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From Adversity to Psychopathology: Long-Term Epigenetic Consequences in Adversity-Divergent Twins

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“Remember,” she repeated, “magic is Chaos, Art and Science. It is a curse, a blessing and progress. It all depends on who uses magic, how they use it, and to what purpose. And magic is everywhere. All around us. Easily accessible.”

Andrzej Sapkowski, *Blood of Elves (The Witcher Saga)*

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Abstract

Background: Mental disorders are one of the leading causes of global disability and represent significant disease burden worldwide. Psychosocial adversity (PSA) has been established as one of the risk factors in the development of psychopathology. DNA methylation (DNAm), particularly at CpG sites, has been proposed as a candidate mechanism through which environmental and lifestyle factors become biologically embedded, potentially acting as a mediating factor in the relationship between PSA and psychopathology. However, these mechanisms are still not entirely understood, and isolating post-natal environmental influences without the confounding of any genetic predispositions poses a major challenge. The extent to which adversity-induced DNAm patterns predict or mediate mental health outcomes – apart from merely assessing exposure history – remains unclear. We hypothesize that (1) PSA leads to differential patterns in DNAm between the exposed and control twins, (2) the link between PSA and mental health outcomes is partially mediated by altered DNAm, and (3) the extent of mental disorder symptoms is linked to DNAm (scale model).

Aims: The overarching aim of this project was to understand the epigenetic mechanisms involved in the relationship between PSA experienced from pre-school age through early adulthood, and mental health outcomes. In order to assess our hypotheses, we (1) developed and validated methodology for quantifying PSA in monozygotic (MZ) and dizygotic (DZ) twin to identify twin pairs that diverge meaningfully to warrant further analyses, (2) examined whether specific CpG methylation sites mediate associations between PSA and psychological symptoms, and (3) evaluated whether aggregate poly-epigenetic scores (PES) predict psychological symptom severity and mediate the PSA – psychopathology relationship.

Methods: Three studies were conducted using data from the German TwinLife cohort. Study I screened 739 twin pairs (349 MZ, 390 DZ; ages 17–32) for PSA, using six validated adversity instruments mapping experiences like social and peer rejection, familial and household conflict, lack of social support, and general negative life events. We applied standardized score rankings, Euclidean distances, and standard deviation sanity checks to identify adversity-discordant pairs that would be invited for subsequent studies within the project. Study II examined 9 MZ twin pairs (N = 18) selected in Study I. Using the Illumina Infinium MethylationEPIC v2.0 BeadChip, we extracted genome-wide DNAm β-values, utilized the PSA scores established in Study I, and collected psychological symptom scores via structured clinical interviews based on the Mini-DIPS. Differential methylation analysis, dimension reduction (PLS-DA, sPLS), and bootstrap mediation models tested whether specific CpG sites quantified as β-values mediated the associations between PSA and psychological symptoms. Study III evaluated whether PES – constructed from 200 CpG sites weighted by variable importance projection (VIP) scores – predicted nine psychological symptom dimensions and mediated adversity effects in 22 participants (9 complete twin pairs plus 4 unpaired individuals).

Results: Study I identified 144 MZ and 173 DZ adversity-discordant twin pairs (approximately 41–44% of the sample). Additive genetic factors, Common (shared) environment, and E (unique or non-shared) environment (ACE) modelling revealed that 43% of adversity variance was attributable to unique environment, 23% to shared environment, and 33% to genetic factors, validating the rationale for the case co-twin design. Study II identified 73 differentially methylated CpG sites between adversity-exposed and control twins. Mediation analyses of biologically relevant candidate sites revealed significant indirect effects for several loci, with a predominant suppression pattern: adversity-induced hypomethylation associated with GTF2I (cg24977276) and PRKAR1B (cg17650397) was

linked to altered depression and anxiety symptom scores, suggesting compensatory epigenetic mechanisms as a response to PSA exposure. In contrast, SS18 promoter hypermethylation (cg15060929) exhibited positive mediation (higher PSA exposure was linked to increased methylation), transmitting 94.9% of the adversity effect on bipolar symptoms. Study III replicated prior findings that PES significantly differentiated adversity-exposed from control twins ($d = 1.12$, $p = 0.017$), with within-pair adversity-PES correlations confirming the association ($r = -0.69$, $p = 0.039$). However, PES showed no significant associations with any psychological symptom dimension (all $r \leq 0.26$, all $p > 0.05$), and mediation analyses across 45 models revealed no significant indirect effects.

Conclusion: These results provide observational evidence that suggests that PSA is biologically embedded through DNAm in the present sample, with specific CpG sites mediating adversity effects on psychological symptoms. The findings provide preliminary evidence that DNAm may function not only as a mechanism transmitting risk but also as a compensatory mechanism conferring protection. This view challenges traditional "molecular scar" interpretations of adversity-related epigenetic modifications, but is in line with emerging body of research that suggests that adversity-induced DNAm is highly context-dependent and not necessarily straightforward. The non-significant results of PES as a predictive or mediating factor in the link between PSA and psychological outcomes may be partially due to sampling limitations, but could also suggest that aggregate methylation scores may function as stress history biomarkers rather than measures of psychological vulnerability/resilience. These findings have implications for the understanding of adversity-induced DNAm as a biomarker and predictor of psychological ill-health development. Accounting for limitations such as small sample sizes, the preliminary results warrant further investigation. Future work should prioritize replication in larger cohorts, longitudinal investigation of perceived adversity rather than memory recounts, and functional validation

of candidate loci rather than the focus solely on numerical quantifiers of DNAm. The identification of both risk-transmitting and protective epigenetic pathways offers a more nuanced understanding of biological embedding and suggests novel targets for epidemiological and diagnostic approaches.

Keywords: psychosocial adversity, DNA methylation, twin study, epigenetics, poly-epigenetic scores, psychopathology, biological embedding

Abbreviations

ACE	Additive genetic factors, Common (shared) environment, and E (unique or non-shared) environment
ACEs	Adverse Childhood Experiences
ACTH	Adrenocorticotropic Hormone
ADHD	Attention-Deficit Hyperactivity Disorder
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
AUC	Area Under the Curve
BCa	Bias-Corrected and Accelerated (bootstrap)
BDNF	Brain-Derived Neurotrophic Factor
BMI	Body Mass Index
BPD	Bipolar Disorder
CAR	Cortisol Awakening Response
CAS	Composite Adversity Score
CASMIN	Comparative Analysis of Social Mobility in Industrial Nations
CCA	Canonical Correlation Analysis
CFA	Confirmatory Factor Analysis
CFI	Comparative Fit Index
CNS	Central Nervous System
CpG	Cytosine-Phosphate-Guanine
CRH	Corticotropin-Releasing Hormone
CRM	Cluster-Robust Mediation
DALYs	Disability-Adjusted Life Years
DNA	Deoxyribonucleic Acid
DNAm	DNA methylation
DOHaD	Developmental Origins of Health and Disease
DSM-5	Diagnostic and Statistical Manual of Mental Disorders
DZ	Dizygotic
ELA	Early-Life Adversity
EWAS	Epigenome-Wide Association Study
FDR	False Discovery Rate

GAD	Generalized Anxiety Disorder
GL	Glucocorticoid
HPA axis	Hypothalamic-Pituitary-Adrenal axis
ICC	Intraclass Correlation Coefficient
KMO	Kaiser-Meyer-Olkin Measure
MD	Major Depression
mPFC	medial Pre-Frontal Cortex
MRS	Methylation Risk Scores
MZ	Monozygotic
OLS	Ordinary Least Squares
PCA	Principal Component Analysis
PES	Poly-Epigenetic Score
PKA	Protein Kinase A
PLS-DA	Partial Least Squares Discriminant Analysis
PSA	Psychosocial Adversity
PTSD	Post-Traumatic Stress Disorder
RMSEA	Root Mean Square Error of Approximation
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
ROPE	Region of Practical Equivalence
SES	Socioeconomic Status
SHRP	Stress Hypo-Responsive Period
sPLS	Sparse Partial Least Squares
TSS	Transcription Start Site
UTR	Untranslated Region
VIP	Variable Importance Projection
YLD	Years Lived with Disability

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1. General Introduction

1.1. The Global Burden of Mental Disorders

Mental disorders represent one of the greatest public health challenges of the present day, affecting individuals across all socioeconomic areas, geographic regions, and demographic groups. As a society, we are transitioning from an era defined by communicable diseases to one increasingly dominated by non-communicable disorders that are influenced by genetics, lifestyle, and the environment, within which mental disorders have emerged as primary drivers of global health-related disability. Today, mental disorders represent approximately 15% of the total years lived with disability globally (Fan et al., 2025; HealthData.org, n.d.). The structure of global disease burden is quantified through disability-adjusted life years (DALYs), a metric combining years of life lost to premature mortality and years lived with disability (YLD). According to these metrics, mental disorders constitute approximately 14% of the global burden of disease (Arjadi et al., 2015; Chadda, 2016). Importantly, mental disorders are the leading cause of YLDs worldwide, accounting for one in every six YLDs (Liu et al., 2025).

As of 2025, more than a billion people worldwide live with mental disease (World Health Organization, 2025). Between 1990 and 2019, mental disorders rose from the 13th to the 7th leading cause of global DALYs (Fan et al., 2025). This 37.6% increase in the burden of mental and substance use disorders over two decades could be partially attributed to better and more accessible diagnostic tools, but it is also driven largely by population growth and aging (Chadda, 2016; Porsdam Mann et al., 2016). While mortality attributed directly to mental disorders remains underreported due to diagnostic and operationalizing challenges, it holds true that suicide – a leading cause of premature death in the mental disease spectrum – claimed roughly 727 000 lives in 2021 alone (World Health Organization, 2025).

Furthermore, individuals with mental disorders such as bipolar disorder (BPD) and schizophrenia face significantly reduced lifespans relative to healthy population, due to physiological comorbidities such as cardiovascular disease, metabolic disorders, nutritional deficiencies, infectious diseases, and substance use, which are additionally often exacerbated by risk behaviors (Chadda, 2016; Stelmach et al., 2022). Depressive and anxiety disorders are among the most prevalent mental disorders globally, across all geographical locations and populations. Between 1990 and 2001, the number of people living with an anxiety disorder rose from approximately 78 million to 138 million, closely followed by major depression, that increased from 72 million to 121 affected individuals (GBD 2019 Mental Disorders Collaborators, 2022; Xu et al., 2025; Brody & Hughes, 2025).

Taken together, these findings underscore an urgent need to advance scientific understanding of the etiology and pathophysiology underlying mental disorders, and their correlates in physiology, neurobiology, and genetics. A comprehensive understanding of the biological, psychological, and environmental foundations of mental disease is essential for translating population-level findings into clinically meaningful outcomes. Addressing the global rise in mental disease therefore requires coordinated efforts that connect psychology, biology, genetics, neuroscience and public health, with an explicit emphasis on prevention and scalable interventions. Last but not least, advances in understanding the complex foundations of mental disorders could help reduce persistent stigma, affirming that mental illness is neither a choice nor a character flaw, but a biological and medical reality warranting compassion and care.

1.2. Psychosocial and Early Life Adversity

Early life adversity (ELA) encompasses a broad spectrum of events that are generally defined as stressors that pose a direct threat to a child's physical or psychological well-being,

often requiring significant behavioral or neurobiological adaptation by the individual. These experiences are typically not isolated, but appear in an interrelated manner, where one type of adversity significantly increases the likelihood of experiencing other hazards (Duffy et al., 2018; Buchanan et al., 2023). In contemporary clinical and epidemiological literature, psychosocial adversity is observed across multiple domains, experienced throughout early life to young adulthood. The present research has multitudes of definitions, categories, and criteria for adversity, on one hand presenting a wealth of knowledge and data, on the other hand posing significant operational challenges, especially in the research setting. For the sake of this thesis and all manuscripts included, we have opted for adversity definitions that appear in peer-reviewed literature consistently, and are supported by sufficient empirical data. One such category of adversity is direct maltreatment that encompasses intentional acts such as emotional, physical, or sexual abuse, as well as physical or emotional neglect (Kuhlman et al., 2018; Buchanan et al., 2023; Jangid et al., 2025). Other work defines adversity as household dysfunction, or more specifically issues linked to the home environment such as parental divorce or separation, parental incarceration, witnessing domestic violence, and caregiver mental illness or substance abuse (Brennan et al., 2024). Interpersonal adversity describes stressors linked to the social environment including peer rejection, discrimination, bullying victimization, or intimate partner violence (Bierman et al., 2015; Warburton, 2026; Woerner et al., 2020;). Other adversities are context-dependent and specific to individuals, including but not limited to poverty, food insecurity, bereavement, partner substance abuse, partner incarceration, displacement due to natural disasters, or any other type of forced migration (Jacob, 2013; Lotzin et al., 2023). Adversity can begin in utero – often facilitated by the expectant mother's own history of adversity or mental health struggles – and can alter fetal neurodevelopment and the offspring's stress-response systems (Kurbatfinski et al., 2024). These early stress pathway modifications – mostly in connection with the

hypothalamic-pituitary-adrenal (HPA) axis – have been suggested to be a catalyst for future pathological developments, and may partially explain individuals' varying propensities for mental disease vulnerability or resilience later in life (Álvarez-Mejía et al., 2025).

The epidemiology and prevalence rates of psychosocial adversity vary significantly by geographic region, perhaps not surprisingly; studies report childhood adversity rates between 31% and 93.5% in China, 46.2% in young Europeans, and a substantial 72% to 82% in Sub-Saharan Africa (Abate et al., 2024). A significant disparity exists between socio-economic settings, where approximately 59% of children in developing countries were victims of abuse or witnessed domestic/community violence in the previous year compared to 44% in developed countries (Berens et al., 2017). Large-scale adversity prevalence research shows that 58% of individuals across 18 studied countries experience at least one adverse childhood experience, that is, 22% experience one, 13% experience two, 8% experience three, and 15% experience four or more (Jangid et al., 2025). Systemic inequalities, rather than socioeconomic factors, also function as important drivers of adversity, disproportionately affecting marginalized populations. Black young adults report substantially more exposure to caregiver death and neighbourhood violence than their white counterparts, and individuals living in poverty are also at a significantly higher risk for poly-adversity. Sexual and gender minority groups experience higher rates of physical abuse and emotional neglect than heterosexual individuals, often in combination with comorbid substance use and other mental health complaints (Raghunathan et al., 2024; Kirkbride et al., 2025; Kurbatfinski et al., 2024; McCabe, 2020).

Earlier work has shown gender-based differences in the prevalence, category, pattern, and experience of psychosocial adversity, particularly depending on the type and context of the adversity (Akram et al., 2025). Females are significantly more likely to be exposed to sexual abuse, with an approximate victim ratio of 2.5 relative to males. In Western contexts,

this type of abuse is about 20.4% higher for girls. Emotional abuse, maltreatment, and neglect was reported twice as much for girls compared to boys. This disparity, however, is less apparent in other regions, such as Asia, where males report higher median prevalence rates of emotional abuse (33.2%) than females (26.9%), highlighting the sociocultural and gender role context across different regions and societies (Kim et al., 2023; Haahr-Pedersen et al., 2020; Moody et al., 2018). Conversely, physical abuse is consistently more prevalent in boys across multiple cultural settings. In Africa and Europe, physical abuse is often reported at significantly higher rates for boys than girls, and in Middle Eastern contexts like Qatar and Saudi Arabia, boys also report more physical abuse and a higher total number of ACEs overall (Moody et al., 2018). In their work on adversity differences between genders, Haahr-Pedersen et al., propose two models of complex adversity experience, specifically the two- and four-class model. According to their data, female experiences of psychosocial adversity can be better described by the more complex four-class model (High Adversity, Child Abuse and Neglect, Dysfunctional Home, and Low Adversity), while their male counterparts' experiences fit the simpler two-class model better (Low vs. High Adversity). The distinction between these two models lies in the pattern of experiences, whereas females report exposure to multiple adversities than males, and males report less variety in exposure, but higher rates of significant stressors such as community violence (Haahr-Pedersen et al., 2020).

The biological correlates of sexual dimorphism in adversity experience lie in the brain structure, psychological embedding, particularly the HPA axis. In their study on pre-natal stress exposure, Carpenter et al. have demonstrated that the femal placenta is more permeable towards maternal glucocorticoids under strass than the male placenta. As a result, girls who experienced in-utero stress exhibit higher cortisol awakening response (CAR) and stronger reactions to social stress tests than their male counterparts (Carpenter et al., 2017; Hollanders et al., 2017). In contrast, males often show blunted or attenuated cortisol responses following

chronic maltreatment, which may represent a form of physiological system exhaustion (Gunnar & Quevedo, 2008; Hagan et al., 2011; Hagan et al., 2014). Another proposed mechanism are differential epigenetic signatures between males and females. In a rodent model, Yehuda & Lerner demonstrate that orenatal stress alters DNAm of the glucocorticoid (GL) receptor gene (*NR3C1*) in female mice, while impacting *de novo* methylation of the corticotropin-releasing factor (*CRF*) promoter in the brains of male mice (Yehuda & Lerner, 2018). Granted, rodent models must be interpreted with caution when extrapolating findings to human populations, but in combination with human studies investigating the stress-induced DNAm of these particular genes, they add empirical support to existing biological models.

Taken together, it is imperative that the assessment of adversity must be conducted with all the demographic, sociocultural, and sex differences in mind. Psychosocial adversity varies not only in its broad and narrow definition and evaluation criteria, but also in terms of experience, type, and severity. Recognizing this heterogeneity is essential for developing theoretical and methodological frameworks that account for the full complexity of individual stress exposure rather than reducing adversity to a single score or checklist.

1.3. The Relationship between Adversity and Psychopathology is Context-Dependent

The role of adversity in mental health is best understood through a context-dependent perspective. The body of research on adversity and its far-reaching consequences on human organisms reveals that even though there is sufficient data to describe psychosocial adversity exposure as a primary driver of psychopathology, its effects are not always uniformly catastrophic (Nelson et al., 2020; Sisk et al., 2025). Multiple models describe how the “right” dose of stress or adversity can act as biopsychological inoculation, that may render individuals more resilient and better prepared to face and respond to challenges. The resilience paradigm conceptualizes mental resilience as a dynamic and multi-faceted process

of successful adaptation in the face of significant adversity, that stems from the interaction between current stress exposure and the availability of protective resources (Zheng et al., 2024; Gonzalez-Mendez et al., 2023; Roos et al., 2026). Interestingly, the Steeling Effect posits that repeated exposure to stressors that an individual can withstand – moderate, somewhat manageable stress – can strengthen their mental resilience systems, improving neurobiological and behavioral responses to adversity exposure. This concept complements other models that suggest that overwhelming stress leads to emotional dysregulation, whereas the complete lack of stress leads to understimulation of the development of endogenous resilience resources (Southwick et al., 2015; Liu, 2015). The predictive adaptive response model, aligned with the match/mismatch hypothesis, argues that early exposure to mild adversity primes the brain for the anticipated future environment. If the later-life environment matches this early programming, the individual remains resilient, if they mismatch, vulnerability is enhanced (Daskalakis et al., 2013). Recent longitudinal studies have identified specific DNAm sites that act as compensatory or protective mechanisms, differentially methylated because of adversity exposure, and in turn mitigating psychopathological outcomes (Lussier et al., 2024). Finally, research in squirrel monkeys demonstrates that brief intermittent separations (moderate stress) led to larger ventromedial prefrontal cortical (vmPFC) volumes and increased white matter myelination. The expansion of the vmPFC facilitates top-down inhibition of the amygdala, reducing anxiety and fear (Lyons et al., 2009; Southwick et al., 2015)

Combined, these findings complete the complex picture of psychosocial adversity as a prominent determinant of mental health outcomes, with different outcomes. As it seems, the relationship between stress exposure and psychopathology isn't linear and definable by a single paradigm, rather, it seems to be highly dependent on the degree of exposure, and the overall context. Thorough understanding of psychosocial adversity and its exact role in

vulnerability and resilience towards mental health outcomes may help shape intervention approaches and mitigation strategies.

1.4. The Challenge of Measuring Adversity

Despite the clear importance of PSA as a determinant of mental health, quantifying adversity remains methodologically challenging. There are few accepted definitions or frameworks for operationalizing adversity, resulting in diverse measurement approaches and inconsistent findings across studies. Retrospective self-report measures, while practical, are susceptible to recall bias and may be influenced by current mood state. Empirical comparisons often struggle with agreement between various measures recorded at the time of the event, and retrospective reports, often capturing two distinct groups of individuals. Prospective designs, though more rigorous, are resource-intensive and may miss adversities that were not assessed earlier (Thurston et al., 2025). Furthermore, reported perceptions of event vary from individual to individual making their objective impact difficult to capture without including additional assessments (Kalmakis & Chandler, 2014). An example of this is the cumulative risk score proposed by the Adverse Childhood Experiences scale, which assumes adverse experiences without accounting for the possibility that they may be perceived as more adverse by some individuals, and less so or not at all by others (Anda et al., 2020). The biological sensitivity model highlights genetic differences in susceptibility to environmental influences, suggesting that the same level of adversity exposure may have divergent effects depending on an individual's genetic background. This gene-environment interplay introduces genetic confounding that complicates efforts to isolate the specific contribution of environmental adversity to health (Belsky & Pluess, 2009). As a result, accurately quantifying adversity requires methodological frameworks that move beyond single-source or static measures and account for individual differences in perception, timing, and biological susceptibility.

1.5. Biological Embedding of Psychosocial Adversity

Biological embedding of adversity refers to the processes, in which environmental influences become integrated into an organism's biological systems, permanently altering them, particularly during developmental sensitive periods (Berens et al., 2017). One of such processes is the toxic stress response, which is the chronic, maladaptive activation of the body's stress response systems such as the HPA axis. The HPA axis is a neuroendocrine pathway between the brain's hypothalamus and pituitary gland and the adrenal glands and serves as the central command center for the neuroendocrine stress response via a precise signaling cascade. This cascade – in a healthy nervous system – starts with the detection of a real or perceived threat, which triggers the release of the corticotropin-releasing factor (CRF), which in turn stimulates the pituitary gland to secrete the adrenocorticotropic hormone (ACTH) into the bloodstream. The ACTH then reaches the adrenal cortex, inducing the synthesis and release of glucocorticoids, specifically cortisol, which binds to receptors in the brain to initiate a negative feedback loop, signaling the hypothalamus and the pituitary gland to terminate the stress response once the threat has passed. In the context of continuous adversity, however, the HPA axis becomes chronically over-activated, and the feedback mechanisms often fail or become desensitized. This gives rise to hypercortisolism, persistently high levels of circulating cortisol, which contributes to an increase in allostatic load, the so-called "wear and tear" on the organism's physiological systems (Nelson et al., 2020; Ring, 2025; Kurbatfinski et al., 2024; Berens et al., 2017; Liu, 2015; Remes et al., 2021). Severe or chronic adversity can also lead to other detrimental structural and functional changes in the brain including reduced gray matter volume in the prefrontal cortex (PFC) and hippocampus, and increased sensitivity in the amygdala and other nodes of the salience network, leading to over-reactivity to emotional stimuli and poor risk assessment capability (Nelson et al., 2020; Duffy et al., 2018).

Adversity can also become biologically embedded through epigenetic modifications, notably the DNA methylation (DNAm) of stress-regulatory genes (Aristizabal et al., 2020; Kurbatfinski et al., 2024). Epigenetics refers to long-term changes in gene function and transcriptional potential, without changes to the underlying DNA sequence. Some epigenetic processes alter gene expression patterns via environmental and lifestyle influences, altering physiological and mental health outcomes through life, and potentially across generations, since these changes can be heritable (Gibney & Nolan, 2010; Alvarado-Cruz et al., 2018). The DNAm of CpG Biological embedding occurs primarily during sensitive windows in very early childhood (from birth to about age 3), that are marked by heightened neuroplasticity but can take place throughout the entire lifespan (Berens et al., 2017).

Continuous psychosocial adversity triggers chronic low-grade inflammation, which can be observed in elevated biomarkers such as C-reactive protein (CRP) and Interleukin-6 (IL-6). These inflammatory processes have been shown to increase the risk for depression in a dose-response relationship, in which the risk for certain mental disorders increases exponentially with the severity of adversity exposure (Dosanjh et al., 2025; Zajkowska et al., 2021).

1.6. Sensitive Developmental Windows for Biological Embedding

The impact of psychosocial adversity on mental health outcomes via biological mechanisms is modulated by several important factors, one of which is the developmental timing of exposure. The earliest critical window for biological embedding begins *in utero*. The epigenome undergoes extensive recombination during two primary phases: gametogenesis (the formation of eggs and sperm) and early embryo preimplantation, and detrimental environmental influences during this phase, such as maternal smoking, malnutrition or infection, can disrupt the normal establishment of the fetal epigenome (Alvarado-Cruz et al., 2018; Álvarez-Mejía et al., 2025). A well-known example of this is the

Dutch Hunger Winter study, which has shown that famine exposure specifically during the first pregnancy trimester led to persistent hypomethylation of the insulin-like growth factor 2 (*IGF2)* gene even 60 years later, whereas later gestation exposure did not produce the same effect (Álvarez-Mejía et al., 2025). Even during the first years of life, children exhibit distinct susceptibility to inconsistent parental signals or erratic household routines. According to Short et al., this household and parental unpredictability in the first year of life correlates with specific DNAm signatures that predict deficits in executive function and emotional regulation by age five (Short et al., 2024).

Two critical frameworks have been identified to conceptualize developmental periods of heightened sensitivity. The "Barker window", based on the Barker hypothesis or the Developmental Origins of Health and Disease (DOHaD) framework, focuses on the intrauterine environment as the primary site of biological modification. This window is characterized by rapid organogenesis and neurodevelopment, where the fetus relies on signals from the mother that "forecast" the postnatal environment and adaptively adjusts its physiology (Talge et al. 2007; *The Future of Children Editorial Board*, 2020). This framework is well aligned with other sensitive period models of stress regulation, that posit that maternal stress exposure primes the offspring's HPA axis for hypersensitivity to perceived or real threats (Talge et al., 2007). Disruptions during the Barker Window, such as poor fetal nutrition resulting in low birth weight, are linked to a biological memory that can manifests later in life in the form of increased risk for coronary heart disease, type 2 diabetes, stroke, and hypertension (Barker, 2004). Epigenetically, stress experienced within the Barker window is associated with the hypermethylation of the glucocorticoid receptor gene (*NR3C1*), which silences its expression and may lead to lifelong HPA hyper-reactivity (Champagne et al., 2024; Al Shehab et al, 2025).

Conversely, the “Gunnar window” encompasses early infanthood (ages 0 to 3), focusing specifically on the so-called Stress Hypo-Responsive Period (SHRP), which typically occurs between 12 and 24 months of age (Gunnar, 2000; Agorastos et al., 2019). This window is characterized by the infant’s significantly reduced cortisol response to common stressors (e.g., vaccinations, mild maternal separation). This is hypothesized to be a neuroprotective mechanism designed to shield the rapidly developing brain from the potential neurotoxicity of high glucocorticoid levels (Agorastos et al., 2019; Rash et al., 2016; Bernard et al., 2015; Smith &Pollak, 2020; Lewis et al., 2007). The maintenance of the SHRP has an additional psychosocial component, in which a sensitive and responsive caregiver can provide “social buffering”, thus serving as a as an "external HPA axis," regulating the child's physiology and preventing HPA over-activation specifically in infants with secure attachment (Laurent et al., 2016; Shirtcliff et al., 2017). Conversely, in children who experience severe adversity during the SHRP, for instance those in institutionalized care, the social buffering system fails, resulting in “epigenetic scars” such as disturbances in diurnal cortisol rhythms and long-term deficits in inhibitory control (Bakermans-Kranenburg et al., 2011; Tarullo & Gunnar, 2025; Pollak et al., 2010; Bakermans-Kranenburg et al., 2008; Bernard et al., 2015).

