Aus dem Institut für Medizinische Psychologie
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DISSERTATION

Early-Life Stress and Epigenetic Ageing in Preschool-Aged Children

Lebensgeschichtlich Frühe Stresserfahrungen und Epigenetische Alterung bei Kindern im Vorschulalter

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ELS	–	Early-life stress
PedBE	–	Pediatric buccal epigenetic
CRP	–	C-reactive Protein
CpG	–	Cytosine-phosphate-guanine sites
DNA	–	Desoxyribonucleic acid
BerlinLCS	–	Berlin Longitudinal Children Study
PAPA	–	Preschool Age Psychiatric Assessment
AUCg	–	Area under the curve with respect to ground
BMI	–	Body mass index
MPIP	–	Max Planck Institute of Psychiatry
DSM-IV	–	Diagnostic and Statistical Manual of Mental Disorders fourth edition
ANCOVA	–	Analysis of covariances
OLS	–	Ordinary least square regression
SES	–	Socioeconomic status
SD	–	Standard deviation
CI	–	Confidence interval
OR	–	Odds ratio
DNMT1	–	DNA methyltransferase 1

Abstract

Background: Research of stress-related ageing may be crucial to tackle future burdens for health care systems. Chronic stress exposure during childhood such as exposure to maltreatment is associated with ageing-related diseases and global population as well as life expectancy is steadily increasing. However, understanding of underlying mechanisms remains cloudy. The current study investigates epigenetic ageing in children in association with various manifestations of stress exposure including psychopathology and maltreatment and biological stress. Further, epigenetic ageing is assessed in relation to stress-related biological factors including glucocorticoid signaling and inflammation.

Methods: Hypotheses were tested in 158 children aged 3 to 5 years. The impact of psychopathology on epigenetic ageing was tested and maltreatment was integrated as moderator in this relationship. The association between epigenetic ageing and biological correlates of stress, i.e., Cortisol and CRP, was examined overall and in groups of differential risk for accelerated epigenetic ageing. Lastly, it was evaluated whether the pediatric buccal epigenetic (PedBE) clock is responsive to glucocorticoids.

Results: Internalizing disorder significantly accelerated epigenetic ageing ($F_{1,147}$ = 6.67, p = 0.011) while externalizing disorder had no effect on epigenetic ageing. Maltreatment gradually increased the effect of internalizing disorder on epigenetic ageing (b = 0.49, 95% CI [0.073, 0.909], t = 2.322, p = 0.022). Further, epigenetic ageing significantly correlated with cortisol (r [32] = 0.36, p = 0.043) and CRP (r [32] = 0.42, p = 0.016) in maltreated children with internalizing disorder. A significant amount of the PedBE clock CpG sites (18/94) was responsive to glucocorticoid exposure (OR = 4.36, $p = 1.65*10^{-6}$).

Conclusion: The current study identified maltreatment as catalyzer for accelerated epigenetic ageing in children with internalizing disorder and thereby expanded knowledge on stress-related ageing very early in life. This relationship may depend on inflammation and glucocorticoids. Determination of epigenetic age could depict a relevant future tool for identification of children at increased risk for stress-related morbidity and may thereby enable and support early targeted interventions.

Zusammenfassung

Hintergrund: Die Erforschung stressbedingten Alterns kann entscheidend sein, um künftigen Herausforderungen für Gesundheitssysteme zu begegnen. Chronischer Stress in der Kindheit wird mit altersbedingten Krankheiten in Verbindung gebracht. Dabei steigen die Weltbevölkerung sowie die Lebenserwartung der Menschen stetig an. Ein Verständnis der zugrunde liegenden Mechanismen bleibt jedoch weitgehend unklar. Die aktuelle Studie untersucht das epigenetische Altern bei Kindern im Zusammenhang mit verschiedenen Manifestationen von Stressexposition, einschließlich Psychopathologie, Misshandlungserfahrungen und biologischem Stress. Darüber hinaus wird getestet, inwieweit stressbiologische Korrelate wie Glukokortikoide und Inflammation zur epigenetischen Alterung beitragen.
Methodik: Für die vorliegende Studie wurden 158 Kindern im Alter zwischen 3 und 5 Jahren untersucht. Der Einfluss der Psychopathologie auf die epigenetische Alterung wurde getestet und Misshandlungserfahrungen als Moderator in diesem Zusammenhang berücksichtigt. Der Zusammenhang zwischen epigenetischer Alterung und biologischen Korrelaten von Stress, Cortisol und CRP, wurde insgesamt und innerhalb von Gruppen mit unterschiedlichem Risiko für eine beschleunigte epigenetische Alterung untersucht. In einem letzten Schritt wurde in einer unabhängigen Stichprobe untersucht, ob die CpG-Dinukleotide der PedBE clock reaktiv gegenüber Glukokortikoiden sind.
Ergebnisse: Kinder mit internalisierender Psychopathologie wiesen eine beschleunigte epigenetische Alterung im Vergleich zu Kindern ohne internalisierende Psychopathologie auf ($F_{1,147}$ = 6.67, p = 0.011), wobei externalisierende Psychopathologie keinen Effekt auf die epigenetische Alterung hatte. Der Schweregrad der Misshandlungserfahrungen erhöhte graduell den auf die epigenetische Alterung beschleunigenden Effekt von internalisierender Psychopathologie (b = 0.49, 95 % KI [0.073, 0.909], t = 2.322, p = 0.022). Darüber hinaus war bei Kindern mit internalisierender Psychopathologie und Misshandlungserfahrungen die epigenetische Alterung signifikant mit Kortisol (r [32] = 0.36, p = 0.043) und CRP (r [32] = 0.42, p = 0.016) assoziiert. In der PedBE clock waren signifikant mehr CpG-Dinukleotide reaktiv gegenüber Dexamethason (18 von 94) als es bei 94 CpG-Dinukleotiden zu erwarten wäre (OR = 4.36, p = $1{,}65*10^{-6}$).

