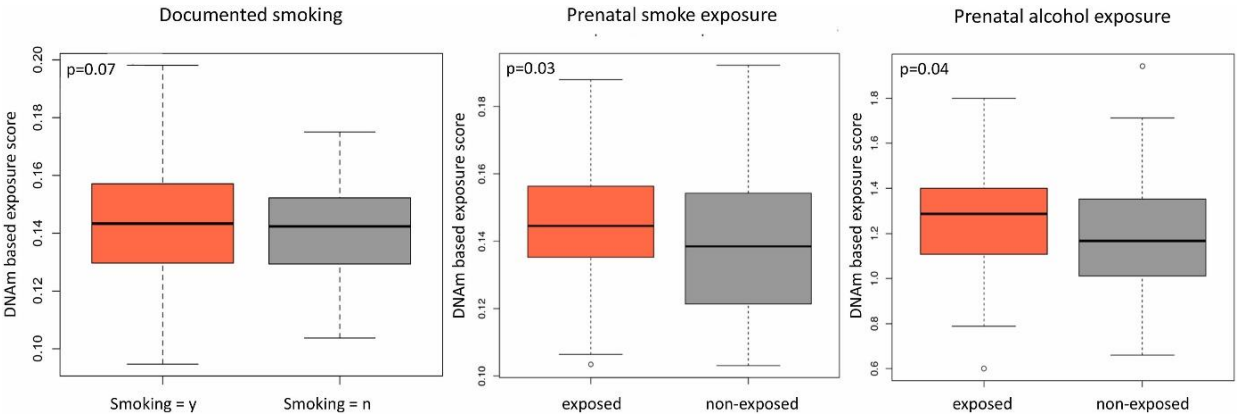


Figure 7: Epigenetic scores reflecting prenatal exposure. DNAm-based scores for prenatal exposure were contrasted between the group exposed to CM (red, n = 84) and the non-maltreated group (grey, n = 83). In general, cases had higher levels of smoke exposure (middle panel, nominal p = 0.03) and alcohol exposure (right panel, nominal p = 0.04) compared to controls. Further, the prenatal smoke exposure score (n = 23 + 36 with missing information) was compared between children with documented exposure (red) and non-exposed children (grey)

(n = 120). The children with documented smoke exposure also presented with higher epigenetic scores (left panel).

3.1.4. Correlation between DMRs, prenatal exposure, and outcome measures

Next, the relationship between the scores, DMRs, and the clinical or developmental measures was investigated. The epigenetic prenatal smoke exposure score positively correlated with baseline cortisol AUC (Pearson correlation $r = 0.14$, nominal $p = 7.6*10^{-4}$), and the psychopathology (Pearson correlation $r = 0.16$, $p = 0.04$). Both DMRs measures at baseline from Model 2 and 3 DMRs of Model 3 that reflect additive or interactive associations of time and adversity score correlated with prenatal smoke exposure (strongest correlation $r = 0.78$, nominal $p < 2.2 \times 10^{-16}$). There was no overlap between the CpGs included in the prenatal smoke exposure epigenetic score and the CpGs within the DMRs identified.

In addition, the prenatal alcohol exposure score positively correlated with the AUC of cortisol at baseline (Pearson correlation $r = 0.09$, nominal $p = 0.03$), CRP levels (Pearson correlation $r = 0.09$, nominal $p = 0.03$), AA levels (Pearson correlation $r = 0.15$, nominal $p = 0.04$) and low maternal SES (Pearson correlation $r = -0.12$, nominal $p = 0.002$). The prenatal alcohol exposure score also correlated with developmental scores such as the WPSSI (Pearson correlation $r = -0.14$, nominal $p = 0.05$). Children with high prenatal alcohol exposure scores scored lower on the WPSSI. Children with high prenatal exposure scores additionally presented with significantly more externalizing symptoms captured by the PAPA subscale (Pearson correlation $r = 0.19$, nominal $p = 0.01$).

All DMRs except one (dmr2_m2) were significantly correlated with both the prenatal alcohol and smoke exposure (strongest Pearson correlation $r = 0.71$, nominal $p < 2.2*10^{-16}$), again pointing towards a convergence of prenatal and postnatal exposures on DNAm. There was no overlap between CpGs included in the prenatal alcohol exposure score and CpGs within the identified DMRs. Correlations of DMRs, exposures (CM, adversity score, prenatal exposure scores, SES), bio data, and clinical/developmental outcomes are summarized in Figure 8.

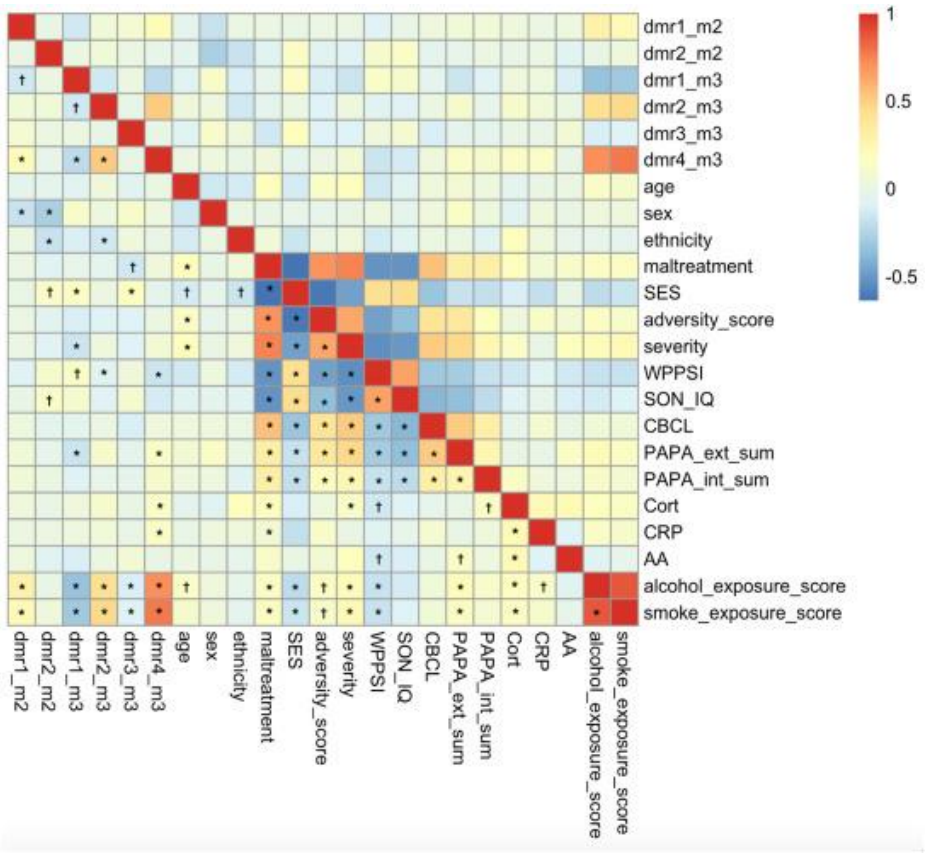


Figure 8: Correlations of the baseline data (T0). Correlations at baseline between significant hits from differentially methylated regions (DMRs; across 3-time points), age, sex, ethnicity, maltreatment, socioeconomic status (SES), composite adversity score and maltreatment severity, developmental outcomes (SON_IQ, WPPSI), psychiatric assessments (CBCL, PAPA internalizing subscale, PAPA externalizing subscale), biodata (alpha-amylase (AA), C-reactive protein (CRP), cortisol (Cort)) as well as prenatal exposure. Significant correlations are marked "*", correlations with 0.05 ≤ nominal p ≤ 0.10 are marked "†".

Finally, to disentangle the effects of prenatal exposure and maltreatment on the DMRs identified, Models 2 (time + adversity score) and 3 (time x adversity score) were re-run including the DNAm based prenatal exposure scores (smoking + alcohol score) as covariates. After correcting for prenatal exposure, 13 of 17 CpGs from Model 2 (additive model) remained significant with some CpGs having even lower p-values as compared to when not correcting for prenatal exposures (lowest nominal p = $1.1*10^{-12}$). Similarly, 25 of 31 CpGs from Model 3 (interaction model) remained significant after correcting for prenatal exposures (lowest nominal p = $1.4*10^{-38}$).

3.1.5. WGCNA reveals maltreatment specific differential methylation

In the next step, the regional analysis was expanded to a network-based approach. Weighted gene co-expression network analysis (WGCNA) was applied to our DNAm

data to obtain a correlation network of the baseline DNAm levels. Due to the computational complexity of this analysis, only 10% most variable CpGs (n = 83,021) were used as input in order to cluster changes in the variable methylome and associate obtained CpG modules with differences in environmental factors including CM, adversity score, SES, and prenatal exposure scores. This analysis identified 9 co-methylated modules (plus one module containing unassigned CpGs), the number of CpGs in the respective modules ranged from 56 to 56,344. All modules are described in Supplementary table S3. A module stability analysis was performed by resampling 50 times and repeating network construction with 66% of the samples. Nine modules remained stable across all 50 runs and were consistent with modules identified in the complete data set. The tenth module (magenta module, which included only 56 CpGs) was unstable and thus excluded from downstream analyses. Stability analysis of the modules is summarized in Supplementary Figure S2. Additionally, I tested if the distribution of the beta-values affected the module detection as there was a significant difference in the methylation levels ($p < 2.2*10^{-16}$) of the CpGs within the modules and in the MAD-score of the corresponding CpGs ($p = 1.95*10^{-15}$) (Supplementary Figure S3). However, re-running WGCNA with standardized Z-scores showed no difference in the modules (lowest p = 0.28) obtained and using M-values as input yielded no good scale-free topology fit (with scale-free fit index $r^2 \geq 0.8$). Networks with scale-free topology retain network characteristics independent of the size of the network (e.g., like the number of nodes), meaning that when the network grows the underlying structure remains unchanged. (Supplementary Figure S4). Therefore, the analysis was performed with beta values.

One module (green module) was selectively correlated with CM (Pearson correlation r = 0.17, nominal p = 0.04) and not significantly correlated neither with adversity nor prenatal exposure. This module consisted of 268 CpGs mapping to 165 unique genes. Functional annotation of genes within the green module showed that they were not enriched for any specific pathway or significantly overlapped with hits from any relevant GWAS for psychiatric disorders. However, a large portion of the genes in this module mapped to non-coding RNAs (47 lincRNA, 3 snoRNA, and 3 microRNA). The genes within the green module also included interesting genes from the C21-steroid pathway which is involved in the biosynthesis of GCs such as cort, *CYP1A1, CYP2A6, CYP2A7*) and genes which have been reported to be important in early development (such as *DNM1, FOXR1, ZNF570*). The mean methylation of the green module was lower in maltreated children across all five-time points as compared to non-maltreated children (see Figure 9). Maltreated children show demethylation

over time for the average methylation levels in the green module, while non-maltreated children show stable methylation levels.

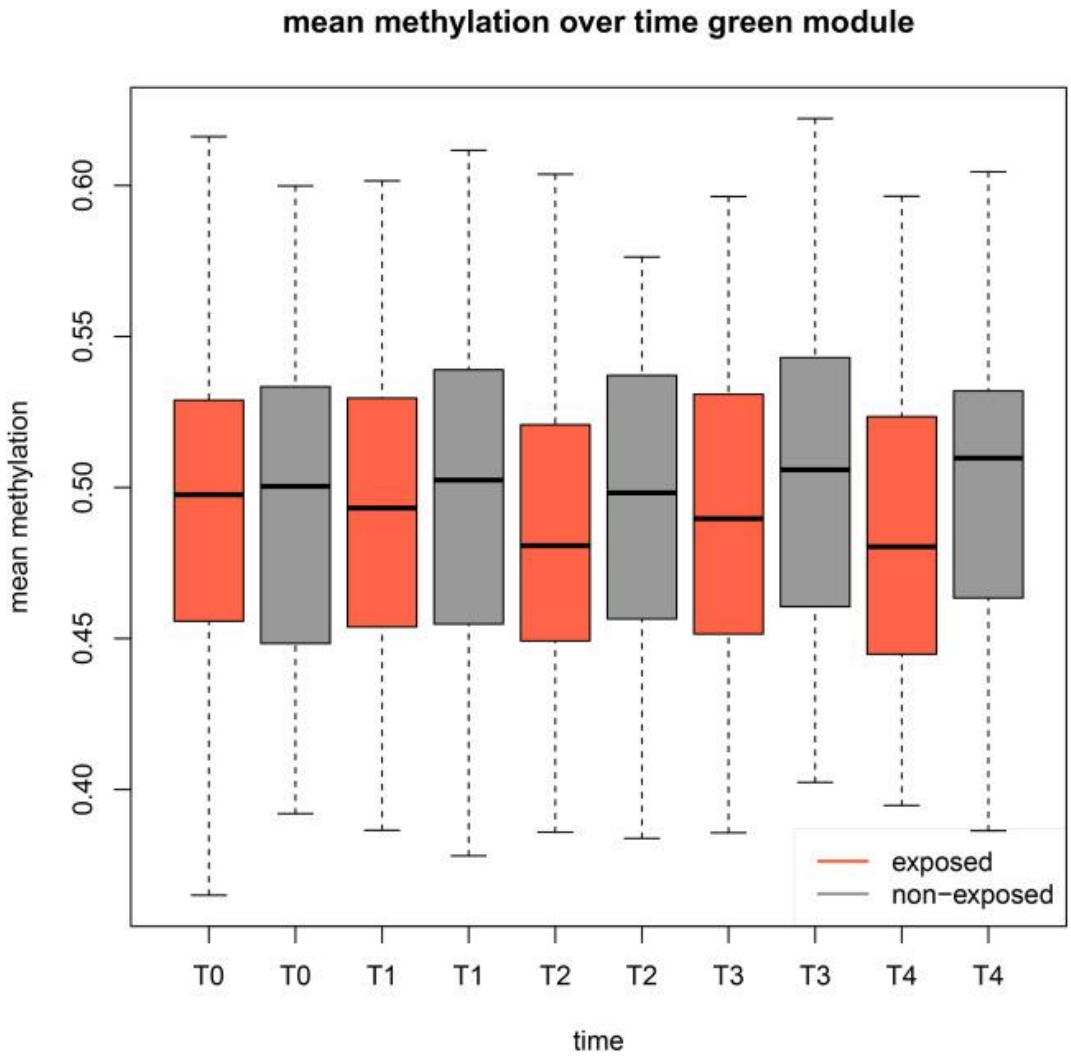


Figure 9: Green module mean over time split by CM. Across all time points mean methylation of CpGs in the green (n = 268) module is lower in children exposed to maltreatment (red) than in non-maltreated children (grey).

Interestingly, the sample module that correlated with CM (green module) also showed a strong correlation with sex (Pearson correlation r = −0.79, nominal $p = 2*10^{-35}$). Mean DNAm for the green module was lowest in exposed boys and highest in non-exposed girls. Two other modules (red and brown) were correlated with developmental (SON_IQ, WPPSI) or behavioral measures (CBCL). None of the modules were significantly associated with the adversity score or SES. The turquoise module (the largest module, consisting of 56,344 CpGs) was nominally significantly associated with the AUC of cort and externalizing symptoms (PAPA subscale).

Finally, the modules that were associated with interesting outcomes or environment variables were functionally annotated and examined for overlaps with top

hits from GWASA summary statistics (summarized in Supplementary tables S3 – S5). The smaller modules (black, pink, red, yellow, and green) with less than 500 CpGs showed no significant enrichments. The other modules were mainly enriched for immune signaling and cytoskeleton organizational pathways.

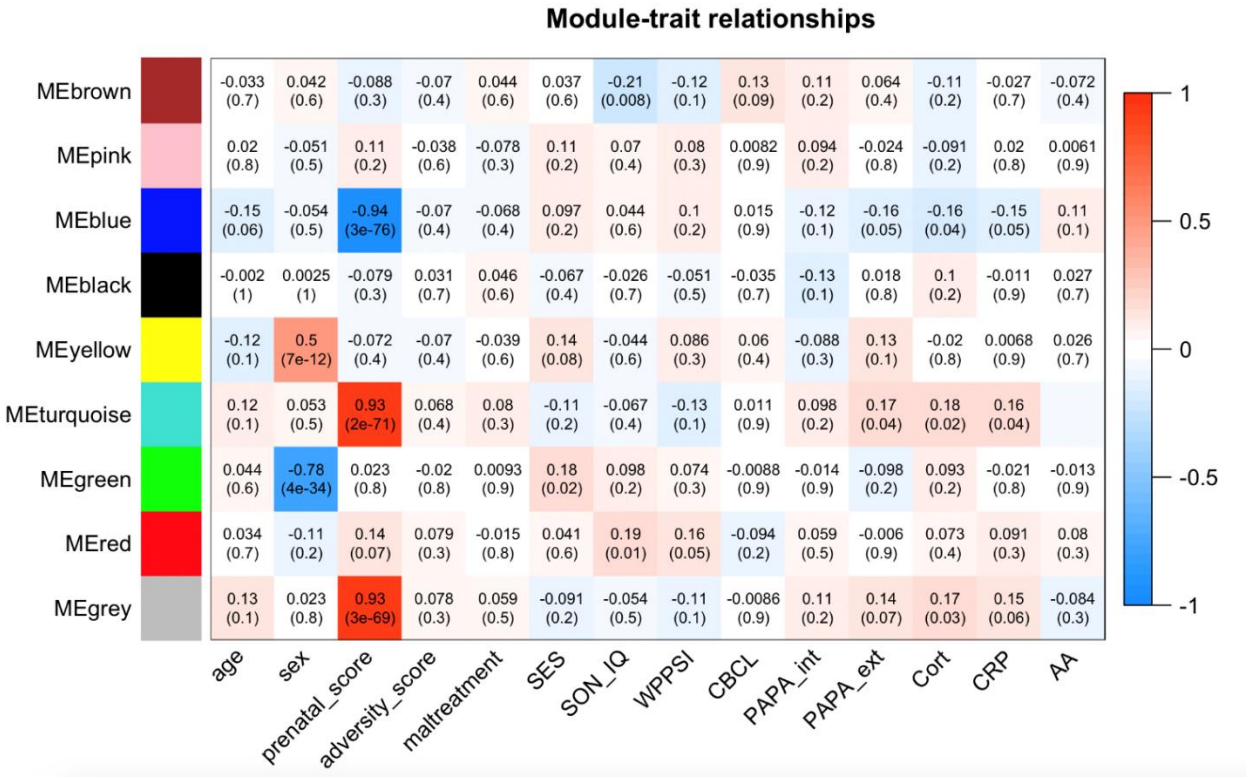


Figure 10. Module Trait Relationship in the Berlin LCS cohort. Associations at baseline between assigned weighted correlation network analysis (WGCNA) modules and general traits, different measures of exposure: adversity score, maltreatment, socioeconomic status (SES), and prenatal exposure proxies: alcohol and smoking. Additionally, correlations between the modules and developmental outcomes (SON_IQ, WPPSI), psychopathology (CBCL, PAPA internalizing subscale, PAPA externalizing subscale), and biodata (alpha-amylase (AA), C-reactive protein (CRP), cortisol (Cort)) are shown. Additionally, correlations of WGCNA modules and polygenic risk scores (PRS) are presented. Shown on the right is the number of CpGs included in each module. The coloring of the tiles reflects the Pearson correlation, the number within the tile is the nominal p-value.