1.7. Epigenetics: The Molecular Bridge Between Environment and Biology

Epigenetics refers to the study of changes in gene expression that occur without alterations to the underlying DNA sequence and can be heritable. Epigenetic mechanisms enable cells with identical genomes to adopt distinct phenotypes by selectively activating or silencing genes in response to environmental cues (Bird, 2007). The three principal epigenetic mechanisms are DNA methylation, histone modifications, and non-coding RNA regulation, each operating through distinct biochemical pathways to influence chromatin structure and transcriptional activity (Gibney & Nolan, 2010).

DNA methylation (DNAm) is the most extensively studied epigenetic modification in the context of environmental influences on mental health. DNAm involves the covalent addition of a methyl group to the fifth carbon position of cytosine residues, almost exclusively at CpG sites, where a cytosine is followed immediately by a guanine. The reaction is catalyzed by a family of enzymes known as DNA methyltransferases (DNMTs). DNMT3a and DNMT3b are responsible for *de novo* methylation, establishing new patterns during development or in response to environmental cues, while DNMT1 serves a maintenance function, copying existing patterns onto daughter strands during cell division (Gibney & Nolan, 2010; Dupont et al. 2009). In mammalian genomes, approximately 70–80% of CpG sites are methylated, except for CpG islands – regions of high CpG density typically located in gene promoters – which are generally unmethylated in active genes. DNA methylation usually acts as a "silencer", repressing gene expression by preventing transcription factors from binding to the DNA or by attracting proteins that promote a closed chromatin structure (Gibney & Nolan, 2010; Kurbatfinski et al., 2024; Dosanjh et al., 2025; Dupont et al., 2009). This process translates into mental health consequences by silencing the *NR3C1* gene promoter for example, which codes for the glucocorticoid (GL) receptor. Consequently, the GL-dependent negative feedback loop within the HPA axis will be disabled, and will lead to permanent neuroendocrine dysregulation (Berens et al., 2017).

The functional consequences of DNAm are context dependent. Although DNAm within gene promoters and CpG islands is generally associated with gene silencing through recruitment of methyl-binding proteins and subsequent chromatin compaction, DNAm within gene bodies is associated with active transcription, meaning that methylation in this region is positively associated with gene expression rather than silencing (Schübeler, 2015). This context-dependence underscores the importance of precise genomic annotation, and biological relevance when interpreting DNAm findings.

1.8. Evidence of the Link Between DNA Methylation and Mental Disorders

DNAm is a well-established mechanism that acts as a direct driver of psychopathological development. Hypermethylation at the promoter region of the brain-derived neurotrophic factor (*BDNF*) gene leads to decreased expression, thereby impacting neuroplasticity. This is associated with hippocampal atrophy and synaptic dysfunction observed in major depression. The *SLC6A4* gene, responsible for the reuptake of serotonin (5-HT) from the synaptic cleft, is highly sensitive to environmental influences, and DNAm-associated silencing is associated with increased emotional reactivity and susceptibility to stress-induced anxiety (Montel Hayes et al., 2025). Similarly, hypermethylation at the promoter regions of *SLC6A4* and the stress regulator gene *FKBP5* also contributes to depressive disorders (Rasmi et al., 2022). Aside of its role in the HPA axis, the GL receptor-encoding gene *NR3C1* was found to be hypermethylated in the postmortem brain tissue of suicide victims with a history of abuse, further evidencing the biological embedding of complex trauma (Montel Hayes et al., 2025; Hirai et al., 2025). The role of DNAm in schizophrenia lies in the dysregulation of neurotransmitter systems and synaptic plasticity. Hypermethylation and subsequent silencing of the *RELN* and *GAD1* genes impairs synaptic plasticity and disrupts the balance between excitatory and inhibitory signaling, possibly contributing to the hallucinations and cognitive deficits characteristic of this disorder (Younesian et al., 2022). Because of maternal smoking, DNAm in the newborn genome, for example at the *AHRR* gene, correlates with risk for neurodevelopmental disorders, specifically attention-deficit hyperactivity disorder (ADHD) (Edgar et al., 2017). The oxytocin receptor gene (*OXTR*) is emerging as an essential regulator of social behavior and emotion processing. Higher levels of methylation have been identified as potential biomarkers for anxiety risk following trauma. Conversely, high-quality and positive social environments were linked to lower methylation, evidencing adaptive emotional regulation

and social bonding. In infants, specific methylation patterns in *OXTR* are associated with the development of emotion-processing circuits in the brain (Montel Hayes et al., 2025).

Both hyper- and hypomethylation can act as significant disruptors of normal functioning of the stress pathways and cognitive performance. Hypomethylation of the catechol-O-methyltransferase (*COMT*) has been implicated in both schizophrenia and bipolar disorder as a mechanism that leads to enhanced dopamine dysregulation in the prefrontal cortex, contributing to the symptom characteristic of these disorders (Yang et al., 2023; Thurston et al., 2025). The *FKBP5* gene helps modulate receptor sensitivity, and demethylation of *FKBP5* increases its expression, decreasing the neuronal sensitivity to glucocorticoids, further dysregulating glucocorticoid signaling and amplifying the physiological burden of stress (Montel Hayes et al., 2025). While hypermethylation of the glucocorticoid receptor gene (*NR3C1*) is a common epigenetic signature of trauma, some studies of adults with history of childhood adversity have found hypomethylation of the *NR3C1* exon 1F promoter, suggesting that the HPA axis can be epigenetically primed towards different states of dysregulation depending on the overall context (Persaud et al., 2023). In children with ADHD-like behaviors, hypomethylation at the *SPTBN2* gene, which stabilizes the glutamate transporter EAAT4, has been found and linked to poorer performance on test assessing ADHD symptoms (Ehlinger et al., 2023).

1.9. Poly-Epigenetic Scores: Aggregating Epigenetic Information

Recognizing that individual CpG sites quantified in ß-values explain only a small fraction of variance in mental disease phenotypes, researchers have developed poly-epigenetic scores (PES) – also termed methylation risk scores (MRS) – that aggregate methylation information across multiple loci into a single summary measure. PES are typically constructed by weighting methylation levels at informative CpG sites by coefficients derived from EWAS or machine learning algorithms. (Hüls & Czamara, 2020).

This approach has shown promise in predicting mental health outcomes and treatment response. Blood-based DNAm scores have predicted depression with modest accuracy, differentiated PTSD cases from controls, and identified individuals at risk for schizophrenia relapse (Barbu et al., 2021; Wani et al., 2024; Segura et al., 2026). Beyond psychiatric applications, PES have demonstrated utility in predicting cardiometabolic risk factors and biological aging, suggesting broad applicability as biomarkers of environmental exposure and health risk (Lin et al., 2025).

However, methodological challenges remain. PES are typically derived from specific discovery cohorts and may not generalize across populations. The choice of CpG sites, weighting schemes, and construction methods significantly influences predictive performance. Furthermore, it remains unclear whether PES primarily index environmental exposure history, current biological state, or predisposition to future disease – distinctions with important implications for their interpretation and clinical application.

1.10. Co-Twin Research Design

Co-Twin design has significant utility particularly in research studies that seek to investigate post-natal influences on biological and psychological systems of individuals, because it allows to control for any confounding influences of unique genetic profiles or the prenatal environment. By comparing the similarity of genetically identical twin pairs, who share 100% of their genes, to fraternal pairs who share roughly 50% of their genes, researchers can study the relative contributions of "nature" and "nurture" across multiple domains (Iacono et al., 2018). The application of twin models has evolved from simple heritability estimates to sophisticated multivariate and molecular frameworks. The classical twin design categorizes phenotypic variation into additive genetic influences (A), common or shared environmental influences (C), and unique environmental or residual influences (E) (ACE). This has been used to establish the genetic basis for diverse conditions, including

autism (approximately 60% heritability), intelligence (about 50%), and depression (about 40%) (Tan et al., 2015; Boomsma et al., 2002). Through the co-twin control design, researchers can mathematically leverage a twin pair discordant for an exposure (such as substance use) to evaluate possible neurodevelopmental scenarios. If a substance-abusing twin shows a neurocognitive deficit not found in their healthy co-twin, the results support the possibility that the exposure caused the disorder (Iacono et al., 2018; Sahu & Prasuna, 2016). Using the co-twin design can improve the statistical power of genetic studies by reducing environmental and/or genetic variability. Additionally, co-twins are particularly useful because they are siblings of the exact same age, which decreases variance for traits that change over time (Sahu & Prasuna, 2016; Boomsma et al., 2002).

Despite their remarkable contribution to understanding multivariate mechanisms, twin studies come with significant challenges. Many twin databases depend on voluntary participation, which can lead to "volunteer bias" or the over-inclusion of identical and female twins. Additionally, recruiting large enough samples for studied traits or outcomes remains a persistent hurdle for achieving adequate statistical power (Sahu & Prasuna, 2016; Niu et al., 2023). Additionally, in epigenetic co-twin research, high tissue-specificity of DNAm markers poses a challenge. Because direct sampling of the living brain is impossible, researchers must rely on tissues like blood, saliva, or buccal cells, which may have a tenuous connection to brain-based DNAm (Edgar et al., 2017; Doom & Gunnar, 2013). All in all, twin research warrants informed caution and careful planning, like all methodological approaches.

1.1. Research Aims

Correlation does not imply causality, making even the most intuitive relationships in nature difficult to argue without adding causal inference. The overarching aim of this project

was understanding the epigenetic mechanisms governing the links between adverse experience and psychopathological developments. Specifically, we were examining a population of young adults who were able to describe experiences from the pre-schooling age, all the way to young adulthood. To isolate the experiences which give rise to mental disease, we opted for co-twin research. In three interrelated studies, we have examined correct methodologies for assessing the twin experience, mediating factors of epigenetic modulations, and the utility of PES scores in predictive modelling. The results of this project have the potential to add to the body of knowledge that informs public health policies and diagnostic and preventative approaches.

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2. Study I: Case Co-Twin Study Design as a Methodology To Quantify the Effects of Life and Social Adversities: The ImmunoTwin Cohort

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Abstract

Life and social adversities experienced before adulthood are decisive drivers of later life health inequalities, which hinge on immune, endocrine and epigenetic changes that reflect an individual's interaction with the environment. Since monozygotic (MZ) twins share the same genetic background and perinatal environment, they provide a robust approach to identify environmental influences on biological features while controlling genetic confounders. We screened MZ and dizygotic (DZ) twin pairs (ages 17 - 32) from the German TwinLife study for recent and time-distant adversities in their psychosocial environment. Here, we present three statistical approaches we developed to quantify adverse experiences in 739 twin pairs (349 MZ, 390 DZ). We calculated divergence scores based on score differences between each twin pair. By combining these approaches, we identified adversity-divergent twins (144 MZ, 173 DZ), suggesting that in approximately 41% - 44% of twin pairs, there was a clear divergence in their adversarial experiences and how they interpreted their psychosocial environment. Variance modelling suggested that 43% of the adversity experienced by the twins was due to their unique environment, while 23% was from their shared environment while 33% of the variance was explained by genetic factors.

Introduction

Psychosocial adversity (PSA) is a broad term encompassing various adverse experiences, such as parental separation, childhood maltreatment, and low socioeconomic status (SES). These experiences are linked to an increased risk of both physical and mental health disorders, including cardiovascular diseases, type 2 diabetes, allergies, asthma, autoimmune diseases, obesity, migraine, psychiatric conditions (e.g., depression), substance use disorders, and personality disorders (Anda et al., 2008; Eriksson et al., 2014; Gern et al., 2009; Nelson & Gabard-Durnam, 2020; Nelson et al., 2020; Spitzer et al., 2013; Tomasdottir et al., 2015). Additionally, early-life psychosocial adversity has been associated with accelerated biological aging, including changes in pubertal onset (Bleil et al., 2013), ageing of cells, epigenetic ageing (Hamlat et al., 2021; Holuka et al., 2024), and cortical thinning in childhood and adolescence (Islam et al., 2024). Alarmingly, PSA is widespread, with the World Health Organization estimating that 39% of the global population has experienced at least one childhood adversity, with similar prevalence rates across high-, middle-, and low-income countries (Kessler et al., 2010).

Adversity is commonly described as a deviation from the expected environment, encompassing atypical experiences (e.g., emotional or physical abuse), deprivation (e.g., inadequate caregiving), and household exposure to substance abuse, psychological illness, or incarceration (Bair-Merritt et al., 2015; Nelson & Gabard-Durnam, 2020). Not all adverse experiences are overtly dramatic; routine daily interactions may be significantly more important than previously assumed, as continual exposure to maltreatment may have a more profound effect than isolated instances of abuse (Bair-Merritt et al., 2015). Furthermore, the long-term effects of adversity on health outcomes are complex, varying by adversity type and exposure period. For example, threat-based adversity (e.g., exposure to harm or violence) is linked to accelerated pubertal development and epigenetic aging, while socioeconomic

adversity is associated with cortical thinning in frontoparietal, visual, and default mode brain networks (Colich et al., 2020). Childhood abuse is linked to decreased prefrontal and temporal cortical thickness, which predicts antisocial behavior and generalized anxiety (Busso et al., 2017). Verbal abuse, whether from parents or peers, is also associated with heightened psychiatric symptoms, including depression, anxiety, and dissociative disorders (Polcari et al., 2014; Teicher et al., 2010).

The timing of adversity exposure plays a crucial role, with two critical periods identified: the "Barker window" (the first 1,000 days of life) and the "Gunnar window" (pubertal period) (Gunnar et al., 2019). For example, post-institutionalized (PI) youths exhibit blunted hypothalamic-pituitary-adrenal (HPA) axis stress reactivity, which can be recalibrated through adoption into a supportive environment. By late puberty, HPA axis reactivity may return to levels comparable to non-adopted controls. Conversely, negative exposures during this period can have lasting detrimental effects (King et al., 2017).

With the general understanding that adversity is a potential determinant of negative phenomena in health and well-being, the question arises how to measure adversity and its consequences. The problem is that there are several definitions and theoretical frameworks, resulting in various classifications of adversity and differences in the patterns of their effects. Moreover, for all theoretical frameworks, quantifying adversity remains a significant challenge.

Despite the clear impact of PSA on health and well-being, measuring adversity and its consequences remains challenging. There is no single universal definition or framework, leading to diverse classifications and inconsistent findings. Quantifying adversity requires consideration of multiple factors, including stressor type, timing, accumulation, and individual susceptibility. Experience-expectant and experience-dependent models propose

different mechanisms for adversity's effects, with some emphasizing cumulative risk, while others focus on sensitive periods (Gabard-Durnam & McLaughlin, 2019; Nelson & Gabard-Durnam, 2020).

Additionally, the biological sensitivity model ("three-hit model") highlights genetic differences in susceptibility to environmental influences, suggesting that genetic underpinnings may act as a confounding factor in adversity's impact on health (Belsky & Pluess, 2009; Daskalakis et al., 2013; Grova et al., 2019).

This study adopts a psychosocial perspective on adversity and its role in health inequalities, leveraging a life-course approach and twin study methodology. Monozygotic (MZ) twins share nearly 100% of their genetic makeup and perinatal environment, making them an ideal model for disentangling environmental influences from genetic confounds. By examining phenotypic discordance within MZ twin pairs, we can robustly quantify the impact of environmental adversity while controlling for genetic predisposition.

A critical challenge in PSA research is consolidating multiple adverse experiences into a single, quantifiable measure. We have previously proposed using a case co-twin design to assess the effects of social environment while controlling for genetic and early developmental influences, particularly during the "Barker window" (Turner et al., 2020). Indeed, two recent studies examined twins divergent in SES. The first associated SES-divergence with mental-health (Lam et al., 2019) and the second with gut microbiome changes (Bowyer et al., 2019). Lam et al. (Lam et al., 2019) demonstrated that the higher-SES twin had considerably less psychological distress. However, these studies primarily examined between-family differences rather than within-family variation.

Our study builds on these findings by identifying adversity divergence within MZ twin pairs and comparing these results with dizygotic (DZ) twin pairs to estimate genetic effects. We

develop cumulative measures of adversity that can in the future be linked to biological markers, allowing us to quantify the contribution of different psychosocial environmental factors to observed biological phenotypes. By refining adversity measurement and leveraging the twin design, we aim to provide novel insights into the mechanisms by which psychosocial adversity shapes health outcomes and developmental trajectories.

Materials and Methods

Here we report how we determined our sample size, all data exclusions, all manipulations, and all measures in the study.

TwinLife study

We investigated the formulated research question in the 6th version data of TwinLife (Diewald et al., 2024), the ongoing German twin family study that focuses on the development of social inequality and its psychosocial mechanisms (Hahn et al., 2016). With a cross-sequential design for 4 available birth cohorts, the TwinLife study includes cross-sectional and longitudinal psychological, sociological and genetic data of twin families currently from age 5 up to age 32 (Hahn et al., 2016). We examined the presence of adversity and discordance, firstly, in a sample of MZ twin pairs (N = 349 pairs) and then, in a sample of DZ twin pairs (N = 390 pairs) aged above 17. These twins stem from the second, third and fourth TwinLife birth cohorts who at the moment of the measurement were 17-19, 23-25, and 28-32 years old respectively.

The research received the ethical approval from the Ethical Committee of Bielefeld University no. 2020-184-W1. The personal data collection and its analysis were compliant with correspondent data protection regulations. The statistical analysis was performed on pseudonymized data on the servers of Bielefeld University, University of Luxemburg, and Luxemburg Institute of Health.

Screening interview period

The screening interview took place in the first half of 2022 and collected data on past negative experiences during the life course and the degree of current stress. Each twin was invited to take part in the screening interview via e-mail. The twins and the chance to fill in questionnaires online or receive a paper version. Over a period of 4 months, questionnaires

were sent to 4085 individuals (1898 MZ twins; 2189 DZ twins). From 2094 families, we received 2079 responses (952 MZ twins, 49.1%; 1127 DZ twins, 50.7%). 739 complete twin pairs who filled out all questionnaires were selected, of which 349 pairs were MZ twins and 390 pairs were DZ twins. Among the 349 MZ twin pairs, 308 participants belonged to the second cohort (Mn age = 17.9), 186 to the third one (Mn age = 24.1) and 204 to the fourth cohort (Mn age = 30.0). 60.4% of the MZ sample were females. Within 390 DZ twin pairs, 412 participants belonged to the second cohort (Mn age = 17.9), 234 to the third one (Mn age = 23.9) and 134 to the fourth cohort (Mn age = 29.8). 59.7% of the DZ sample were females. The social economic statuses of families of the twin pairs differed from low (11%) to medium (37%) and high (56%) based on a comparative analysis of social mobility in industrial nations (CASMIN) educational scale (Brauns et al., 2003).

Adversity mapping inventories and questionnaires

To assess each individual twin pair's psychosocial environment, we opted for 6 questionnaires addressing various aspects that the literature defines as adverse, stressful, or negative. These cover not only the physical presence of adversity but the individuals' emotion interpretation of the exposure (Supplementary Table 1). All six questionnaires were administered and scored according to their original authors' guidelines.

Life events. Firstly, we compiled a set of 6 items mapping out impactful events in one's life, such as financial hardships, death of a close person, or disease (Life Events Questionnaire) (Turner et al., 2020). This questionnaire was rated by participants on a binary yes orno scale, with additional indication of one or more stages of life where each event occurred.

Humiliation. Following this, participants were presented with an adjusted version of the Humiliation Scale (Hartling & Luchetta, 1999), which is a set 4 of items rated in terms of negative impact on a 5-point Likert scale, with least points indicating least negative impact,

and most points indicating most negative impact. The Humiliation Scale illustrates life events such as peer disapproval and social exclusion. Additionally, we included indicators for the life stage in which these events took place; however, these indicators were not considered in the post-hoc data analysis.

Daily hassles. Next, we examined daily hassles with brief daily stressors screening tool (BDSST) (Scholten et al., 2020). BDSST is a 10-item scale that is also rated on a 5-point Likert scale increasing according to their negative impact. This questionnaire examines difficulties with day-to-day situations such as social and familial obligations, or conflict with close persons, which have been occurring during the past 12 months.

Shame. Consequently, we opted for the Other as Shamer Scale 2 (OAS2) (Matos et al., 2015) from which we selected all of the 8 items, again rated on a 5-point Likert scale increasing according to their negative impact. OAS2 is an 8 items scale, again rated on a 5-point Likert scale according to how often the events described in each item occur. In this questionnaire, current self-views within a social group were evaluated, such as feelings of inadequacy compared to others, insecurity, or insignificance.

Perceived social support. Next, we looked at the lack of social support. Here, we used 3 modified items from the Social Support Scale (Zimet et al., 1988) in which participants were asked to indicate how they currently feel about receiving emotional support from their family, co-twin, or reciprocity from their friends. The possible answers varied from 1 – "Strongly disagree" to 5 – "Strongly agree". In the post-hoc analysis, these scores were reversed to describe the severity of the lack of social support.

Bitterness (Covid-19 context). Eventually, we looked at how participants were impacted by the recent Covid-19 pandemic. Here, we considered two items about participants' feelings

towards their government's and peers' response to the Covid-19 pandemic on a 5-point forced-choice Likert scale (1-"Strongly disagree" to 5-"Strongly agree").

Data pre-processing

Data cleaning and preparation. During the initial data cleaning, we first excluded twin pairs for which only one twin participated in the study. Second, we excluded twin pairs where at least one twin failed to answer ≥51% of the items on at least one of the survey waves. Answers of never experiencing an adverse event in any life phase were coded as "one", meaning no negative experiences at all.

To establish a composite total score not driven by any one construct for every participant, individual scores were stretched on a 0-10 scale to provide an equal contribution to this composite total and then added.

$$Y = \left(\frac{X - Xmin}{Xrange}\right)10$$

The stretched score is represented by Y, whereas the raw questionnaire score is represented by X. Xmin reflects the minimum questionnaire score within the entire sample, and Xrange is the subtraction of Xmin from the maximum score within the entire sample.

The bias correction was subtle, and stretched score totals had an almost perfect correlation with raw score totals (correlation coefficient = 0.95).

Data Structure. To examine whether chosen negative experiences are valid representations of adversity, we first performed principal component analysis (PCA) to identify latent variables that we later validated using confirmatory factor analysis (CFA). Initially, we performed a Varimax rotation PCA on the unbiased indicators, with the standard Eigenvalue of >1, i.e. only components with an Eigenvalue greater than 1 would be considered as factors.

Additionally, we ran the Bartlett's Test of Sphericity ($p<0.05$) and Kaiser-Meyer-Olkin Measure (KMO) with a standard cut off of ≥ 0.6, to establish whether our data meets the criteria for a meaningful factor analysis.

CFA on the latent variable of adversity was measured with standardized b coefficients from a structural equation model. At least a weak effect (>0.1) as a statistical confirmation of the validity of the measure as an indicator of adversity was expected (Gana & Broc, 2019). To estimate the model, full information maximum likelihood under the missing at random assumption was applied (Schafer & Graham, 2002). For making inferences regarding the fit of the model and significance of associations, we applied a threshold p-value equal to 0.05. The confidence intervals for the root mean square error of approximation (RMSEA) fit indices should cross 0.08 and the comparative fit indices (CFI) should be above 0.90 (Gana & Broc, 2019).

Power Calculation for Adversity Divergence. To future-proof our cohort, we performed a linear multiple regression power analysis in G*Power 3.1.9 (Faul et al., 2009). With medium effect size (0.15), one predictor (degree of within-pair discordance) and an error probability of $\alpha = 0.05$, an amount of 70 discordant twin pairs provided a power of 0.89, which is above the acceptable threshold of 0.8. We opted for a linear multiple regression approach with the intention to plot adversity divergence scores as a predictor for biomarker divergence scores in a future study.

Calculation of Adversity Divergence

We calculated the within-pair divergence, based on the results of structural equation modelling and exploratory factor analysis. Based on the literature and the multivariate nature of the data, three approaches were applied to define divergence: score ranking, Euclidean distance and standard deviation-based ranking.

Score ranking. We grouped our sample into twin pairs and subtracted Twin 1 unbiased composite total score from Twin 2 unbiased composite total score. We converted these difference scores into absolute numbers, eliminating negative values. After we obtained the absolute differences in the unbiased composite total score, we ranked twin pairs from least within-pair difference to highest within-pair difference.

Euclidean distance. Compared to other multivariate distance measures, such as Mahalanobis distances (Mahalanobis, 1930) or Penrose´s measure (Penrose, 1954), Euclidean distance focuses on the multivariate distances between individuals (Blumenthal, 1970). It is a common measure for the twin discordance in epigenetic and immune studies (Kaczorowski et al., 2017; Wang et al., 2018). For multivariate cases, Euclidean distance is equal to the square root of the sum of the squared differences for each variable between two individuals, given that scores are standardized (Cronbach & Gleser, 1953). It can be represented by the formula:

$$\mathrm{d_{ij}} = \left\{\left(x_{i1} - x_{j1}\right)^2 + \left(x_{i2} - x_{j2}\right)^2 + \left(x_{i3} - x_{j3}\right)^2\right\}^{\frac{1}{2}}$$

where d_{ij} is Euclidean distance for three arbitrary variables, x1, x2, and x3 – scores for the correspondent variables, i and j – are twins of a twin pair. We applied this formula to the composite scores for six indicators of adversity for pairs of twins and defined pairs with the largest distances.

Finally, as a "sanity check" on our two principal divergence scores, we performed the Standard deviation (SD) based ranking. Standard deviation (SD) ranking accounts for the part of the distribution of within-pairs differences that is higher than 1SD from the mean, defining the most discordant pairs. Compared to the two aforementioned approaches, Standard deviation ranking implies the threshold (>1SD), that to some degree limits the number of discordant pairs. Defining discordance based on the SD is shown to be an efficient tool in the

studies of behaviour problems (Caspi et al., 2004) or anxiety (Asbury et al., 2008). To calculate SD based ranking, we evaluated within-pair differences based on the composite scores for each indicator. Pairs with a within-pair difference of at least 1SD higher compared to the mean differences on at least 1 of the 6 indicators were considered divergent.

Data Analysis

To explore the divergence, while controlling for possible genetic confounding, the main analysis was performed in the subsample of MZ twins and then repeated in the subsample of DZ twin pairs as a supplementary analysis. ACE models of heritability were calculated using OpenMx(Neale et al., 2016). All data analysis was performed in SPSS (28.0.0.0, IBM, Armonk, NY: IBM Corp) and R Studio (RStudio 2022.07.2+576 "Spotted Wakerobin") running R (Version 4.2.2) with the packages “vegan” (Dixon, 2003), “lavaan” (Rosseel, 2012), “dplyr” (Wickham et al., 2020), “sjmisc” (Lüdecke, 2018), “epitools” (Aragon, 2020), “ggplot” (Wickham, 2016), OpenMx. We confirm that this study’s design and its analysis were not pre- registered.