Bei einem Gruppenvergleich nach Misshandlungsstatus und dem Vorhandensein einer internalisierenden Störung wiesen zwei spezifische CpG-Dinukleotide eine aberrante DNA-Methylierung auf.

Schlussfolgerung: Die aktuelle Studie identifizierte Misshandlungserfahrungen als Wegbereiter für eine beschleunigte epigenetische Alterung bei Kindern mit internalisierender Störung und konnte dadurch den Wissensstand über stressbedingtes beschleunigtes biologisches Altern, das bereits sehr früh im Leben stattfindet, erweitern. Dieser Zusammenhang könnte zudem mit erhöhten Konzentrationen von inflammatorischen Parametern und Glukokortikoiden zusammenhängen. Die Bestimmung des epigenetischen Alters könnte damit ein wichtiges Werkzeug zur Identifizierung von Kindern mit erhöhtem Risiko für stressbedingte Morbidität im Kindes- und Erwachsenenalter darstellen und dadurch frühzeitige und gezielte Interventionen ermöglichen.

1 Introduction

Early-life stress (ELS) such as child maltreatment, war-related trauma as well as suffering from psychiatric disorders during childhood can critically increase vulnerability for somatic diseases and psychopathology later in life. Child maltreatment is one aggravated form of ELS and includes experiences of physical neglect and abuse, witnessing domestic violence, sexual abuse as well as emotional maltreatment (e.g., caretakers threatening their child with putting it up for adoption). From a psychodynamic perspective, ELS can be understood as traumatic experiences that critically exceed an individual's defense mechanisms producing psychological and biological stress. A growing body of literature has collected evidence for measurable biological alterations following ELS exposure and due to increased neuroplasticity during maturation children represent a vulnerable risk group in this regard (Heim & Binder, 2012; Lupien et al., 2009). It is believed that such biological alterations forge a link between ELS and increased risk for pathogenesis.

1.1 Current relevance of ELS research

The risk to be exposed to ELS has not merely decreased, but rather increased during the COVID-19 pandemic as indicated by epidemiological studies.
In Germany the prevalence for psychiatric disorders in children aged 7 to 17 years increased from overall 10% to nearly 18% during the pandemic and the increase was particularly higher (from 7.4% to 26.8%) in younger children aged 7 to 10 years (Ravens-Sieberer et al., 2021). Further, based on a nationwide survey it is estimated that over 30% of children in Germany have experienced at least one category of maltreatment (Witt et al., 2018) and the number of cases of child maltreatment is believed to have increased during COVID-19 pandemic (Griffith, 2020). Risk factors making maltreatment more likely may be increased perceived stress of parents and parental burnout (Griffith, 2020) and in fact, parents reported that familiar conflicts escalated quicker during the COVID-19 pandemic than they have before (Ravens-Sieberer et al., 2021). However, the true number of child maltreatment cases remains unknown.

Provision of early help for children experiencing ELS relies on detailed knowledge on whether and how ELS contributes to clinically relevant health outcomes. Increased Neuroplasticity during childhood not only indicates higher susceptibility for stress, but on the flipside, implies higher chances for successful stress-targeting interventions. In this regard, it is important to translate study finding into psychiatric and psychotherapeutic interventions and to implement these interventions into the health care system, one of the main actors in child protection. To establish and improve early targeted interventions, evaluation of ELS effects in pediatric study samples is needed. In future medicine, underlying biological correlates may allow identification of children at increased risk for stress-related adverse health outcomes later in life such as ageing-related diseases and mortality that are consistently shown to be associated with stress exposure (Brown et al., 2009; Druss et al., 2011; Eriksson et al., 2014; Kelly-Irving et al., 2013; Walker et al., 2015).