3.1.6. Effects of polygenic risk scores on clinical outcome measures

PRS for educational attainment (EA), MDD and SCZ were computed at different P-value thresholds (0.001, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and1). No significant correlations were found between SCZ risk scores and psychometric outcomes.

PRS at all cut-offs for EA were significantly correlated with SON_IQ (strongest Pearson correlation: r =0.39 p = $1.75*10^{-7}$, PRS with P-value cut-off at 0.001) and WPPSI (strongest Pearson correlation: r = 0.43, p = $6.98*10^{-9}$, PRS with P-value cut-off at 0.001). The PRS with cut-off at 0.001 (EA_0.001) was used for downstream analyses.

The PRS at this cut-off included the top 10.215 SNPs from the GWAS summary statistics. EA_0.001 was significantly correlated with SES ($r = 0.29$, $p = 1.15*10^{-4}$) and CM ($r = -0.19$, $p = 0.01$), and showed a trend for high adversity ($r = -0.13$, $p = 0.08$). This showed that exposed children also presented with lower PRSs for EA.

The MDD risk scores were not significantly correlated with psychiatric symptoms (strongest Pearson correlation: $r = 0.14$, $p = 0.08$, PRS with P-value cut-off at 0.3), there was a trend that individuals with higher risk scores also showed more symptoms in the PAPA. The PRS with p-value cut-off 0.3 best correlated with the PAPA and was used for downstream analyses. The PRS at this cut-off encompassed the top 181.285 SNPs from the GWAS summary statistic. The MDD_0.3 score also correlated with CM ($r = 0.15$, $p = 0.04$), SES ($r = -0.24$, $p = 0.002$), SON_IQ ($r = -0.25$, $p = 0.001$) and maternal depression captured by the BDI ($r = 0.15$, $p = 0.05$).

The EA score with the best association (EA_0.001) was used to compute an additive and interaction models between environmental measures by the PRS and SON_IQ. Significant additive effects were found with maltreatment ($p = 1.417*10^{-15}$), SES ($p = 4.063*10^{-11}$) and the adversity score ($p = 1.892*10^{-10}$). Interaction effects were stronger for the all Es tested: SON_IQ were found for EA_0.1 x maltreatment ($p < 2.2*10^{-16}$), EA_0.1 x SES ($p = 1.773*10^{-11}$) and EA_0.1 x adversity score ($p < 4.975*10^{-10}$) and using the interactive model as a null model revealed a significant improvement (best for maltreatment: $F = 5.499$, $p = 0.02$). Non-exposed children with high educational attainment PRS presented with the highest SON_IQ scores, while exposed children with low PRS had the lowest SON_IQ scores (see Figure 11). The number of symptoms reflected by the PAPA best correlated with maltreatment and adding the PRS for MDD (MDD_0.3) to the model yielded no significant improvement.

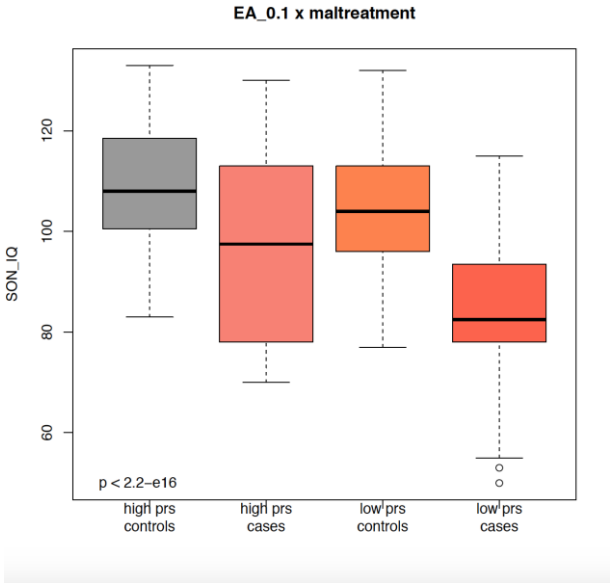


Figure 11: Interaction effect of EA PRS and maltreatment. Non-exposed children with high educational attainment PRS (first box in grey) had the highest SON_IQ scores, while exposed children with low PRS (last box in red) showed the lowest SON_IQ scores.

3.1.7. Correlation between PRS and DNAm changes following adversity

In order to investigate the genetic contribution to the DNAm changes associated with exposures (parental scores, CM, adversity in general), the association of the PRS for EA and MDD and DMRs and WGCNA modules were examined. The PRS for SCZ was not considered for downstream analyses.

First, the correlation between the best PRS for EA and MDD and the DMRs from the models including E (Model 2 and Model 3) was tested using the mean methylation levels across the DMRs. There were no significant correlations between the PRS for EA and the DMRS. The risk score for MDD was correlated with one DMR from the additive model (dmr2_m2, p = 0.03, r = -0.17) and one DMR from the interactive model (dmr1_m3, p = 0.02, r = -0.19). In both DMRs, individuals with a higher PRS also showed demethylation following exposure.

Second, the best PRS for EA and MDD were in the following tested for associations with WGCNA modules. EA_0.001 was significantly correlated with the pink module (p = 0.005, r = 0.22), the black module (p = 0.03, r = -0.17) and the red module (p = 0.05, r = 0.15). Interestingly, the red module was also associated with the developmental measures (SON_IQ and WPPSI, see Figure 10) and the prenatal exposure scores. MDD_0.3 exclusively showed a significant correlation with the green module, which was also correlated with CM (p = 0.04, r = -0.16).

3.2. Validation of findings in an independent cohort

In order to further test the stability of the findings obtained immediately following CM in the Berlin LCS cohort, the analyses were repeated in the LMU cohort consisting of 640 adolescents aged 8 to 18 years with and without a diagnosis for MDD. This cohort includes participants with (n=392) and without exposure to childhood abuse (n=248). Replication of the findings from the child cohort would not only show the stability of these findings over time but also their reliability across different tissues as the DNAm measurements in the LMU cohorts was performed on DNA extracted from blood samples.

3.2.1. No overlap with DMRs at a different age range

A linear model was run to identify differentially methylated CpGs between the adolescents who have been exposed to CA and the non-exposed group. We detected 170 CpGs significantly associated with CA after correction for multiple testing (lowest $p = 5.02*10^{-8}$). Single CpGs were aggregated to regions using the same settings as in the Berlin LCS cohort. While aggregating the CpGs from this model to DMRs yielded over 25 significant regions passing the multiple testing correction (lowest $p = 6.63*10^{-11}$), none of these DMRs overlapped with the DMRs from the additive (Model 2) and interactive model (Model 3) in the Berlin LCS cohort.

3.2.2. Validation of the prenatal exposure scores

The epigenetic scores for prenatal alcohol and smoke exposure based on weights from previous studies were computed in the LMU cohort and tested for discriminative power between cases and controls as well between individuals exposed to CA and non-exposed individuals. The parental smoke exposure score was significantly higher in cases ($p = 0.005$) and exposed individuals ($p = 0.01$) as compared to the control group or the non-exposed group respectively. While there were significant differences between groups for the prenatal smoke exposure score, the prenatal alcohol exposure score only showed a trend with cases and individuals with adverse childhood experiences presenting with higher scores (p (case-control) = 0.09 and p(risk) = 0.06). Results are summarized in Figure 12.

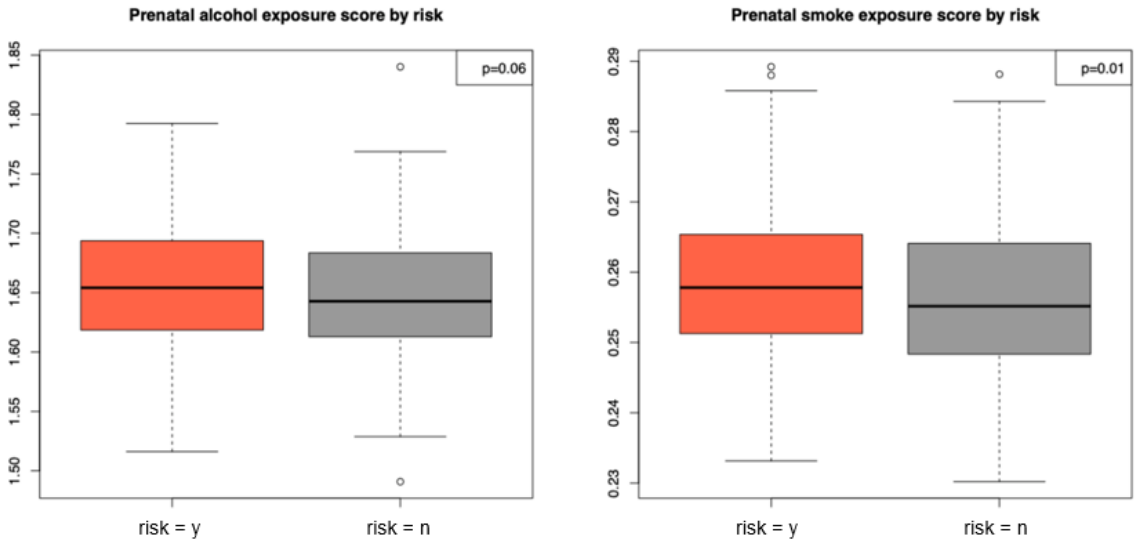


Figure 12: Prenatal exposure scores in the LMU sample. The DNAm based prenatal exposures scores for alcohol and smoke exposure were computed in the LMU cohort and compared between groups. The “risk = y” group denotes participants which were exposed to childhood abuse (shown in red) while the “risk = n” group reflects unexposed participants. The exposed groups on average showed higher prenatal alcohol exposure scores (left panel) and present with significantly higher prenatal smoke exposures scores (right panel) as compared to the non-exposed group.

3.2.3. Co-methylation modules in the LMU sample and their correlation with exposure

WGCNA was run on the most variable CpGs from the LMU sample using the same parameters as for the Berlin LCS sample. The most variable 10% of CpGs from the Berlin LCS cohort only partially overlapped with the most variable 10% of CpGs in the LMU cohort (n=146,85), most likely due to the difference of samples in tissue and age. Therefore, the same CpGs selected for running WGCNA in the Berlin LCS cohort were used as input (n = 74,307, available after preprocessing in the LMU cohort). While these CpGs were not the most variable in the LMU sample, they still presented with some variation with MAD scores of these CpGs ranging from 0.11 to 0.45.

WGCNA revealed 23 co-methylated modules with the sizes of the modules included ranging from 32 to 41,665 CpGs. Next, the association of the modules with exposure or case-control status was investigated. Of these modules three significantly correlated with childhood abuse: the purple module (r = 0.12, p = 0.002), the cyan module (r = 0.12, p = 0.003) and the dark red module (r = -0.09, p = 0.03). The dark red module was also the only module that correlated with a history of childhood abuse (r = 0.13, p = 0.001). The majority of modules, including the dark red module, correlated with the prenatal exposure scores for alcohol and smoking (strongest correlation: r = -0.8, p =

$6*10^{-145}$). The cyan and the magenta modules both significantly correlated with the prenatal exposure scores (strongest correlation: r = -0.44, p = $8*10^{-4)}$. Both of the modules were additionally correlated with age (strongest correlation: r = -0.2, p =$3*10^{-7}$) and sex (strongest correlation: r = 0.16, p = $3*10^{-5}$). Correlations between all modules and traits are summarized in Figure 13.

The CpGs within all modules were mapped onto genes and used as input for FUMA in order to functionally annotate the co-methylation modules in the LMU sample. Of the modules which correlated with childhood abuse, the cyan module was enriched in pathways related to the neuronal system (lowest p = 0.034), the dark red module was enriched in oxidative stress (p = 0.026) and TGF signaling pathways (p = 0.026) and the magenta module was enriched in chromatin organization (p = 0.013) and mitochondrial pathways (p = 0.038). The majority of the remaining modules showed significant overlaps with pathways from KEGG or Reactome, the modules without significant overlap with pathways mostly were small (number of CpGs included in the module < 100, for most of the unannotated modules) and mapped to only a few genes. The overlap with specific pathways for all modules is summarized in Supplementary table S8.

Interestingly, the top five enrichments for two of the modules (blue and grey 60) were genes that were associated with long-term depression (strongest enrichment: p = $2.31*10^{-5}$). While the blue module was also significantly correlated with the prenatal scores (strongest correlation: r = 0.44, p =$7*10^{-32}$) and sex (r = 0.11, p = 0.005), the grey 60 module was not correlated with any trait.

Finally, the overlap between the exposure-associated modules from the Berlin LCS cohort and the LMU cohort was examined. Most of the CpGs (n = 139) within the green module of the LCS analysis overlapped with the tan module of the LMU cohort, which was not associated with childhood abuse. The tan module, however, was significantly associated with age (r = 0.19, p = $2*10^{-6}$), sex (r = -0.78, p = $6*10^{-130}$) and both DNAm based prenatal exposure scores (strongest r = 0.2, p = $7*10^{-7}$). CpGs in the tan module did not overlap with any significant pathway. Only a few of the CpGs from the green LCS module overlapped with the dark red LMU module (n = 16), the remaining CpGs overlapped with the grey module.

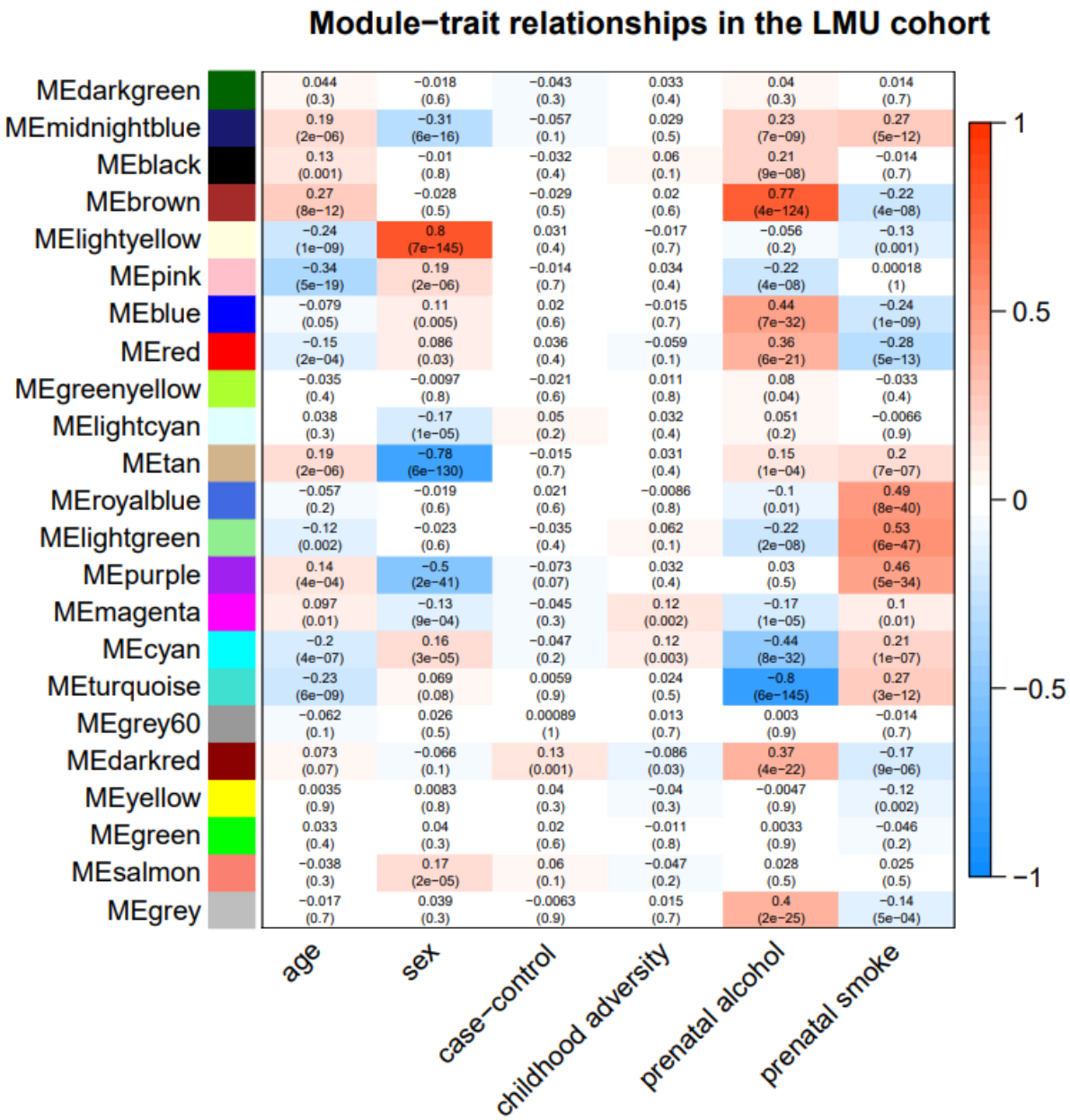


Figure 13. Module Trait Relationship in the LMU sample. Correlations between DNAm leaves in the WGCNA modules and exposures (child adversity reflecting exposure to childhood abuse, prenatal alcohol, and smoke exposure scores) and case-control status (MDD diagnosis) are shown in this figure. The majority of modules are correlated with age and sex.