Results

Questionnaire data distribution and exploration in the MZ twins

Upon first data inspection, we have found that the Daily Hassles scale (dhs) and the Bitterness scale (bit) were normally distributed while the Life Events (lev), Humiliation (hum), Other as Shamer (oas) and the Lack of Social Support (sup) scales were skewed to the right. From the literature, these response distributions were expected given the parameters of the questionnaires used and the sample population (Hartling & Luchetta, 1999; Matos et al., 2015; Scholten et al., 2020; Turner et al., 2020; Zimet et al., 1988). Similarly, when all scores for six adversity indicators were summed up, the results showed a right skewed continuous distribution (Figure 1). Furthermore, we examined the internal reliability of our questionnaires. Humiliation scale, Daily Hassles scale, and Other as Shamer scale showed high internal consistency. Lack of social support scale and bitterness scale showed lower internal consistency, partially due to a small number of questionnaire items. The Humiliation scale, Daily Hassles scale, and Other as Shamer scale showed a high standard deviation (> 5), indicating large response spread from each questionnaire's respective population mean. Responses on the life events scale, lack of social support scale, and bitterness scale were more centered on their respective mean, with ≤ 2.1 standard deviations (Table 1).

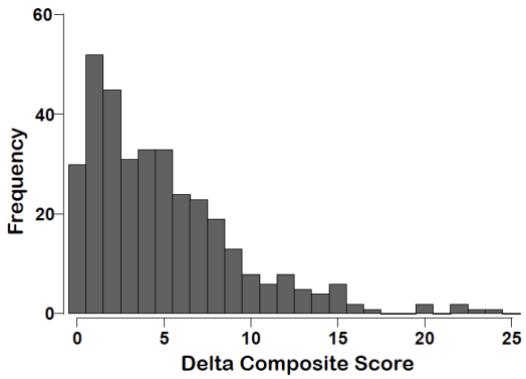


Figure 1: Histogram displaying the distribution of delta composite scores between twins in each of the 349 MZ twin pairs.

Table 1 : Questionnaire data distribution and exploration

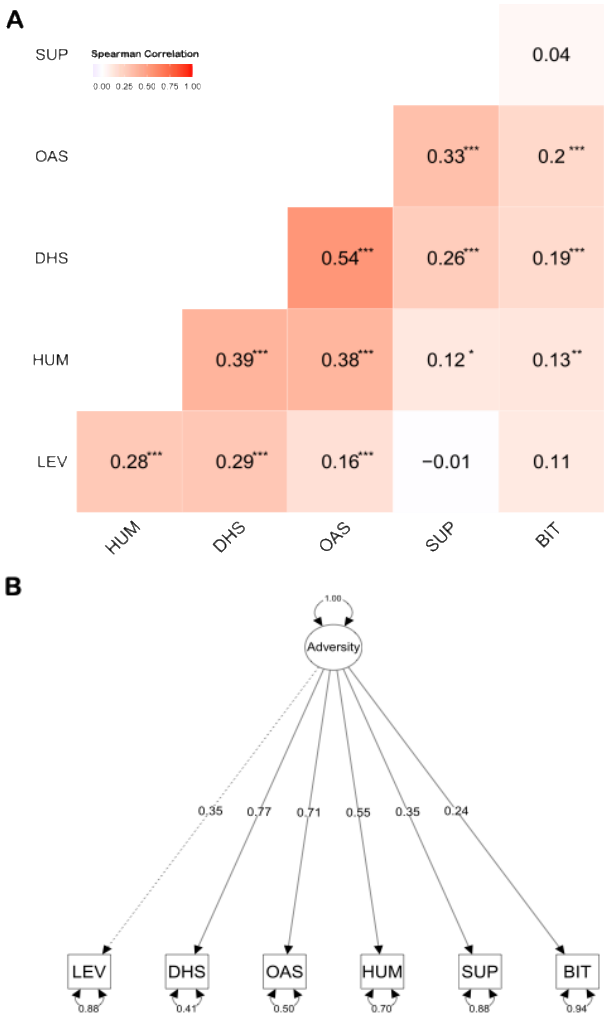
		LEV	HUM	DHS	OAS	SUP	BIT	ALL
N	**Valid**	698	698	698	698	698	698	**698**
Mean		2.15	7.63	18.32	15.44	5.29	6.55	**55.38**
Median		2.00	6.00	17.00	14.00	5.00	7.00	**53.00**
Std. Deviation		1.68	4.70	6.06	6.15	2.13	1.76	**15.41**
Skewness		1.16	1.25	0.93	1.06	1.15	-0.34	**0.88**
Kurtosis		1.77	0.42	0.79	0.94	1.39	-0.08	**0.79**
Cronbach's Alpha		**N/A**	**0.85**	**0.77**	**0.90**	**0.64**	**0.35**	**N/A**

Abbreviations : negative life events, LEV, humiliation, HUM, daily hassles, DHS, others as shamers, OAS, lack of social support, SUP, bitterness, BIT

Inter- and Intra-questionnaire reliability and correlations in the MZ twins

Spearman correlations. The indicators of adversity correlated weakly to moderately with each other (Figure 2A-B). Three of the indicators showed no significant correlation with each other: Life events, Lack of Social Support, and Bitterness scale. Despite this, they were significantly correlated with other variables: Daily hassles, Other as Shamer, and Humiliation Scale. We did not observe any extremely high correlations (> 0.8) among our indicators, which suggests the absence of multicollinearity. All chosen indicators potentially measured the different adversity experiences.

Figure 2: Spearman correlations and path diagram derived from factor analysis for six indicators of adversity.



(A) Spearman correlations for scores of six indicators of adversity in the subsample of MZ twins (N = 349). Rho estimates are represented as numbers and graphically (color). *, p < .05; **, p < .01; ***, p < .001. (B) Path diagram derived from the confirmatory factor analysis and standardized factor loadings for the model with the adversity single factor observed in the subsample of MZ twins (N = 349). Circles represent latent variables; rectangles represent observed variables. The double-headed arrows on the observed variables represent variances, while the double-headed arrows between latent variables denote covariances. Dashed straight arrows indicate marker variables used for coefficient estimation. LEV, the total number of reported negative life events; HUM, humiliation scale score; DHS, daily hassles scale score; OAS, other as shamer scale score; SUP, level of the perceived social support scale (reversed score); BIT, the mean score for bitterness due to the Covid-19. All scores were standardized.

Factor Analysis. We assumed inter-relatedness of the scores, so we applied Promax rotation on the unbiased total scores of each questionnaire. We were able to extract two factors, which taken together, accounted for 55.11% of the total cumulative explained variance. The Bartlett Test of Sphericity showed that the questionnaire scores are significantly correlated at $p < 0.001$. Further, the KMO revealed that our sample was adequate for factor analysis, with a value of 0.71.

The Humiliation scale, the Daily Hassles scale, and the Other as Shamer scale had high respective correlations with factor 1, suggesting they represent a common underlying construct, which accounts for 28.6% of the explained variance. The scores in the Life Events scale loaded slightly more strongly on factor 2, representing a different construct. The finding that certain questionnaires tend to group together is supported by our Spearman correlation matrix, where higher correlations roughly correspond to component placement taken from the PCA. This result suggests two latent variables, which are both determined by life adversity. The results are summarized in Table 2.

Table 2: Principal Component Analysis

Component	Eigenvalues	Explained Variance (%)	Cumulative Variance (%)
1	2.24	37.37	**37.37**
2	1.06	17.74	**55.11**
3	0.91	15.21	**70.32**
4	0.72	11.95	**82.28**
5	0.63	10.48	**92.76**
6	**0.43**	**7.24**	**100**

Confirmatory factor analysis in the MZ twins. As PCA did not arrive at the solution with the simple structure, we performed CFA to examine whether a theory-driven one factor structure with six indicators of adversity fits the data. The structural equation model with six chosen variables is represented in Figure 2B. The fit of the model was acceptable ($x2 = 61.74$, $p = 0.05$, comparative fit index (CFI) = 0.92, the root mean square error of approximation (RMSEA) = 0.092, RMSEA 90% confidence intervals (CI) [0.071; 0.114]), indicating that

model represents data relatively well. All indicators of adversity had significant beta coefficients. “Daily hassles” variable demonstrated the highest standardized coefficient (b = 0.77, p <0.001) and “Bitterness due to COVID-19” demonstrated the lowest standardized coefficient (b = 0.24, p < 0.001). Based on this, we performed further analysis using six indicators of adversity.

Identification of Adversity-divergent MZ twins.

Ranking by differences in total scores

After ranking every twin pair’s stretched composite total score difference from lowest to greatest, we were able to identify a population (N >100) of twin pairs with the greatest adversity divergence scores. The overall range of adversity divergence scores in our sample was 0.00 to 23.58. Following up on this, we selected twin pairs ranging from an adversity divergence score of 9.00 to 23.58 for our experimental subsample. The divergence scores in this subsample all had a SD of at least 1.

Ranking by Euclidean distances

The Euclidean distances (MnEclD = 4.74 (SDEclD = 2.26)) for the 349 final MZ twin pairs were not normally distributed ((Shapiro-Wilk test) W = 0.96, p < 0.01). The Q-Q chart plot demonstrated a higher number of cases with shorter and longer distances (Figure 3A-B). Similar results were obtained for each indicator separately (Supplementary Figure 1). The distribution of Euclidean distances for six indicators of adversity was significantly, positively skewed. Both sex and age were not significantly associated (Spearman correlation) with Euclidean distances (r = 0.01 for sex, r = 0.03 for age).

Within-pair score difference distribution. After exploring the distribution of within-pair difference in scores, we found that differences in all scores were skewed to the right. Composite total difference scores, in contrast, were only moderately skewed to the right.

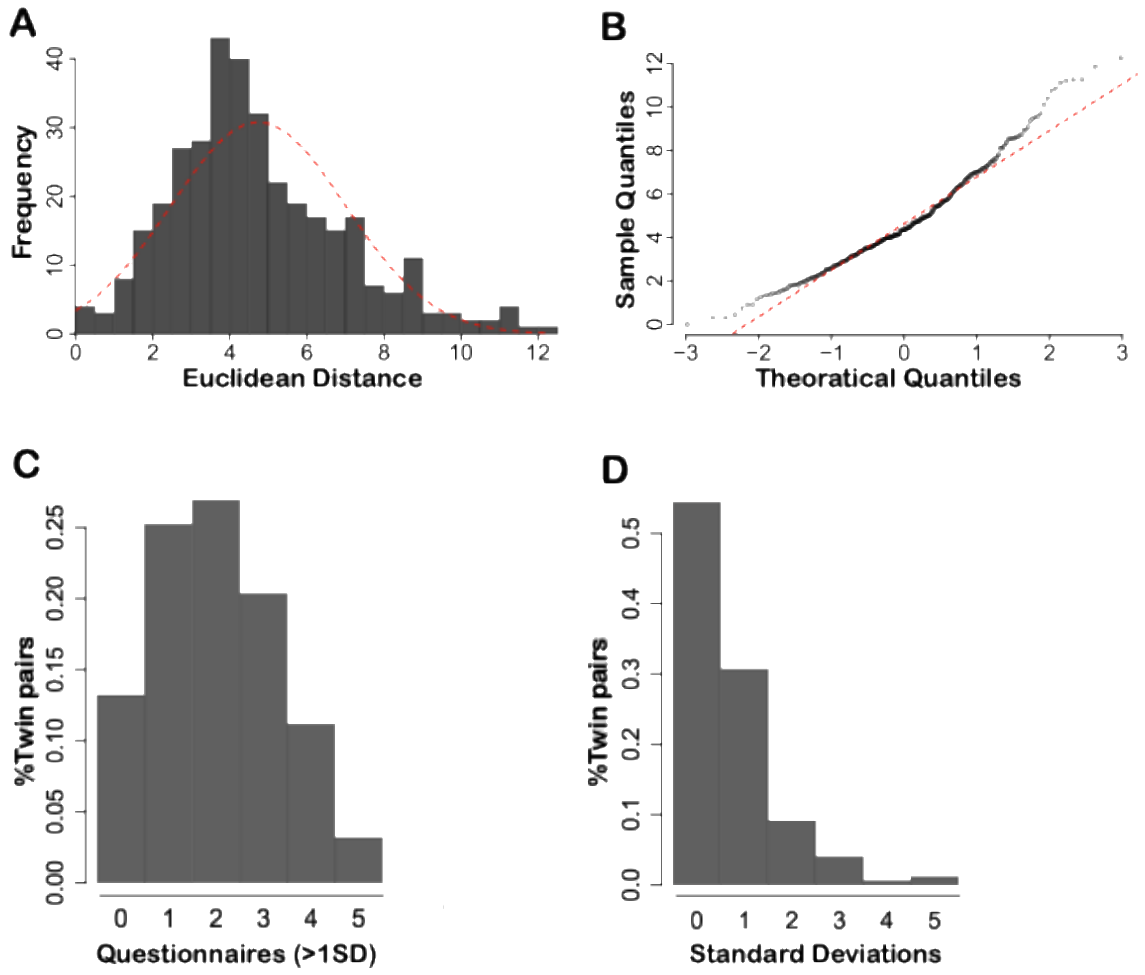


Figure 3: Histogram (A) and Q-Q chart plot (B) displaying the distribution of Euclidean distances in a sample of MZ twins (N = 349), based on six indicators of adversity. The red line represents the expected data density, assuming the data follow a normal distribution. Bar graphs displaying the distribution of (C) the number of questionnaires divergent by one standard deviation (1SD) or more (D) standard deviations between MZ twin pairs.

MZ Twin pairs show multiple differences in multiple questionnaires.

As a final "sanity check", we used statistical inference to confirm that the twin pairs identified as divergent above had measurable differences in their responses to the indicators of adversity. We calculated the mean and standard deviation for each questionnaire. We then expressed the difference between the twins in terms of the number of SD between their responses. As can be seen from Figure 3C-D, approximately 20% of twins diverged by 1SD on each individual questionnaire score, and approximately 10% of the twins diverged by 2SD. The results demonstrated that MZ twins' lives were more divergent than anticipated. Only 13% of twins did not differ by >1SD on any questionnaire, while 25% of twins differed by 1SD on 1 questionnaire, peaking at 27% of twins differing by 1SD on 2 questionnaires, decreasing almost linearly to 4% of twin pairs differed by 1SD on 5 or the 6 questionnaires (Figure 1D). When widening the difference in questionnaire responses to 2SD, 45% of twins did not diverge, while 30.5% of twins differed by 2SD on at least 1 questionnaire. 2.5% of twins diverging by 2SD on 4 questionnaires.

MZ twin pairs show cross-list concordance across all 3 divergence quantifiers.

To complete the analyses, we finally compared the lists of the most discordant twin pairs identified by three approaches. By taking the 150 most divergent twins from each of the applied approaches, we identified 78 twin pairs that were divergent in all three approaches and an additional 66 twin pairs that could be considered divergent based on two of the approaches bringing the total to 144 MZ twin pairs (Figure 4A- B).

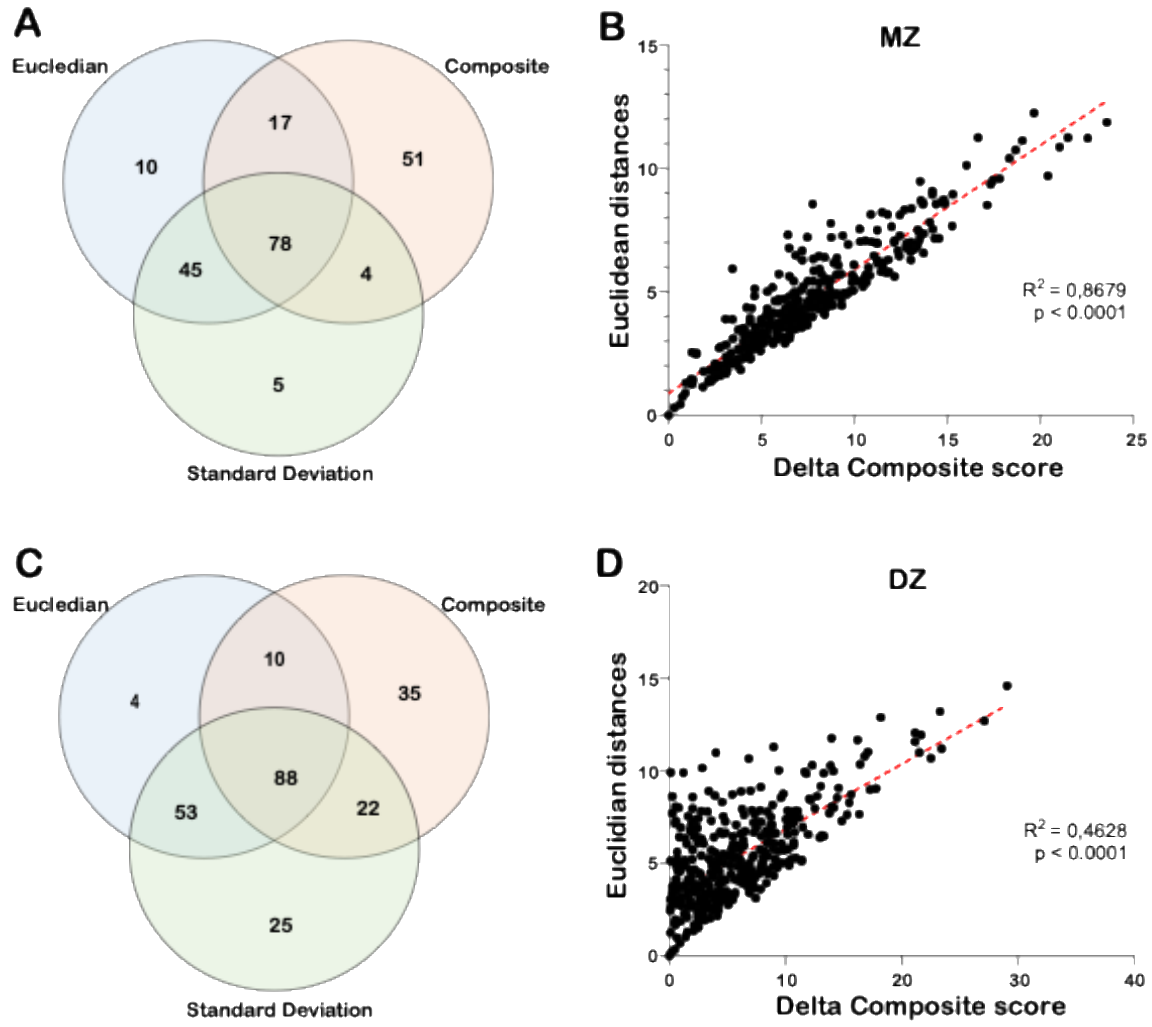


Figure 4: Comparison of divergence analysis methods for MZ and DZ Twins. (A) Venn diagram and (B) correlation graph for MZ twins comparing divergence identified by the Euclidean distance approach and the composite score approach. The analysis focuses on the 150 most discordant twin pairs identified by each method including the Standard deviation approach. (C) Venn diagram and (D) correlation graph for DZ twins comparing divergence identified by the Euclidean distance approach and the composite score approach. Analysis focuses on 155 most discordant twin pairs and shows the overlaps amongst the three methods for DZ twins.

DZ twins show greater levels of diversity than MZ twins.

In order to access the level of genetic confounding we repeated the complete set of analyses above with data from the 390 DZ twin pairs. Like the MZ twin pairs, there was a clear divergence between the twins in the individual questionnaires. Furthermore, the factor analysis for six indicators of adversity demonstrated that the data structure for DZ twins

almost mirrored what was observed earlier for MZ twins. The fit of the model was acceptable ($x2 = 52.26$, $p < 0.001$, CFI = 0.93, RMSEA = 0.078) The CI 90% RMSEA crossed the threshold of 0.08 [0.059; 0.100], indicating that the model represented data relatively well. All indicators of adversity had significant beta coefficients. The loading or beta coefficient for the life event scores for DZ twins ($b = 0.22$) was lower compared to MZ twins ($b = 0.35$) possibly reflecting a larger divergence in life experiences within DZ twins compared to MZ twins. Using both the Euclidean distance and ranking techniques, we identified a clear set of twins that diverged in their PSA. For these twins, we were able to confirm from number of questionnaires in which there was greater than 1SD in the reply of the twins within a pair. Comparing the top 155 divergent twin pairs identified by each approach, we found a total of 88 DZ twin pairs, that were divergent in all three approaches and 85 twin pairs that could be considered divergent on two analysis techniques bringing the total to 173 DZ twin pairs (Figure 4C - D). Furthermore, to show that DZ twins were more discordant than MZ twins we conducted a comparison of frequency distribution of divergence scores between the two groups (Figure 5).

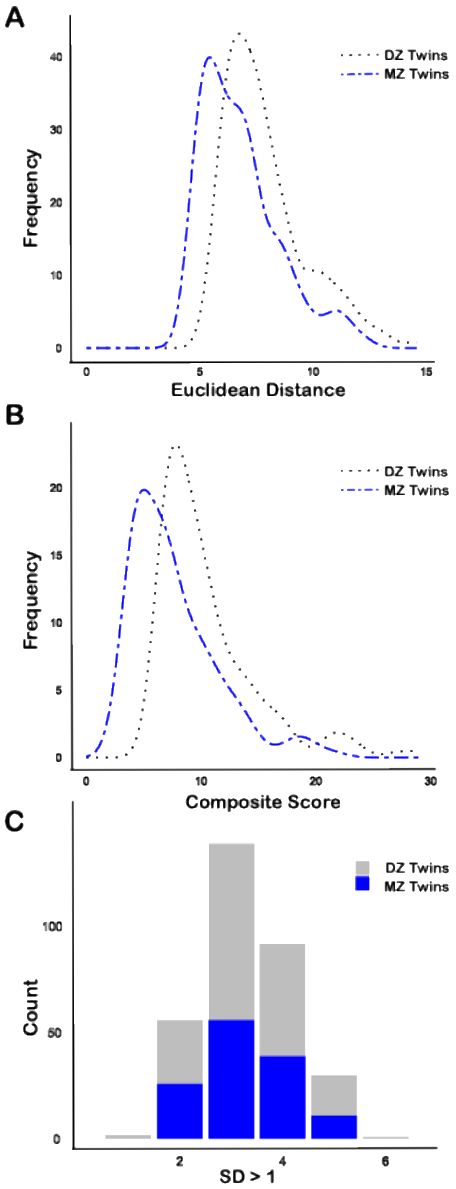


Figure 5: Comparison of frequency distribution of divergence scores between monozygotic and dizygotic twins. (A) Euclidean Distance, (B) Composite Score, and (C) Standard Deviation.

Estimation of the effect of heritability, shared and unique environments on adverse experiences.

Using the sum scores of all the individual indicators of adversity, we generated ACE models for MZ and DZ twins. If our observed diversity in appreciation of the social environment within a twin pair is not an artefact, ACE models should identify a strong influence of the unique environment, the factor E2. After partitioning the observed variance into its genetic, shared environmental, and residual or unique environmental variation we used a standard MZ vs DZ model to estimate the percentage of the total variance that was heritable (H2), or due to the shared (C2) and unique (E2) environments (Table 3). Overall, the unweighted sum of all indicators of adversity suggests that the unique environment explains 43% of the observed difference in adverse experiences, while 33% was heritable and 23% was due to shared experiences. This trend was common to the daily hassles, other as shamer, and perceived social support scales. The closeness of twin lives was reflected in the high heritability of humiliation (49,9%) and life events (62%).

Table 3: ACE variance modelling of MZ and DZ twins.

	Individual unweighted questionnaires						**Sum scores**
ACE component	**HUM**	**DHS**	**OAS**	**SUP**	**BIT**	**LEV**	**Unweighted questionnaires**
Heritability (H^2)	0.4995	0.3408	0.3404	0.3965	0.4186	0.6245	**0.3345**
Shared Environment (C^2)	0.2495	0.2251	0.0865	0.1457	0.005	-0.0101	**0.2354**
Unique Environment (E^2)	**0.251**	**0.4341**	**0.5731**	**0.4578**	**0.5764**	**0.3856**	**0.4301**

Discussion

The primary objective of this study was to recruit a cohort of adversity-divergent monozygotic (MZ) and dizygotic (DZ) twins to investigate biological and psychological differences induced by PSA while controlling for genetic effects. Here, we report the pre-screening of participants in the German TwinLife study aged over 18 years. From an initial sample of 4085 individuals (1898 MZ twins; 2189 DZ twins) twins, we successfully identified 144 MZ and 173 DZ twin pairs who demonstrated clear divergence in their life trajectories and had experienced at least one form of adversity or stressor.

Growing evidence supports the use of disease- or exposure-discordant twins in a "case co-twin" design to identify underlying pathophysiological mechanisms (Castillo-Fernandez et al., 2014; Gatz et al., 2006; Turner et al., 2020; van Dongen et al., 2016). This approach enables control for genetics, sex, and age, and critically, each twin pair serves as their own in-utero and early- life environment control (Bell & Spector, 2011; Boomsma et al., 2002; Castillo-Fernandez et al., 2014; Petronis, 2010; Turner et al., 2020). The shared rearing environment (Craig, 2013; Plomin et al., 2013) also mitigates numerous confounding environmental variables that remain difficult to quantify (Turner et al., 2020). Here, we leverage this advantage, and use the shared developmental environment of twins to not only eliminate genetic effects, but to "close the Barker window". As the twins have a largely identical first 1000 days, this only leaves open the "Gunnar window" for adaptation to the environment and for twins to diverge. While prior work has focused on supportive environments to counteract early-life adversity (Gunnar et al., 2019; Gunnar & Quevedo, 2008; Hostinar et al., 2015a, 2015b; King et al., 2017; VanTieghem et al., 2021) (reviewed in (Howland, 2023)), evidence from rodent studies suggests that this window can also encode negative experiences, with long-term consequences (Meaney, 2001; Morley- Fletcher et al., 2003; Romeo, 2018; Weaver et al., 2004).

In our case co-twin paradigm, twin lives start to diverge in adolescence. Adolescence is a time of increased emotional sensitivity, particularly with stress, from significant changes within biological and environmental settings (Shiner et al., 2017). Adolescents are also more likely to perform impulsive or risky behaviours, potentially increasing their likelihood of psychosocial stress exposures (Kuhlman et al., 2020). Teenage retrospective models offer valuable approaches for adversity studies as questionnaires addressing threat and deprivation have been shown to be reliable methods for reporting recent and childhood experiences in adolescence (Berman et al., 2022). This is particularly relevant for monozygotic twin studies as these individuals start to show epigenetic differences during this period (Fraga et al., 2005), and environmentally-induced differences in methylation become visible (Planterose Jimenez et al., 2021).

Despite the developmental changes in adolescence, disclosing personal experiences of maltreatment or abuse can be challenging (McKay et al., 2022). Self-reporting tools that offer additional non-responses and indirect items have been shown to help high risk populations with disclosing sensitive information (Carmel & Widom, 2020). While retrospective reports are subject to recall bias (Müggenburg, 2021), they remain a valuable method for assessing adversity exposure and its long-term effects (Berman et al., 2022). Given that early-life adversity is associated with alterations in immune function (Cunningham et al., 2022; Elwenspoek et al., 2017; Fernandes et al., 2021) and increased risk for psychopathologies (Heim & Nemeroff, 2001) (Reid & Danese, 2020), understanding these experiences is crucial for identifying developmental risk factors.

Our previous analysis of the German TwinLife study suggested that twin life trajectories are more divergent than traditionally assumed. Retrospective data revealed that measurable divergence in twins' experiences begins prior to adolescence, with differences observable by age 11 (Turner et al., 2020). In this study, we focused on older twin pairs who consented to

participate in a large-scale research project, using dedicated screening interviews to assess their subjective interpretation of adversity. Given Germany's general socioeconomic stability, our cohort did not experience extreme hardships such as war, food insecurity, or severe poverty. Instead, we examined adversities relevant to adolescent development and the "Gunnar window," including parental transitions, changes in caregivers, family bereavement, serious illness or injury, financial instability, and social exclusion (Ellis et al., 2022). The screening questionnaires were designed to capture these adversities, which are common in middle-class Western populations and are known to influence health (Danese & McEwen, 2012) and its biomarkers, such as physiological, psychological, and biological aging, immune and endocrine functioning (Belsky, 2019; Belsky et al., 1991; Graf et al., 2022) outcomes across the lifespan.