For instance, in individuals that have been exposed to ELS or suffer from psychiatric disorders epidemiological studies demonstrated higher prevalence rates of ageing-related diseases including cardiovascular diseases (Eriksson et al., 2014). These studies showed that psychiatric disorders are associated with a mortality rate that is over twice as high and occurs on average 10 years earlier as in individuals without psychiatric disorders. Rather than unnatural deaths the majority (>67 %) of deaths in individuals with psychiatric disorders is due to diseases associated with ageing (Druss et al., 2011; Walker et al., 2015). Moreover, ageing-related diseases may become an immense burden for future health care systems as it is estimated that the proportion of people worldwide aged 60 years and older will have doubled by 2050 (United Nations, 2019). Identification of early risk factors for ageing-related morbidity including ELS and its treatment therefore constitute global health concerns.

1.2 Biological ageing as potential risk indicator for ageing-related diseases

Increased risk for ageing-related diseases and excess mortality rate in individuals suffering from psychiatric disorders is believed to be mediated by accelerated biological ageing (Danese & McEwen, 2012; Gassen et al., 2017). Acceleration of biological ageing may reflect a premature onset of deficient molecular processes naturally emerging with increasing chronological age including accumulation of DNA damage and cell deaths as well as defects in cellular functions and DNA repair mechanisms.

Acceleration of biological ageing early in life, however, may be a warning signal for increased disease risk later in life. To identify incongruencies between chronological ageing and biological ageing researchers developed indicators for biological age estimation and, long before, clock makers facilitated chronological age estimation.

There are two commonly applied indicators for biological age estimation including epigenetic age and DNA telomere shortening. For the latter, biological ageing is mirrored by natural attrition of telomeres, the protective caps at the end of a chromosome, as with each cell division the length of telomeres decreases. Excessive telomere shortening (i.e., accelerated biological ageing) has been evidenced for various adult psychiatric disorders (Darrow et al., 2016). The other widely applied and valid measure for biological age is the epigenetic clock enabling epigenetic age estimation (Hannum et al., 2013; Horvath, 2013).

1.3 Biological age determination: Epigenetic age and epigenetic ageing

The epigenome allows us to derive new information from our DNA instead of only reading the genetic code itself. The enzyme methyltransferase attaches methyl groups to cytosine nucleotides forming methyl cytosine at cytosine-phosphate-guanine (CpG) sites. Methyl groups can hinder reading the genetic code and thereby DNA methylation is a mechanism for gene regulation without changing the genome.
It has been found that certain CpG sites become either hypermethylated or hypomethylated with increasing chronological age (see Figure 1). The conglomerate of all CpG sites whose DNA methylation significantly correlates with chronological age is referred to as epigenetic clock.
Hannum et al. (2013) firstly provided a method to determine an individual's epigenetic age in blood whereas Horvath (2013) followed with his method of determining epigenetic age in multiple tissues. This method provides an algorithm resulting from an elastic net regression analysis that computed population-based regression coefficients carrying the information on to which extend the DNA methylation status of each CpG site of the genome significantly contributed to the prediction of an individual's chronological age. Epigenetic age estimation in study samples is subsequently enabled by measuring the percentage of DNA methylation across multiple tissues at each CpG site of the epigenetic clock. The percentages of DNA methylation for the respective CpG sites are then entered as scores and the population-based weighted regression

coefficients as factors into regression formula to estimate an individual's epigenetic age. The pace of epigenetic ageing is computed by putting the estimated epigenetic age in relation to an individual's chronological age. If an individual's epigenetic age exceeds its chronological age accelerated epigenetic ageing is exhibited. Deceleration of epigenetic ageing is indicated if the epigenetic age is lower than an individual's chronological age.