3.3. Targeted bisulfite sequencing of the *FKBP5* locus reveals stable changes following childhood maltreatment

Increased *FKBP5* activation via genetic or epigenetic factors has previously been associated with increased stress sensitivity and risk for psychiatric disorders in human as well as animal studies. The human locus of *FKBP5* is located on the short arm of chromosome 6 (6p21.31) and spans around 155 kb. The locus encompasses 13 exons and 12 introns. Transcription of the *FKBP5* gene is steroid-regulated and mediated by binding of the GR to GREs, which are located in a region spanning over 100 kb and range from upstream of *FKBP5* promoter to introns 2, 5, and 7 of the gene. As transcriptional regulatory sites with enhancer regions including GREs are distributed across the locus, investigating DNAm changes at the *FKBP5* locus following CM using a fine-mapping approach is of particular interest. Using TBS, regulatory regions within the *FKBP5* locus that are not covered in commercially available DNAm arrays (shown in Figure 14) can be systematically assessed. In a first step, I investigated differential DNAm directly following CM (at baseline) in regulatory regions of the *FKBP5* locus and then analyzed the stability of these effects at a one-year follow-up measurement (at T2).

The best-characterized polymorphisms within the locus comprise a haplotype spanning the entire gene and containing up to 18 SNPs in strong LD in Caucasians ($r^2 > 40.8$, distance 4500 kb, 1,000 genomes next-generation sequencing project), and is commonly tagged by the SNPs rs3800373, rs9296158, or rs1360780. A mechanism for the GxE of childhood abuse and genetic variants of the *FKBP5* gene has been suggested by Klengel et al. (2013). In this GxE mechanism, functional polymorphisms, especially rs1360780, within GRE in intron 2 can alter the 3D chromatin structure of the *FKBP5* locus and embed CT-dependent epigenetic modification. Therefore, in a second step, I investigated the genotype-dependent effects of rs1360780 on DNAm in all regions covered. Additionally, additive effects (G + CM) and interaction effects were investigated.

Finally, I tested if the change in DNAm levels between the baseline timepoint (T0) and the one-year follow-up timepoint (T2) were associated with any changes in the other biodata levels using linear models. In addition, I examined which DNAm changes following CM overlapped with DNAm differences between children with and without psychiatric symptoms.

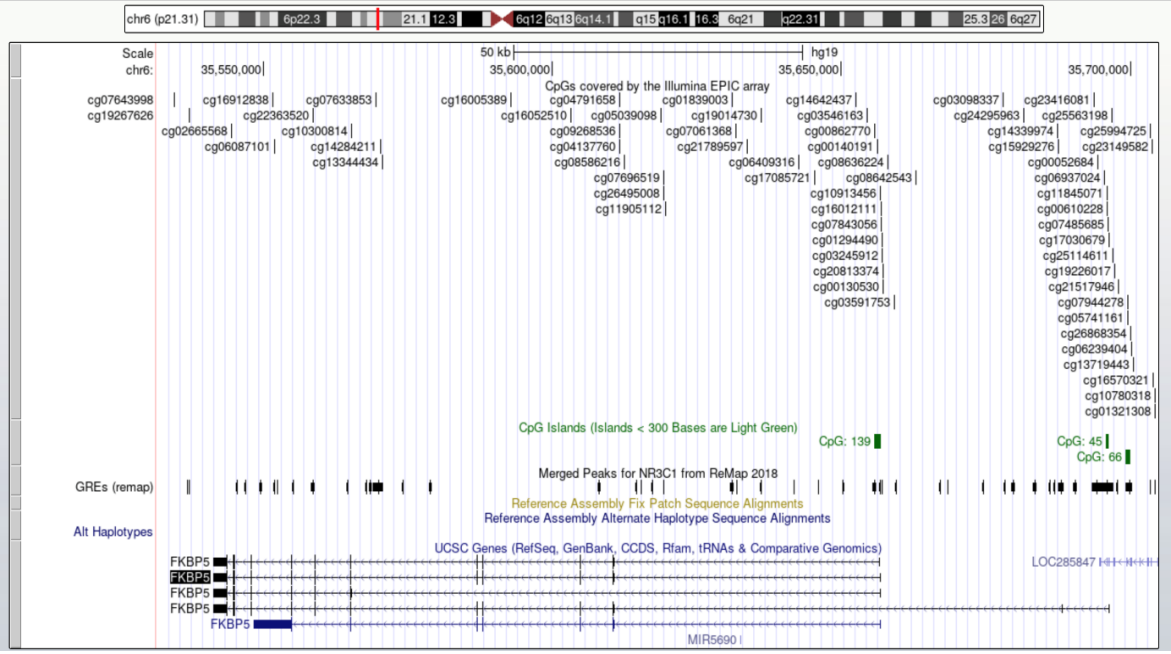


Figure 14: Genome Browser view of the *FKBP5* locus. The first track represents the CpGs covered by the Illumina EPIC array. The second track shows ChIP-Seq peaks for NR3C1 obtained from ReMap (Chèneby et al., 2018), a database integrating DNA-binding experiments across various transcription factors. The last track shows the *FKBP5* gene.

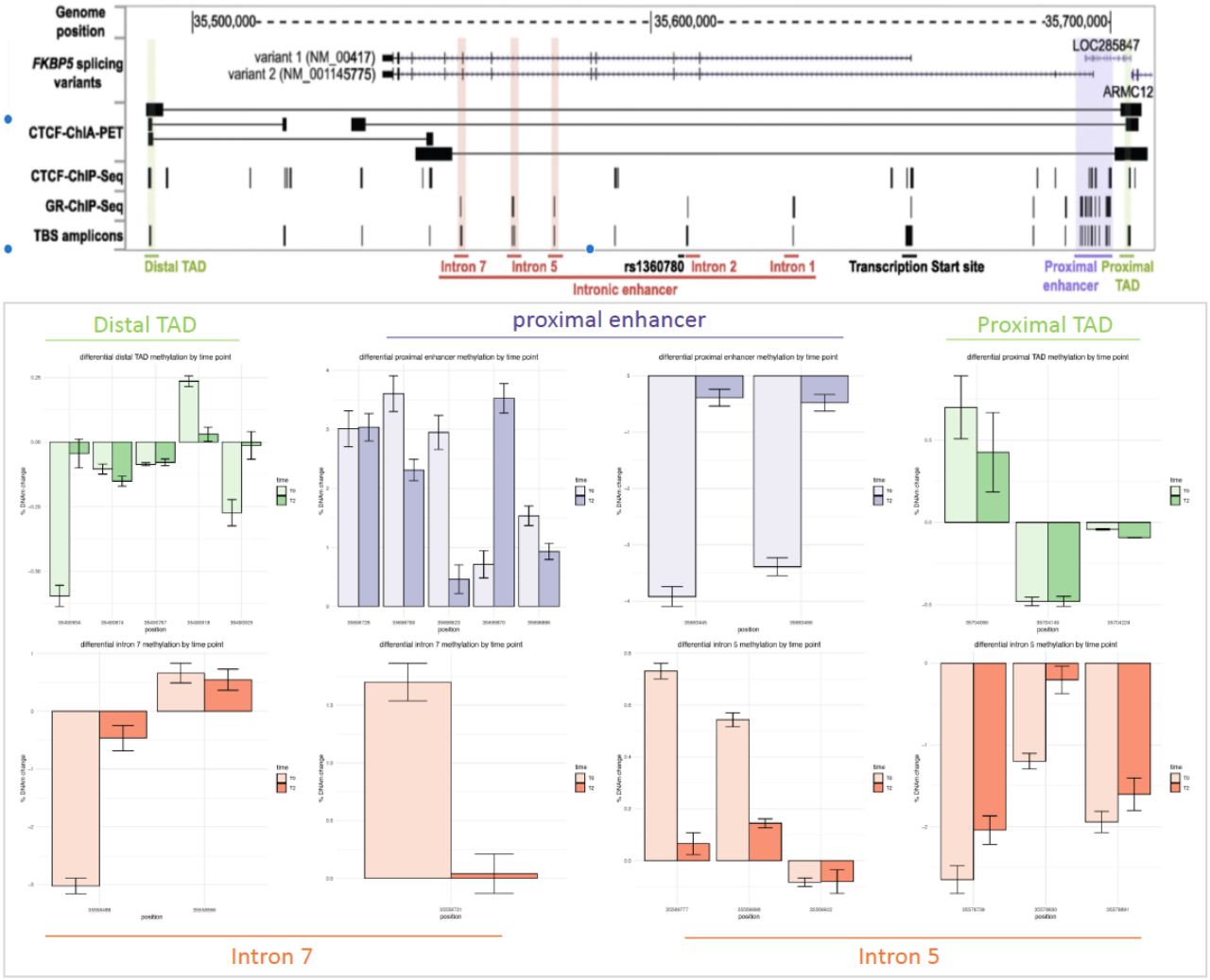
3.3.1. Early life stress induces stable DNAm changes in *FKBP5* regulatory regions

For each CpG covered by the amplicons selected, differential DNAm between maltreated children and non-maltreated children at baseline (T0) was analyzed. The majority of the CpGs within regulatory regions of the *FKBP5* locus (n=41) showed significant differential DNAm changes with the largest methylation differences being 4,6% (within the proximal enhancer region) and the lowest adjusted p-values being $p < 1*10^{-350}$. Differentially methylated CpGs at baseline are listed in Supplementary table S9.

Next, the stability of the DMPs at baseline was investigated at the one-year follow-up timepoint (T2). Of the CpGs with significant differential methylation at baseline, 25 showed differential DNAm with the same direction of effect at the follow-up timepoint. These CpGs were considered to be stable over the timeframe of one year. However, due to the drop of the sample size at the follow-time point (n=111) compared to baseline (n=168), no CpGs remained significant after correction for multiple-testing.

Stable CpGs were located within intronic and proximal enhancers as well as the topologically associated domains (TADs). TADs are self-interacting genomic regions, where DNA sequences within this region have a higher probability of interacting with each other than

outside of this region. While stable DMPs located within intron 5 and intron 7 were mostly



demethylated in exposed children, stable DMPs located within the proximal enhancer region were predominantly hypermethylated in this group. DMPs in the proximal and distal TAD showed mixed directions of effects. Baseline and one-year follow-up DNAm levels of the stable CpGs are summarized in Figure 15, sorted by location.

Figure 15: Differential methylation of the *FKBP5* Locus following Childhood Maltreatment. Top panel genomic location and variants of the *FKBP5* locus, genes located within the locus, locations of CTCF factor-mediated chromatin interactions (ChIA-PET data) extracted from lymphoblastoid cell lines (GM12878, (Tang et al., 2015). Chromatin interactions are shown by blocks connected by a line, transcription factor binding (CTCF & GR) obtained from chromatin immunoprecipitation (ChIP) experiments in multiple cell lines from the ENCODE project; locations of targeted bisulfite sequencing (HAM-TBS, (Roeh et al., 2018)) amplicons. Bottom panel: Bar plots with differential DNAm for each amplicon showing differential methylation at baseline (T0, light bars) and at the one-year follow-up time point (T2, dark bars). Plots are color-coded by region (TAD: green, proximal enhancer: purple, intronic enhancers: orange).

3.3.2. Genotypic effects of rs136780 on *FKBP5* DNA methylation

First, genotypic effects of the tag SNP rs1360780 within the *FKBP5* locus on DNAm within functional regions of *FKBP5* were investigated. This SNP has previously been associated with differential up-regulation of *FKBP5 expression* and increased GR sensitivity (Klengel et al., 2013; Xie et al., 2010; Zimmermann et al., 2011). In this sample, the rs1360780 genotype was significantly associated with two CpGs, located in the 3' distal TAD region. These two CpGs (positions 3549608 and 3549619) showed significantly lower DNAm levels ($p(3549608)=1.78*10^{-3}$ and $p(3549619)=2.83*10^{-3}$) in carriers of the risk genotype (CT/TT), (Figure 16). Two additional CpGs showed small correlations with the genotype but did not pass the multiple testing correction (Table 2).

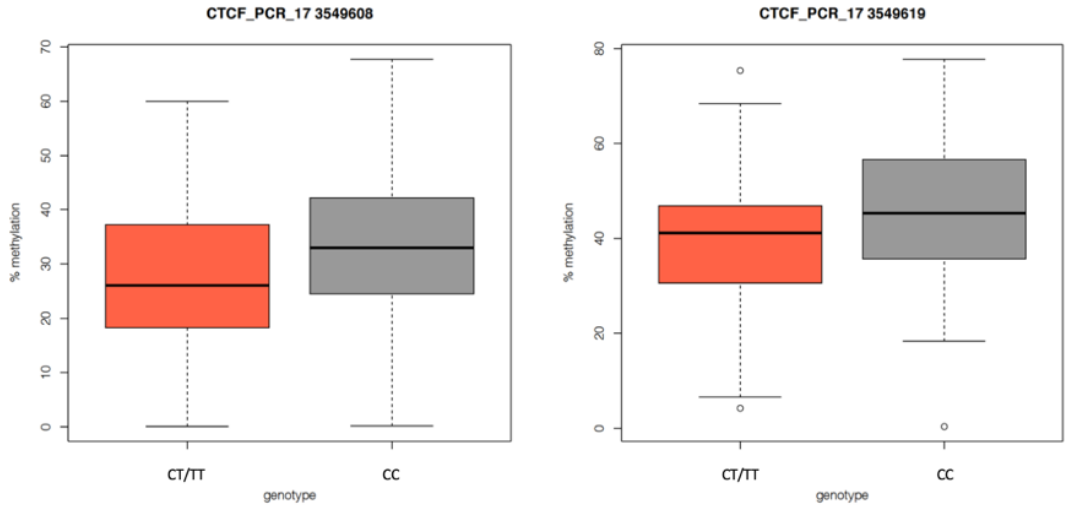


Figure 16: Genotype-dependent CTCF methylation. Rs1360780 showed a significant effect on two CpGs within the 3'TAD. These two CpGs showed significantly lower methylation levels in carriers of the risk genotype (AA/AG, shown in red) as compared to carriers of the protective genotype (GG, shown in grey).

Table 2: Genotype effects of rs1360780 on *FKBP5* methylation. Two CpGs within the 3'TAD showed significant genotype effects.

PCR name	position	P	Adj R^2	AIC	Functional region
CTCF PCR 17	35490608	$1.8*10^{-3}$	0.52	716.85	3' TAD
CTCF PCR 17	35490619	$2.7*10^{-3}$	0.56	745.13	3' TAD

Next, I tested if CM added to the genotype effect on *FKBP5* DNAm. Testing for additive effects (G + E) revealed significant effects at 7 CpGs located throughout the locus. One of these CpGs was located in the 5'TAD (35704149), two were located in the proximal enhancer (35683363 and 35683445), the remaining CpGs are lying within the enhancer

region of intron 5 (35569751, 35569757. 35569777 and 35570224). The 5'TAD CpG showed the lowest DNAm levels in the group of risk allele carriers (CT/TT) who were also exposed to CM and the highest DNAm levels for the unexposed control group. This was also the case for one CpGs in the proximal enhancer region (35683363). For all CpGs in Intron 5, risk allele carriers exposed to CM showed the highest methylation levels. Additive effects are summarized in Table 4.

Table 4: Additive effects of rs1360780 genotype and maltreatment

PCR name	position	P	Adj R^2	AIC	Functional region
CTCF PCR 5.1	35704149	$2.7*10^{-2}$	0.04	248.51	5' TAD
FKBP5 PCR 12	35683363	$3.9*10^{-2}$	0.01	881.54	Proximal Enhancer
FKBP5 PCR 12	35683445	$4.6*10^{-2}$	0.01	883.08	Proximal Enhancer
FKBP5 cg14284211	35570224	$4.9*10^{-2}$	0.07	109.45	Intronic Enhancer
FKBP5 PCR 2	35569751	$4.5*10^{-3}$	0.07	389.22	Intron 5 Enhancer
FKBP5 PCR 2	35569757	$1.6*10^{-2}$	0.06	339.16	Intron 5 Enhancer
FKBP5 PCR 2	35569777	$1.8*10^{-2}$	0.04	316.04	Intron 5 Enhancer

Finally, the CpGs were also tested for an interaction effect between rs1360780 genotype and CM for one CpGs. Analyzing the interaction effects on DNAm yielded two significant CpGs (at position 35569751 in Intron 5, adjusted $r^2 = 0.10$, $p = 2.8*10^{-2}$). Carriers of the protective genotype and without exposure to CM had the highest

methylation levels, while carriers of the risk allele without exposure to CM had the lowest methylation levels (shown in Figure 17 and Table 5).