The divergences we identified span a broad range of environmental factors with potential long- term phenotypic consequences, particularly through epigenetic modifications (Mill & Petronis, 2007) (Copeland et al., 2022; McCrory et al., 2022; Rampersaud et al., 2022). Phenotypic differences were evident in both physical and mental health outcomes. Factor analysis of adversity exposures in MZ and DZ twins indicated that adversity factor structures remained consistent across zygosity, suggesting minimal genetic confounding. However, one notable exception was the total number of life events, which loaded more strongly on the adversity factor in MZ twins compared to DZ twins. Additionally, DZ twins exhibited greater Euclidean distances and composite total adversity scores than MZ twins, suggesting that DZ twins experience greater divergence in life experiences and subjective interpretations of adversity.

The greater divergence observed in DZ twins may be partly attributable to differences in genetic and epigenetic similarity. DNA methylation has been implicated in learning and memory(Yu et al., 2011), risk-taking behavior (Kaminsky et al., 2008), and both social and

stress responses (McGowan et al., 2009) (Kader et al., 2018). Methylation-induced impairment of glucocorticoid receptor function (NR3C1 gene) disrupts hypothalamic-pituitary-adrenal (HPA) axis regulation, altering stress responsiveness (Schneider et al., 2016; Weaver et al., 2004). Since MZ twins exhibit greater similarity in DNA methylation patterns than DZ twins (Fraga et al., 2005) (Hannon et al., 2018), this could partly explain the reduced divergence observed in MZ twin pairs. Our findings were further validated using ACE variance modeling. While differences emerged between questionnaire responses, the results underscore the importance of unique environmental influences in shaping adversity interpretation.

As with all human studies, there are limitations. While we assembled what the prior literature suggested was a suitable series of questionnaires, no reasonable study design can cover all types of imaginable and unimaginable adversities that can be encountered. Instead, we focused on measures that are common to everyone (daily hassles, bitterness) as well as measures that could be a projective proxy for other negative life experiences (humiliation can be a part of bullying, discrimination, and abuse). Additionally, recall bias remains an inherent limitation in retrospective self-reports; however, online survey formats have been shown to partially mitigate this issue (Holuka et al., 2021) (van Gelder et al., 2020).

There is now compelling evidence that case co-twin study designs are a powerful tool for detecting and evaluating subtle environmental effects. Our study demonstrates that twins' lives, particularly in the context of adversity exposure, are sufficiently divergent to warrant further investigation. Recruiting adversity-divergent twins offers a unique opportunity to explore the role of the adult social environment in shaping lifelong health trajectories.

Adversity and resilience are deeply interconnected. Broadly, resilience is the ability of the individual to regain, or at least maintain their function when faced with adversity. Unlike the

current adversity literature, there has been significant work performed to come to a clear quantifiable definition of resilience, as the "residual variance in psychosocial functioning that remains after accounting for adversity exposure" (Cahill et al., 2022). When this theoretical construct was examined in the ALSPAC dataset, its many putative protective factors, with the notable exception of socioeconomic elements, showed strong construct and predictive validity (Cahill et al., 2022).

The growing body of adversity research increasingly emphasizes the need for quantification, yet significant debate persists regarding whether adversity is cumulative or multidimensional. While a dose-response relationship between adversity and later-life outcomes is often assumed, this is not universally observed (McLaughlin et al., 2014). Traditional cumulative models sum adversity exposures, whereas dimensional models categorize adversity based on shared neurodevelopmental effects (Sheridan & McLaughlin, 2014). However, recent dimensional models have largely neglected biological correlates, although they have proven highly successful in identifying the psychopathological effects of a threatening or deprived environment. Despite our individual questions mapping to the different dimensions, analysis of the underlying factor structure did not reveal clear clustering within adversity dimensions, suggesting that adversity may be more amorphous than previously thought.

In conclusion, despite the assumption that twin lives are largely identical, our findings reveal substantial divergence in their psychosocial environments. By focusing on early-adolescence to early-adulthood, we identified significant differences in the psychosocial environment and the perception of stress, daily hassles, and adversity. The identification of adversity-divergent twin pairs enables future research into the biological, neurodevelopmental, and health consequences of these environmental exposures, independent of genetic and early-life confounds.

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Conflict of Interest: The authors declare that they have no known conflict of interest.

Ethical statement: The research received the ethical approval from the Ethical Committee of Bielefeld University no. 2020-184-W1, and performed according to the Declaration of Helsinki. All participants provided written informed consent. Participants received a small monetary reward for participation.

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Supplementary Table 1 : Outline of Questionnaires

1. Life events questionnaire (LEV)

		Before starting school	During elementary school	During secondary education	Before university/college	After university/college
Item 1	Parental separation/divorce	0 - no; 1 - yes				
Item 2	Financial issues					
Item 3	Severe illness/accident					
Item 4	Death of a close person					
Item 5	Own separation/divorce	N/A	N/A			
Item 6	Job loss	N/A	N/A			

2. Humiliation Scale (HUM)

Item 1	Being excluded	1 - not at all impacted; 5 - very strongly impacted
Item 2	Being mocked/made fun of	1 - not at all impacted; 5 - very strongly impacted
Item 3	Being put down	1 - not at all impacted; 5 - very strongly impacted
Item 4	Being bullied	1 - not at all impacted; 5 - very strongly impacted

3. Daily Hassles Scale (DHS)

Item 1	Difficulties with social obligations	1 - not at all impacted; 5 - very strongly impacted
Item 2	Difficulties with familial obligations	1 - not at all impacted; 5 - very strongly impacted
Item 3	Health problems	1 - not at all impacted; 5 - very strongly impacted
Item 4	Financial limitations	1 - not at all impacted; 5 - very strongly impacted
Item 5	Dissatisfaction with higher education or job	1 - not at all impacted; 5 - very strongly impacted
Item 6	Difficulties with miscellaneous activities	1 - not at all impacted; 5 - very strongly impacted
Item 7	Dissatisfaction with housing situation	1 - not at all impacted; 5 - very strongly impacted
Item 8	Frequent conflict with close persons	1 - not at all impacted; 5 - very strongly impacted
Item 9	Frequent conflict with other persons	1 - not at all impacted; 5 - very strongly impacted
Item 10	Other (unspecified) difficulties	1 - not at all impacted; 5 - very strongly impacted

4. Other as Shamer Scale (OAS)

Item 1	I feel other people see me as not good enough.	1 - never; 5 - almost always
Item 2	Other people see me as small and insignificant.	1 - never; 5 - almost always
Item 3	People see me as unimportant compared to others.	1 - never; 5 - almost always
Item 4	Other people see me as not measuring up to them	1 - never; 5 - almost always
Item 5	I think that other people look down on me.	1 - never; 5 - almost always
Item 6	I feel insecure about others' opinions of me.	1 - never; 5 - almost always
Item 7	Others think there is something missing in me	1 - never; 5 - almost always
Item 8	Other people see me as somehow defective as a person.	1 - never; 5 - almost always

5. Social Support Scale (lack thereof was coded in reverse scores) (SUP)

Item 1	I receive the emotional support from my family that I need.	1 - strongly disagree, 2 - disagree, 3 - don't agree or disagree, 4 - agree, 5 - strongly agree
Item 2	I can talk to my twin sibling about my problems.	1 - strongly disagree, 2 - disagree, 3 - don't agree or disagree, 4 - agree, 5 - strongly agree
Item 3	I have friends with whom I can share my joys and my problems.	1 - strongly disagree, 2 - disagree, 3 - don't agree or disagree, 4 - agree, 5 - strongly agree

6. Covid-19 Pandemic Impact Scale (BIT)

Item 1	I feel bitter about how the government dealt with the challenges brought about by the pandemic so far.	1 - strongly disagree, 2 - disagree, 3 - undecided, 4 - agree, 5 - strongly agree
Item 2	I feel angry when I see how inconsiderately and selfishly many peers behaved during the pandemic so far.	1 - strongly disagree, 2 - disagree, 3 - undecided, 4 - agree, 5 - strongly agree

2. Study II: From Adversity To Psychopathology: Long-Term Epigenetic Consequences in Adversity-Divergent Twins

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Abstract

Objectives:

This study aimed to investigate whether postnatal psychosocial adversity (PSA) is biologically embedded through DNA methylation (DNAm) and whether PSA-induced epigenetic alterations mediate the association between PSA exposure and psychological ill-health. By using monozygotic (MZ) twins discordant for individual PSA experience, the study isolated environmentally driven epigenetic effects while controlling for genetic and prenatal confounding factors.

Materials and Methods:

Nine pairs of MZ twins (N = 18) were selected from a non-clinical twin cohort based on within-pair differences in PSA experienced during childhood, adolescence, and early adulthood. PSA was assessed using standardized questionnaires capturing social, familial, and socioeconomic stressors. Psychopathological symptoms were evaluated via structured clinical interviews based on criteria established in the DSM-5. DNAm biomarkers were extracted from whole-blood samples and analyzed using the Illumina Infinium MethylationEPIC v2.0 BeadChip. Dimension reduction techniques, differential methylation analyses, and nonparametric mediation models - including twin-controlled within-pair analyses - were applied.

Results:

Adversity-exposed twins exhibited significant differential DNAm across multiple CpG methylation sites, with both hypermethylation and hypomethylation observed in genomic regions implicated in neurodevelopmental, neurotransmission, and immune-related pathways. Mediation analyses identified several CpG methylation sites which significantly mediated the association between PSA and psychological symptom severity. Notably, hypomethylation

within genes such as *GTF2I* and *PRKAR1B* consistently showed suppression (protective) effects across multiple internalizing symptom domains, whereas methylation at other loci amplified adversity-related manic symptoms. Several mediating CpGs overlapped with loci previously identified in epigenome-wide association studies of psychiatric disorders.

Conclusions:

Our findings suggest that postnatal psychosocial adversity is associated with lasting epigenetic modifications that partially mediate the development and severity of psychopathological symptoms. DNAm appears to function both as a vulnerability and compensatory mechanism, depending on genomic context. The use of adversity-discordant MZ twins underscores the role of environmentally driven epigenetic regulation in mental health and highlights DNAm as a potential biomarker linking lived experience to psychopathology.

Keywords: psychosocial adversity; DNA methylation; monozygotic twins; psychopathology; epigenetics; mediation analysis

1. Introduction

Mental ill-health is one of the leading global challenges, affecting millions of individuals across all socioeconomic backgrounds. Depression, anxiety, and other mental disorders contribute significantly to the global burden of disease, with mental disorders being among the primary causes of disability worldwide (GBD 2019 Mental Disorders Collaborators, 2022). These disorders are characterized by disruptions in emotion regulation, cognitive function, and social behaviors, which can profoundly affect an individual's quality of life (World Health Organization, 2025). This highlights the need for a better understanding of the etiology of mental disorders to target high-risk groups and establish preventative measures. The link between life adversity and mental ill-health is well-documented in the literature; however, the identification and understanding of biomarkers and physiological mediating factors and mechanisms remain an ongoing area of research.

In an effort to identify some of the factors contributing to the interplay between adversity and the etiology of mental disorders, epigenetics has emerged as a pivotal field. Epigenetic modifications such as DNA methylation (DNAm), histone modification, and non-coding RNA interactions are dynamic processes influenced by environmental factors and life experiences (Kubota et al., 2012). DNAm, a prominent factor in the framework of biological embedding, is a process in which methyl groups are added to the fifth position of cytosines, typically at cytosine-guanine dinucleotides (CpG), silencing or enhancing gene expression without altering the DNA sequence. This impact on gene expression is critical for mammalian development throughout life, making DNAm potential mediating factor between adversity and mental disorders. Importantly, CpG methylation has been proposed as a mechanism used by mammals to silence deleterious and parasitic DNA (Jones et al., 2001). Epigenetic modifications, including DNAm, are associated with inflammaging and immunosenescence, promoting a chronic, low-grade inflammatory systemic state (Zhu et al.,

2021). These immune changes can disrupt brain function through neuroinflammation (Wu et al., 2023), hypothalamic-pituitary-adrenal (HPA) axis dysregulation Hassamal et al., 2023), and neurotransmitter imbalances (Rawani et al., 2024), thereby contributing to the development and progression of mental disorders. Specifically, DNAm has been implicated as a biomarker or mediating factor in a broad spectrum of mental disorders, including major depression, bipolar disorder, schizophrenia, and alcohol dependence disorder (Starnawska et al., 2021; Lussier et al., 2024). These mechanisms provide a model for understanding how adverse life events contribute to the onset and severity of psychological symptoms.

DNAm patterns are particularly responsive to external factors from conception throughout prenatal development. Multiple maternal lifestyle factors - such as stress, smoking, and nutritional supplementation - can influence DNAm, making it challenging to compare adversity-induced methylation patterns across individuals (Starnawska et al., 2021). Postnatally, DNAm has also been proposed to be the result from lifestyle factors such as stress, offspring nutrition, and environmental exposures (Bekdash, 2021). However, it is difficult to pinpoint the exact role of external factors from birth to adulthood on DNAm and its outcomes due to the variety of genetic predispositions that make methylation patterns unique to each individual. To the best of our knowledge, no study has comprehensively examined the broad range of mental disorders outlined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (5th ed.; American Psychiatric Association, 2013) in relation to adversity-induced DNAm as a mediating factor, while isolating postnatal influences. To explore methylation effects that occurred exclusively in childhood, adolescence, and early adulthood, we collected blood samples from nine pairs of monozygotic (MZ) twins (N = 18). To investigate the role of adversity-induced DNAm in the development of psychological symptoms, we selected twin pairs with unequal experiences of adversity across various life stages. The aim of this study is to examine which CpG

methylation sites or patterns - and their associated genes - act as mediating factors in the interplay between psychosocial adversity (PSA) and the development of mental disorders. The results of this study have the potential to inform at-risk populations about interventions to counter the effects of immune aging and immunosenescence that emerge as a result of PSA, leading to improved mental health outcomes.

2. Materials and Methods

2.1. Ethics Information

This study was approved by the respective ethics committees of the University of Bielefeld (approval number: EUB2020-184) and the University of Luxembourg (approval number: ERP 22-078). All participants provided informed consent and gave permission for data collection and processing prior to the study. All collected data underwent pseudonymization, and no individual persons are identifiable in any published results. All participants received a compensation of 40€ for providing their biological samples, and 50€ for participating in the clinical psychological interviews.

2.2. Participants

All participants (N = 18) were monozygotic (MZ) twin pairs sampled from the 3 oldest waves of the TwinLife study (N=710, $mnage_{cohort2}$ = 18.0, $mnage_{cohort3}$ = 24.1, $mnage_{cohort4}$ = 30.0) REF, belonging to a German genetic study investigating the socioeconomic and health inequalities across multiple stages of life (Diewald et al., 2024; Hahn et al., 2016). We utilized the entire sample of N = 18 individuals for individual-level analyses, which formed N = 9 twin pairs for delta analyses. We assessed socioeconomic indicators of childhood (pre-schooling age), adolescence, and young adulthood adversity, using a common stressor questionnaire, and selected items from the Humiliation Scale (Hartling et al., 1999), Other as Shamer Scale – 2 (Matos et al., 2015), Brief Daily Stressors Screening Tool (Scholten et al., 2020), and Social Support Scale (Zimet et al., 1988). These questionnaires explore perceived social rejection, financial difficulty, familial and household conflict, and job dissatisfaction. Twin pairs who completed the full pre-screening and whose within-pair total score difference was at least 1 standard deviation from the sample mean, were recruited for subsequent biological sampling and clinical psychological assessment. Composite adversity score (CAS)

for each participant was created by standardizing and aggregating all individual adversity subscale scores.

2.3. DNA Extraction

Blood of the selected MZ ImmunoTwin participants was collected at local medical facilities by general practitioners or nurses drawing circulatory blood. Peripheral blood was collected into Paxgene Blood RNA Tubes (BD Biosciences, Franklin Lakes), preserving RNA integrity during transportation and storage, and 10ml into EDTA blood tube (KS Medical, Seoul). DNA isolation was performed on 22 blood samples, out of which 18 belong to 9 twin pairs, and 4 samples are unpaired, stored in PaxGene RNA tubes (BD Biosciences, Franklin Lakes). The DNA extraction protocol is based on QIAamp DNA Blood Midi Kit (Qiagen, Hilden) recommendations although some adjustments were made. 2ml of PaxGene (BD Biosciences) whole blood mix per sample was used. 350 to 500ng of DNA was used for bisulfite conversion following the EZ DNA Methylation Kit (Zymo research, Irvine, California, US) recommendations. To assess DNA methylation patterns we used the Infinium Methylation EPIC v2.0 BeadChip (Illumina) and iScan (Illumina). Samples were processed according to the Infinium HD Methylation Assay Reference Guide's recommendations (Illumina) using GenomeStudio Software 2.0 (Illumina) for quality control.

2.4. Clinical Psychological Screening

Clinical-psychological interviews conducted online were used to assess psychological symptom scores. Each participant completed the same item protocol based on the German version of the Mini-DIPS clinical interview (Margraf et al., 2017) via video call, which assesses the presence, severity, duration, and frequency of psychological symptoms along DSM-5 domains such as depressive disorders, anxiety disorders, or eating disorders. Symptom scores were computed across all symptom categories for each participant, and within-pair score deltas (Δ) for subsequent analyses were calculated for each twin pair.

Additionally, items indicative of clinically relevant disorders, as defined by the DSM-5, were summarized.

2.5. DNAm Data Analysis

Data collected from blood samples was analyzed with R using SeSAMe pipeline (Sensible step-wise analysis of DNA methylation bead chips) (Zhou et al., 2018) for pre-processing. β - values ranging from 0 to 1 were used for further analysis where a value of 0 indicates that none of the copies of a specific CpG site are methylated, while a value of 1 means that all copies are methylated (Pidsley et al., 2016).

2.6 Statistical Power Analysis

Given that the sampling was completed before the statistical analyses, we conducted sensitivity power analyses using G*Power 3.1 (Faul et al., 2009) to determine the minimum detectable effect size given the modest sample sizes. It is important to note that the reported power analyses reflect sensitivity for simple association tests and are provided for orientation – they do not estimate power for the bootstrapped mediation analyses conducted in this study.

Individual-level analyses (N = 18)

A two-tailed sensitivity analysis for Pearson correlation ($\alpha = 0.05$, power = 0.80) indicated that the study was sufficiently powered at N = 18 to detect correlations of $r \geq 0.46$. This indicates that the sample size could reliably detect moderate-to-large correlations

Delta-level analyses (N = 9)

For within-pair delta analyses treating each twin pair as a single data point (N = 9), a two-tailed sensitivity analysis for a one-sample t test ($\alpha = 0.05$, power = 0.80) indicated that effects of at least Cohen's d = 1.07 could be detected. Only very large, consistent within-pair effect could be detected reliably with this sample size.

3. Results

3.1. Identification of Candidate DNA Methylation Sites Through Dimension Reduction

Preliminary analysis identified over 800.000 methylated CpG sites across each participant in our sample. To reduce this β-matrix into a subsample of values that meaningfully separate the ELA sample from non-ELA controls, we performed a Partial Least Squares Discriminant Analysis (PLS-DA), which is a supervised dimension reduction model with pre-established parameters (Buchanan et al., manuscript in preparation; Mposhi et al., manuscript in preparation). This approach yielded a reduced β-matrix of 7.260 CpG methylation sites, that we used for further downstream analysis. Sparse Partial Least Squares (sPLS) regression was subsequently applied on the reduced β-matrix to identify biologically meaningful CpG methylation clusters that load strongly on the respective psychosocial adversity scales utilized in this study.

Each dimension reduction model yielded one component of candidate CpG methylation sites associated with each respective PSA subscale. These CpG methylation sites were associated with genes specifically in the 3'UTR, 5'UTR, gene body, and promoter regions, involved in synaptic function, GABA-signaling, susceptibility to psychological and neurodevelopmental disorders, neurotransmitter function, release and regulation, and neuronal development (Tuñon-Ortiz et al., 2025; Shennib et al., 2025; Lohoff et al., 2008; Katrancha et al., 2019; Cruceanu et al., 2016; Li et al., 2023; Gao et al., 2021; Li et al., 2022) (KIRREL3, SLC18A1, RIMS2, TRIO, NFASC, SYN3, DIP2C). Furthermore, meaningful loadings were observed across CpG DNAm sites associated with genes involved in immune or inflammatory processes (Zhang et al., 2022; Klaus et al., 2021; Wu et al., 2024) (CYBA, TNFSF10, PGF). The CpG DNAm sites retained in the dimension reduction were used for subsequent mediation analyses.

Annotation of CpG probes was conducted in R (v4.x) using the minfi (Aryee et al., 2014) package to access official Illumina manifest files from the IlluminaHumanMethylationEPICanno.ilm10b4.hg19 (EPIC v1, hg19 build) and IlluminaHumanMethylationEPICv2anno.20a1.hg38 (EPIC v2, hg38 build) Bioconductor packages. Where probes were present in both manifests, annotation from the newer EPIC v2 (hg38) build was prioritized. The final dataset included probe genomic coordinates, island relations, and UCSC gene annotations, providing biological and functional context for CpG sites identified through subsequent analyses.

3.2. Case-Controlled Analysis Shows Differential Methylation Dependent on Adversity Exposure in Monozygotic Twins

To establish CpG sites meaningful for further analysis, we applied aggregate PSA scoring to discriminate between exposed and control twin in each twin pair based on the degree of exposure. We then used the Wilcoxon signed-rank test in the combination with Analysis of Covariance (ANCOVA) to identify CpG methylation β's that differ meaningfully between twins that were exposed to a higher degree of PSA as opposed to their sibling, with sex as a covariate. In total, 73 CpG sites were found to be differentially methylated depending on adversity exposure. Consistent with established epigenetic models, promoter/CpG-island hypermethylation is generally associated with reduced gene expression, while hypomethylation increases gene expression, however, outside promoters, the relationship is context-dependent (Jones et al., 2012; Deaton et al., 2011; Moore et al., 2013; Schübeler et al., 2015; Jjingo et al., 2012; Kang et al., 2019; Kreibich et al., 2023; Kaluscha et al., 2022). Significant hypermethylation (r = 0.36–0.83, p = 0.001–0.04) linked to higher PSA exposure in our sample was observed for 34 DNAm sites, out of which 18 could be meaningfully linked to gene regions according to Illumina (Illumina). In summary, PSA-exposed twins exhibited hypermethylation at multiple genomic sites, predominantly in gene promoters/first

exons. The hypermethylation of these genes has been implicated in malfunctions of the nervous or immune system, neurobehavioral dysregulation (Comasco et al., 2015), and even suicidal behavior (Keller et. al, 2010) (ADRA2A, BDNF-AS/BDNF). SYNGR3 and PDE1B, hypermethylated in the PSA-exposed twins, are implicated in dopaminergic neurotransmission, and cAMP signaling and cognition (Egaña et al., 2009, McQuown et al., 2019). Conversely, significant hypomethylation (r = –0.46 to –.086, p = 0.001–0.04), associated with higher PSA exposure was observed in 39 DNAm sites, out of which 24 could be traced back to genomic regions. Although adversity exposure is normally associated with greater methylation, there is growing evidence that hypomethylation in stress-exposed individuals occurs as well, and may act as a protective mechanism, especially in mediating psychopathologies such as mood disorders (Lussier et al., 2024).

3.3 Associations Between Psychosocial Adversity and DNA Methylation

Exploratory analyses examining adversity subscales revealed differential associations with PSA across biologically relevant CpG sites. We examined correlations between individual adversity subscales and methylation at each CpG site (Figure 1). Consistent with the mediation results, cg17148317 (CHST13) showed the strongest correlation with Daily Hassles Scale (r = -0.68, p = 0.002), followed by Other as Shamer Scale (r = -0.52, p = 0.026). Similarly, cg20698667 (TGFB2) demonstrated significant negative correlations with Other as Shamer Scale (r = -0.55, p = .019) and Daily Hassles Scale (r = -0.52, p = 0.026), with a marginal association with Humiliation Scale (r = -0.44, p = 0.068). Additional CpG methylation sites examined revealed further patterns of adversity differentiation. Most notably, cg00299036 (GFI1B) exhibited a particularly strong negative correlation with Other as Shamer Scale (r = -0.78, p < 0.001), representing the strongest single adversity-methylation association observed in the dataset. Additionally, cg05124073 (NFASC) showed robust negative correlations with Daily Hassles Scale (r = -0.79, p < 0.001) and Other as

Shamer Scale (r = -0.58, p = 0.011). Interestingly, while most CpG sites showed negative correlations with adversity measures, cg15060929 (SS18) displayed a contrasting pattern of positive associations, particularly with Daily Hassles Scale (r = 0.71, p = 0.001) and Other as Shamer Scale (r = 0.59, p = 0.010). Among the remaining CpG sites examined, cg24977276 (GTF2I) showed significant negative correlations with Other as Shamer Scale (r = -0.57, p = 0.014) and Humiliation Scale (r = -0.51, p = 0.030), while cg08461802 (DIP2C) was most strongly associated with Humiliation Scale (r = -0.60, p = 0.009). The Lack of Social Support Scale showed consistently weaker associations across all CpG sites, suggesting that the absence of social support may operate through different biological pathways than the presence of social stressors.

3.3 Mediation Analysis of Differentially Methylated Candidate Loci

Due to the relatively small and non-normally distributed sample size of 18 MZ twins yielding 9 pairs, we ran nonparametric bootstrap with 500 - 1.000 simulations to assess whether there are indicators of mediation. Our mediation model assumed that the association between aggregate adversity exposure score and respective psychological symptom (PSY) scores would be mediated by hyper- or hypomethylation of each candidate CpG methylation β-value. First, we used Ordinary Least Squares (OLS) mediation model on within-pair deltas as predictor (Δ_PSA), outcome (Δ_PSY), and mediator (Δ_DNAm). Subsequently, Cluster-Robust Mediation (CRM) analysis was used on individual participants, taking into consideration the previously established differential methylation results. Direct, indirect, total effects, and path coefficients were calculated to investigate the direction of each mediation pathway. We decided to interpret mediation with non-significant direct effects as meaningful according to Shrout & Bolger (2002), who argue that path c (the total effect of X on Y) does not need to be statistically significant to test for and establish mediation, particularly if the sample is small and not normal, and theoretical arguments for mediation are sufficient

(Shrout & Bolger, 2002). Notably, most of the mediation established confirmed hypomethylation relative to PSA exposure, suggesting a dose-dependent suppression effect of adversity on psychological symptoms by means of DNA methylation. Notably, all within-pair mediation effects were consistent (positive indirect effects), indicating that methylation changes *transmitted* rather than *buffered* the adversity–symptom relationship. This pattern reflects the negative path *b* coefficients observed in the delta analysis, whereby the twin with higher methylation tended to have *lower* symptoms—thus, adversity-induced hypomethylation contributed to increased symptom risk. The results are summarized in Figure 2.

3.4 Within-Pair Difference Mediation Analysis

To leverage the MZ twin design, we conducted within-pair difference mediation analyses using delta scores (Twin1 − Twin2) for adversity, DNAm β-values, and PSY score symptoms. This approach controls for shared genetic and early environmental factors, isolating the effect of within-pair differences in adversity exposure on within-pair differences in psychopathology through epigenetic mechanisms.