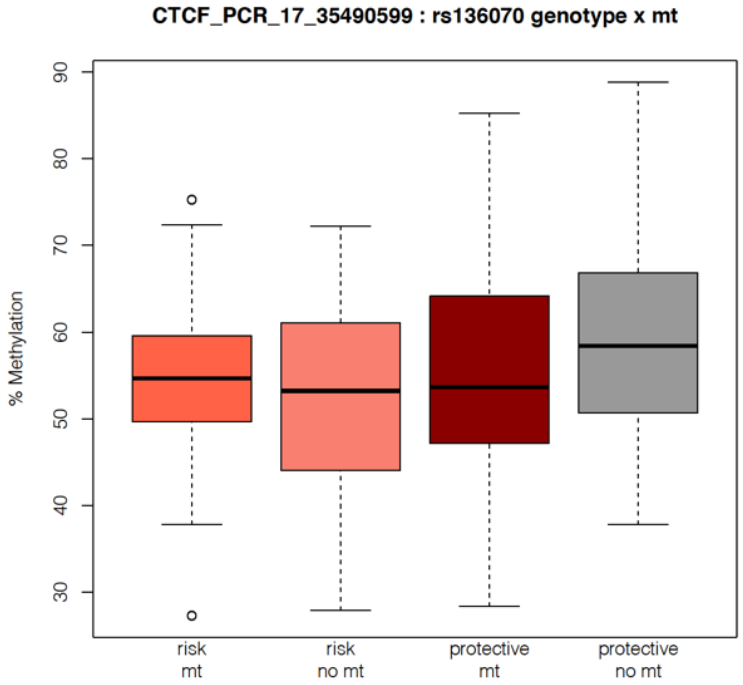


Figure 17: Interaction effect of rs1360780 genotype and maltreatment (mt). One CpG with the 3'TAD showed an interaction effect between CM and rs1360780. The carriers of the protective genotype and without exposure to CM (shown in grey) had the highest methylation levels, while carriers of the risk allele without exposure to CM had the lowest methylation levels (shown in salmon color).

Table 5: Interaction effects of rs1360780 genotype and maltreatment

PCR name	position	P	Adj R^2	AIC	Functional region
FKBP5 PCR 2	35569751	$2.8*10^{-2}$	0.10	106.24	Intron 5 Enhancer

3.3.3. Association between of *FKBP5* methylation changes and biodata changes

Additionally, associations of the biodata (Cort, CRP) and AA) and DNAm at baseline were investigated. Salivary AA activity has been suggested to be relevant as a stress-related biomarker and possibly is associated with lifestyle characteristics (Nagata et al., 2011). Exposure to different stressors has been shown to promote elevations in inflammatory markers such as CRP (Broyles et al., 2012; Gouin et al., 2012). Dysregulation of cort levels in children and adults has been associated with exposure to CA, however, there is little agreement on the direction of these associations (Iob et al., 2021). In this study biodata changes over time were linked to changes in *FKBP5* DNAm levels in children directly following CM. Interestingly, in this cohort baseline biodata levels for all measurements did not strongly correlate with biodata levels at the one-year follow-up timepoint (T0-T2 correlations: r(Cort) = -0.12, r(CRP) = 0.01, r(AA) = 0.07).

First, associations with DNAm in regulatory regions of *FKBP5* and salivary biodata were investigated. AA levels were significantly associated with DNAm levels of one CpG in the 3'TAD (position: 35490713, adjusted r^2 = 0.003, p = $4.4*10^{-3}$). CRP levels at baseline were associated with DNAm of another CpG in the 3'TAD (position: 35490812, adjusted r^2 = 0.05, p = $4.0*10^{-2}$) and one CpG in the intronic enhancer region of intron 5 (cg14284211, position: 35570224, adjusted r^2 = 0.03, p = $4.1*10^{-2}$). Cort levels were associated with two CpGs in the 3'TAD (position: 35490787, adjusted r^2 = 0.03, p = $4.0*10^{-2}$ and position2: 35490818, adjusted r^2 = 0.004, p = $1.7*10^{-2}$). Baseline correlations between CM, DNAm levels, and the biodata levels are summarized in Figure 18.

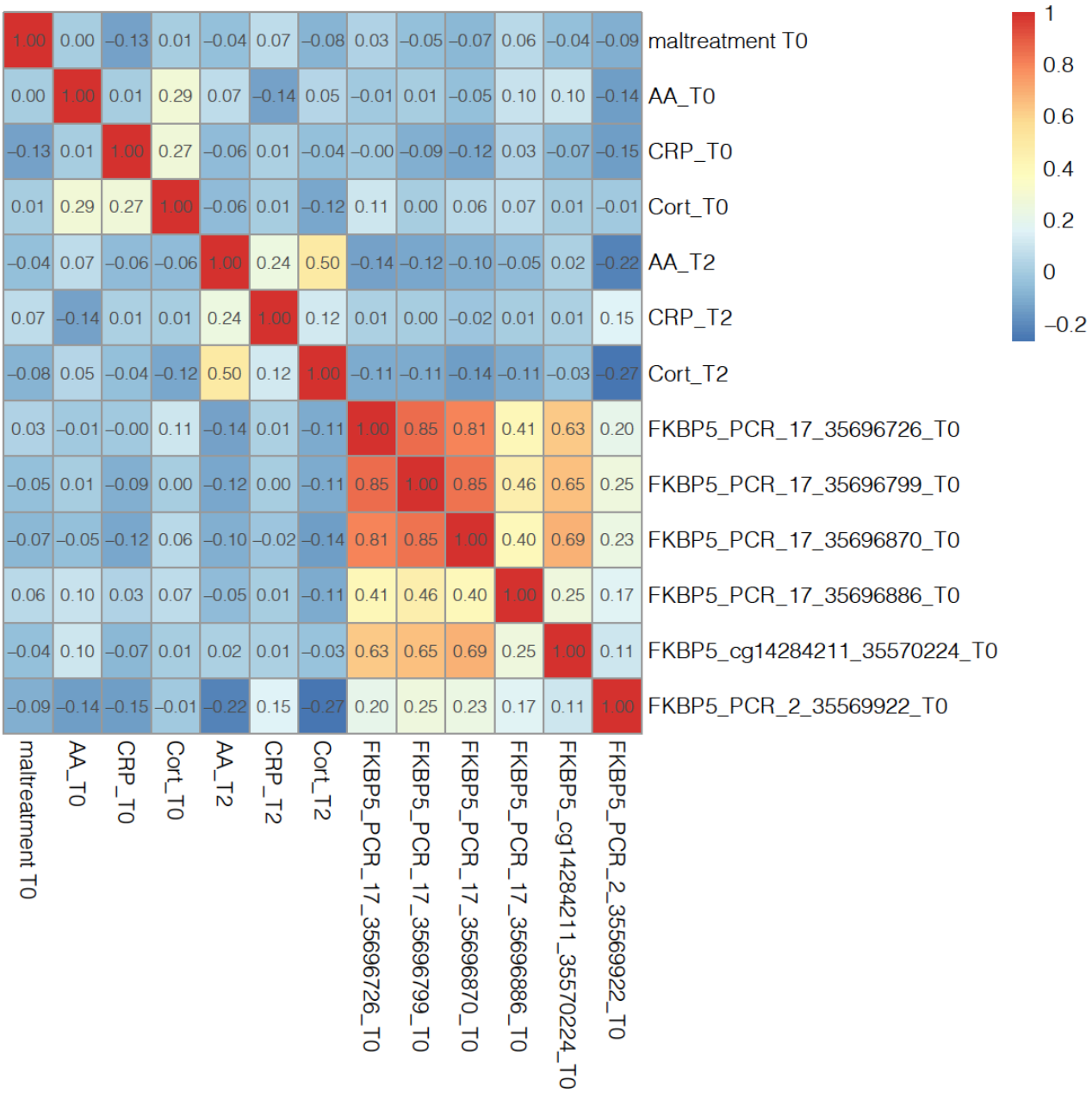


Figure 18: Correlation of baseline methylation levels, biodata levels at both time points, and baseline maltreatment. Maltreatment is coded as 0 for controls and 1 for cases, negative correlation with methylation level signifies demethylation in maltreated children. All CpGs reported show a correlation with methylation at baseline and cortisol levels at T2.

Next, I examined if the DNAm changes between baseline and the one-year follow-up time point were associated with changes of the biodata levels within the same timeframe. On average, cort levels (AUCg) and CRP levels were reduced after one year, while AA levels were increased at T2. Cort and CRP changes were associated with DNAm changes at the same six CpG-sites, AA changes were associated with 5 of these positions. Four of the CpGs were located within the proximal enhancer region, with the strongest association being with CRP level changes and the CpG at position 35696726 ($r = 0.14$, $p = 3.5*10^{-3}$). DNAm levels of the four sites within the proximal enhancer were higher at T2 as compared

to baseline, while the CpG within the intron 5 and cg14284211 were demethylated at T2. Results are summarized in Table 6.

Table 6: Associations between differential methylation and biodata. Changes of alpha-amylase (AA), Cortisol (Cort), and C-reactive protein (CRP) levels between T0 and T2 were associated with DNA methylation changes between T0 and T2 at similar positions, mostly within the proximal enhancer region covered by *FKBP5* PCR 17.

PCR name	position	AA (adj. R^2, P)	Cort (adj. R^2, P)	CRP (adj. R^2, P)	Functional region
FKBP5 PCR 2	35569922	n.s.	0.002, $3.7*10^{-2}$	0.03, $7.8*10^{-3}$	Intron 5 Enhancer
FKBP5 cg1428421	35570224	0.05, $1.9*10^{-3}$	0.004, $2.2*10^{-2}$	-0.006, $4.2*10^{-2}$	Intron 5 Enhancer
FKBP5 PCR 17	35696726	0.09, $3.5*10^{-3}$	0.13, $1.0*10^{-2}$	0.14, $3.5*10^{-3}$	Proximal Enhancer
FKBP5 PCR 17	35696799	0.01, $1.2*10^{-2}$	0.04, $1.6*10^{-2}$	0.07, $1.3*10^{-2}$	Proximal Enhancer
FKBP5 PCR 17	35696870	0.08, $2.1*10^{-2}$	0.12, $5.7*10^{-3}$	0.10, $1.8*10^{-2}$	Proximal Enhancer
FKBP5 PCR 17	35696886	0.05, $4.4*10^{-3}$	0.12, $1.7*10^{-3}$	0.11, $1.4*10^{-3}$	Proximal Enhancer

3.3.4. Shared differential DNAm patterns of CM and psychopathology

Finally, differential DNAm between the children with and without psychopathology (at least one symptom using the PAPA questionnaire) were computed in order to check the overlap with the differentially methylated CpGs following CM. At baseline, the majority of CpGs (42 of the 57 covered) were differentially methylated between children that scored high on any symptom on the PAPA and children without symptoms, with the largest DNAm difference being 5% change and the lowest p-value being $p = 3.1*10^{-254}$. 38 CpGs overlapped with the differentially methylated CpGs following maltreatment. Overall, directions of effect compare to the differential methylation analysis: children with symptoms show predominantly demethylation in intron 5 and intron 7, high methylation in the proximal enhancer region, and mixed direction in TADs. In general, the mean % methylation difference was larger when comparing the groups at the symptom level. A visual representation of the comparison at baseline between

differential methylation between maltreated and non-maltreated children and differential methylation between children with and without psychiatric symptoms is shown in Figure 19.

For example, the mean methylation changes across intron 7 in maltreated vs. non-maltreated was -1.1% (demethylation in the maltreated group) as compared to -2.2% (demethylation in the group with symptoms). However, due to small sample sizes, any subgroup-specific differential methylation analysis did not yield significant results. In addition, CM and symptoms were tested for additive or interaction effects on DNAm. For 7 CpGs, the model was significantly improved by adding symptoms. Four of these CpGs were located in intron 7, one intron 5, one in the proximal enhancer region, and one in the 3'TAD. This data showed nominal significant interaction effects of CM and symptoms on DNAm for 3 CpGs of which two were located in intron 7 and one was located in the 3'TAD. The subgroup with symptoms that were also exposed to CM also had the lowest methylation levels across intron 7 (mean: 65.33% compared to 68.87% for the group without symptoms or CM exposure). However, these results did not pass the multiple testing correction.

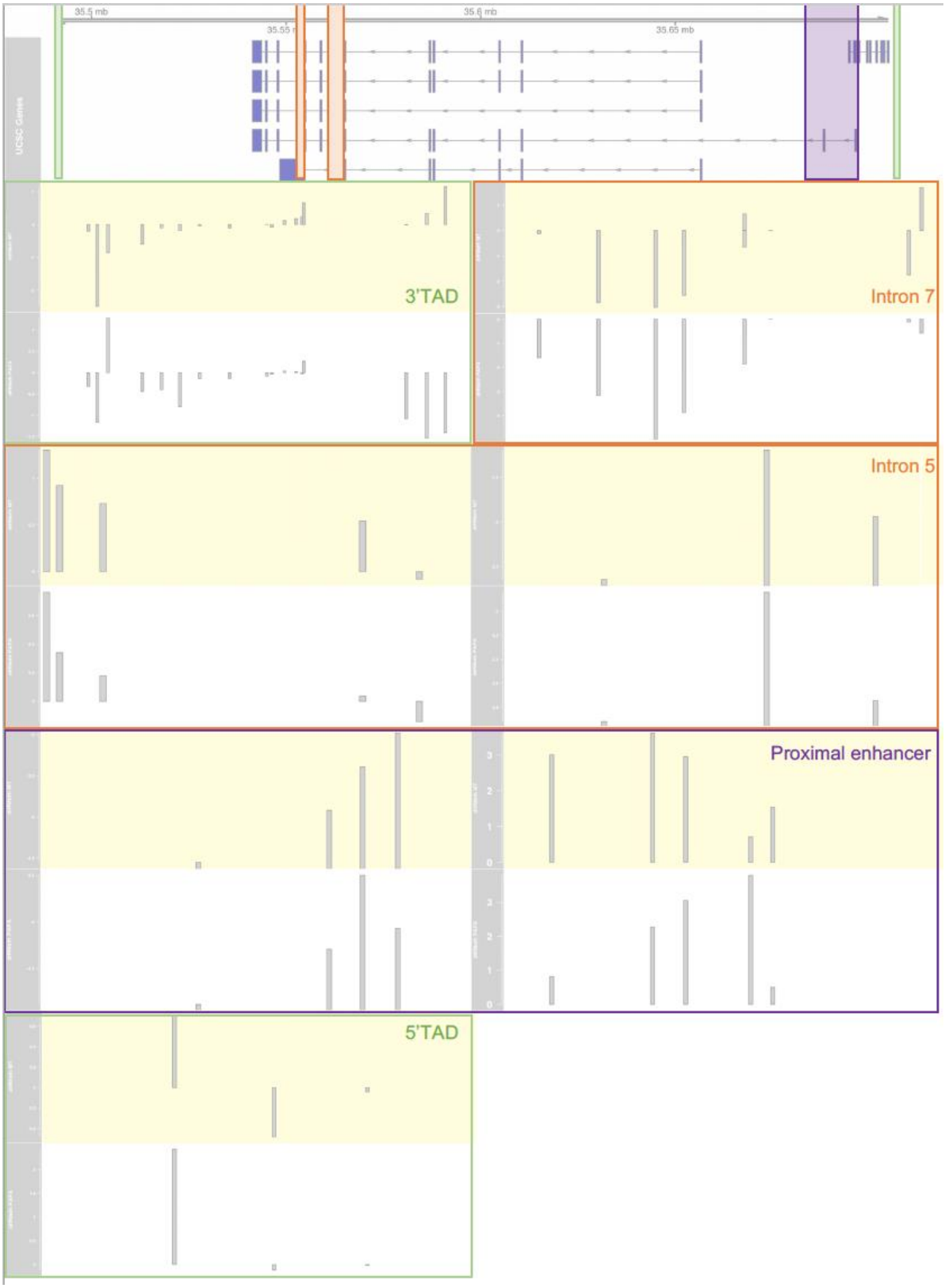


Figure 19: Comparison between differential methylation at baseline. For each region, the top track (yellow background) represents the differential methylation between maltreated and non-maltreated children, while the bottom track shows the differential methylation of children with and without symptoms (PAPA).

4. Discussion

CM is one of the strongest environmental risk factors for chronic and severe mental and physical health problems across the lifespan (Parade et al., 2021). Increasing evidence suggests that genetic variants interact with environmental risk factors in a complex interplay and that long-lasting effects depend on both the individual's genetic background and environmental factors (Stephens & Wand, 2012; A. S. Zannas & Binder, 2014). Epigenetic mechanisms, in particular DNAm, have been proposed as a mechanism of how environmental exposures are embedded in the genome (Cecil et al., 2020a). Numerous studies support the hypothesis that CM is associated with epigenetic changes that may subsequently serve as mechanisms of disease (Cicchetti et al., 2016; Parade et al., 2017; Parent et al., 2017; Suderman et al., 2014). A systematic review (Parade et al., 2021), analyzed over 100 empirical studies that focused on the relationship between CM and DNAm, including 69 articles focused on candidate genes and 31 articles leveraging epigenome-wide data. Within the candidate-focused studies, the most common genes were those that regulate GC signaling, such as *FKBP5,* which modulates the sensitivity of the GR. The majority of studies also measured DNAm in adulthood using retrospective measures of CM, while only a few investigated DNAm in childhood. Additionally, only two longitudinal findings were presented for two candidate genes in children, also focusing *FKBP5* (Parade et al., 2017).

While findings from the studies broadly back the association between CM and altered patterns of DNAm, limitations such as the lack of longitudinal data, low comparability across studies as well as pre-exposure environmental confounding, such as prenatal exposures, currently limit the conclusions that can be drawn (Cecil et al., 2020a; Dunn et al., 2016). Potential improvements for future work include the harmonization with regards to phenotypic and genetic data. Certain effects potentially can only be observed in a certain phenotype or within individuals with a specific ethnic origin. Exposure information has been gathered differently across previous studies, ranging from using retrospective self-reported measures in adults to prospective measures in children. Future studies would benefit from a harmonized assessment of phenotypic measures and exposures including the pre-exposure environment.

Addressing the lack of longitudinal data, this work is, to the best of our knowledge, the first epigenome-wide study on longitudinal effects of CM on DNAm at the critical time period of early childhood (C. A. Nelson & Gabard-Durnam, 2020).