Three CpG sites were examined as candidate mediators: cg20698667 (*TGFB2, gene body*), cg24977276 (*GTF2I, gene body*), and cg17148317 (*CHST13, gene body*). All 3 methylation sites had differential β-values between exposed and control twins (($F(1, 15) = 37.64$, $p < 0.001$, $\eta^2p = 0.72$, $\Delta\beta = -0.020$), ($F(1, 15) = 49.69$, $p < 0.001$, $\eta^2p = 0.77$, $\Delta\beta = -0.009$), ($F(1, 15) = 34.38$, $p < 0.001$, $\eta^2p = 0.70$, $\Delta\beta = -0.024$)). Bootstrap mediation analyses (500 simulations) revealed significant indirect effects for all three CpG sites when using the composite adversity measure as the predictor (Table 2).

For cg20698667, within-pair differences in total adversity significantly predicted within-pair differences in methylation (path $a = -0.00176$, $p = 0.006$), and methylation differences predicted bipolar disorder symptom differences controlling for adversity (path $b = -292.60$, $p = 0.035$). The indirect effect was significant ($ab = 0.514$, $p = 0.044$), indicating consistent mediation whereby adversity-induced hypomethylation partially transmitted the effect of adversity on bipolar disorder symptoms.

Similarly, for cg17148317, both path coefficients were significant (path $a = -0.00177$, $p = 0.039$; path $b = -206.38$, $p = 0.018$), yielding a significant indirect effect (0.365, $p = 0.036$).

The path mediated by cg24977276 showed a significant path a (−0.00082, $p = 0.003$) and significant indirect effect (0.490, $p = 0.040$), though path b did not reach significance (−594.96, $p = 0.114$), therefore robust mediation could not be reported.

3.5 Individual-Level Mediation Analysis

3.5.1 GTF2I-Associated Hypomethylation Mediates the Relationship Between Psychosocial Adversity and Major Depression Symptoms

Methylation at a CpG site located within the gene body of *GTF2I* (cg24977276) mediated the relationship between PSA and major depression (MD) symptom severity. In the resent sample, higher PSA exposure was significantly associated with lower methylation at cg24977276 (path a: $b = -0.0004$, $p = 0.005$), and a significant group-level difference in methylation was observed between adversity-exposed and control twins (ANCOVA: $p = 0.028$). The methylation–symptom path was also significant (path b: $b = 795.31$, $p = 0.034$), indicating that higher methylation was associated with higher MD scores. Mediation analysis revealed a significant negative indirect effect ($ab = -0.31$, $p = 0.012$), with 68.0% of the total effect mediated through cg24977276 methylation. The direct effect of adversity on MD symptoms remained significant after controlling for methylation ($c' = 0.76$, $p = 0.002$). No

significant sex effects were observed. These results are summarized in Table 3a and Figure 3a.

The negative indirect effect indicates a suppression pattern: while adversity was directly associated with increased MD symptoms (positive direct effect), it simultaneously triggered hypomethylation at cg24977276, which was in turn associated with *lower* symptom severity (negative indirect effect). This suggests that *GTF2I*-linked epigenetic changes may partially buffer the impact of adversity on depressive symptoms.

Exploratory analysis of generalized anxiety disorder (GAD) symptoms revealed a similar pattern, with a significant path *a* ($b = -0.0004$, $p = 0.005$) and a significant indirect effect ($ab = -0.21$, $p = 0.016$). However, path *b* was only marginally significant ($b = 549.25$, $p = 0.061$), therefore no robust mediation was observed. Indirect effects for both MD and GAD did not survive Benjamini-Hochberg correction for multiple comparisons.

3.5.2 Preliminary Evidence for PRKAR1B-Associated Hypomethylation as a Potential Mediator of Psychosocial Adversity and Major Depression

Methylation at a CpG site located within the gene body of *PRKAR1B* marginally mediated the relationship between PSA and symptoms of MD. Within twin pairs, higher adversity exposure was associated with lower methylation at cg17650397, though this relationship was only marginally significant (path *a*: $b = -0.0005$, $p = 0.069$). Exposed and control twins showed differential methylation β-values for cg17650397 ($F(1, 15) = 34.47$, $p < 0.001$, $\eta^2p = 0.70$, $\Delta\beta = -0.016$). Mediation analyses revealed nominally significant negative indirect effects on symptom scores for MD ($b = -0.18$, $p = 0.040$); however, these effects did not survive BH correction for multiple comparisons. The proportion of the total effect mediated by cg17650397 was 40.3% for MD. The negative indirect effects indicate a suppression pattern: higher adversity was associated with lower methylation at cg17650397, whereas

lower methylation was associated with lower symptom scores. Notably, the methylation → symptom path (path *b*) was significant for MD ($b = 367.49$, $p = 0.035$), suggesting a mechanistic link between *PRKAR1B* gene body methylation and depressive symptoms. The direct effects of adversity on symptom scores remained significant ($b = 0.64$, $p = 0.003$) after controlling for methylation. No significant sex effects were observed. These results are summarized in Table 3b and Figure 3b. These findings suggest that *PRKAR1B*-linked epigenetic regulation may modulate the relationship between stress exposure and psychological symptoms, though the marginally significant path *a* and the lack of significance after multiple comparison correction warrant cautious interpretation and replication in larger samples.

3.6 Mediation Effects Without Differentially Methylated Candidate Loci

3.6.1 SS18-Associated Methylation Mediates the Link Between Psychosocial Adversity and Bipolar Disorder Symptoms

Although we did not observe differential methylation of cg15060929 (TSS200 promoter region of *SS18*) between exposed and control twins ($F(1, 15) = 2.38$, $p = 0.143$, $\eta^2p = 0.14$, $\Delta\beta = 0.007$), it emerged as a significant mediator of the relationship between PSA and bipolar disorder symptoms (BPD). The CpG methylation site demonstrated consistent mediation pattern, in contrast to the suppression patterns observed for other CpG sites in this study. For cg15060929, adversity was significantly associated with increased methylation (path *a*: $b = +0.0008$, $p = 0.002$), and higher methylation was significantly associated with higher symptom scores (path *b*: $b = +253.38$, $p = 0.020$). The indirect effect was significant ($b = +0.20$, $p = 0.008$), with a proportion mediated of 94.9%, indicating near-complete mediation. The direct effect was non-significant ($b = 0.01$, $p = 0.924$), suggesting that the effect of adversity on bipolar symptoms operates almost entirely through methylation at this locus. No significant sex effects were observed ($ps > 0.15$). These results are summarized in Table 3c,

and Figure 3c. These findings suggest that SS18 methylation may serve as a mechanistic pathway through which adversity influences bipolar symptomatology.

4. Discussion

In this study, we investigated the role of DNA methylation (DNAm) in the relationship between psychosocial adversity and mental health outcomes in young adults. Using a monozygotic (MZ) twin design with adversity-divergent co-twins, we aimed to isolate the effects of differential post-natal exposures on biological embedding while controlling genetic predispositions as confounders. Twin design has been used by multiple previous studies to investigate epigenetic mechanisms encoding environmental exposure when randomized-controlled trials are impossible, because it controls for genetic, age, sex, and shared early-life/in-utero confounding factors (Turner et al., 2020). This approach made it possible to examine whether PSA-induced DNAm changes serve as biological mechanisms through which environmental stressors become embedded in the biological system.

Dimension Reduction Identifies Key CpG Methylation Site Candidates

First, we conducted dimension reduction to distil a large pool of CpG methylation sites yielded from data collection into candidates that were meaningfully linked to PSA indicators in our sample. We identified 73 differentially methylated CpG sites between adversity-exposed and control twins, with both hypermethylation and hypomethylation patterns observed depending on genomic context. We created a smaller candidate pool out of those which were meaningfully associated with particular gene regions and biologically relevant.

CpG Methylation Sites Show Dose-Dependent Relationship with Psychosocial Adversity

In an exploratory correlation analysis between candidate CpG methylation sites and PSA subscales, we established statistically significant DNA methylation (quantified by β-values) differentiation in dose-dependent relationships with various PSA subscales. This analysis was conducted to partially replicate findings from studies examining biological embedding and to evaluate support for our candidate CpG methylation pool. Several candidate sites showed

significant positive or negative correlations across various genomic regions, highlighting the diverse biological interpretations of DNA methylation depending on both direction and genomic location.

Notably, cg05124073, located in the 3' untranslated region (3'UTR) of the *NFASC* gene, showed significant negative correlations with the Daily Hassles Scale and Other as Shamer Scale ($p < .001$, $r = -0.79$; $p = .011$, $r = -0.58$), indicating that higher exposure to daily difficulties and peer rejection is associated with *NFASC* hypomethylation at the 3'UTR. According to topographic mapping of the human genome, the 3'UTR region contains the highest density of DNA methylation, and its hypermethylation signifies that a gene is being actively transcribed (Luo et al., 2018). Furthermore, we observed significant negative associations between cg00299036, linked to the 5' untranslated region (5'UTR) of *GFI1B*, and both the Daily Hassles and Other as Shamer scales. This region of the *GFI1B* gene is normally unmethylated, so hypomethylation is generally associated with active gene expression by removing repression (Luo et al., 2018).

Perhaps most notably, we found a significant association between hypomethylation of cg17650397 and the Other as Shamer Scale ($p = .040$, $r = -0.49$), located in the *PRKAR1B* gene body. The *PRKAR1B* gene encodes the RIβ regulatory subunit of Protein Kinase A (PKA), a critical cAMP-dependent serine/threonine kinase implicated in synaptic plasticity (Glebov-McCloud, 2024). This subunit is predominantly expressed in the central nervous system (CNS), particularly within the brain stem, pituitary, hypothalamus, and the dentate gyrus and pyramidal cells of the hippocampus (Glebov-McCloud, 2024). Contrary to the classic paradigm of promoter hypermethylation being associated with reduced gene expression, DNA methylation at the gene body—a transcribed region—is typically positively correlated with gene expression (Lev Maor et al., 2015; Bewick et al., 2016; Yang et al., 2014). This suggests that adversity-induced hypomethylation in *PRKAR1B* may actually

decrease its expression, potentially exerting deleterious effects on synaptic transmission in the nervous system.

Another significant adversity-induced gene body hypomethylation was observed at cg08461802, located in the gene body of *DIP2C* ($p = 0.009$, $r = -0.60$). *DIP2C* is implicated in maintaining genomic stability through the regulation of DNA methylation and plays an essential role in early neurodevelopment (Ha et al., 2024). Additionally, it has been suggested as a candidate cancer gene, found mutated in approximately 5% of breast cancers and 9–14% of small-cell lung cancers (Larsson et al., 2017). Methylation at cg17148317, located in the *CHST13* gene body, was negatively associated with the Daily Hassles and Other as Shamer scales ($p = 0.002$, $r = -0.68$; $p = 0.026$, $r = -0.52$). Aside from its role in cellular signaling, *CHST13* is also a cancer candidate gene that exhibits significantly higher mRNA expression in malignant ovarian tumors compared to non-malignant tissues (Begolli et al., 2023). Since hypomethylation at the gene body is associated with reduced expression, this may suggest a stress-induced protective mechanism.

The gene body of *GTF2I* (cg24977276) showed hypomethylation in response to stress, particularly on the Humiliation Scale and Other as Shamer Scale ($p = 0.030$, $r = -0.51$; $p = 0.014$, $r = -0.57$). Similarly, the *TGFB2* gene body (cg20698667) was hypomethylated in response to adversity, particularly on the Daily Hassles and Other as Shamer scales ($p = 0.026$, $r = -0.52$; $p = 0.019$, $r = -0.55$).

Contrary to the overall trend of DNA methylation being negatively correlated with psychosocial adversity, cg15060929, located at the TSS200 region of the *SS18* gene, was positively correlated with the Daily Hassles and Other as Shamer scales ($p = 0.001$, $r = 0.71$; $p = 0.010$, $r = 0.59$), indicating hypermethylation in response to stress. TSS200 is a genomic region situated within the gene promoter, extending from the transcription start site (TSS) to

200 nucleotides upstream. Hypermethylation of this region typically functions as a mechanism for transcriptional repression or gene silencing (Li et al., 2024).

Mediation Analysis of Within-Pair Δ-Values

To leverage the unique advantage of the monozygotic twin design, we conducted within-pair difference mediation analyses using delta scores (Twin1 − Twin2) for adversity exposure, DNA methylation β-values, and psychiatric symptom measures. This calculation assumes that depending on the directionality of the Δ-values, overall association directionality can be derived. If the same twin consistently shows higher values in two or three variables, the relationship or mediation path will be positive. Conversely, a negative path means that a twin who shows a higher value in one domain, will show lower value in another domain. It is important to take into consideration that not all the twin pairs in the sample will exhibit the same directionality, but an aggregate, statistically significant result determines the overall trend. This approach controls for shared genetic sequence and early environmental factors, thereby isolating the effect of differential adversity exposure on psychopathology through epigenetic mechanisms. By examining within-twin-pair differences rather than individual values, we effectively removed genetic and shared environmental confounding, allowing us to attribute observed effects specifically to divergent post-natal environmental experiences.

Three CpG sites located in gene bodies were examined as candidate mediators: cg20698667 (*TGFB2*), cg24977276 (*GTF2I*), and cg17148317 (*CHST13*). All three sites showed significant differential methylation between adversity-exposed and control twins in a previous analysis, with large effect sizes: *TGFB2*, $F(1, 15) = 37.64$, $p < 0.001$, $\eta^2p = .072$, $\Delta\beta = -0.020$; *GTF2I*, $F(1, 15) = 49.69$, $p < 0.001$, $\eta^2p = 0.77$, $\Delta\beta = -.0009$; *CHST13*, $F(1, 15) = 34.38$, $p < 0.001$, $\eta^2p = 0.70$, $\Delta\beta = -0.024$. Notably, all three sites exhibited hypomethylation

in the adversity-exposed twin relative to the control twin, consistent with adversity-induced downregulation of DNA methylation at gene body regions, and overall trends in this sample.

Bootstrap mediation analyses (500 simulations) were conducted to test whether these methylation differences mediated the relationship between within-pair adversity differences and within-pair psychiatric symptom differences. When using composite adversity as the predictor, significant indirect effects emerged for all three CpG sites, providing evidence that adversity-driven methylation changes mechanistically contribute to psychiatric outcomes.

For cg20698667 (*TGFB2*), within-pair differences in total adversity significantly predicted within-pair differences in methylation (path $a = -0.00176$, $p = 0.006$), indicating that the twin with higher adversity exposure exhibited lower methylation at this locus. Methylation differences, in turn, significantly predicted bipolar disorder symptom differences after controlling for adversity (path $b = -292.60$, $p = 0.035$). The indirect effect was statistically significant ($ab = 0.514$, $p = 0.044$), demonstrating consistent mediation whereby adversity-induced hypomethylation partially transmitted the effect of adversity on bipolar disorder symptom severity. The negative path *b* coefficient indicates that the twin with lower methylation (due to higher adversity) tended to exhibit higher symptom scores, confirming that hypomethylation at *TGFB2* contributed to increased psychopathological risk.

Similarly, for cg17148317 (*CHST13*), both path coefficients were statistically significant (path $a = -0.00177$, $p = 0.039$; path $b = -206.38$, $p = 0.018$), yielding a significant indirect effect ($ab = 0.365$, $p = 0.036$). This pattern suggests that adversity-driven hypomethylation at this locus could mechanistically link environmental stress exposure to psychopathological outcomes, independent of genetic predisposition.

For cg24977276 (*GTF2I*), the adversity–methylation pathway was significant (path $a = -0.00082$, $p = 0.003$), and the overall indirect effect reached statistical significance ($ab =$

0.490, $p = 0.040$). However, the methylation–symptom pathway (path $b = -594.96$, $p = 0.114$) did not achieve conventional significance thresholds. Based on these results, no mediation effect could be demonstrated.

Importantly, all within-pair mediation effects demonstrated consistent patterns (positive indirect effects), indicating that methylation changes transmitted rather than buffered the adversity–symptom relationship. The negative path *b* coefficients observed across all three sites reveal a directional pattern: the twin with higher methylation tended to have lower symptom scores. Thus, adversity-induced hypomethylation contributed to increased symptom risk, supporting a model in which environmental stress downregulates DNA methylation at these gene body loci, which in turn elevates vulnerability to psychopathology. These findings are aligned with the paradigm of functional genomics, that at the gene body, rather than upregulation, hypomethylation is generally associated with gene downregulation, and hypermethylation in turn with gene upregulation (Yang et al., 2014). This could potentially serve to help explain the deleterious effects of downregulating gene expression involved in cellular signaling and neurotransmission, however, given the small sample size and the observational nature of these results, conclusions should be made with caution.

Individual-Level Mediation Analyses

Building on the within-pair difference findings, individual-level cluster-robust mediation analyses identified additional CpG sites that mediated relationships between psychosocial adversity and specific psychiatric symptom domains. This complementary analytical approach allowed us to observe every participant as an individual data entry and thus mitigate some of the limitations associated with small samples, increasing statistical power. It is important to note however, that in this approach, we could not control for genetic confounders. These individual-level analyses revealed that several CpG sites exhibited

suppression effects, and adversity-induced DNAm changes appeared to buffer rather than transmit psychiatric risk – a pattern with implications for understanding epigenetic regulation as a potentially adaptive response to environmental stress.

Methylation at cg24977276, located within the *GTF2I* gene body, emerged as a significant mediator of the relationship between psychosocial adversity and major depression symptom severity. Higher adversity exposure was significantly associated with hypomethylation at this locus (path *a*: $b = -0.0004$, $p = 0.005$), with adversity-exposed and control twins showing significant group-level differences in methylation (ANCOVA: $p = 0.028$). The DNAm–symptom pathway was also statistically significant (path *b*: $b = 795.31$, $p = 0.034$), indicating that lower methylation predicted lower depression scores. Mediation analysis revealed a significant negative indirect effect ($ab = -0.31$, $p = 0.012$), with 68.0% of the total adversity effect on depression symptom scores mediated through *GTF2I* methylation. Notably, the direct effect of adversity on depressive symptom score remained significant after controlling for DNAm ($c' = 0.76$, $p = 0.002$), indicating partial rather than complete mediation. No significant sex effects were observed.

The negative indirect effect demonstrates a suppression pattern rather than consistent mediation. While adversity was directly associated with increased depressive symptom score (positive direct effect), it simultaneously facilitated hypomethylation at cg24977276, which was in turn associated with *lower* symptom severity (negative indirect effect). This bidirectional pattern suggests that *GTF2I*-linked epigenetic changes may partially buffer the deleterious impact of adversity on depressive symptoms. In other words, adversity-induced hypomethylation at this gene body site appears to function as a compensatory mechanism that attenuates – rather than amplifies – the psychological consequences of environmental stress. The precise biological or protective interpretation of these results is up to further research,

however, consistently with overall trends, we can confidently report stress-induced suppression.

Exploratory analysis of generalized anxiety disorder symptom scores revealed a similar suppression pattern, with a significant adversity–methylation pathway (path *a*: $b = -0.0004$, $p = 0.005$) and a significant negative indirect effect ($ab = -0.21$, $p = 0.016$). However, the methylation–symptom pathway was only marginally significant (path *b*: $b = 549.25$, $p = 0.061$), precluding definitive claims of robust mediation for generalized anxiety. Furthermore, neither the depression nor anxiety indirect effects survived Benjamini-Hochberg correction for multiple comparisons, indicating that these findings should be considered preliminary and require replication in larger, adequately powered samples.

Methylation at cg17650397, located within the *PRKAR1B* gene body, showed preliminary evidence of mediating the relationship between psychosocial adversity and major depression symptom scores. Within twin pairs, higher adversity exposure was associated with lower methylation at this locus, though this relationship was only marginally significant (path *a*: $b = -0.0005$, $p = 0.069$). Nevertheless, adversity-exposed and control twins exhibited significant differential methylation at cg17650397, $F(1, 15) = 34.47$, $p < 0.001$, $\eta^2p = 0.70$, $\Delta\beta = -0.016$, suggesting that this site is sensitive to environmental stress exposure, and warranting further replication.

These findings suggest that epigenetic regulation may modulate the relationship between stress exposure and depressive symptoms through a compensatory mechanism. However, the marginally significant adversity–methylation pathway (path *a*) and the failure to survive multiple comparison correction warrant cautious interpretation. Replication in larger, independent as well as co-twin samples is essential to determine whether this protective epigenetic response represents a robust biological phenomenon.

In contrast to the suppression patterns observed for *GTF2I* and *PRKAR1B*, cg15060929 – located in the TSS200 promoter region of *SS18* – demonstrated a consistent mediation pattern wherein DNAm changes transmitted rather than buffered psychopathological manifestations. Notably, this site did not show differential methylation between adversity-exposed and control twins in the within-pair analysis, $F(1, 15) = 2.38$, $p = 0.143$, $\eta^2p = 0.14$, $\Delta\beta = 0.007$, yet it emerged as a statistically significant mediator in the individual-level analysis.

For cg15060929, adversity was significantly associated with *increased* methylation (path *a*: $b = 0.0008$, $p = 0.002$), and higher methylation was significantly associated with higher bipolar disorder symptom scores (path *b*: $b = 253.38$, $p = 0.020$). The indirect effect was statistically significant ($ab = 0.20$, $p = 0.008$), with a proportion mediated of 94.9%, indicating near-complete mediation. The direct effect of adversity on bipolar symptoms was non-significant after controlling for methylation ($c' = 0.01$, $p = 0.924$), suggesting that adversity's influence on bipolar symptomatology operates almost entirely through *SS18* promoter methylation. No significant sex effects were observed (*ps* > .15).

Apart from the exception of SS18, this pattern is largely inconsistent with traditional models of stress-induced epigenetic dysregulation, wherein adversity triggers promoter hypermethylation, leading to transcriptional silencing and downstream psychopathological consequences. The TSS200 region – extending from the transcription start site to 200 nucleotides upstream – is a critical regulatory domain where DNA methylation typically functions as a repressive mark (Li et al., 2024). Thus, adversity-induced hypermethylation at the *SS18* promoter likely downregulates gene expression, potentially disrupting neurobiological processes that maintain mood stability and contributing to bipolar disorder symptom expression.

The contrasting directionality between *SS18* (consistent mediation with promoter hypermethylation) and *GTF2I*/*PRKAR1B* (suppression effects with gene body hypomethylation) underscores the critical importance of genomic context in determining whether epigenetic changes amplify or attenuate risk for psychopathological developments. While gene body methylation appears to exert potentially compensatory effects on gene expression, promoter methylation at *SS18* follows the canonical pathway of transcriptional repression, consistently transmitting adversity-related risk to psychiatric outcomes.

4.1 Limitations

A principal limitation of this study was a relatively small sample of MZ twin pairs (N = 9; N = 18), which challenges the overall statistical power and outcome robustness. In order to partially overcome this limitation, we have utilized statistical models that account for limited samples such as bootstrap mediation, individual-level cluster-robust mediation, and false-discovery rate (FDR) correction. Furthermore, in order to increase the sample, we additionally performed individual-level analyses, in which participants did not appear as twin pairs, but independent individuals. Therefore, the findings of this study are not yet generalizable to large populations and serve rather as pilot results that may guide further work with larger samples. We, however, observe, that these findings are well-aligned with previous work that solidifies the role of DNAm (particularly within CpG islands), as a mechanism that may explain the interplay between life stressors and mental resilience.

An additional limitation is a relatively low severity of the adverse events that we observed in our sample. We specifically assessed day-to-day stressors such as financial insecurity, household conflict, or familial struggles, to social disapproval and peer rejection. We did not investigate severe types of adversities such as war, displacement by natural disasters, famine, or sexual abuse. While our results suggest that minor stressors may act as a mechanism that

increases individuals' resilience towards mental health complaints, it is true that we do not have observations for the whole spectrum of life adversities, warranting further research.

Finally, we have reported that none of our results survived statistical correction. As this effect is largely due to our small sample size, a larger sample should be taken into account for further work.

4.4 Conclusion

By utilizing the rigorous adversity-divergent twin design, we provide preliminary evidence that psychosocial adversity acts as a driver of specific DNA methylation changes that mediate psychiatric outcomes. The replication of the "suppressive" mediation pattern in larger cohorts (Lussier et al., 2024) suggests that we should move beyond viewing epigenetic modifications solely as markers of pathology. Instead, DNAm appears to act as a double-edged sword – capable of mediating risk through promoter hypermethylation of homeostatic genes (e.g., *ADRA2A*) while simultaneously deploying gene-body hypomethylation (e.g., *GTF2I*) as a compensatory attempt to preserve psychological function. Future interventions may eventually leverage these biomarkers not only to identify at-risk individuals but to quantify the biological efficacy of resilience-promoting treatments.

Figure 1. Association heatmap between CpG methylation and PSA domains

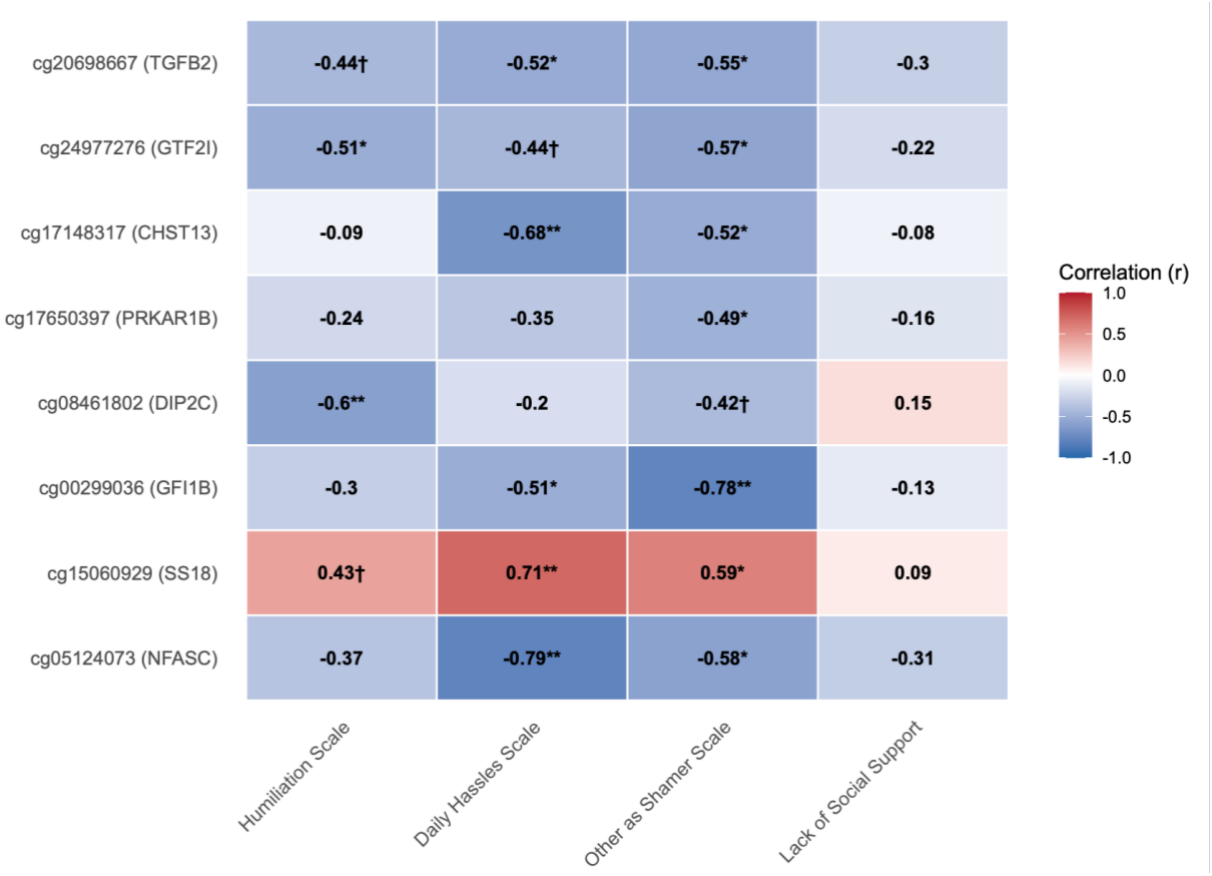

Figure 1. Heatmap showing Pearson correlations between CpG methylation β-values and psychosocial adversity (PSA) subscale scores across MZ twin pairs (N = 18). Rows represent individual CpG sites; columns represent PSA domains (Humiliation, Daily Hassles, Other as Shamer, Lack of Social Support). Color scale: red = positive correlation (hypermethylation with adversity); blue = negative correlation (hypomethylation with adversity). Only correlations reaching |r| > .40 are displayed. *p < .05, **p < .01.