4.1. Summary of results

In the first part of this thesis, I investigated whether CM-specific variation in DNAm measured using the EPIC array exists and if it is more accentuated in the context of additional adversities, such as low SES or exposure to life events. The effects of CM were assessed in the Berlin LCS cohort, a longitudinal child cohort, consisting of 173 DNAm saliva samples of children aged between 3 and 5 years, of which 86 were exposed to CM. The presence of pre-exposure adversities was assessed using DNAm-based scores as proxy measures for prenatal alcohol and tobacco exposure. Additionally, findings were compared with results using a broader adversity score which, apart from CM, also included contextual stressors. Finally, the association of CM-induced variation in DNAm with behavioral or biological outcomes was tested and CM-associated variation in DNAm changes over time were characterized. Genotype effects on the outcomes were investigated by computing PRS x CM interactions.

In the second part of this thesis, I aimed to validate the longitudinal array-based findings in an independent cohort (the LMU cohort) of 640 adolescents with and without MDD and/or history of childhood abuse. Here, I tested if CM-specific DNAm patterns identified in the Berlin LCS cohort could be replicated across different age groups and tissues. Additionally, I investigated if the effects of prenatal exposure were also still present at a later point in life.

In the third part of this thesis, I focused on *FKBP5,* one of the best-studied candidates in the context of DNAm changes following CM. A potential molecular mechanism incorporating *FKBP5* variants and CA was proposed by Klengel et al., highlighting rs1360780 as a functional variant in the disease-associated haplotype, which showed allele-specific local demethylation of CpGs in a specific GRE enhancer (Klengel et al., 2013). While Illumina DNAm arrays provide a method to obtain DNAm levels at base-pair resolution in a cost-efficient manner, they do not cover key enhancer regions, which are important for the regulation of *FKBP5* gene expression and epigenetic mechanisms contributing to the development of psychiatric diseases (Wiechmann et al., 2019).

Therefore, I systematically analyzed the effects of CM on DNAm by employing targeted bisulfite sequencing (Roeh et al., 2018), in order to replicate previously reported findings in a child cohort and to investigate the stability of DNAm changes in regulatory regions of *FKBP5*. Additionally, the interaction between rs1360780 and CM, reported by Klengel et al., on *FKBP5* DNAm was tested. Additionally, the correlation between DNAm changes, genotype, and biodata (including cort) was investigated.

Finally, key challenges in the study of GxE and thoughts on future perspectives, including methodological considerations for future study design were discussed.

4.2. Differentially methylated regions over time in the context of childhood maltreatment

As a first step, I explored the association of DNAm levels with exposure to CM and a more global adversity score using data from the baseline measurements. A limited EWAS, in which the most variable CpG sites on the array were tested individually for associations with exposure to identify DMPs, was run for CM and the adversity score. While the analysis for adversity yielded no significant CpGs after correction for multiple testing, the model using CM yielded four differentially methylated CpGs, of which two mapped to the genes *TBCD* and *NT5C1B*. *TBCD* mutations have been associated with infantile neurodegenerative disorders (Edvardson et al., 2016). While *NT5C1B* has been associated with eating disorders and is involved in the regulation of adenosine levels (Wade et al., 2013).

Next, a longitudinal analysis across all time points was conducted by computing linear mixed models in order to test for associations of CM or adversity (adversity score) with epigenetic trajectories: a model over time (Model 1), an additive model (Model 2) and an interactive model (Model 3). While each of the models yielded significant CpGs after correction for multiple testing, detected effect sizes were small (< 2% methylation change between exposed and non-exposed groups) and therefore the downstream analysis was directed towards grouping the single CpGs to DMRs. Additionally, it can be argued that observed changes in DMRs can be considered more credible as neighboring sites show similar changes, and CpGs are thought to function in groups to regulate gene expression.

4.2.1. Changes over time

The region-centered analysis examining changes over time returned 9 DMRs changing over 24 months and 7 changing over 12 months, suggesting that overall DNAm pattern in saliva is rather stable over this developmental time frame. The changes over time mostly mapped to prenatal exposures and impairments during early development (Jiang et al., 2020; Kunkle et al., 2017). The DMRs exposure to CM or adversity added to or moderated the changes over time and mapped to genes that have previously been associated prenatal exposures (tobacco (M. Alexander et al., 2013), alcohol (Nguyen et al., 2018), lead (Engström et al., 2015) and beta-blockers (Rojas et al., 2015)) and with psychiatric disorders (Roberson-Nay et al., 2020; Zhu et al., 2019). While some differences in trajectories could be identified for this short time frame (12 or 24 months), future work would need to validate if these changes remained stable beyond childhood

as DNAm is a reversible process. A study investigating intraindividual time-dependent changes in DNAm, found that 8% to 10% of individuals show changes greater than 20% over an 11- to 16-year span. Both losses and gains of DNAm were observed over time, with various environmental factors potentially contributing to these changes over time (Bjornsson et al., 2008).

4.3. Genetic and pre-exposure environmental confounding

Attributing effects specifically to one exposure, such as CM, poses challenges such as identifying confounding factors and establishing directionality (Parade et al., 2021). One strength of the Berlin LCS cohort is the longitudinal study design and the measurement of DNAm directly following the exposure to CM. However, the CM-associated DMRs may also reflect preceding or concurrent exposures (Bosch et al., 2012; Dong et al., 2004; Green et al., 2010; Sosnowski et al., 2018). CM is known to be reflective of a suboptimal caregiving environment and is often correlated with a range of prenatal exposures such as maternal substance use (Putnam-Hornstein et al., 2016), smoking (Taha et al., 2014), and stressful life events (Kelley, 1992). Additional exposures after birth include low SES (Isumi et al., 2021) or community violence (Cicchetti & Lynch, 2016), which are less likely to be shared by non-maltreated children. These additional exposures may partially explain the association between DNAm changes and CM.

4.3.1. Prenatal exposure as a confounder

Given the fact that CM and prenatal exposure are often correlated and that a number of the identified DMRs lie in regions that were previously associated with prenatal exposures, I was prompted to next investigate epigenetic proxies of such exposures in the Berlin LCS cohort. Two DNAm exposure scores, for prenatal tobacco exposure (Richmond et al., 2018) and prenatal alcohol exposure (Portales-Casamar et al., 2016) were computed based on previous findings. This was done as there was no information on alcohol consumption during pregnancy available and the information on smoking was incomplete. Additionally, DNAm-based scores have been shown to be less biased than self-reports (Langdon et al., 2020).

Indeed, CM-exposed children presented with both significantly higher DNAm based smoking scores and significantly higher alcohol exposure scores. In addition, of the six DMRs of Model 2 and Model 3 associated with additive and interactive effects of time and maltreatment or adversity score, five significantly correlated with both prenatal exposure measures (strongest correlation $r = 0.71$, $p < 2.2 \times 10^{-16}$). These findings suggest that maltreated children might present with a higher extent of prenatal

exposures as compared to controls and that this might also influence DNAm patterns, possibly with larger effect sizes than CM itself.

Next, I aimed to disentangle the effects of adversity/CM from prenatal exposures. To do so, I re-ran Models 2 and 3 correcting for prenatal exposure and found that the majority of CpGs within the DMRs remained significantly associated with adversity/CM. This supports independent effects of adversity/CM on DNAm, even if there seems to be a correlation of DNAm at these sites with prenatal exposures (strongest correlation dmr4_m3 and epigenetic smoke exposure score: $r = 0.78$, nominal $p < 2.2 \times 10^{-16}$). While some studies investigated the co-occurrence of prenatal exposures and CM, and their individual effects on DNAm none of these studies investigated nor disentangled the effects of the exposures on DNAm levels. As prenatal exposure and CM were strongly correlated in the Berlin LCS cohort, it was challenging to disentangle the effects of the individual exposures. Maltreated children also showed higher prenatal scores, while non-maltreated children had lower prenatal scores (groups using a median split: n(CM + prenatal exposure) = 48, n(no CM + prenatal exposure) = 33, n(no CM + no prenatal exposure) = 47, n(CM + no prenatal exposure) = 34). Future work would need to assess both exposures in larger cohorts and investigate the joint effects on the DNAm level. Additionally, a pregnancy cohort with longitudinal data would be required to establish the effects of prenatal exposure alone and investigate the dynamics of their embedding.

4.3.1.1. Prenatal exposure and cortisol reactivity

In the Berlin LCS sample, the cort response (AUC) was positively correlated with the prenatal exposure scores for smoking ($r = 0.14$, $p = 7.6*10^{-4}$) and alcohol ($r = 0.09$, $p = 0.03$). The majority of studies present an attenuated cort response following prenatal tobacco exposure in humans and mice (Azar et al., 2010; Eiden et al., 2015). Studies examining the relationship between prenatal alcohol exposure and infant stress reactivity reported an increased response after light to moderate exposure (Haley et al., 2006; Keiver et al., 2015; May & Gossage, 2011). Based on previous findings of altered cort reactivity associated with adversity and CM (Agorastos et al., 2019; Cecil et al., 2020a) converging effects of prenatal exposure on the stress system need to be considered. Such effects may be mediated by epigenetic mechanisms such as DNAm given the joint correlations of adversity, prenatal alcohol and smoking scores, and cort response with, for example, dmr4_m3.

One limitation of the Berlin LCS study is that, while the clinical visit would be perceived as a stressful event for the children, the repeated cort measurements obtained do not

reflect the cortisol response following a standardized stress test. For a more established measure of the HPA-axis activity, for instance, the awakening response, samples would be needed to be collected at home, which could not be requested from these families. Additionally, children (3–5 years at baseline) were too young to perform a Trier Social Stress Test (Kirschbaum et al., 1993). Although this measure of cort is more difficult to interpret compared to a standard test, it can be argued that it nonetheless allows a comparative evaluation of HPA-axis activity.

4.3.2. Socioeconomic status

Similarly, to prenatal exposures, low SES represents a confounder that might partly explain or compound the association of adversity/CM on DNAm. In the Berlin LCS cohort, low SES and exposure to CM were significantly correlated ($r = -0.63$, $p < 2.2 * 10^{-16}$, see Figure 8). Therefore, I re-ran Models 2 and 3 including SES as a covariate. Correcting for SES showed that the majority of CpGs remained significantly associated with adversity/CM. Running the models with SES as the only exposure (Time + SES for Model 2 and Time * SES for Model 3), showed that only the CpGs within one of the DMRs of Model 3 (dmr1_3) were significantly associated with SES, with the p-values being larger than using the adversity score (strongest association for SES: $p = 9*37*10^{-4}$). Maltreated children presented with lower SES than non-maltreated children (groups using a median split: n(CM + low SES) = 64, n(CM * high SES) = 18, n(no CM + low SES) = 20, n(no CM + high SES) = 60). Studies have estimated the effects of low SES on DNAm (McDade et al., 2019; Needham et al., 2015) with some of the effects overlapping with CpGs with differential DNAm following CM (Cecil et al., 2020a) such as FKBP5 and OXTR. However, most of the previous studies investigating the effects of CM on DNAm did not account for SES (Parade et al., 2021). Future work assessing contextual stressors alongside CM will be needed to separate the effects of various environmental exposures.

4.3.3. Genetic contribution to DNAm

Family and twin studies have shown that all psychiatric disorders are heritable to varying degrees. Over time, the cohort sizes increased and more risk variants with smaller effect sizes were identified, yet there is a knowledge gap in the understanding of how these variants contribute to the pathophysiology of psychiatric disorders (Starnawska & Demontis, 2021). The majority of the identified SNPs by GWAS are non-coding but are enriched in regulatory regions of the genome (Hill et al., 2016). It, therefore, has been of great interest to estimate the effects of the identified risk SNPs on DNAm.

Heritability studies have shown that individual genetic variation can influence DNAm levels across the genome, with heritability estimates for brain DNAm within a 50kb window ranging from 3-4% (Quon et al., 2013). This estimate differs from DNAm heritability estimates in blood, which were previously reported to be around 18% (Bell et al., 2012). Nevertheless, heritable DNAm loci across tissues are enriched in open chromatin regions, DNAase I hypersensitive sites, binding sites of transcriptional repressor CTCF, and near histone modifications (Banovich et al., 2014). This enrichment suggests that heritable DNAm loci may play an important role in the regulation of chromatin packaging.

A study aiming to determine whether there are global longitudinal changes in DNAm within individuals found that methylation maintenance demonstrated familial clustering, which suggests genomic control (Bjornsson et al., 2008). In this work, the first three PCs of the genotype were added to all models to correct for population structure and ethnicity. Additionally, known polymorphic probes were excluded from downstream analysis. Nevertheless, genetic control on the sites of interest could only be shown by determining the best model (G, E, G+E, GxE) for the CpGs of interest using the approach as described by Czamara et al. (2021). The Berlin LCS cohort was included in the study by Czamara et al. and showed that variable methylation at the majority of CpGs (61.25%) were best explained by the interaction model (GxE), while only a small fraction was best explained by E alone (0.1%).

To date, the most studied phenomenon through which genetic variation impacts epigenetic regulation is methylation quantitative trait loci (mQTLs). mQTL refers to a significant association between the genotype at a SNP and DNAm at a CpG at a nearby (*cis*-) or distant (*trans*-) position (Gamazon et al., 2012). Intraindividual comparisons of DNAm patterns between different brain regions and blood reported that correlations between DNAm levels across tissues were likely to result from mQTLs. mQTL studies found that several risk SNPs for common psychiatric disorders act as mQTLs, for example, a study performed on dorsolateral prefrontal cortex samples from SZ patients and matched controls confirmed the abundance of *cis*-mQTLs in this tissue and reported the detection of mQTL interactions to be independent of the case-control status (Numata et al., 2014). In MDD, evidence was presented that some of the MDD GWAS-associated risk SNPs act as *cis*-mQTLs (Barbu et al., 2020; N. R. Wray et al., 2018). Overall, mQTL studies show that interpretation of GWAS results can benefit from including mQTL approaches (Starnawska & Demontis, 2021). Future work to understand the impact of risk SNP would require more systematic studies incorporating DNAm information across lifetime and tissues.

4.4. Polygenic risk scores

In this work, the genetic contribution to some of the outcomes measured (developmental scores and psychiatric symptoms) was assessed using polygenic risk scores for educational attainment (EA), MDD, and SCZ and were computed. Within the field of GxE studies, the combination of PRS and environmental phenotypes (PRSxE) investigates the possible increase or decrease of PRS effects if a specific environmental risk factor is present (Mullins et al., 2016). In our study, the PRS only moderately correlated with the respective outcomes. Testing the interaction of the PRS with CM, the SCZ PRS showed no significant correlations. The MDD PRS did not improve the model using CM. This matches the findings from a previous meta-analysis of 5,765 individuals with depression, where no interaction effects of PRS and CT could be found (Peyrot et al., 2017). The failure to find significant PRS x E interactions might stem from the way PRSs are defined. They are typically derived from GWAS based on a case-control setting and are constructed to model additive effects of genetic variants, which does not incorporate environmental risks very well (Meisner et al., 2019). Interestingly, the EA PRS showed a significant interaction with all exposures (CM, adversity score, and SES) on all developmental outcomes, with the strongest interaction being EA PRS x CM ($p < 2.2*10^{-16}$). Previous studies have reported the interaction of EA PRSs and SES to predict EA (Bates et al., 2018). The PRS for EA, however, seems also to be associated with intermediate phenotypes, such as cognition (Richards et al., 2020; Smith-Woolley et al., 2019), which might explain the interaction effects.

4.5. Identifying maltreatment-specific methylation changes in children

WGCNA, a module-centric approach, was performed to identify co-methylated modules of CpG-sites when there was a group of CpGs that were specifically associated with CM, but not with any of the other exposures. This analysis was limited to the 10% most variable CpGs in terms of DNAm levels, as variable CpGs are enriched for functional regions and correlate with gene expression (Allum & Grundberg, 2020; Lioznova et al., 2019). This approach offers the advantage of not only focusing on functionally relevant DNAm regions but also alleviating the multiple testing problem. The WGCNA revealed one module (green module, consisting of 268 CpGs) which was exclusively associated with CM ($p = 0.04$, $r = 0.17$) and did not correlate with adversity in general ($p = 0.8$, $r = -0.2$), prenatal exposure ($p = 0.8$,

r = 0.023) nor SES (p = 0.9, r = 0.009). Although no strong effects on the individual CpG level could be observed, this finding suggests that a specific set of co-methylated CpGs is correlated with CM. CpGs in this module were on average demethylated in exposed children and this remained stable across time. This module also showed a strong association with sex (p = −0.79, r = $4*10^{-34}$), with the lowest DNAm presented in exposed boys. Previous studies have reported sex differences in resilience to CM as well as moderating effects of sex on the consequences of CM (Samplin et al., 2013; White & Kaffman, 2019). Annotation of the green module yielded no strong functional enrichments of the CpGs within this module, but a large proportion of genes (54 of 164 of the genes) mapped to long non-coding RNAs. Long non-coding RNAs are known to regulate gene expression by multiple mechanisms and are considered important players in developmental processes such as cell differentiation and genomic imprinting.

4.6. Validation of DNA methylation signatures in the LMU cohort

The next step aimed to externally validate the findings from the Berlin LCS cohort in an independent cohort to show the stability of the findings after childhood. The DMR analyses, the computation of the prenatal exposure scores, and WGCNA were repeated in the LMU cohort consisting of 640 adolescents aged 8 to 18 years with and without a diagnosis for MDD. While there were many DMRs comparing participants with and without a history of childhood abuse in the LMU cohort, none of these DMRs overlapped with the ones identified in the child cohort. The lack of overlap between DMRs might be due to multiple reasons: first the difference in the type of tissue which was used to measure DNAm, second the fact that changes of DNAm trajectories over time cannot be captured at a one-time measurement, and finally, that the DMRs do not remain stable past childhood.