Figure 2a. Negative-negative mediation path with non-significant direct effect (3 observations)

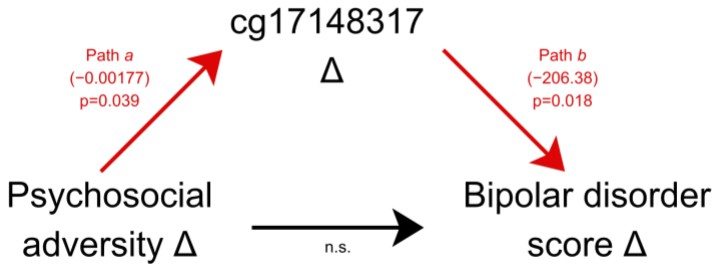

Figure 2b. Negative-positive mediation (3 observations)

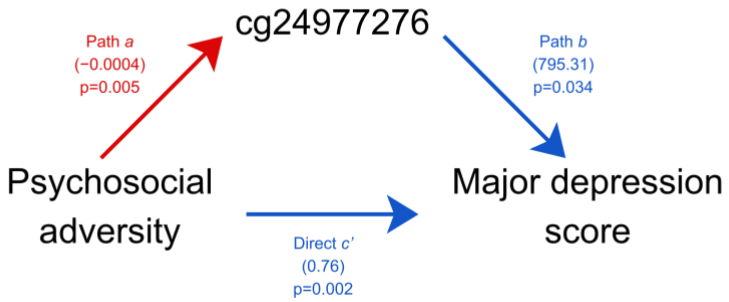

Figure 2c. Positive-positive mediation (1 observation)

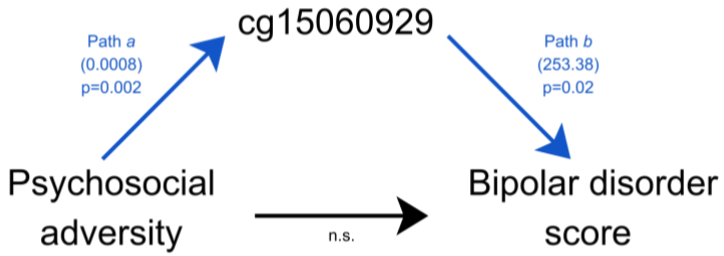

Fig. 2a. Within-pair delta mediation model for cg17148317 (CHST13) showing indirect-only mediation. Within twin pairs, differences in Other as Shamer scale predicted differences in DNAm (path a), which in turn predicted differences in BPD symptom scores (path b). The indirect effect was significant (ab = 0.513, p = .020), despite a non-significant total effect (c = 0.11, p = .404), consistent with indirect-only mediation. Solid arrows indicate significant paths ($p < .05$); red arrows represent negative paths, blue arrows represent positive paths. Black arrow indicates non-significant paths. Unstandardized coefficients shown.
Figs. 2b, 2c. Individual-level mediation model for cg24977276 and cg15060929 as respecive mediators. Aggregate scores on PSA predicted hypo- and hypermethylation, which in turn predicted MD and BPD symptom scores, respectively.

Table 2. Within-Pair Delta Mediation Analysis: Adversity → DNA Methylation → Bipolar Disorder Symptoms

CpG site	Gene	Predictor	*n*	Path *a*	p(*a*)	Path *b*	p(*b*)	Indirect	p(ind)
cg20698667	TGFB2	CAS	9	−0.00176	0.006**	−292.60	0.035*	0.514	0.044*
cg17148317	CHST13	CAS	9	−0.00177	0.039*	−206.38	0.018*	0.365	0.036*
cg17148317	CHST13	Other as Shamer Scale	9	−0.00189	0.005**	−271.50	0 .011*	0.513	0.020*

Note. Delta scores computed as Twin1 − Twin2. Path *a* = effect of Δ_adversity on Δ_methylation; Path *b* = effect of Δ_methylation on Δ_symptoms controlling for adversity. Positive indirect effects indicate consistent mediation (adversity effect transmitted through methylation). Bootstrap confidence intervals based on 500 simulations. *p < .05. **p < .01.

Table 3a. Mediation Analysis Results for cg24977276 (GTF2I) as Mediating Factor

Outcome	Path *a*	p(*a*)	Path *b*	p(*b*)	Direct(*c'*)	p(*c'*)	Indirect (*a* × *b*)	p(ind)	Total(*c*)	p(*c*)	PM(%)	Sex Effect
MD	−0.0004	0.005**	795.31	0.034*	0.76	0.002**	−0.31	0.012*	0.45	0.008**	60.0	n.s.
GAD	−0.0004	0.005**	549.25	0.061†	0.63	0.002**	−0.21	0.016*	0.42	<.001**	51.3	n.s.

Table 3b. Mediation Analysis Results for cg17650397 (PRKAR1B) as Mediating Factor

Outcome	Path *a*	p(*a*)	Path *b*	p(*b*)	Direct(*c'*)	p(*c'*)	Indirect (*a* × *b*)	p(ind)	Total(*c*)	p(*c*)	PM(%)	Sex Effect
MD	−0.0005	.069†	367.49	0.035*	0.64	0.003**	−0.18	0.040*	0.45	0.008**	40.3	n.s.

Table 3c. Mediation Analysis Results for cg15060929 (SS18) as Mediating Factor

Outcome	Path *a*	p(*a*)	Path *b*	p(*b*)	Direct(*c'*)	p(*c'*)	Indirect (*a* × *b*)	p(ind)	Total(*c*)	p(*c*)	PM(%)	Sex Effect
BPD	0.0008	0.002**	253.38	0.020*	0.01	0.924	0.20	0.008**	0.21	<0.001***	94.9	n.s.

Note. MD = major depression symptom score; GAD = generalized anxiety disorder symptom score; BPD = bipolar disorder symptom score. Path *a* = effect of PSA on DNAm; Path *b* = effect of DNAm on psychological score as outcome; *c′* = direct effect of PSA on psychological score as outcome; *c* = total effect; PM = proportion mediated, Sex Effect = the coefficient for female relative to male; n.s. = non significant. Indirect effects were estimated using bootstrap mediation with 500 simulations. Indirect effects did not survive Benjamini-Hochberg correction for multiple comparisons.

*p < .05. **p < .01. † marginal statistical significance

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2. Study III: Predicting Mental Health Outcomes: No Association Between Poly-Epigenetic Scores and Psychological Symptoms in the ImmunoTwin Cohort

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Abstract

Objective:

This study examined whether poly-epigenetic scores (PES), derived from DNA methylation (DNAm) data serve as predictor for mental health outcomes in young adulthood, and mediate the association between psychosocial adversity (PSA) and psychological symptomatology.

Materials and Methods:

Participants were drawn from the ImmunoTwin cohort and included 22 participants, 18 of which constituted full twin pairs (N=9). PSA was assessed using validated psychosocial and stress-related questionnaires, and clinical interviews were conducted according to DSM-5 criteria. Genome-wide DNAm was measured from whole-blood samples using the Illumina Infinium MethylationEPIC v2.0 BeadChip. PES were computed in a previous study by weighting CpG site methylation levels with variable importance projection scores (VIP) from partial least squares-discriminant analysis (PLS-DA).

Results:

Significant group differences in PES were observed between PSA-exposed and control twins, $t(20) = 2.62$, $p = .017$, $d = 1.12$, replicating prior findings of reduced DNAm following moderate psychosocial stress. Bayesian estimation corroborated this effect, with a posterior probability of 99.4% that the exposed group had lower PES (posterior $d = -1.12$, 95% CrI [−1.97, −0.26]). To address the limited statistical power inherent in the sample size ($N = 22$), five complementary analytical strategies were employed: Bayesian estimation with weakly informative priors, multilevel modeling of the twin-pair structure, exact permutation tests, parametric bootstrapping with copula-based confidence intervals, and penalized multivariate estimation. Across all five frameworks, PES showed no significant association with any of the 17 psychological symptom dimensions examined, spanning mood disorders, anxiety

disorders, and eating disorders. Bayesian posterior correlations were uniformly small (all posterior mean $|r| \leq .24$), with 95% credible intervals and bias-corrected bootstrap confidence intervals consistently spanning zero. Exact permutation tests (all $p > .05$) and ridge-regularized multivariate regression confirmed these null associations. Multilevel models decomposing within-pair (genetically unconfounded) and between-pair effects revealed no significant within-pair PES – symptom associations, while intraclass correlations indicated substantial twin-pair clustering for most symptom dimensions (median ICC = .54). Mediation analyses revealed that PES did not significantly mediate the relationship between PSA and any psychological outcome (all indirect effects $p > .05$).

Conclusions:

Although differential PES were observed between adversity-exposed and control MZ twins, convergent evidence across five complementary statistical frameworks indicates that PES did not explain individual differences in psychological symptom severity. Intraclass correlation analyses further revealed that even for symptom dimensions with maximal individual-specific variance – where an epigenetic predictor would be most expected to contribute – PES showed no predictive value. These findings suggest that while DNAm-based poly-epigenetic scores reliably capture biological traces of psychosocial stress exposure, they do not directly predict clinical symptomatology in the present cohort. The convergent null across multiple analytical approaches substantially strengthens this conclusion.

Keywords: DNA methylation, poly-epigenetic score, psychosocial adversity, twins, mental health, epigenetics

1 Introduction

Psychological disorders have emerged as one of the leading global health challenges, and the leading cause of disability worldwide. According to the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2019, mental disorders accounted for approximately 125.3 million disability-adjusted life years (DALYs), or roughly 5% of the global disease burden (Tian et al., 2025; Zhan et al., 2025). However, there is a growing consensus that traditional methodologies substantially underestimate the true prevalence and impact of psychiatric morbidity (Vigo et al., 2016; Arias et al., 2022). This burden is not static–longitudinal analyses reveal a rising trajectory in the prevalence of disorders, particularly among adolescents and young adults, with the prevalence of disorders peaking around age 14. Between 1990 and 2021, the age-standardized DALY rate for mental disorders among individuals aged 10–24 years rose by 14%, a trend significantly exacerbated by the COVID-19 pandemic which triggered a 25% to 27.6% increase in the global prevalence of anxiety and major depressive disorders in 2020 alone (Liu et al., 2025; Tian et al., 2025; Zhan et al., 2025). Furthermore, individuals with severe mental disorders exhibit a mortality rate 2.22 times higher than the general population, often due to comorbid physical conditions and suicide, yet these excess deaths are rarely attributed to the underlying psychiatric conditions in global statistics (Walker et al., 2015).

Understanding the biological underpinnings of psychological disorders remains a central challenge in mental health research, with the aim of mitigating the global challenges put in place by mental disease. One of the central aims of contemporary biopsychological research is to enhance the prediction of mental-health outcomes by identifying biological and environmental markers that indicate vulnerability before clinical symptoms appear. Epigenetics has emerged as a promising field for elucidating the molecular mechanisms that

mediate the interaction between genetic predisposition and environmental stressors in the development of mental health disorders. While genetic factors provide a background of susceptibility, epigenetics serves as the biological "bridge" or molecular correlate that partially explains how exogenous stressors – ranging from early-life adversity and trauma to social environments – are biologically embedded to influence neurobiology and behavior (Varela et al., 2022). The most extensively implicated epigenetic mechanism in biopsychological research is DNA methylation (DNAm), the covalent addition of a methyl group to cytosine residues, which generally results in transcriptional repression when located in gene promoters (Aljabali et al., 2024). Research has consistently demonstrated that adverse environmental exposures can induce lasting DNAm changes in genes regulating the hypothalamic-pituitary-adrenal (HPA) axis, serotonin neurotransmission or neuroplasticity, impacting individuals' mental health (Aljabali et al., 2024; McGowan & Roth, 2015). For instance, childhood maltreatment and prenatal stress have been consistently linked to hypermethylation of the glucocorticoid receptor gene (*NR3C1*), leading to dysregulation of the HPA axis and heightened vulnerability to psychopathology later in life. Similarly, socioeconomic disadvantage and trauma have been associated with epigenetic modifications in the serotonin transporter gene (*SLC6A4*) and the *FKBP5* gene, which regulate stress reactivity and neural plasticity (Hayes et al., 2025; Hirai et al., 2025; Cunliffe, 2016; Howie et al., 2019; Swartz et al., 2017; Raabe & Spengler, 2013).

However, identifying reliable epigenetic biomarkers remains challenging largely due to the complexity of the involved biological and environmental variables. Recent approaches, including the development of poly-epigenetic scores (PES**)**, aim to integrate multiple epigenetic markers into quantifiable scores that may improve the prediction of psychological disorders and help delineate the biological pathways underlying vulnerability and resilience. PES, also known as methylation risk scores (MRS), are a risk assessment tool which

aggregates methylation levels across multiple informative loci into a single summary measure. Specifically, PES are weighted sums of methylation levels at CpG sites identified from epigenome-wide association studies (EWAS), utilizing weights often derived from regression coefficients measure (Hüls & Czamara, 2020; Ohi et al., 2025; Lin et al., 2025). PES have emerged as an advanced tool for risk categorization, helping quantify the biological embedding of environmental influences such as adversity and stress, metabolic health, or lifestyle factors such as maternal smoking (Lin et al., 2025; Taylor & Ternès von Hattburg, 2025; Deng et al., 2024). In the context of biopsychological research, PESs derived from blood and brain tissues have shown specific methylation profiles that differentiate disorders such as schizophrenia and bipolar disorder, particularly in patients with high genetic risk (Ohi et al., 2025; Wani et al., 2024). Previous work has also shown the predictive power of DNAm-derived risk scores throughout psychological disorders such as major depression (Barbu et al., 2021), social anxiety, and panic disorder (Ohi et al., 2024). Moreover, poly-epigenetic scores have been implicated as predictors for psychological resilience in young adults (Lu et al., 2023), and schizophrenia relapse (Segura et al., 2025). Other work has shown consistently differentiated PES between psychosocial adversity-exposed and control monozygotic (MZ) twins, potentially linking to further development of chronic disease (Buchanan et al., manuscript in preparation). Furthermore, recent studies have demonstrated that blood-based DNA methylation risk scores can predict post-traumatic stress disorder (PTSD) with high accuracy in military and civilian cohorts, outperforming models based solely on clinical variables (Wani et al., 2024; Hüls & Czamara, 2020). Beyond their use in diagnostics, PES have shown efficiency in predicting cardiometabolic risk factors, such as body mass index (BMI) and inflammation, which are often comorbid with mental disorders (Lin et al., 2025). By integrating complex information across the epigenome, PESs offer a

promising approach for precision diagnostics and disease prediction, potentially serving as biomarkers for monitoring disease progression and treatment response (Ohi et al., 2025).

However, to our knowledge, no study to this day has examined PES as a mediating factor between psychosocial adversity (PSA) and multiple psychological symptom scores. Furthermore, no study has yet examined whether PES is correlated with differential psychological symptom scores between twin pairs. To test these hypotheses, we utilized the ImmunoTwin cohort ($N = 22$), which includes monozygotic twin pairs discordant for psychosocial adversity exposure. This design offers a unique advantage: by comparing genetically identical twins who differ in environmental stress exposure, we can isolate the effects of adversity on epigenetic variation while controlling for genetic and shared environmental confounds. We examined whether PES (a) differentiate adversity-exposed from control twins, (b) predict individual differences in psychiatric symptom severity across nine diagnostic dimensions, and (c) mediate the pathway from adversity exposure to psychopathological outcomes.

2 Materials and Methods

Data for the present study were drawn from the ImmunoTwin cohort, a large study investigating the epigenetic, psychosocial, and psychological correlates of disease etiology in certain risk groups (Turner et al., 2020). Details of participant recruitment, DNA methylation sample collection, and psychosocial assessments have been described previously (Mposhi et al., manuscript in preparation; Repcikova et al., manuscript in preparation; Buchanan et al., manuscript in preparation). The same data collection procedures were used across all sub-studies from this project.

2.1 Ethics Information

Ethical approval for this study was obtained from ethics committees from the University of Luxembourg (ERP 22-078) and the University of Bielefeld (EUB2020-184). Participants signed the informed consent and data collection and processing form before proceeding with participation. We used pseudonymization for all collected data, and provided a combined compensation of 90€ to all participants.

2.2 Participants

Participants were recruited from the TwinLife database, which is a part of a genetic study assessing socioeconomic inequalities in German-speaking areas. All participants were MZ and DZ twin pairs sampled from the 3 oldest waves of the TwinLife study (N=710, $mnage_{cohort2}$ = 18.0, $mnage_{cohort3}$ = 24.1, $mnage_{cohort4}$ = 30.0), belonging to a German genetic study investigating the socioeconomic and health inequalities across multiple stages of life (Diewald et al., 2024, Hahn et al., 2016). Socioeconomic indicators of adversity across childhood, adolescence, and young adulthood were assessed using a standardized stressor questionnaire, supplemented with selected items from the Humiliation Scale Hartling & Luchetta, 1999), Other as Shamer Scale–2 (Matos et al., 2015), Brief Daily Stressors Screening Tool (Scholten et al., 2020), and Social Support Scale (Zimet et al., 1988). These inventories collectively evaluate perceived social rejection, financial hardship, familial and household conflict, or occupational dissatisfaction. Participants were asked to indicate whether adverse events outlined in the questionnaires occurred, if yes, at what stage in life, and what was the perceived severity of their negative impact. Twin pairs who completed the full pre-screening and exhibited within-pair total adversity score differences of at least one standard deviation from the sample mean were invited to participate in biological sampling and clinical psychological assessment. For each participant, an aggregate psychosocial

adversity (PSA) score was calculated by summing standardized scores across all adversity indicators. Due to the nature of the sample, no clear cut-off score values for exposed and control groups was established. Participants were categorized into groups based on their composite adversity score (CAS) relative to their co-twin.

2.3 Clinical Psychological Screening

Clinical-psychological interviews were administered online to evaluate current and past psychological symptoms, specifically in the domains of mood disorders, anxiety disorders, and eating disorders. All participants completed an identical questionnaire protocol based on the German version of the Mini-DIPS clinical interview (Margraf et al., 2017), which assesses the presence, severity, duration, and frequency of psychological symptoms according to Diagnostic and Statistical Manual of Mental Disorders (5th ed.; APA, 2013) criteria. Symptom scores were calculated across all diagnostic categories for each participant. Total symptom scores and within–twin-pair differences (Δ) were then derived, and summarized for descriptive analyses.

2.4 DNA Extraction

In order to acquire DNAm data, blood samples were collected from selected MZ participants from the ImmunoTwin project, at local medical by certified medical professionals. Peripheral blood was drawn into PAXgene Blood RNA Tubes (BD Biosciences, Franklin Lakes, NJ, USA) to preserve RNA integrity during transport and storage, and an additional 10 mL sample was collected in EDTA tubes (KS Medical, Seoul, South Korea). DNA was isolated from 22 samples - 18 from 9 complete twin pairs and 4 unpaired individuals, whose co-twins did not participate - stored in PAXgene RNA tubes. Extraction followed the manufacturer's protocol for the QIAamp DNA Blood Midi Kit (Qiagen, Hilden), with minor procedural adjustments. Approximately 2 mL of PAXgene whole-blood mixture was used per sample. Between 350 ng and 500 ng of extracted DNA underwent bisulfite conversion using the EZ

DNA Methylation Kit (Zymo Research, Irvine, CA, USA). Genome-wide DNA methylation was then assayed using the Infinium MethylationEPIC v2.0 BeadChip and iScan system (Illumina, San Diego, CA, USA). All processing and quality control steps adhered to the Infinium HD Methylation Assay Reference Guide, implemented in GenomeStudio Software v2.0 (Illumina).

2.5 DNA Methylation Analysis

Data derived from whole-blood DNA methylation profiles were processed and analyzed in R (version 4.4.2) using the SeSAMe pipeline (Sensible Step-wise Analysis of DNA Methylation BeadChips; Zhou et al., 2018) for initial preprocessing and quality control. As detailed in previous work (Mposhi et al., manuscript in preparation; Buchanan et al., manuscript in preparation), β-values ranging from 0 (no methylation) to 1 (complete methylation) were generated using the getBetas() function and annotated with sample metadata and the Infinium manifest for the hg38 genome build (https://zwdzwd.github.io/InfiniumAnnotation#human/). To reduce potential bias, probes associated with known single nucleotide polymorphisms (SNPs) and those located on sex chromosomes (X and Y) were excluded.

Batch effects were adjusted using the *sva* package (version 3.54.0; Buchanan et al., 2026). Differential methylation analysis was performed using the DML() function in SeSAMe, specifying sample group contrasts and including age and sex as covariates. Summary statistics were generated using the summaryExtractTest() function, applying a false discovery rate (FDR) threshold of < 0.05.

2.6 Poly-Epigenetic Scores Calculation

For dimensionality reduction and feature selection, partial least squares-discriminant analysis (PLS-DA) was conducted using mixOmics (version 6.30.0), and hierarchical clustering, gene ontology (GO), and KEGG pathway analyses were performed using clusterProfiler (version

4.14.4). Principal component analysis (PCA) utilized the base stats package (prcomp() function). The CpG sites selected for the PES construction were those identified in Component 1 of the PLS-DA, which were also confirmed as significant (FDR < 0.01) in a concurrent epigenome-wide association study (EWAS). The top 200 CpG sites were selected based on their Variable Importance Projection (VIP) scores,. Receiver Operating Characteristic (ROC) analysis indicated that the Area Under the Curve (AUC) remained stable across these top sites, justifying the inclusion of 200 loci to maintain separation between groups. The score for each individual was calculated by summing the beta value of each selected CpG site multiplied by its corresponding VIP score, which functioned as the weight. To ensure the score's utility across different array technologies, a secondary calculation was performed using a subset of 70 CpGs compatible with the Illumina 450K BeadChip, yielding similar AUC values to the primary score derived from EPIC v2.0 arrays.

2.7 Power Analysis

Sensitivity power analyses were conducted to evaluate the detectable magnitude of associations between PES and psychological symptom measures given the available sample size (N = 22) and a two-tailed significance threshold of $\alpha = .05$. Results indicated that the study had 80% power to detect only large correlations (minimum detectable $|r| \approx .56$). Accordingly, the present analyses were underpowered to detect small-to-moderate associations between PES and symptom scores, and non-significant correlations should be interpreted in light of this limited statistical power.

2.8 Convergent Null Across All Methods

PES did not predict any psychological symptom dimension, and this finding was stable across all statistical approaches. This concludes that the null result is linked to insufficient predictive power of PES for mental health domains in the present cohort, rather than a statistical artifact.

2.8.1 Bayesian Estimation

All 17 posterior correlations (SBS excluded, zero variance) showed 95% credible intervals that include zero. Anorexia nervosa scores showed the largest posterior means at –0.24, however, even there the CrI runs from −0.59 to +0.18. For most symptom scores, roughly 33–36% of the posterior mass falls within |r| < .10 (the "negligible" zone), with the remaining mass spread symmetrically across both positive and negative values. Taken together, this indicates that the data are uninformative about direction. The group comparison, by contrast, is decisive: P(Exposed − Control < 0) = 99.4%, posterior Cohen's d = −1.12 [−1.97, −0.26]. This cleanly dissociates the PES–adversity link (strong) from the PES–symptom link (absent).

2.8.3 Permutation Tests

All 17 permutation values were non-significant (smallest: SP_score at p = .051). With only 512 possible permutations, these tests have limited resolution, but they confirm that no within-pair PES–symptom association emerges even under assumption-free inference. The within-pair group comparison permutation p = .77 suggests that the mean within-pair PES difference is not systematically directional.

2.8.4 Bootstrap

Anorexia nervosa scores again showed the largest effect: BCa 95% CI [−0.77, 0.04]. This is symptom dimension could be worth following up in a larger sample – the bootstrap, Bayesian, and frequentist results all converge on a small negative association that could plausibly reach significance in a larger sample.

2.8.5 Penalized Multivariate Estimation

The CCA canonical r = 0.53, but the permutation p = 0.99 was non-significant – meaning even the best possible linear combination of all symptom scores cannot be predicted by PES. This is a strong multivariate null result.

3 Results

3.1 Within-Twin-Pair Variation in Adversity and Poly-Epigenetic Scores

A central leverage of the co-twin design is that MZ, despite sharing nearly-identical genomes, exhibit meaningful within-pair variation in environmental exposures, enabling these to be studied as differential factors. Before examining group differences and predictive relationships, we first evaluated whether sufficient within-pair variation existed in both PSA and PES. This preliminary analysis included only complete twin pairs (i.e., pairs where both twins participated in all data collection phases); of the 22 participants in the ImmunoTwin sample, 18 individuals constituted 9 complete monozygotic twin pairs, while 4 unpaired individuals were excluded from this analysis. Psychosocial adversity was assessed using five validated instruments: the Humiliation Scale, the Daily Hassles Scale, the Other as Shamer Scale, the Lack of Social Support Scale, and a Negative Life Events questionnaire developed for this study. A composite adversity score (CAS) was computed by standardizing and aggregating the individual subscale scores.

3.1.1 Within-Pair Differences: Descriptive Statistics

Within-pair difference scores were computed by subtracting Twin 02's value from Twin 01's value for PES and all adversity measures. Descriptive statistics for these difference scores are presented in Table 1 and shown in Figure 2. Mean raw differences were close to zero across all variables, indicating no systematic bias in which twin scored higher. However, the substantial standard deviations and ranges demonstrate considerable within-pair variability.

Absolute difference scores, which capture the magnitude of within-pair discordance regardless of direction, revealed meaningful variation across all measures. Twins differed by an average of 7.99 points on PES and 8.82 points on PSA. Among the PSA subscales, the Other as Shamer Scale showed the largest absolute within-pair differences (M = 8.78, SD =

7.84), followed by the Daily Hassles Scale (M = 6.44, SD = 7.32), the Humiliation Scale (M = 4.22, SD = 2.95), the Lack of Social Support Scale (M = 2.78, SD = 2.11), and the Negative Life Events questionnaire (M = 1.33, SD = 1.12). The results are shown in Figure 3.

3.1.2 Normality of Within-Pair Difference Scores

To evaluate whether within-pair difference scores in PES and adversity scales were normally distributed, we conducted Shapiro-Wilk tests for all PSA subscales. Results are presented in Table 2 and Figure 4.

The majority of difference score distributions were consistent with normality ($p > .05$), including PES, the CAS, the Humiliation Scale, the Other as Shamer Scale, the Lack of Social Support Scale, and the Negative Life Events questionnaire. However, the Daily Hassles Scale showed significant deviation from normality for both raw ($W = 0.82$, $p = .032$) and absolute ($W = 0.68$, $p < .001$) difference scores.

Table 1

Descriptive Statistics for Within-Twin-Pair Difference Scores (N = 9 pairs)

Variable	M	SD	Min	Max	Mdn
PES	−1.02	10.17	−18.31	11.70	1.33
CAS (Standardized)	−0.02	10.74	−13.21	19.84	2.52
Humiliation Scale	−2.44	4.69	−10.00	4.00	−3.00
Daily Hassles Scale	2.00	9.79	−7.00	25.00	0.00
Other as Shamer Scale	−1.44	12.07	−18.00	21.00	−1.00
Lack of Social Support	0.11	3.62	−6.00	6.00	1.00
Negative Life Events	0.67	1.66	−2.00	3.00	0.00

Note. Difference scores computed as Twin 01 − Twin 02. PES = poly-epigenetic score.

Table 2

Shapiro-Wilk Tests for Normality of Within-Twin-Pair Difference Scores

Variable	W (Raw)	p	W (Abs)	p
PES	.945	.633	.946	.645
CAS (Standardized)	.928	.465	.925	.437
Humiliation Scale	.953	.722	.944	.623
Daily Hassles Scale	.817	.032*	.682	< .001***
Other as Shamer Scale	.948	.671	.875	.138
Lack of Social Support	.972	.909	.896	.231
Negative Life Events	.922	.405	.845	.065

*Note. W = Shapiro-Wilk statistic; Raw = raw differences; Abs = absolute differences. *p < .05, ***p < .001.*

3.1.3 Association Between Within-Pair Adversity and PES Differences

A critical test of the hypothesis that psychosocial adversity influences DNA methylation patterns is whether twins who differ in adversity exposure also differ in their PES. To address this question, we correlated within-pair adversity differences with within-pair PES differences for each measure; results are presented in Table 3 and illustrated in Figure 1.

A significant negative correlation emerged between raw within-pair CAS differences ($r = -0.69$, $p = 0.039$). Out of the adversity subscales, the Daily Hassles Scale and the Other as Shamer Scale were individually statistically significant and negatively correlated ($r = -0.73$, $p = 0.024$; $r = -0.74$, $p = 0.022$). This finding indicates that the twin with greater adversity exposure tended to have lower PES values relative to their co-twin.

Table 3

Correlations Between Within-Twin-Pair Difference Scores in PSA Subscales and PES

Variable	r	p
CAS (Standardized)	−0.691	0.039*
Humiliation Scale	−0.41	0.267
Daily Hassles Scale	−0.73	0.024*
Other as Shamer Scale	−0.74	0.022*
Lack of Social Support	−0.16	0.675
Negative Life Events	−0.5	0.174

*Note. Raw differences = Twin 01 − Twin 02; absolute differences = |Twin 01 − Twin 02|. *p < .05.*

3.2 Replication Analysis: PES Differences Between Adversity-Exposed and Control Twins in the ImmunoTwin Cohort

Before examining whether the PES predicts psychological symptom severity, we first sought to replicate previous findings demonstrating differential PES between adversity-exposed and control monozygotic twins (Buchanan et al., 2026). This replication analysis serves to validate the PES measure within the ImmunoTwin sample. Of the 22 participants in the sample, 12 (54.5%) were classified as adversity-exposed and 10 (45.5%) as controls. This near-equal distribution provides adequate statistical power for between-group comparisons. Descriptive statistics for PES by group are presented in

Table 4. Control participants exhibited higher mean PES values (M = 558.19, SD = 3.75) compared to adversity-exposed participants (M = 551.60, SD = 7.17). This pattern in which exposed individuals show reduced PES relative to controls is consistent with previous findings indicating suppressive effects of psychosocial adversity on DNA methylation patterns (Buchanan et al., 2026).

Table 4

Descriptive Statistics for Poly-Epigenetic Scores by Adversity Group

Group	n	M	SD	Min	Max	Mdn
Control	10	558.19	3.75	549.49	564.36	558.63
Exposed	12	551.60	7.17	537.80	561.06	551.74

Note. M = mean; SD = standard deviation; Min = minimum; Max = maximum; Mdn = median.

3.2.1 Statistical Assumptions

Prior to conducting inferential analyses, we evaluated the assumptions underlying parametric tests. Shapiro-Wilk tests indicated that PES values were approximately normally distributed within both the control group ($W = 0.87$, $p = .099$) and the exposed group ($W = 0.96$, $p = .723$). Levene's test for homogeneity of variance was non-significant, indicating that the assumption of equal variances was met. Given that both normality and homogeneity assumptions were satisfied, parametric analyses were deemed appropriate. Nevertheless, we also report non-parametric results for robustness.

3.2.2 Group Comparison Results

A one-way analysis of variance (ANOVA) was conducted to examine whether PES differed significantly between adversity-exposed and control participants. Results revealed a statistically significant main effect of group on PES, $F(1, 20) = 6.84$, $p = .017$, $\eta^2 = .26$. This large effect size indicates that group membership (exposed vs. control) accounts for approximately 26% of the variance in poly-epigenetic scores.

To ensure robustness against potential violations of the equal variance assumption, we also conducted Welch's ANOVA, which does not assume homogeneity of variance. Results were consistent with the standard ANOVA, $F(1, 17.15) = 7.62$, $p = .013$, confirming the reliability of the group difference.

An independent samples t-test yielded equivalent results, t(20) = 2.62, p = .017, with a large effect size (Cohen's d = 1.12, 95% CI [0.21, 2.00]). This effect size indicates that adversity-exposed individuals scored, on average, more than one standard deviation lower on PES compared to controls. Welch's t-test, which adjusts for unequal variances, produced similar findings, t(17.15) = 2.76, p = .013.

Finally, to provide a non-parametric alternative that does not assume normality due to the non-normal character of twin studies, we conducted a Mann-Whitney U test. The results (W = 95, p = .021) confirmed that the group difference in PES is robust across analytical approaches.

To further assess this group difference, a Bayesian analysis using conjugate priors estimated the posterior distribution of the mean difference and Cohen's *d*. The posterior mean difference (Exposed − Control) was −6.57 PES units (95% CrI [−11.58, −1.54]), with a posterior probability of 99.4% that the exposed group had lower PES. The posterior Cohen's *d* = −1.12 (95% CrI [−1.97, −0.26]) was consistent with the frequentist estimate. An exact permutation test preserving the twin-pair constraint (512 possible permutations) yielded *p* = .766 for the mean within-pair difference, indicating that while the between-group difference is robust, the mean directional discordance within pairs is not statistically significant—consistent with the differential epigenetic signatures noted in Section 3.2.3.

Table 5

Summary of Statistical Tests Comparing PES Between Adversity-Exposed and Control Groups

Test	Statistic	p	Effect Size
One-way ANOVA	$F(1, 20) = 6.84$	.017*	$\eta^2 = .26$
Welch's ANOVA	$F(1, 17.15) = 7.62$	.013*	$\eta^2 = .26$
Independent t-test	$t(20) = 2.62$	.017*	$d = 1.12$
Welch's t-test	$t(17.15) = 2.76$	.013*	$d = 1.12$
Mann-Whitney U	$W = 95$	.021*	—

Note. η^2 *= eta-squared; d = Cohen's d. Effect size interpretation:* $\eta^2 > .14$ *= large;* $d > 0.80$ *= large. *p < .05.*

3.2.3 Summary of Replication Findings

The replication analysis confirmed significant differences in poly-epigenetic scores between adversity-exposed and control participants. Consistent with prior work (Buchanan et al., 2026), adversity-exposed individuals exhibited significantly lower PES values than controls, with a large effect size ($d = 1.12$, $\eta^2 = .26$). This finding was robust across parametric (ANOVA, t-test) and non-parametric (Mann-Whitney U) approaches, as well as methods robust to assumption violations (Welch's tests).

The observed pattern of reduced PES in adversity-exposed individuals is consistent with the hypothesis that psychosocial stress to some degree induces suppressive or protective epigenetic modifications. This replication provides support for the role of PESs as a measure of meaningful biological variation related to adversity exposure within the current sample.

It is noteworthy that the exposed group exhibited greater variability in PES ($SD = 7.17$) compared to controls ($SD = 3.75$), suggesting that the epigenetic response to adversity may be heterogeneous across individuals. This variability may reflect differences in the nature, timing, or severity of adversity experiences, as well as individual differences in biological

stress reactivity. Despite this heterogeneity, the group difference remained statistically significant across all analytical approaches.

3.3 Sample Characteristics and Descriptive Statistics

The final analytic sample comprised 22 participants, including 18 individuals from 9 complete MZ twin pairs and 4 unpaired individuals (M_{age} = 23.2; Min_{age} = 17; Max_{age} = 31). Participant age was approximately normally distributed, and no extreme outliers were observed. The sample included both male and female participants, with a slightly higher proportion of females (72.7%) than males (27.3%).

Descriptive statistics for psychological symptom scores across all diagnostic domains are presented in Table 6 and Figure 5. Mean symptom severity was generally low to moderate across domains, with substantial variability observed within most symptom categories. Mood and anxiety symptom scores showed comparable levels of dispersion, while eating disorder symptom scores exhibited lower mean values and narrower ranges overall.

Specifically, major depression and generalized anxiety symptoms showed moderate variability, whereas manic symptoms and panic-related symptoms were less prevalent in the sample. Eating disorder symptom dimensions (anorexia nervosa, bulimia nervosa, and binge-eating disorder) showed the lowest mean scores, with wide confidence intervals relative to their means, reflecting considerable interindividual heterogeneity despite low overall symptom severity.

Taken together, these descriptive statistics indicate that the sample exhibited meaningful variability in psychological symptomatology across multiple diagnostic domains.

Table 6

Descriptive and Distribution Statistics for Psychological Symptom Dimensions (N = 22)

Symptom Dimension	N	M	SD	Min	Max
Major Depression	22	6.64	7.57	0	18
Social Anxiety	22	4.59	3.7	0	10
Generalized Anxiety	22	5.18	6.29	0	15
Bipolar Disorder	22	1.77	3.53	0	11
Agoraphobia	22	2.57	4.47	0	12
Panic Disorder	22	3	5.32	0	15
Anorexia Nervosa	22	2.09	3.98	0	15
Bulimia Nervosa	22	1.91	3.08	0	8
Binge-Eating Disorder	22	2.27	3.91	0	11

3.4 No Association Between PES and Psychological Symptom Domains

To evaluate the relationship between PES and psychological symptom severity despite limited sample size, five complementary analytical strategies were applied across 17 symptom dimensions (somatic symptom disorder score was excluded due to zero variance). Results are presented in Table 7 (convergent evidence summary) and Figures 6–7.

3.4.1 Frequentist Correlation Analysis

Pearson correlation analyses conducted at the individual level ($N = 22$) revealed no significant associations between PES and any symptom dimension (all $p > .05$). Effect sizes were uniformly negligible to small, with the largest observed correlation for anorexia nervosa

score ($r = -.26$, $p = .238$). All other correlations fell below $|r| = .20$, and all 95% confidence intervals included zero.

3.4.2 Bayesian Posterior Correlations

Bayesian posteriors were calculated for each PES–symptom correlation using an LKJ(2) prior. Results aligned with the frequentist findings: all posterior mean correlations fell within $|r| \leq .24$, and all 95% credible intervals spanned zero. The Region of Practical Equivalence (ROPE) analysis indicated that for the majority of symptom dimensions, approximately 33–36% of the posterior mass fell within the negligible effect zone ($|r| < .10$), with the remaining mass distributed symmetrically across positive and negative values–indicating that the data are genuinely uninformative about the direction of any effect rather than due to modest sample size. Anorexia nervosa score showed the highest posterior probability of a meaningful association ($P(|r| > .20) = 61\%$), but this did not reach conventional thresholds for inferential confidence.

3.4.3 Permutation Tests

Permutation tests were conducted for the within-pair PES–symptom correlations using all 2^9 = 512 sign permutations. No within-pair correlation reached significance (all $p > .05$). The closest to significance was specific phobia ($r = -.49$, $p = .051$), suggesting a possible within-pair trend that warrants investigation in larger samples but did not survive correction for multiple testing.

3.4.4 Parametric Bootstrap Confidence Intervals

Bias-corrected and accelerated (BCa) bootstrap confidence intervals, derived from a Gaussian copula parametric bootstrap (10,000 replicates), closely tracked the Bayesian credible intervals. All BCa 95% CIs included zero. Anorexia nervosa score again showed the

narrowest margin (BCa 95% CI [−.77, .04]), consistent with a possible small negative association that would require a larger sample to detect reliably.

3.4.5 Multilevel Models: Within-Pair and Between-Pair Effects

Multilevel regression models decomposed PES into within-pair and between-pair components, with cluster-robust standard errors. No within-pair PES effect reached significance for any symptom dimension (all $p > .14$), confirming that twins who differ in PES from their co-twin do not systematically differ in psychological symptom severity. One between-pair effect was significant: hypersomnia ($\beta = -0.40$, $p = .017$, 95% CI [−0.72, −0.09]), indicating that twin pairs with higher average PES had lower hypersomnia scores. However, the corresponding within-pair effect was non-significant ($\beta = 0.19$, $p = .40$), suggesting that this association reflects confounding by shared genetic or environmental factors rather than a causal epigenetic pathway.

Intraclass correlations (ICCs) revealed substantial variation in the twin-pair clustering of symptom scores. Hypersomnia (ICC = .83), binge-eating disorder (.82), anorexia nervosa (.69), persistent depressive disorder (.66), generalized anxiety (.62), obsessive-compulsive disorder (.62), and separation anxiety (.59) scores exhibited high ICCs, indicating that most variance in these domains is attributable to shared genetic and environmental factors. In contrast, specific phobia, body dysmorphic disorder, illness anxiety, and insomnia showed ICCs at or near zero, indicating predominantly individual-specific variance. Notably, PES failed to predict symptom severity even for these low-ICC dimensions, where an individual-level epigenetic predictor would be most expected to contribute.

3.4.6 Penalized Multivariate Estimation

A regularized canonical correlation analysis (CCA) assessed the maximal linear association between PES and the full 17-dimensional symptom profile simultaneously. The observed canonical r = .53, but the permutation-based significance test was non-significant (p = .99, 5,000 permutations), indicating that even the optimal linear combination of all symptom scores could not be predicted by PES above chance levels. The canonical weights were largest for specific phobia (−1.00), anorexia nervosa (−.90), social anxiety (−.89), and hypersomnia (−.88), identifying these as the symptom dimensions most strongly weighted in the best-fitting—but still non-significant—multivariate association.

3.4.7 Convergent Results

Across all five analytical frameworks PES showed no meaningful association with psychological symptom severity. This convergent null substantially strengthens the conclusion that the absence of PES–symptom associations is not attributable to methodological limitations of any single approach or to the limited statistical power of the sample.

Table 7

Pearson Correlations Between Poly-Epigenetic Scores and Psychological Symptom Dimensions (N = 22)

Symptom Dimension	*r*	*p*	95% CI
Major Depression	−0.05	0.813	[−0.46, 0.38]
Social Anxiety	−0.19	0.389	[−0.57, 0.25]
Generalized Anxiety	−0.04	0.845	[−0.46, 0.38]
Bipolar Disorder	−0.08	0.727	[−0.48, 0.35]
Agoraphobia	0.09	0.675	[−0.34, 0.50]
Panic Disorder	0.07	0.754	[−0.36, 0.48]
Anorexia Nervosa	−0.26	0.348	[−0.62, 0.18]
Bulimia Nervosa	−0.08	0.734	[−0.48, 0.36]
Binge-Eating Disorder	−0.01	0.974	[−0.43, 0.42]

Note. df = 20 for all correlations. None of the correlations reached statistical significance (all $p > .05$).

3.5. No Mediating Effect of Poly-Epigenetic Scores on the Relationship Between Psychosocial Adversity and Psychological Symptom Domains

To examine whether PES mediated the association between PSA and psychological symptom dimensions, mediation analyses were conducted using bootstrapping (1,000 samples). Five adversity predictors (composite score and four subscales) were examined in relation to nine symptom outcomes spanning mood disorders, anxiety disorders, and eating disorders, yielding 45 mediation models. All mediation results are presented in Table 8.

A necessary condition for mediation is that the mediator must significantly predict the outcome (the b-path). Across all 45 models, PES did not significantly predict any symptom dimension when controlling for adversity (all b-path $p > .10$). This finding is consistent with

the bivariate correlations reported in Section 3.3, which showed no significant associations between PES and any symptom measure.

Although the a-path (adversity → PES) was significant for the composite adversity score (β = −.51, p = .016) and Daily Hassles Scale (β = −.55, p = .009), indicating that higher adversity was associated with lower PES, this effect was not transmitted to symptoms. For eating disorder outcomes specifically, all indirect effects were non-significant: anorexia nervosa (composite: b = .07, p = .554; Daily Hassles: b = .24, p = .146), bulimia nervosa (all ps > .45), and binge-eating disorder (all ps > .54). The Daily Hassles → PES → Anorexia Nervosa pathway showed the largest indirect effect (b = .24), but this remained non-significant (p = .146), likely reflecting insufficient statistical power.

Consequently, all 45 indirect effects were non-significant (all p > .05), and no evidence of mediation was observed across any symptom domain.

These results indicate that while adversity may influence DNA methylation patterns (as indexed by PES), these epigenetic changes do not, in turn, predict psychological symptom severity in the present sample. PES therefore does not serve as a mechanistic link between adversity exposure and psychopathology—whether mood, anxiety, or eating disorders—in this cohort. The absence of significant b-paths (PES → symptoms) across all 45 mediation models is fully consistent with the multi-method null reported in Section 3.4, which demonstrated that PES does not predict symptom severity under any of the five analytical frameworks applied.

Table 8

Summary of Mediation Analyses: Adversity → PES → Psychological Symptoms (N = 22)

	Models Tested	Sig. *a* Paths	Sig. *b* Paths	Sig. Indirect Effects
CAS (Standardized)	9	9 (100%)	0 (0%)	0 (0%)
Humiliation Scale	9	0 (0%)	0 (0%)	0 (0%)
Daily Hassles Scale	9	9 (100%)	0 (0%)	0 (0%)
Other as Shamer Scale	9	0 (0%)	0 (0%)	0 (0%)
Lack of Social Support	9	0 (0%)	0 (0%)	0 (0%)
Total	45	18 (40%)	0 (0%)	0 (0%)

Note. Mediation model: Adversity → PES (mediator) → Symptoms. Bootstrap samples = 1,000. a-path = adversity → PES; b-path = PES → symptoms (controlling for adversity). Indirect effects ranged from −.17 to .24 (all $p > .05$). For full mediation, a-path and b-path must be statistically significant.

4 Discussion

In this study, we examined PES as a possible predictive factor for psychological symptom prevalence and severity, and a mediating factor in the relationship between PSA and psychological ill-health. The present findings are consistent with earlier work by Buchanan et al. (2026), in that PSA exposure is consistently negatively associated with PES. Lower PES reflects a general trend of hypomethylation (lower DNAm levels) in the CpG sites associated with adversity in the studied cohort, consistent with DNAm patterns that may reflect adaptive or regulatory responses to stress. This effect was observed both at an individual level where each twin acted as an independent participant, as well as in pairwise analyses, in which within-pair deltas were compared. Notably, this association appeared to be independently driven by The Daily Hassles (Scholten et al., 2020) and Other as Shamer (Matos et al., 2015) adversity subscales, suggesting that day-to-day challenges and interpersonal difficulties may be specific factors that contribute to resilience, suggesting that chronic, socially evaluative stressors may be particularly salient drivers of epigenetic variation following PSA exposure. In contrast, PES showed no meaningful association with any psychological symptom dimension across five complementary analytical frameworks–Bayesian estimation, multilevel modeling, exact permutation tests, parametric bootstrapping, and penalized multivariate estimation–and subsequently failed to function as a mediating factor linking the relationship between adversity and psychological symptom domains. This convergent null across methods with distinct assumptions substantially strengthens the conclusion beyond what any single analytical approach could provide at N = 22. Importantly, PES in this study appear to function primarily as markers of stress exposure rather than downstream indicators of psychopathology. Taken together, these results indicate that while PESs are clearly associated with adversity exposure in a surprising negative pattern, it may not represent a reliable mechanism through which PSA facilitates psychological symptoms in this sample.

The present findings replicate a robust association between PSA exposure and lower PES, providing further support for the biological embedding of psychosocial stress via DNAm-based processes. Because MZ twins are matched on genetic background and early shared environment, the observed within-pair associations indicate that adversity exposure itself is linked to epigenetic variation that occurs as a result of post-natal influences. This pattern is consistent with a substantial body of prior work demonstrating enduring epigenetic modifications following adversity, particularly within stress- and emotion-regulation pathways. In the present study, this framework is supported by the observed negative association between PSA exposure and PES, both at the between-group level and within monozygotic twin pairs, indicating that greater stress exposure was associated with lower PES. The concept of biological embedding posits that life experiences and environmental factors are integrated into an organism's physiological state, particularly via epigenetic mechanisms such as DNAm, altering developmental trajectories and health outcomes across the lifespan (Essex et al., 2013; Aristizabal et al., 2019; Non et al., 2016). Through these modifications, the epigenome may act as a molecular interface that translates psychosocial stressors – especially chronic and socially evaluative forms of stress – into persistent alterations in neurobiological and emotion-regulation pathways (Dee at al., 2023). The multilevel modeling results provide additional insight into the structure of the null PES–symptom findings. Intraclass correlations revealed that symptom domains varied considerably in the proportion of variance attributable to twin-pair membership (ICC range: 0–.83). Symptom dimensions with high ICCs–such as hypersomnia (.83), binge-eating disorder (.82), and anorexia nervosa (.69)–are predominantly driven by shared genetic and environmental factors, leaving relatively little individual-specific variance for an epigenetic predictor to explain. However, PES also failed to predict symptoms in dimensions with ICCs at or near zero (specific phobia, body dysmorphic disorder, illness anxiety, insomnia), where

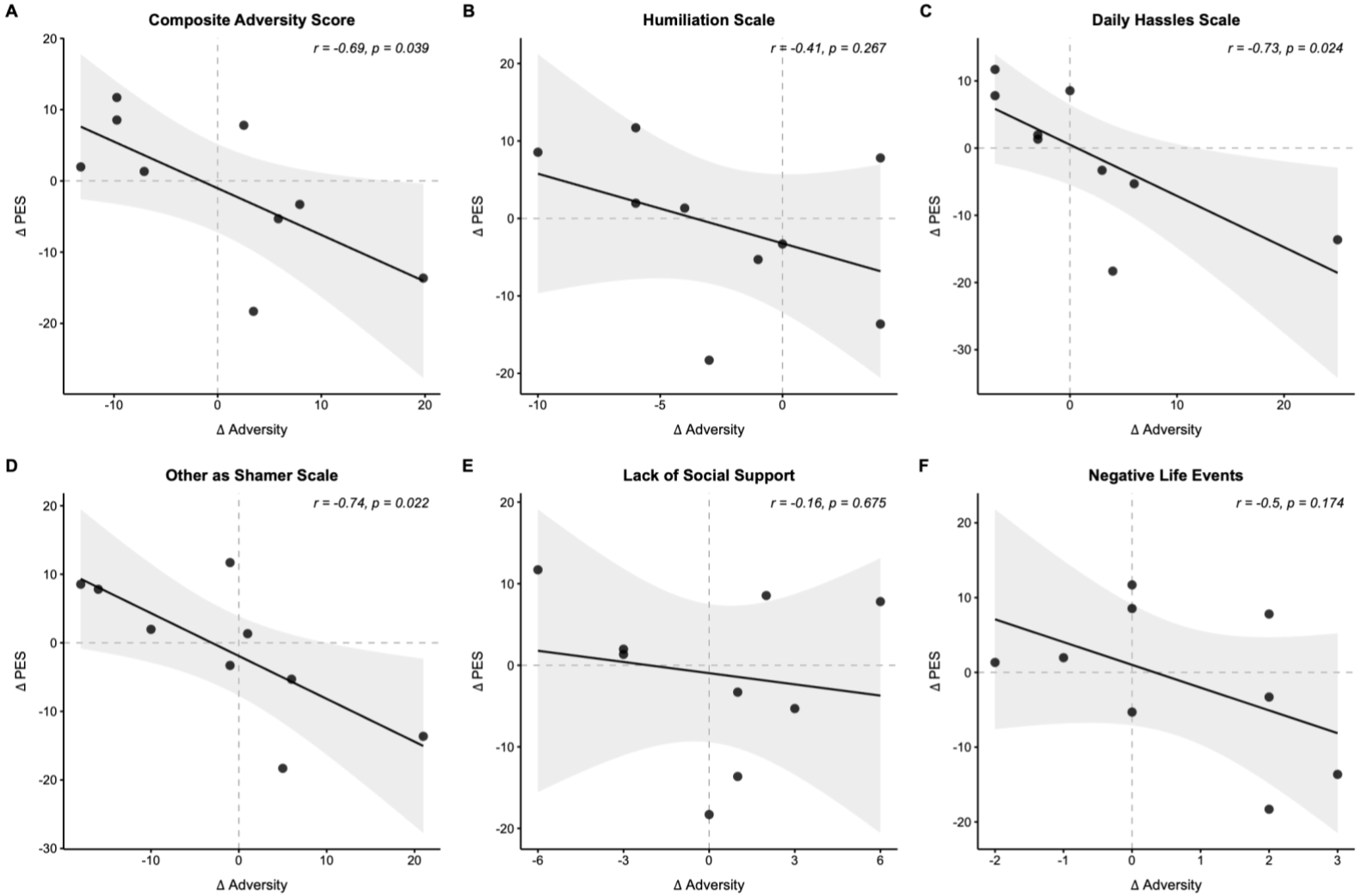
nearly all variance is individual-specific and thus maximally amenable to individual-level epigenetic prediction. This pattern suggests that the null PES–symptom association is not simply a consequence of symptom heritability constraining the available variance, but rather reflects a genuine absence of predictive power in the PES construct as currently operationalized. The one significant between-pair effect—hypersomnia ($\beta = -0.40$, $p = .017$)—was not accompanied by a corresponding within-pair effect, indicating confounding by shared factors rather than a causal epigenetic pathway. This dissociation is consistent with the broader interpretation that PES captures exposure-related biological variation but not symptom-relevant variation.

4.1 Limitations

This study has several important limitations. The modest sample size ($N = 22$) limited the statistical power of individual frequentist tests, providing only 80% power to detect large correlations ($|r| \geq .56$). To address this limitation, five complementary analytical strategies were employed (Section 2.8), yielding convergent null findings across Bayesian estimation, multilevel modeling, exact permutation tests, parametric bootstrapping, and penalized multivariate estimation. This convergence substantially reduces the probability that the null PES–symptom associations are attributable to insufficient power alone, although the possibility of very small effects ($|r| < .15$) falling below the detection threshold of all methods cannot be excluded. The largest observed correlation ($r = -.26$ for PES × anorexia nervosa) showed some indication of a possible small effect across multiple methods—with the Bayesian posterior $P(|r| > .20) = 61\%$ and the bootstrap BCa 95% CI approaching but not excluding zero [−.77, .04]—warranting investigation in larger samples. Replication with $N > 100$ remains essential, both to confirm the null findings for most symptom dimensions and to clarify whether the anorexia nervosa association reflects a true small effect.