WGCNA was rerun using the LMU sample and yielded three modules (cyan, dark red, and magenta) that were associated with childhood abuse. The dark red module was correlated with case-control status (MDD diagnosis) and the prenatal exposure scores. Interestingly, this module also significantly overlapped (p = $3.64*10^{-6}$, number of overlapping CpGs = 18) with the module which was associated with CM in the Berlin LCS cohort (green module). While the green module in the Berlin LCS cohort was exclusively correlated with CM, the dark red module in the LMU cohort was correlated with multiple exposures. This may be again due to the tissue specificity of DNAm, the difference in age ranges, but also might be due to the heterogeneity on how maltreatment was assessed in both cohorts

In contrast to the DMRs and the WGCNA modules, the prenatal exposure scores could be replicated in the LMU cohort. Participants with a history of childhood abuse also presented with significantly higher smoke exposure scores and with higher alcohol exposure scores. The same held for participants with MDD compared to healthy controls. The fact that these scores could be replicated across different tissues and show strong effects well beyond childhood suggests that exposures in the prenatal period of life result in broader and more long-lasting effects on DNAm than CM. This is supported by the fact that the majority of WGCNA modules in both the Berlin LCS and the LMU cohort were correlated with the prenatal exposure scores.

Complementing the external validation in the LMU cohort, I overlapped the CpGs from the DMRs and the green module with findings from a study on an independent adolescent cohort by Cecil et al. (2016). In this study, the authors quantified DNAm in buccal epithelial cell samples from a high-risk sample from the London inner-city youth (n = 124; age = 16–24; 53% female), with 68% of the participants reporting to have experienced at least one form of maltreatment while growing up. CM was retrospectively assessed using the CTQ. Only two DMPs associated with physical neglect in the study from Cecil et al., overlapped with CpGs within the DMRs (cg16210526 and cg10390589). Interestingly, both of these CpGs were also included in the green module associated with CM in the Berlin LCS cohort.

4.7. Improving replication of findings across cohorts

A large number of studies, including candidate-gene and epigenome-wide studies, (Parade et al., 2021) have investigated the effects of CM on DNA and while these studies collectively support an association of CM and other adversities with DNAm, there are still some replication inconsistencies that need to be addressed in future research.

4.7.1. Tissue specificity of DNAm

In most of the studies on CM, DNAm has been measured in several peripheral tissue types, including blood, saliva, and buccal cells (Cecil et al., 2020b). Studies investigating CM in children mostly rely on buccal or saliva samples (Martins et al., 2021; Parade et al., 2017; Parent et al., 2017), while adolescent and adult cohorts measure DNAm in blood (Cecil et al., 2016; Halldorsdottir et al., 2019; Klengel et al., 2013). Some studies have reported good correspondence of DNAm in blood and saliva and the investigation of methylation age has also shown to be consistent across tissues (Horvath, 2013). While it has been proposed that psychiatric epigenetic research to

identify causal alterations should be limited to brain tissue, many studies point to the value of peripheral indicators. According to Langie et al., who performed a whole-genome comparison of DNAm levels in saliva and blood using the 450k platform, DNAm levels of about 96% of the CpGs were comparable between peripheral blood mononuclear cells and saliva (Langie et al., 2016). Even though the correlation of DNAm in the brain with DNAM in saliva, blood, and buccal cells were all reported to be high, the strength of these associations depends on the genomic region of interest (Braun et al., 2019). Although the standard procedure of analyzing DNAm data includes the estimation and correction for the cell-type composition of the tissue, cell type heterogeneity remains a significant challenge for epigenetic research (Parade et al., 2021).

It is important to note that this work was performed in saliva DNAm, which may originate from a combination of blood leukocytes and buccal epithelial cells. There is some evidence showing that salivary DNA may more closely reflect methylation patterns in the brain than DNA from leukocytes (A. K. Smith et al., 2015). Saliva therefore might be a valuable surrogate tissue for the assessment of pathophysiology and could be used to reveal putative peripheral biomarkers. Even though DNAm is tissue-specific, some of the effects of CM on DNAm in peripheral tissues might point towards causal findings. This holds especially true if findings can be replicated across multiple tissues and match effects on other molecular layers, such as gene expression. For example, converging lines of evidence indicate that methylation of GREs in *FKBP5* in DNA from the periphery may have functional effects in the body and may reflect changes to GR sensitivity in the brain (Tyrka et al., 2015).

4.7.2. Power and sensitivity considerations

One major challenge in the study of environmental exposures such as CM is the power required to detect true changes and eliminate false positives. Large sample sizes and replication cohorts with comparable measures are required, however, the very large cohorts typically do not provide the depth of phenotyping and data on the exposure (Dunn et al., 2016). Longitudinal studies additionally include the problem of dropouts.

The Berlin LCS cohort for example presents with a relatively small sample size and a high drop-out rate of 47,97% (drop-out rate of 57,83% for maltreated and 40,22% for non-maltreated) over time. Additionally, it could be observed that more cases dropped out than controls and dropouts had significantly higher adversity scores at baseline than the remaining individuals (mean(dropouts) = 2.92, mean(remaining) = 2.18,

p = 0.02). Some of these children might have been removed from their social context and therefore were excluded from the study. Another explanation could be that some of the children reached school age and attending the follow-up assessments might have been too time-intensive for the families. In particular, the interaction models over all time points are underpowered to detect small effects, as the last time point only included 90 samples.

In order to investigate the effects of dropouts on the study, I performed a sensitivity analysis. To do so the linear mixed models, as well as the aggregation step, were re-run using only the complete samples for time point 4 (n = 102) and time point 5 (n = 83). The findings with completers at time point 4 were very consistent with the findings on the complete data, however lost significance using only completers at time point 5. It needs to be pointed out that here only 38 cases remained in the analysis.

To address the concerns of sample size, a power analysis tailored to longitudinal models was performed. A pilot model of the CpG with the median effect was used to estimate power to detect a 5% change in methylation levels attributed to the fixed effect of interest (time, adversity, time: adversity). The power of the model over time was calculated to be 76,2%, for the additive model (Time + E) it was 79,79% (time) and 15,9% (adversity). For the interaction model, the power calculated for the interaction term was 47%. Although the study was underpowered for testing interactions, the findings highlight the importance of the effect of CA specifically on DNAm trajectories.

4.7.3. Harmonizing exposure measures

The variability of measures and sample sizes feasible makes it difficult to compare findings between cohorts and trade-off between deep phenotyping and sample size. Overall, very few studies investigating the effects of CM on DNAm included an independent replication sample or meta-analyzed findings from different samples (Cecil et al., 2020b). Most of the different information on exposure to CM is collected across cohorts, ranging from prospectively to retrospectively assessed questionnaires and information from official registries to self-reports. Subjective measures of CM are not always in accordance with objectively documented cases of maltreatment but show stronger relationships with psychopathology than only objectively ascertained cases of maltreatment that are not subjectively recalled as maltreatment (Danese & Widom, 2020). Additionally, different subtypes of CM (sexual, physical, emotional) might have different effects on DNAm (Cecil et al., 2016; Saito et al., 2020). In summary, well-defined measures of CM that include dimensions of severity, timing, and subjective impact would be required across cohorts.

4.7.4. Reliability and coverage of DNAm measurement methods

While array-based technologies are already widely used to measure DNAm, the reliability of the data generated is surprisingly variable (Pidsley et al., 2016). A study comparing probes on the 450K and EPIC, found that BeadChip reliability correlations ranged from −0.34 to 0.95 with a median value of 0.15, and only 2.6% of the ~420,000 probes assayed had reliability correlations above 0.8 (Sugden et al., 2020). This finding shows patterns of uneven reliability in the repeated measurement of DNAm. This needs to be considered when making comparisons with data from older studies.

Another aspect that needs to be acknowledged when investigating the effects of environmental exposure epigenome-wide and specifically on candidate genes is the coverage of CpGs. Although commonly available array technologies allow us to measure DNAm levels across hundreds of thousands of CpG sites, they only cover 2–4 % of CpG sites across the genome (Flanagan, 2015), leaving a large proportion of CpG sites unmeasured. Moreover, the EPIC array offers improved but still suboptimal coverage of regulatory elements (Pidsley et al., 2016). Using FKBP5, an important modulator of the stress hormone system, as an example: The EPIC array covers 29 CpGs within the gene, of which the majority is located near the TSS. Only a few of the CpGs covered within *FKBP5* are located within Intron 5 or Intron 7, for which the majority of significant findings following CM have been reported (Klengel et al., 2013; Klinger-König et al., 2019; Saito et al., 2020). WGBS can capture more than 28 million CpGs (Stevens et al., 2013), but the feasibility remains low for the population-based EWAS due to the high cost and large genomic DNA input requirements to compensate for degradation during DNA bisulfite treatment (Heiss et al., 2020). High-accuracy targeted approaches using bisulfite sequencing in candidate loci would increase the power to detect and replicate previously reported effects as well as allow researchers to perform time-course experiments in large numbers of samples to understand the stability of the environmentally induced changes during development (Roeh et al., 2018).

4.8. Focusing on *FKBP5* using a fine-mapping approach

Epigenetic alterations following CM have been documented and could be replicated for some candidate genes, with the most commonly studied candidates being those that regulate GC signaling, including *NR3C1* and *FKBP5,* which modulate the sensitivity of the GR (Parade et al., 2017). Klengel et al (2013) previously suggested the following model for the *FKBP5* × childhood abuse interaction: genetic differences, such as the

well-studied polymorphism rs1360780, lead to divergent chromatin conformations and interactions of long-range enhancers with the TSS. This induces a differential transcriptional activation of *FKBP5* by GR activation in response to CM. DNAm in the intronic enhancer regions adds to the differential responsiveness of *FKBP5* to GR activation (Klengel et al., 2013). While these effects were replicated by some studies (Misiak et al., 2020; Tozzi et al., 2018), several studies did not find significant associations of CA and *FKBP5* methylation (Yeo et al., 2017) or moderation by the risk allele (Bustamante et al., 2018; Klinger-König et al., 2019).

The last part of this dissertation focused on employing a fine-mapping approach in order to investigate DNAm changes in key regulatory regions of *FKBP5*. Targeted-bisulfite sequencing was applied to a subset of samples from the Berlin LCS. DNA originated from saliva samples from two time-points: at baseline (n=162, 83 maltreated children, 79 controls) and one-year follow-up (n=117, 54 maltreated children, 63 controls).

As already discussed, most of the studies investigating the effects of early-life adversity or specifically CM, including the study by Klengel et al., were performed in adults using retrospective measures such as the CTQ. Few studies investigated the effects of CM on *FKBP5* methylation in children. Tyrka et al. previously presented evidence for significant demethylation following CM at two CpGs within intron 7 of *FKBP5* in children aged 3-5 years and a trend for interaction with the *FKBP5* polymorphism rs1360780. Parade et al. (2017) investigated if CM contributed to changes in DNAm at the same two sites in intron 7 over 6 months in a sample of preschoolers and found an association of *FKBP5* methylation over time exclusively when the children were exposed to contextual stressors and report a genotype-dependent effect for child-welfare service utilization. To my best knowledge, this study is the first to systematically investigate DNAm alterations following CM at the *FKBP5* locus directly in children with documented cases of maltreatment. Given that the interaction with the genotype and adversity seems to be restricted to early life, as no relationship between *FKBP5* DNAm and adversity has been reported in adults (N. Alexander et al., 2020), this work represents an important addition.

4.8.1. Stability of DNAm changes in *FKBP5* regulatory regions

Out of the 50 CpGs covered by the amplicons selected, differential methylation between maltreated and non-maltreated at baseline could be observed for the majority of CpGs (n = 41, lowest p = $2.04*10^{-281}$, largest methylation change: 4.22%). Exploring

the direction of effects, the majority of CpGs in the intronic enhancer regions (Intron 5, Intron 7) were demethylated in children exposed to maltreatment (lowest $p = 4.63*10^{-179}$, largest methylation change: -3.02%). This matches the findings for the two CpGs investigated in previous studies: CpG1 at position 35558488 (largest demethylation in our data with the lowest p-value, methylation change: -3.02, $p = 4.63*10^{-171}$) and CpG2 at position 35558514 (methylation change: -2.57, $p = 6.83*10^{-31}$). These two CpGs showed some of the strongest effects and were located within a consensus GRE motif. Reduced intron 7 methylation has previously been associated with higher induction of FKBP5 by GR activation, leading to increased GR resistance. Although DNAm marks are tissue-specific, GC-induced demethylation of regulatory regions of *FKBP5* in blood was associated with anxiety-like behavior (R. S. Lee et al., 2011) and levels of *FKBP5* methylation in blood were significantly correlated with both methylation and gene expression in the hippocampus (Ewald et al., 2014). Additionally, demethylation of intron 7 was also shown in a human hippocampal progenitor cell line following GC exposure (Provençal et al., 2020). These findings, together with the findings in the Berlin LCS cohort, suggest that this specific peripheral marker might be informative of brain function. To date, no studies reported the association of the intron 5 DNAm with a psychiatric disorder and the study by Klengel et al. did not find any significant association with childhood abuse (Mendonça et al., 2021). This finding might point to the fact that intron 5 methylation changes following CM might be tissue-specific. However, a human cell experiment line treated with cort showed that changes in intron 5 CpG methylation and *FKBP5* expression were inversely associated (Duis et al., 2018). Additionally, most of the CpGs in the proximal enhancer regions were hypermethylated in the children exposed to maltreatment, while the CpGs within the CTCF binding sites (in the flanking TAD regions) showed mixed direction effects. While there are no studies specifically investigating the effects of DNAm changes within the CTCF sites flanking the FKBP5 locus, a DNAm increase directly at CTCF binding sites can lead to loss of CTCF binding and therefore disruption of chromatin interactions, which can lead to dysregulated gene expression. The changes at CTCF sites following CM were rather small (0.5-1.0%) and a study by Wiechmann et al, investigating these sites following dexamethasone (a synthetic GC) treatment showed similar changes and proposed that they might alter enhancer function but not disrupt the chromatin 3D structure (Wiechmann et al., 2019).

Next, the repeated measure at the one-year follow-up time point was used to assess the stability of the CM effects detected at baseline. Differentially methylated CpGs at baseline were considered stable if they showed the same direction of effect after one year. This held for 25 CpGs of 41 CpGs across all regulatory regions covered,

with some of the CpGs even showing larger methylation differences between the groups than compared to baseline. The direction of effect of CpG1 remained stable after one year (uncorrected p-value: 0.02, diffMeth: -0.46%), however, this was not the case for CpG2. The stable differentially methylated CpGs detected in the Berlin LCS cohort overlapped with differentially methylated CpGs following acute dexamethasone treatment (1 h, 3 h, 6 h) reported by Wiechmann et al. and co-localizing with GC receptor binding sites (Wiechmann et al., 2019). These findings point towards early exposure to CM changing the set point of future *FKBP5* dynamics in response to stress by inducing stable DNAm changes. This supports the proposition that CM increases the risk of developing psychopathology due to a sensitization of the neurobiological systems implicated in stress adaptation and response (Heim et al., 2008).

While the repeated measure represents an advantage of this work, as it allows the assessment of the stability of CM induced DNAm changes, the smaller sample size at the follow-up time point (n=111) might be the cause of the differential methylation analysis at this time point to yield no significant effects after correction for multiple testing. Although we observed that the effects remained stable after one year, our finding needs to be replicated in a larger study. Additionally, in the Berlin LCS cohort maltreatment events were still present between the baseline and the follow-up measurement, therefore it would be interesting to examine if differential methylation effects remain stable without CM and how DNAm changes relate to fluctuating exposure to adversity. Finally, it would be necessary to extend the time frame of the longitudinal studies to assess to what extent the effects of CM on *FKBP5* maltreatment are long-lasting.

4.8.2. Genotype effects at the *FKBP5* locus

As a next step, potential genotype effects of rs1360780 on DNAm in functional regions of *FKBP5* were investigated. This SNP has previously been associated with differential up-regulation of *FKBP5* expression and increased GR sensitivity (Klengel et al., 2013; Xie et al., 2010; Zimmermann et al., 2011). Carriers of the risk genotype in the Berlin LCS cohort showed significantly lower methylation levels at 2 CpGs within the CTCF binding site at 3'TAD. Investigating the sites where CM adds to the genotype effect on DNAm, we found 4 CpGs in the intronic enhancer, 2 in the proximal enhancer, and 1 in the CTCF binding site at the 5'TAD. From these CpGs, one significant interaction was identified for one CpGs within intron 5 (at position 35569751 in intron 5, adjusted r^2 = 0.10, p = $2.8*10^{-2}$). Carriers of the protective genotype and without exposure to CM had the highest methylation levels, while carriers of the risk allele without exposure to CM had the lowest methylation levels.

The genotype-specific effects in the Berlin LCS cohort do not match the findings of Klengel et al., who reported that *FKBP5* risk allele carrier status and early trauma exposure lead to demethylation of intron 7 CpGs in *FKBP5.* While in the Berlin LCS cohort intron 7 CpGs were demethylated in children exposed to maltreatment, there was no significant genotype effect observed for these CpGs. Nevertheless, the Berlin LCS cohort showed significant genotype effects or moderation effects on the topologically associated domains, which support the proposed mechanism of genotype-mediated long-term environmental reactivity via chromatin interactions. Genotype effects of rs1360780 on intron 5 were reported by Duis et al., who performed a study using cord blood samples (Duis et al., 2018). They reported an association of the risk allele (TT) was associated with methylation at multiple CpGs in the *FKBP5* intron 5 region. These findings support that rs1360780 might influence *FKBP5* intronic methylation by acting as cis-mQTL. However, the tissue specificity and stability of these findings need to be investigated over a longer time frame and past childhood.

4.8.3. *FKBP5* DNAm and biodata changes are linked

Next, I explored the relationship between *FKBP5* methylation and biodata levels available in our cohort, including cort). In addition to cort, AA and CRP were measured in the Berlin LCS cohort. AA is considered an important salivary biomarker of stress, with an abrupt increase of AA concentrations following acute stress (Chojnowska et al., 2021). CRP, among other inflammatory markers, has been reported to be elevated following chronic stress (Miller et al., 2019) and is associated with increased risk for stress-related disorders (Wium-Andersen et al., 2013).

At baseline AA, CRP and cort were mainly associated with DNAm of a few CpGs in the 3'TAD. These CpGs overlapped with some of the CpGs what also showed significant associations with the rs1360780 genotype, which might suggest that the salivary biomarkers associated with stress are linked to GR activation. Additionally, changes between baseline and the one-year follow-up of biodata and DNAm changes over time were associated: correlations could be observed for 4 CpGs in the proximal enhancer and 2 in the intronic enhancer in Intron 5 (lowest $p = 1.7*10^{-3}$, adjusted $r^2 = 0.10$).

Interestingly, there was no correlation between cort and any CpG in intron 7 that could be detected. This matches recent findings by Alexander et al. who tested the link between chronic and acute cort output and *FKBP5* methylation in healthy individuals and found no significant association, even if the genotype was taken into consideration (N. Alexander et al., 2020). The lack of effects of CM on *FKBP5* DNAm in a healthy

cohort might be explained in the way that it might be a modulator of the mental state and can only be observed in individuals with psychopathology. This would need to be tested in a larger sample with cases only.

4.8.4. Overlapping DNAm pattern between CM and psychopathology

DNAm changes at the *FKBP5* locus have been reported for various mental disorders such as MDD (Park et al., 2019) and PTSD (Kang et al., 2019) and could be attributed to CA (Parade et al., 2021). Demethylation following CA has been shown in regulatory elements in intron 2 and 7 of *FKBP5*, consequently increasing gene expression following GR signaling and impeding the negative feedback mechanism of the HPA axis (Klengel et al., 2013).

In the last part of the work on DNAm change within regulatory regions of *FKBP5*, I tested if the differential methylation patterns following CM overlapped with DNAm differences associated with psychopathology. At baseline, the majority of CpGs (42 of the 57 covered) were differentially methylated between children that scored any symptom on the PAPA and children without symptoms, with the largest methylation difference being 5% change and the lowest p-value being $p = 3.1*10^{-254}$. 38 CpGs overlapped with the differentially methylated CpGs following maltreatment and overall the direction of effects (demethylation in the intronic enhancers in children with symptoms, hypermethylation in the proximal enhancer, and mixed effects in the CTCF binding sites).

While the small sample size (smallest group: maltreated children without symptoms = 15) did not allow a subgroup analysis, the overlap between differential methylation following CM and differential methylation in psychopathology supports a potential overlap in the underlying molecular mechanisms.

4.9. Conclusion and future directions

Overall, the findings in the Berlin LCS cohort highlight the fact that CM often occurs in an environment that also includes various other types of adversities and environmental factors that mean CM cannot be investigated as an isolated exposure. The presence of additional adversities such as prenatal exposures, SES, and other life events and adversities likely contribute to changes observed with CM and possibly also add to the overall risk and resilience trajectories. Most of the findings were associated with both CM and the adversity score, with stronger effects in some time series models of the latter. The findings additionally suggest the convergence of prenatal and postnatal

adverse exposures on DNAm, with most of the DMRs following CM correlating with the prenatal exposure scores. Only a small DNAm module was exclusively associated with CM and did not correlate with the other exposures. While the first part of this work identifies some interesting loci, especially located in long non-coding RNAs, its main message is focused on the importance of not only mapping the epigenome but also the environment, extending the timeframe to well before birth.

The second part of this thesis, which was directed towards extending the findings from the Berlin LCS cohort beyond childhood, revealed key challenges to be addressed by future research in CM and epigenetics. None of the CM and adversity-related findings could be replicated in the LMU cohort, this is most likely due to the heterogeneity of both cohorts. Considerations for future study design include the tissue in which DNAm is sampled, measures of exposure, time frame, and sample size, unmeasured environmental and genetic influences, and the functional characterization of epigenetic findings. The fact that the prenatal exposure scores remained stable across tissues and age ranges emphasizes the importance of including the pre-exposure environment, especially the prenatal period, in the study design. Finally, the possibilities and limitations of the tools used to measure DNAm need to be considered. The choice of measuring method defines the space for the discovery of CpG loci associated with CM and while the EPIC platform will remain a central tool, targeted approaches might be more suitable to support findings in candidate genes.

The last part of this thesis, the systematic study of DNAm changes at the *FKBP5* locus following CM, validated previous findings and yielded novel insights into the epigenetic regulation of *FKBP5* dynamics. The stability of the findings after one year supports that long-term effects of CM may be mediated by DNAm changes. It will be crucial for future investigations to disentangle shared and distinct patterns of CM and psychopathology and include potential moderators in the associations identified. More work investigating the regulatory effects of other epigenetic mechanisms on *FKBP5* expression changes will be required.

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6. Supplement

Supplementary figures and tables include supporting information on the analyses using the Berlin LCS data. These figures and tables have previously been published in the supplementary material of Martins et al., 2021.

6.1. Supplementary Figures

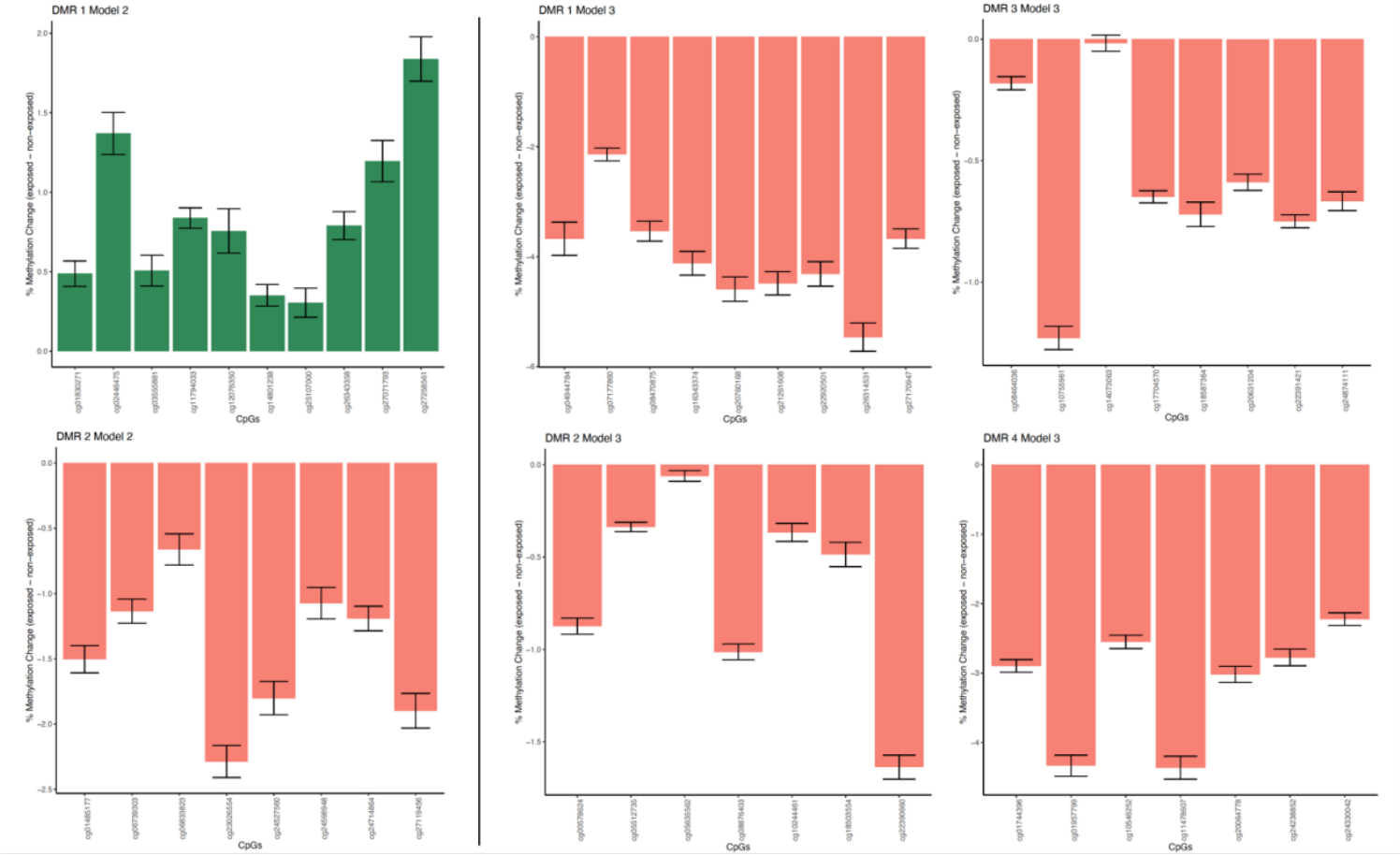

Figure S1: Direction of effect for the differentially methylated regions (DMRs). Differential methylation for each CpG within the significant DMRs obtained from models 2 and 3 for T0-T2. DMRs obtained from model 2 are shown in the left panel, DMRs from model 3 in the right. Most of the regions are hypomethylated (shown in orange) in subjects with high adversity scores, only one DMR from model 2 is hypermethylated in the exposed group.

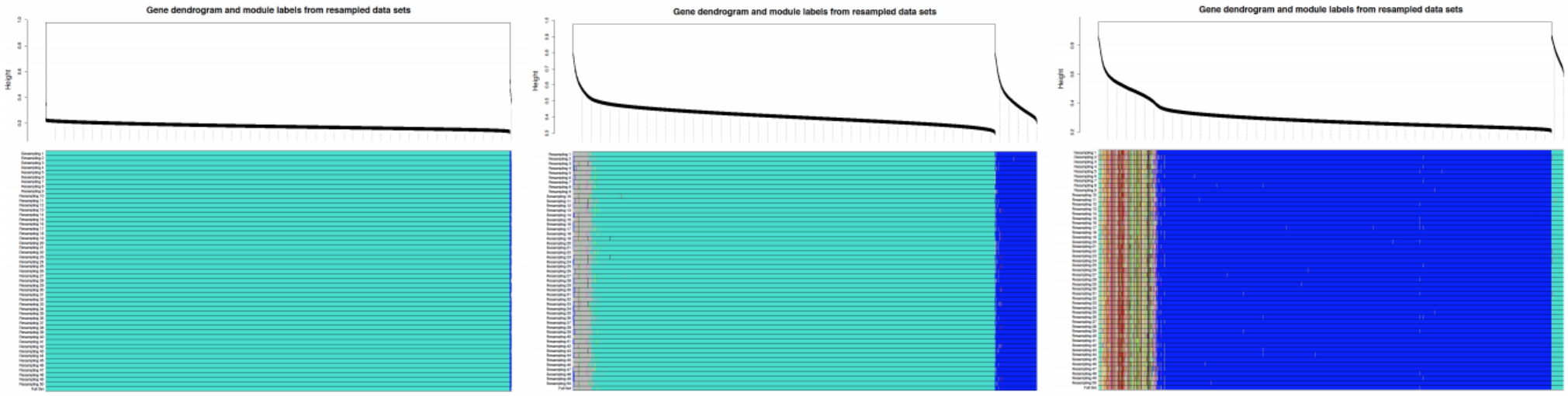

Figure S2: Module stability analysis in the Berlin LCS sample. Block-wise module construction with a maximum module size of 10,000 (due to computational complexity) was repeated (n=50) with 66% of the samples. The full data set was computed using 3 blocks at maxBlockSize of 30,000. Shown here are the dendrograms (blocks1-3) of the full data set with the matched assignments of the resampled networks. The top of the plot shows the dendrogram for each of the blocks, the bottom shows the assignment to the modules for each CpG, where each row represents one resampled network.

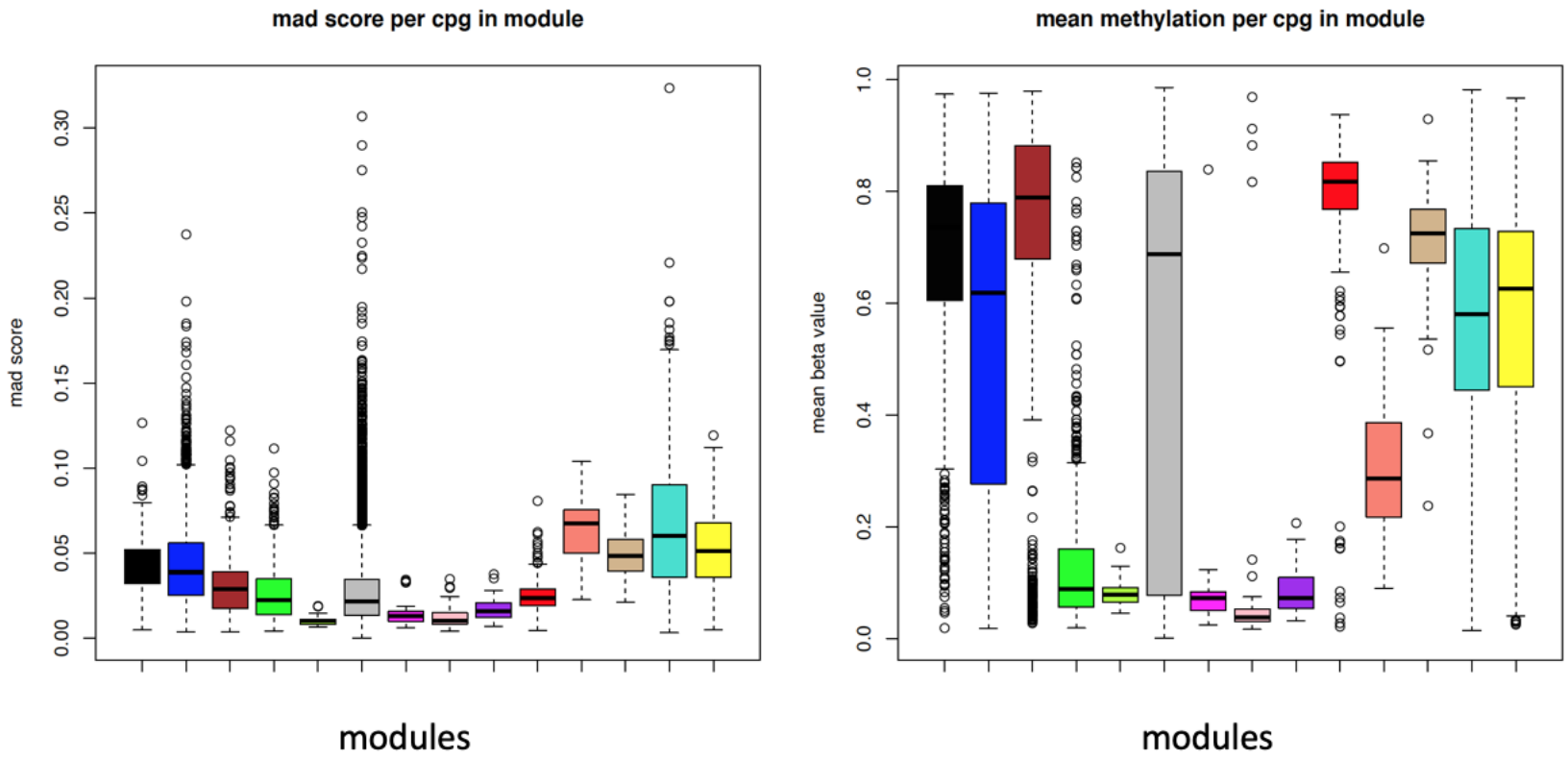

Figure S3: Mean methylation and median absolute deviation (MAD) score by module. In order to investigate if the distribution of beta-values impacted the module detection. Differences in MAD scores (left) and methylation levels (right) between the modules were analyzed. Both showed significant differences between the modules.