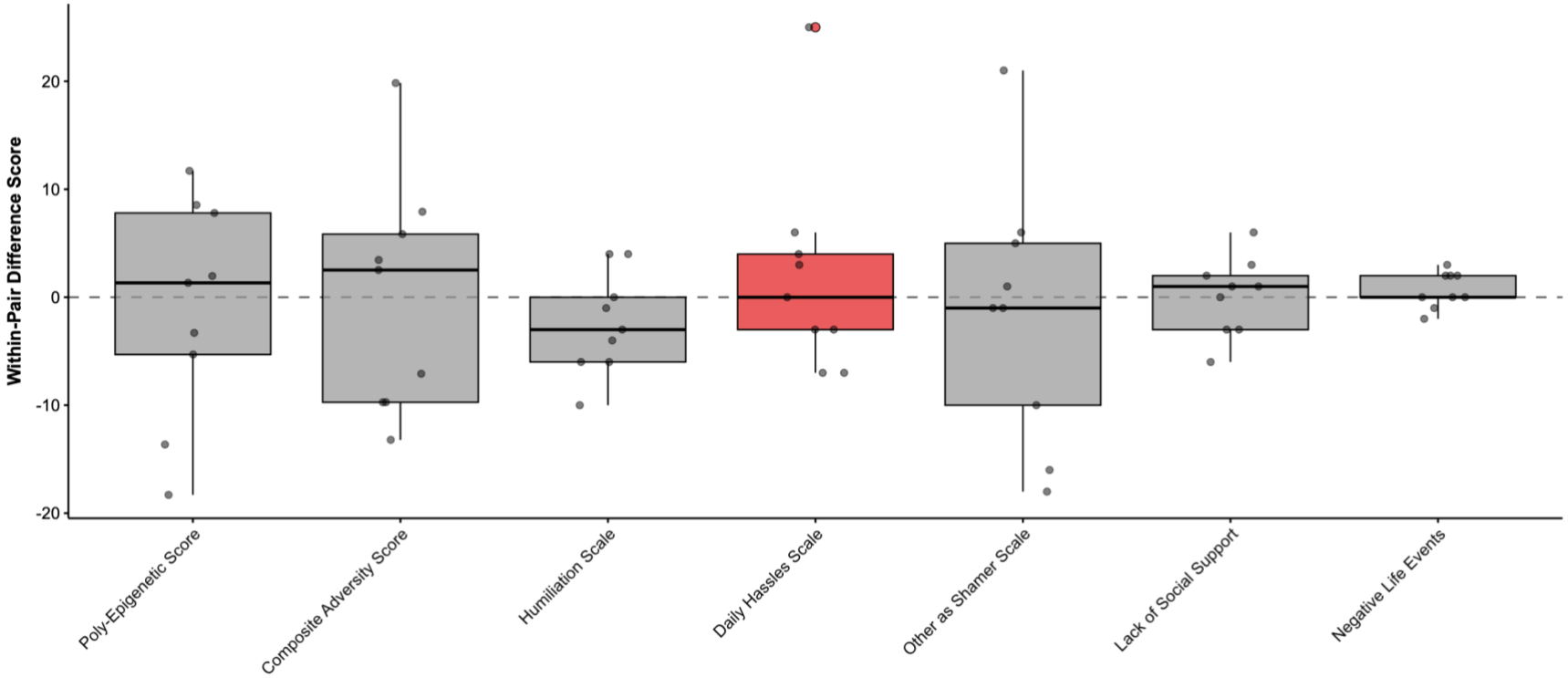
Second, the PES construction methodology may explain the observed pattern of results. PES were derived from CpG sites selected to maximize discrimination between adversity-exposed and control individuals, not to predict psychiatric symptoms. This optimization for exposure classification may explain why PES robustly differentiate adversity groups ($d = 1.12$) yet show negligible symptom associations (all $r < .26$). Future studies should construct PES specifically optimized for symptom prediction using symptom-based EWAS, candidate gene approaches, or machine learning methods.

Figure 1 a-f. *Within-Twin-Pair Association Between Adversity Sub-Scales and Poly-Epigenetic Score Differences.*



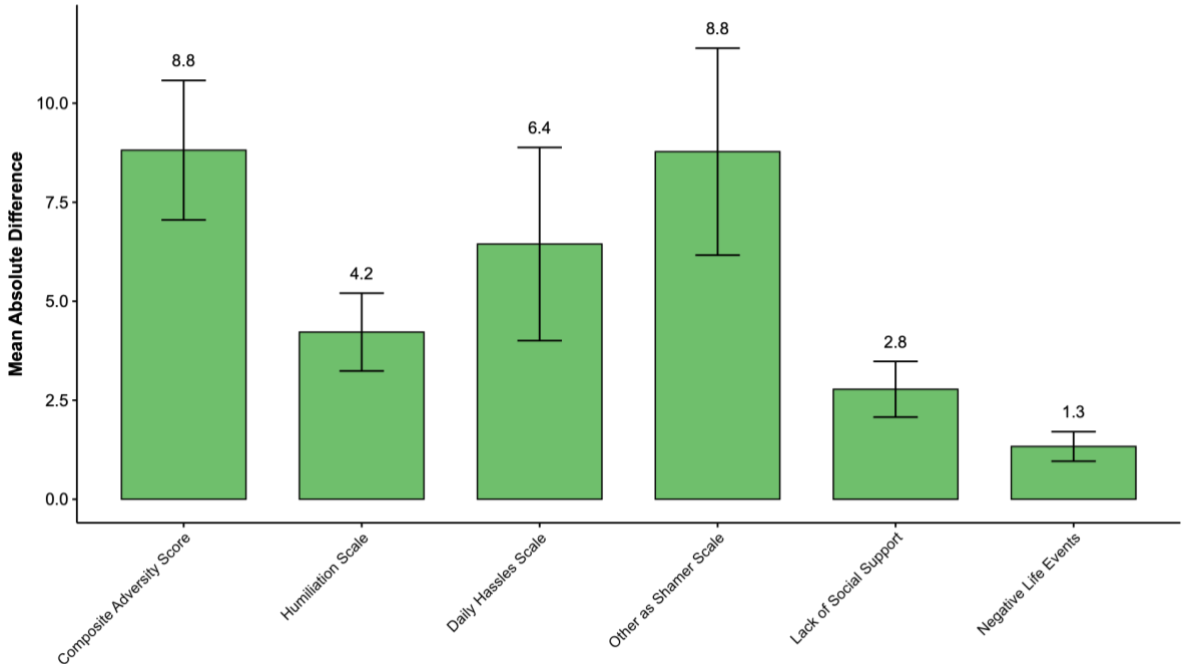
Note. Each point represents one monozygotic twin pair (N = 9). Difference scores calculated as Twin 01 − Twin 02. Shaded area represents 95% confidence interval. Overall, greater within-pair adversity discordance is associated with lower poly-epigenetic scores.

Figure 2. *Boxplots of Within-Pair Difference Scores in Poly-Epigenetic Score and Adversity Subscales.*



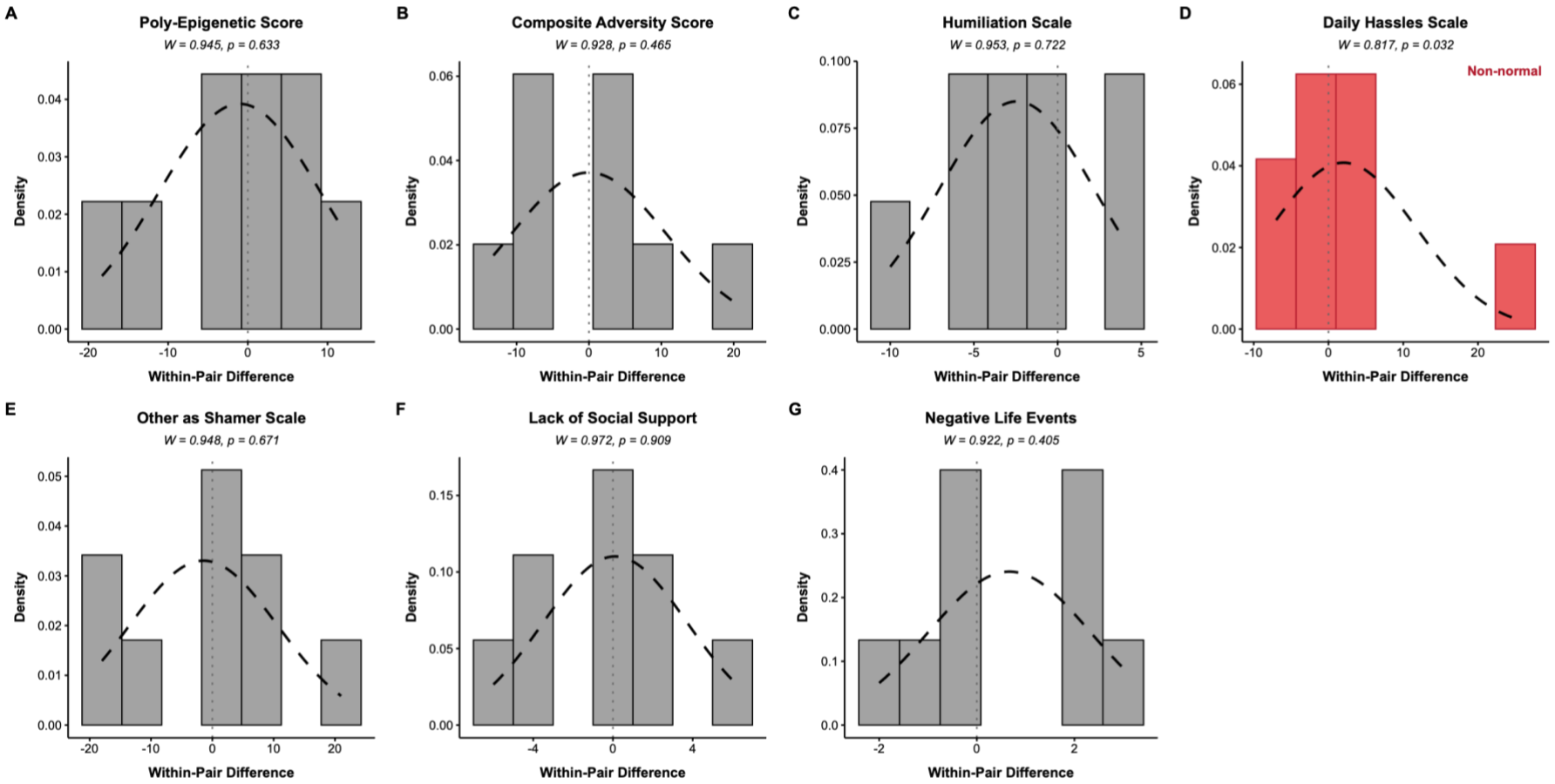
Note. The Daily Hassles Scale (red) shows an extreme outlier driving non-normal distribution. Individual points represent twin pairs (N=9).

Figure 3. *Mean Absolute Within-Pair Differences by Adversity Subscale.*



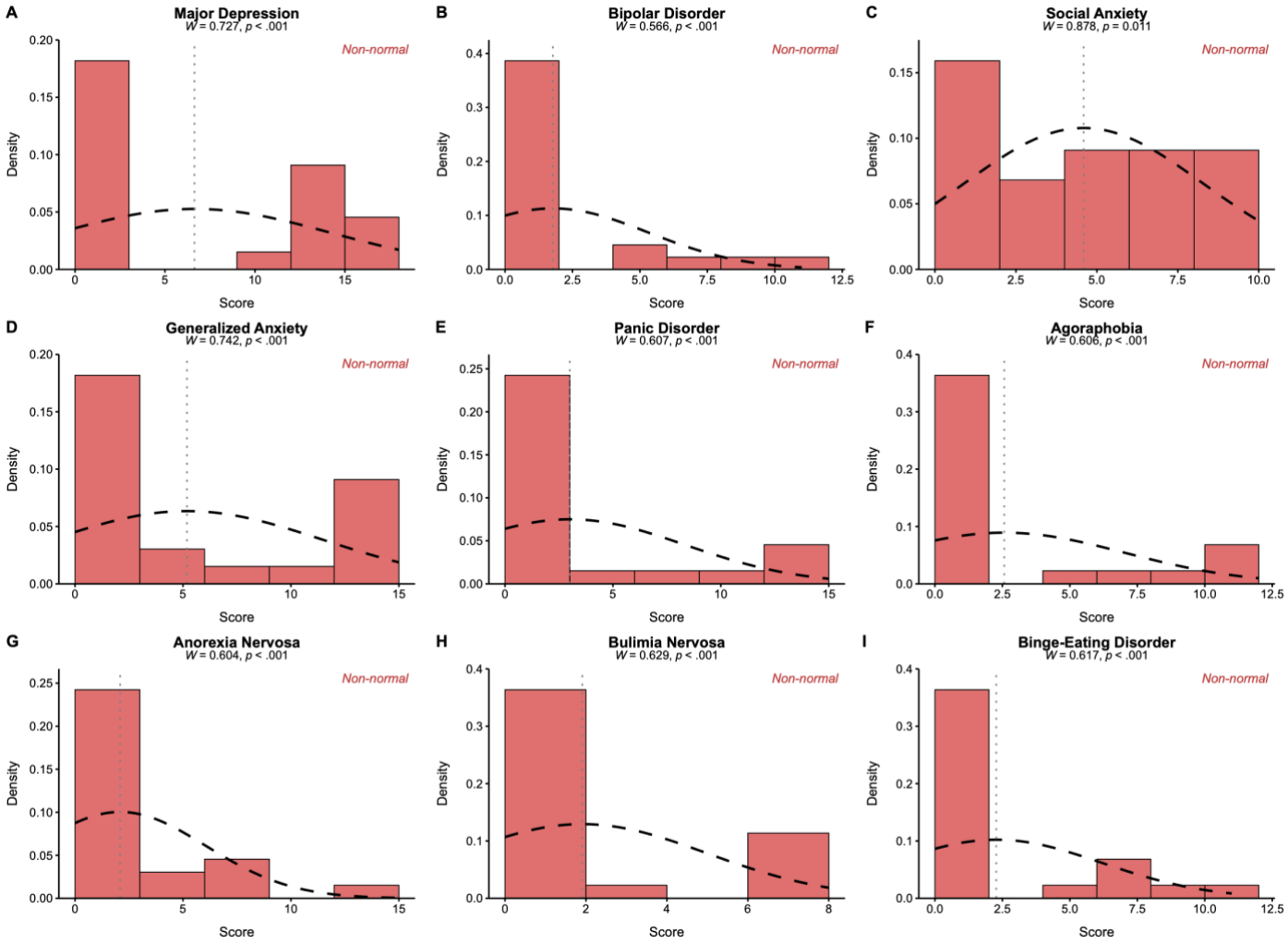
Note. Error bars represent +/- 1 standard error (SE). Higher values indicate greater within-pair discordance on that adversity measure.

Figure 4 a-g. *Distributions of Within-Pair Difference Scores with Normal Curve Overlay.*



Note. The Daily Hassles Scale (red) indicates significant deviation from normality (Shapiro-Wilk $p < 0.05$). Dashed curves represent theoretical normal distribution.

Figure 5. *Distributions of Psychological Symptom Scores With Normal Curve Overlay.*



Note. All distributions show significant deviation from normality (Shapiro-Wilk $p < 0.05$). Dashed curves represent theoretical normal distribution. Dotted line indicates mean. N = 22.

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3 General Discussion

In this research project, we investigated the epigenetic mechanisms linking psychosocial adversity (PSA) to psychological symptom development in young adults, utilizing a case co-twin design in monozygotic (MZ) twins. We opted for a co-twin design in order to isolate postnatal environmental influences, particularly early life stressors, while controlling for genetic confounding. Across three interconnected empirical studies, this work yielded findings that both advance and complicate our understanding of the adversity-epigenetics-psychopathology pathway. We have developed statistical approaches and frameworks that may help researchers disentangle environmentally induced epigenetic variation from genetic confounding in studies of early life adversity and mental health outcomes.

3.1 Study I: Establishing the Methodological Foundations

The first methodological study established the framework for operationalizing adversity in a co-twin design. We developed and validated approaches for quantifying psychosocial adversity as reported by the participants, and identifying adversity-discordant twin pairs within the German TwinLife cohort (reference). Analysis of 739 twin pairs (349 MZ, 390 DZ) verify demonstrated that adversity, as assessed through six validated instruments capturing negative life events, humiliation, daily hassles, shame, lack of social support, and COVID-19 pandemic-related bitterness (Hartling & Luchetta, 1999; Matos et al., 2015; Scholten et al., 2020; Zimet et al., 1988), formed a coherent overall adversity experience measure, confirmed through confirmatory factor analysis (CFI = 0.92, RMSEA = 0.092). Three complementary statistical approaches – score ranking, Euclidean distance, and standard deviation-based sanity check – converged to identify 144 MZ and 173 DZ pairs (approximately 41–44% of the sample) exhibiting meaningful within-pair adversity discordance, adequate for further research.

Critically, statistical variance modelling revealed that 43% of variance in adversity scores could be attributed to unique (non-shared) environment, 23% to shared environment, and 33% to genetic factors. This substantial non-shared environmental contribution validates the fundamental rationale for the case co-twin design: if adversity experiences were entirely genetically determined or driven by shared family environment, within-pair discordance would be minimal and uninformative. The identification of robust within-pair variation confirms that MZ twins, despite genetic identity and even shared upbringing, contrary to adoption studies, diverge meaningfully in their adversity experiences from pre-school age to young adulthood – providing the necessary foundation for quantitative investigation.

3.2 Study II: Individual CpG Methylation Sites as Possible Mediators

The second study examined whether specific CpG methylation sites mediated the association between PSA and psychological symptoms in 9 MZ twin pairs (N = 18). Differential methylation analysis identified 73 CpG sites exhibiting significant differential methylation patterns between adversity-exposed and control twins, with both hypermethylation (34 sites) and hypomethylation (39 sites) observed. Genes associated with the identified candidate CpG sites were implicated in neurodevelopment (DIP2C, GTF2I), neurotransmitter function (SLC18A1, SYNGR3, PDE1B), synaptic plasticity (BDNF-AS, RIMS2), stress response (ADRA2A), and immune regulation (CYBA, TNFSF10, PGF) (Tuñon-Ortiz et al., 2025; Shennib et al., 2025; Lohoff et al., 2008; Katrancha et al., 2019; Cruceanu et al., 2016; Li et al., 2023; Gao et al., 2021; Li et al., 2022; Zhang et al., 2022; Klaus et al., 2021; Wu et al., 2024)

Bootstrap mediation analyses revealed significant indirect effects for several CpG sites. The central finding was a predominant "suppression" mediation pattern: while adversity directly increased symptom severity (positive direct effect), adversity-induced hypomethylation at specific loci was associated with lower symptom severity (negative indirect effect). This

could be attributed to the perhaps surprising finding, that the specific type of adversity identified in our sample consistently decreased methylation, rather than increasing it. Hypomethylation at cg24977276 (GTF2I gene body) significantly mediated the association between adversity and major depression symptoms, with 68% of the total effect operating through this epigenetic pathway. Similar suppression patterns were observed for PRKAR1B-associated methylation. In contrast, SS18 promoter methylation exhibited a positive mediation pattern for bipolar symptoms, with adversity-induced hypermethylation transmitting rather than buffering the adversity effect, accounting for 94.9% of the total effect through near-complete mediation. A major limitation of the study was the relatively modest sample of only 9 twin pairs, where both co-twins completed all phases of the study. In order to mitigate this limitation, we ran supplemental analyses on individual level, assessing every participant uniquely. We have used statistical analyses that perform well on small samples, and we report all findings as preliminary and indicative.

3.3 Study III: Investigating the Role of Aggregate Poly-Epigenetic Scores

The third study evaluated whether aggregate poly-epigenetic scores (PES) – weighted sums of methylation levels across 200 CpG sites identified through PLS-DA (Buchanan et al., manuscript in preparation) – predicted psychological symptoms and mediated adversity-psychopathology associations in 22 participants (9 complete twin pairs plus 4 unpaired individuals). Replicating findings from previous work, we have found that PES significantly differentiated adversity-exposed from control twins, with exposed individuals exhibiting lower PES values (Cohen's $d = 1.12$, $p = .017$). Within-pair analyses confirmed a significant negative correlation between adversity discordance and PES discordance ($r = -0.69$, $p = 0.039$), indicating that the twin with greater adversity exposure tended to have lower aggregate methylation scores. This was well in line with our previous results that have also shown that adversity had a generally suppressive rather than enhancing effects on DNAm.

However, contrary to hypotheses, PES showed no significant associations with any of the 17 psychological symptom dimensions examined, spanning mood disorders, anxiety disorders, eating disorders, somatic symptom domains, and sleep disorders. This null finding was confirmed across five complementary analytical strategies: Bayesian estimation with LKJ(2) priors yielded uniformly small posterior correlations (all $|r| \leq .24$) with 95% credible intervals spanning zero; exact permutation tests across 512 exhaustive sign permutations were non-significant for all within-pair correlations; parametric bootstrap BCa confidence intervals consistently included zero; multilevel models revealed no significant within-pair (genetically unconfounded) PES effects; and regularized canonical correlation analysis showed that even the optimal multivariate combination of symptoms could not be predicted by PES above chance ($p = .99$). Intraclass correlation analysis further revealed that PES failed to predict symptom severity even for dimensions with near-zero ICC (specific phobia, insomnia), where individual-level epigenetic prediction would be most feasible. Consequently, mediation analyses across 45 models revealed no significant indirect effects, indicating that PES did not transmit adversity effects to symptoms despite reliably indexing adversity exposure itself.

3.4 Indicative Evidence for Biological Embedding

A central contribution of this project is the preliminary evidence across studies I, II and III, supporting the biological embedding of psychosocial adversity through DNA methylation mechanisms to a degree. Study I demonstrated that unique environmental experiences account for 43% of variance in adversity exposure, establishing the environmental variation necessary for epigenetic investigation. Study II found that this environmental variation is associated with differential methylation at specific genomic loci, indicating within-pair differences despite genetic identity, even in small samples. Study III extended these findings by replicating that aggregate PES reliably differentiated exposed from control twins with a

large effect size (d = 1.12), even though predictive and mediating role of PES could not be established.

The within-pair co-twin design suggests that these associations reflect post-natal environmental influences rather than pre-existing genetic or prenatal factors. Because MZ twins share virtually identical genomes, shared prenatal environment, and typically shared early childhood experiences, any systematic within-pair covariation between adversity and methylation must arise from divergent post-natal experiences – precisely the environmental influences this thesis sought to isolate. The convergence of findings across individual CpG analyses (Study II) and aggregate scoring approaches (Study III) strengthens confidence that the observed epigenetic variation is genuinely related to adversity exposure.

3.5 Clinical Implications

In the scope of this project, we have identified a consistent and robust suppression pattern, in which psychosocial adversity seemed to be linked with reduced rather than increased methylation. These findings could support the validity of the clinical application of the suppression pattern: adversity-induced hypomethylation at certain loci may represent protective epigenetic adaptations that could inform resilience-based interventions, even though precise practical application remains to be determined. Specific identified CpG methylation sites should therefore be assessed in follow-up studies, specifically in terms of their exact roles in suppressing psychopathology development.

Conversely, the findings in Study III suggest that poly-epigenetic scores (PESs) could potentially serve as blood-based biomarkers for identifying individuals at heightened risk following adversity exposure, provided that replication studies are conducted. While PES did not predict symptom severity in the present sample, their robust differentiation between

adversity-exposed and control twins indicates that they reliably capture biological traces of environmental stress. First, PESs could complement traditional clinical interviews in assessing cumulative stress burden, particularly in populations where self-report may be unreliable (e.g., children, individuals with memory impairments, or those reluctant to disclose health details). Additionally, aggregate methylation scores may help identify individuals who have experienced significant adversity but have not yet developed clinical symptoms, enabling targeted preventive interventions. Moreover, longitudinal monitoring of PESs changes could potentially serve as an objective metric for evaluating the biological efficacy of trauma-focused therapies or stress-reduction programs, though this application requires validation in intervention studies.

The divergent mediation patterns observed in Study II – protective suppression at gene body sites (GTF2I, PRKAR1B) versus risk-transmitting hypermethylation at the SS18 promoter – underscore the importance of genomic context in determining whether epigenetic changes confer vulnerability or resilience, aside from sufficient sampling. This complexity has important implications for future biomarker development: rather than assuming all adversity-induced methylation changes are pathological "molecular scars," clinical applications must account for site-specific functional consequences.

Finally, these results contribute to the emerging framework of precision psychological research, which seeks to tailor prevention and treatment strategies to individual biological profiles. While current findings are preliminary and observational, they suggest a future in which epigenetic profiling could inform personalized risk assessment, guide the timing and intensity of interventions, and monitor treatment response. However, translating these research findings into clinical practice will require substantial additional work, including replication in larger, diverse samples; validation of biomarker sensitivity and specificity; and

demonstration that epigenetic information improves clinical outcomes beyond standard assessment methods.

3.6 Limitations

The prominent limitation recognized in the scope of this project was a modest sample size (N = 22; N = 18; N = 9). This significantly reduces the statistical power of respective studies II and III, and limits reporting to observational and indicative results In Study III, this limitation was addressed through five complementary analytical strategies (Bayesian estimation, multilevel modeling, exact permutation tests, parametric bootstrapping, and penalized multivariate estimation), which yielded convergent null findings for PES–symptom associations. While this convergence substantially reduces the probability that the nulls are attributable to power alone, replication in larger cohorts (N > 100 pairs) remains essential to exclude the possibility of very small effects. Based on the pilot results yielded in studies I – III, it can be said with confidence that replication with more statistical power is justified. While significant effects in studies II and III should be interpreted with appropriate caution, the null findings in Study III are supported by convergent evidence across multiple analytical frameworks, lending them greater credibility than a single underpowered frequentist test would provide. Another important limitations involves the cross-sectional and retrospective design of studies I – III. This introduces the possibility of recall bias, and the single-timepoint methylation measurement precludes causal inference. Future replication might therefore not only benefit from a larger sample, but also from integrating longitudinal measures.

Psychosocial adversity assessment used in the scope of this project focused mostly on moderate adversities, and it is already well established that adversity and its psychopathological consequences function in a dose-dependent, context-based relationship (Nelson et al., 2020; Sisk et al., 2025). Our sampling focus was also limited to the German

middle-class population, which limits generalizability to other cultural contexts where adversity experiences and coping mechanisms differ. To investigate the full scope of adversities, their epigenetic correlates, and their psychopathological outcomes, it is therefore important to incorporate a broad spectrum of stressors.

Transparent Artificial Intelligence Use Statement

All use of artificial intelligence (AI) tools in the scope of this thesis was in accordance with the latest *Guidelines on the use of Generative AI tools for learning and teaching* issued by the University of Luxembourg. The models used were ChatGPT, Claude, Grammarly, and Microsoft Copilot, and the specific uses included: text proofreading, clarity improvements, formatting according to APA 7th edition publishing standards, creating text structure outlines during early drafting, conducting targeted literature search, and coding assistance. All output, including literature references, was verified by the author for accuracy and adherence with the scope of the thesis, and manually adapted. All hypotheses and research questions, as well as methodologies and designs are the original work of the authors of the studies included in this thesis. No participant/study data or confidential information was uploaded into AI systems.

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Affidavit / Statement of originality

I declare that this thesis:

- is the result of my own work. Any contribution from any other party, and any use of generative artificial intelligence technologies have been duly cited and acknowledged;
- is not substantially the same as any other that I have submitted, and;
- is not being concurrently submitted for a degree, diploma or other qualification at the University of Luxembourg or any other University or similar institution except as specified in the text.

With my approval I furthermore confirm the following:

- I have adhered to the rules set out in the University of Luxembourg's Code of Conduct and the Doctoral Education Agreement (DEA)[1], in particular with regard to Research Integrity.
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[1] If applicable (DEA is compulsory since August 2020)