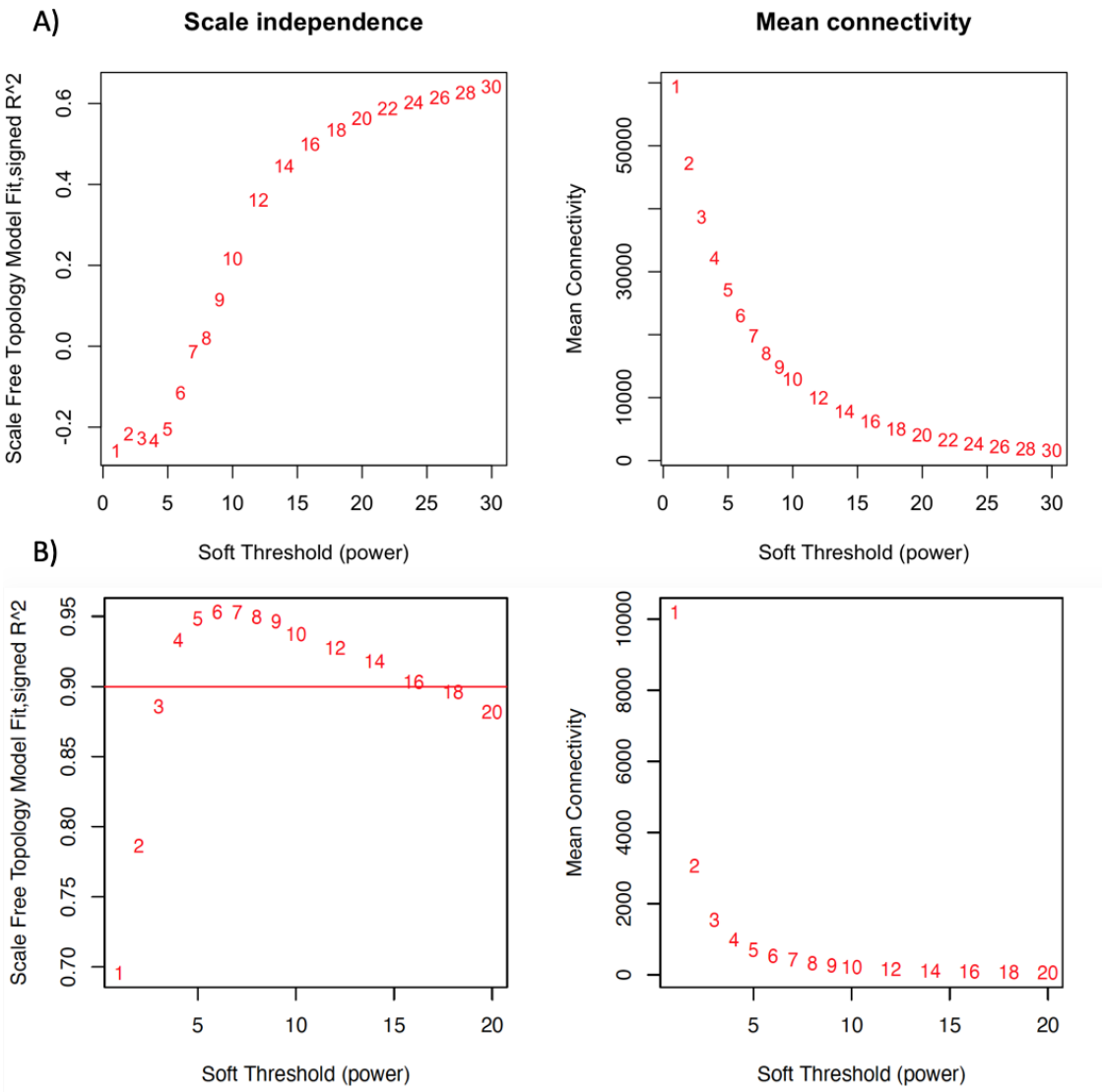

Figure S4: Scale-free topology fit and mean connectivity using M-values compared to beta values. Comparison of scale-free topology fit (left) and mean connectivity (right) for standardized M-values (top half) and beta values (bottom half). The first power to reach an r^2 of 0.8 with mean connectivity in the hundreds is considered good.

6.2. Supplementary Tables

Table S1: Differentially methylated regions aggregated from linear mixed models (Model 1-3) using all time points (T0-T5). Only regions with more than 5 CpGs that passed the multiple testing correction are reported with the nominal p-value. No significant hits were found for models 2 and 3.

Models	**DMRs**	**# CpGs**	**p-value**	**genes**	**annotation**[1]
Time (Model 1)	chr5:126409006-126409311 chr5:178986130-178986831 chr6:30095135-30095496 chr7:24323674-24323940 chr7:63386225-63387148 chr7:130125763-130125985 chr8:22132562-22133357 chr14:24780550-24780891 chr21:45705617-45705743	8 13 17 7 7 7 13 9 7	$2.97*10^{-4}$ $4.23*10^{-6}$ $9.83*10^{-9}$ $1.24*10^{-3}$ $1.43*10^{-10}$ $3.21*10^{-2}$ $1.23*10^{-8}$ $1.18*10^{-2}$ $1.70*10^{-2}$	C5orf63 exon 1 RUFY1 intron 1 Multiple HLA-REs* NPY exon 1 RP11-73B2.6 MESTIT1 exon 1 PIWIL2 exon 1 CIDEB exon 1 AIRE exon 1	Prenatal phthalate exposure Prenatal lead exposure Immune-related Maternal stress, neuroendocrine function Angiogenesis Maternal stress Prenatal lead exposure Cell death & inflammation Autoimmune regulator
Time + Adversity (Model 2)	n.s	-	n.s.		
Time + Maltreatment (Model 2)	n.s	-	n.s.		
Time x Adversity (Model 3)	n.s.	-	n.s		
Time x Maltreatment (Model 3)	n.s.	-	n.s.		

* regulatory element, n.s.: not significant

Table S2: Differentially methylated regions aggregated from linear mixed models (Model 1-3) using the first three-time points (T0-T2). Only regions with more than 5 CpGs that passed the multiple testing correction are reported with the nominal p-value. No significant hits were found for the time x CM interaction (Model 3).

Models	**DMRs**	**# CpGs**	**adjusted p-value**	**gene**	**annotation**[3]
Time (Model 1)	chr1:110254678-110254920	8	$3.18*10^{-3}$	GSTM3/5	maternal smoking, prenatal solvent exposure, FAS[1]
	chr3:182817189-182817627	12	$6.40*10^{-9}$	enhancer	
	chr8:144635259-144635611	10	$8.15*10^{-6}$	MCCC1 exon 1	brain volume in schizophrenia
	chr11:2721242-2721633	12	$2.71*10^{-8}$	GSDMS exon 1	pyroptotic cell-death
	chr14:106938233-106938452	6	$8.09*10^{-3}$	KCNQ1 intron 11	neuronal excitability, prenatal arson exposure
	chr19:57742259-57742445	7	$1.51*10^{-3}$	LINC22001	-
	chr20:36148603-36148780	11	$1.59*10^{-3}$	AURKC exon 1	Prenatal lead & prenatal tobacco exposure
				BLCAP intron 1	FAS
Time + Adversity (Model 2)	chr6:31275147-31275808	10	$7.02*10^{-7}$	HLA-B intron 2	Immune-related gene
	chr10:42862977-42863595	8	$9.83*10^{-9}$	ZFP91 pseudogene	Cell proliferation / migration
Time + Maltreatment (Model 2)	chr6:31275147-31275808	10	$1.35*10^{-5}$	HLA-B intron 2	Immune-related gene
	chr10:42862977-42863595	8	$2.25*10^{-9}$	ZFP91 pseudogene	Cell proliferation / migration
Time x Adversity (Model 3)	chr2:26401597-26402319	10	$1.88*10^{-8}$	GAREML intron 1	FAS, childhood abuse
	chr3:189839037-189839358	7	$3.13*10^{-4}$	P3H2 intron 1	Childhood abuse
	chr19:9785646-9786078	8	$9.32*10^{-4}$	ZNF562 exon 1	Early-onset MDD[2]
	chr22:24384104-24384401	8	$1.28*10^{-4}$	GSTT1 exon 1	FAS
Time x Maltreatment (Model 3)	-	-	n.s.		

[1] Fetal alcohol syndrome
[2] Major depressive disorder

Table S3: Overview of WGCNA modules in the Berlin LCS sample

Detailed information on associations with exposure variables and other outcomes of interest summarized in Figure 10, information on GWAS overlap and enrichment for pathways (KEGG, GO and Reactome) can be found in Supplementary tables S4 and S5.

Module	#CpGs / genes	Associations	Enrichment
Brown	659 / 637	-	-
Pink	64 / 49	-	-
Blue	22,531 / 8.371	prenatal scores	cell signaling / immune related
Black	91/ 81	-	diabetes type I
Yellow	317 / 143	sex	-
Turquoise	56.344 / 14.736	prenatal scores	-
Green	268 / 164	maltreatment, sex	-
Red	107 / 87	-	-

Table S6: Module annotation in the Berlin LCS sample: Overlap with GWAS top hits. All CpGs within a module were mapped to genes based on the annotation of the EPIC array (hg19) and then used as input for enrichment tests with FUMA. Shown here are the top five overlapping GWAS hits per module, where enrichment passed the multiple testing correction.

Module*	GWAS	#genes (overlap)	Adjusted p-value
Black	-	-	n.s.
Blue	Heel bone mineral density Systolic blood pressure Body-Mass-Index Monocyte percentage white cells Mean platelet volume	389/767 349//746 492/1209 100/147 132/233	$1.56*10^{-46}$ $2.78*10^{-32}$ $1.92*10^{-27}$ $5.57*10^{-25}$ $5.18*10^{-21}$
Brown	Type II diabetes Systolic blood pressure Modic change Atrial fibrillation Asthma	248/458 36/746 4/7 16/221 18/311	$6.94*10^{-5}$ $2.44*10^{-4}$ $1.92*10^{-3}$ $1.92*10^{-3}$ $6.15*10^{-3}$
Green	-	-	n.s.
Grey	Pneumonia Drug induced liver injury IgE levels Asthma Neuromyelitis optica	8/8 9/13 10/18 43/311 7/9	$3.01*10^{-8}$ $5.48*10^{-7}$ $9.39*10^{-7}$ $9.39*10^{-7}$ $4.43*10^{-6}$
Pink	-	-	n.s.
Red	-	-	n.s.
Yellow	-	-	n.s.

* the turquoise module was not annotated as it contained over 50% of the CpGs from the analysis

Table S7: Module annotation in the Berlin LCS sample: Enrichment in specific pathways. The top five pathways per module from the functional annotation analysis using FUMA.

Module*	Pathway	#genes (overlap)	Adjusted p-value
Black	-	-	n.s.
Blue	Chemokine signaling Focal adhesion Regulation of actin skeleton Leukocyte migration B-cell receptor signaling	91/177 96/195 98/205 60/111 42/72	$4.31*10^{-11}$ $1.82*10^{-10}$ $7.40*10^{-10}$ $1.17*10^{-8}$ $1.61*10^{-7}$
Brown	Diabetes Mellitus Type I Allograft rejection	6/40 5/34	$1.93*10^{-2}$ $2.71*10^{-2}$
Green	-	-	n.s.
Pink	-	-	n.s.
Red	-	-	n.s.
Yellow	-	-	n.s.

* the turquoise module was not annotated as it contained over 50% of the CpGs from the analysis

Table S8: Functional annotation of modules in the LMU cohort. The top five pathways per module in the LMU cohort that were associated with any type of exposure using FUMA.

Module*	Pathway	#genes (overlap)	Adjusted p-value
Black	T-cell receptor signaling	11/106	$5.71*10^{-5}$
	Natural killer cell-mediated cytotoxicity	11/121	$1.09*10^{-4}$
	Adherens junction	7/73	$4.67*10^{-3}$
	Cytokine-cytokine receptor interaction	12/235	$6.24*10^{-3}$
	Chemokine signaling	10/177	$7.99*10^{-3}$
Blue	Adherens junction	25/73	$3.28*10^{-8}$
	Tight junction	33/128	$1.08*10^{-7}$
	Axon guidance	31/126	$4.56*10^{-7}$
	Calcium signaling	35/170	$5.56*10^{-6}$
	Long term depression	19/68	$2.31*10^{-5}$
Brown	T-cell receptor signaling	25/106	$8.63*10^{-10}$
	Neurotrophin signaling	22/126	$2.16*10^{-6}$
	Natural killer cell-mediated cytotoxicity	20/121	$1.69*10^{-5}$
	Focal adhesion	25/191	$5.98*10^{-5}$
	Leukocyte transendothelial migration	17/111	$2.08*10^{-4}$
Cyan	Protein-Protein interaction at synapses	5/85	$3.43*10^{-2}$
	Neuronal system	9/394	$3.43*10^{-2}$
	Neurexins and neuroligins	4/53	$3.77*10^{-2}$
Dark green	-	-	n.s.
Dark red	Reactive Oxygen Species Pathway	2/46	$2.66*10^{-2}$
	TGF Signaling	2/53	$2.66*10^{-2}$
Green	Rho GTPase cycle	28/421	$9.56*10^{-3}$
	Cell-cell communication	13/127	$1.58*10^{-2}$
	Extracellular matrix organization	21/288	1-$63*10^{-2}$
	Collagen formation	10/87	$3.39*10^{-2}$
	Cell junction organization	10/90	$3.39*10^{-2}$
Green-yellow	-	-	n.s.
Grey60	MAPK signaling	15/295	$1.77*10^{-2}$
	Long term Depression	3/68	$2.11*10^{-2}$
	Phosphatidylinositol signaling	3/76	$2.11*10^{-2}$
	Gap junction	3/86	$2.27*10^{-2}$
	GNRH signaling	3/100	$2.82*10^{-2}$
Light cyan	-	-	n.s.
Light green	-	-	n.s.
Light yellow	-	-	n.s.
Magenta	Phosphatidylinositol Signaling	6/40	$1.32*10^{-2}$
	Chromatin Organization	12/252	$3.81*10^{-2}$
	Mitochondrial Calcium Ion Transport	4/22	$3.81*10^{-2}$
	Glycosaminoglycan Metabolism	8/118	$3.81*10^{-2}$
Midnight blue	-	-	n.s.
Pink	Developmental biology	31/1091	$4.04*10^{-3}$
	Cell-cell junction organization	7/63	$1.09*10^{-2}$
Purple	Extracellular matrix organization	12/288	$3.02*10^{-2}$
	Laminin interaction	4/30	$3.16*10^{-2}$
	PTK2 signaling	4/30	$3.16*10^{-2}$
Red	RhoA regulation	9/46	$4.20*10^{-4}$
	Cell junction organization	12/90	$4.20*10^{-4}$
	Rho GTPase cycle	14/135	$7.43*10^{-4}$
	Extracellular matrix organization	21/288	$7.43*10^{-4}$
	Collagen formation	11/87	$9.17*10^{-4}$
Royal blue	Huntington disease	3/163	$2.42*10^{-2}$
	Inositol phosphate metabolism	2/54	$4.72*10^{-2}$
Salmon	-	-	n.s.
Tan	-	-	n.s.

Turquoise	Chemokine signaling	75/177	$3.36*10^{-11}$
	Focal adhesion	78/195	$2.71*10^{-10}$
	Regulation of actin skeleton	79/205	$1.36*10^{-9}$
	MAPK signaling	91/259	$1.22*10^{-8}$
	Wnt signaling	57/146	$2.18*10^{-7}$
Yellow	Focal adhesion	22/195	$9{,}41*10^{-4}$
	Receptor tyrosine kinase signaling	36/453	$9{,}41*10^{-4}$
	Adherens junction	12/73	$2.41*10^{-3}$
	Gaba B receptor activation	8/36	$4.85*10^{-3}$
	Cell-cell communication	15/127	$4.84*10^{-3}$

Table S9: Differential Methylation at baseline measured by TBS. The significantly differentially methylated CpGs (n=41) between maltreated and non-maltreated children.

PCR name	position	P	Adj P	diffMeth
CTCF_PCR_17	35490599	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	-0.21
CTCF PCR 17	35490608	$2.2*10^{-6}$	$2.7*10^{-6}$	-2.49
CTCF_PCR_17	35490619	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	-0.85
CTCF_PCR_17	35490654	$2.7*10^{-170}$	$5.4*10^{-170}$	-0.60
CTCF_PCR_17	35490674	$6.3*10^{-57}$	$1.0*10^{-56}$	-0.10
CTCF_PCR_17	35490713	$1.1*10^{-7}$	$1.4*10^{-7}$	-0.04
CTCF PCR 17	35490787	$6.7*10^{-12}$	$9.1*10^{-12}$	-0.09
CTCF_PCR_17	35490818	$1.6*10^{-203}$	$3.8*10^{-203}$	0.24
CTCF_PCR_17	35490820	$1.5*10^{-321}$	$4.1*10^{-321}$	0.66
CTCF_PCR_17	35490825	$8.4*10^{-31}$	$1.2*10^{-30}$	-0.02
CTCF_PCR_17	35490946	$1.3*10^{-173}$	$2.7*10^{-173}$	0.34
CTCF_PCR_17	35490965	$5.6*10^{-50}$	$8.8*10^{-50}$	1.16
CTCF_PCR_5.1	35704069	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	0.70
CTCF_PCR_5.1	35704149	$6.2*10^{-19}$	$8.6*10^{-19}$	-0.48
CTCF PCR 5.1	35704224	$3.7*10^{-7}$	$4.6*10^{-7}$	-0.04
*FKBP5*_PCR_12	35683363	$8.2*10^{-34}$	$1.2*10^{-33}$	-4.55
FKBP5_PCR_12	35683445	$9.7*10^{-74}$	$1.7*10^{-73}$	-3.92
FKBP5_PCR_12	35683466	$3.7*10^{-141}$	$7.1*10^{-141}$	-3.39
FKBP5_PCR_12	35683488	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	-2.98

FKBP5_PCR_17	35696726	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	3.01
FKBP5_PCR_17	35696799	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	3.61
FKBP5_PCR_17	35696823	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	2.95
FKBP5_PCR_17	35696870	$1.1*10^{-281}$	$2.9*10^{-281}$	0.71
FKBP5_PCR_17	35696886	$4.8*10^{-176}$	$1.0*10^{-175}$	1.54
FKBP5 cg14284211	35570224	$1.6*10^{-11}$	$2.1*10^{-11}$	0.37
FKBP5 PCR 2	35569751	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	1.30
FKBP5 PCR 2	35569757	$2.4*10^{-121}$	$4.5*10^{-121}$	0.93
FKBP5 PCR 2	35569777	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	0.73
FKBP5 PCR 2	35569896	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	0.54
FKBP5 PCR 2	35569922	$1.4*10^{-234}$	$3.5*10^{-234}$	-0.08
FKBP5 PCR 3	35578830	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	-1.19
FKBP5 PCR 3	35578891	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	-1.94
FKBP5 PCR 1.2	35558386	$2.1*10^{-25}$	$2.9*10^{-25}$	-0.12
FKBP5 PCR 1.2	35558438	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	-2.85
FKBP5 PCR 1.2	35558488	$2.8*10^{-179}$	$6.4*10^{-179}$	-3.03
FKBP5 PCR 1.2	35558513	$2.5*10^{-70}$	$4.1*10^{-70}$	-0.45
FKBP5 PCR 1.2	35558566	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	0.66
FKBP5 PCR 1.4	35558513	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	-2.57
FKBP5 PCR 1.4	35558566	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	-0.65
FKBP5 PCR 1.4	35558710	$7.1*10^{-94}$	$1.2*10^{-93}$	-1.75
FKBP5 PCR 1.4	35558721	$< 1.0*10^{-350}$	$< 1.0*10^{-350}$	1.69

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