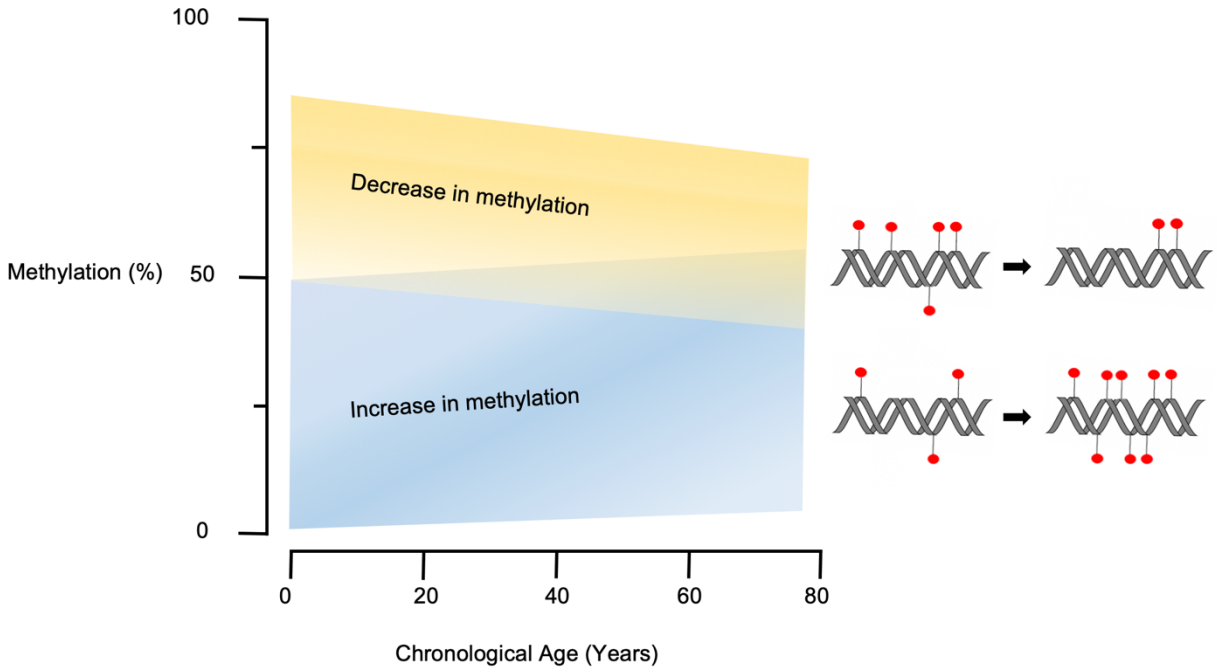

Figure 1. Empirically identified CpG sites of the DNA show either an increase or decrease in DNA methylation with increasing chronological age (Figure modified from Figure 6 in Hannum et al. [2013]).

1.4 Accelerated epigenetic ageing and risk for pathogenesis

Several studies reported associations of accelerated epigenetic ageing with ageing-related morbidity including increased obesity risk, ischemic strokes, diabetes, dementia, cardiovascular disease, and overall mortality (Fransquet et al., 2019; Horvath et al., 2014; Nevalainen et al., 2017; Perna et al., 2016). For instance, using blood samples of over 1800 individuals Perna et al. (2016) found a by 19, 22 and 23% increased risk for cardiovascular-related mortality, cancer-related mortality and all-cause mortality, respectively, per 5 years associated with accelerated epigenetic ageing. Another study using whole blood samples of 86 elderly twins found an over 2-

fold increased mortality risk for the twin with the oldest epigenetic age (Christiansen et al., 2016). As suggested by this finding in twins the influence of non-genetic risk (i.e. environmental) factors may play a crucial role for accelerated epigenetic ageing and associated adverse health outcomes. In fact, accelerated epigenetic ageing has been shown to be 100% heritable in a sample of newborns, but only 39% heritable when determined in on average 63-year-old individuals (Horvath, 2013).

A few studies in children have shown that specific manifestations of ELS had an impact on the pace of epigenetic ageing. Children aged 6 to 16 years that have experienced neighborhood violence, but not children who witnessed neighborhood violence, exhibited accelerated epigenetic ageing as measured in saliva (Jovanovic et al., 2017). Similar results using saliva samples were found by Sumner et al. (2019) showing that threat-related experiences such as violence, but not experiences of deprivation such as physical or emotional neglect predicted accelerated epigenetic ageing in 8 to 16 years old children and adolescents. Further, studies using blood samples found that ELS in terms of financial hardship of households accelerated epigenetic ageing in 7-year-old children (Marini et al., 2020) and early-life socioeconomic disadvantage was associated with accelerated epigenetic ageing later in life (Austin et al., 2018).

Another form of stress exposure that impacts on epigenetic ageing is suffering from psychiatric disorders. Various studies in adults reported associations between accelerated epigenetic ageing as determined across multiple tissues including blood, postmortem brain tissue and buccal cells in adult samples and psychiatric disorders. Important work and evidence was demonstrated for major depression (Han et al., 2018), bipolar disorder (Fries et al., 2017) and posttraumatic stress symptoms including arousal, avoidance and numbing (Shenk et al., 2021; Wolf et al., 2019) accelerating epigenetic ageing. One study found accelerated epigenetic ageing to be associated with internalizing symptoms, but not externalizing symptoms in buccal cells of 148 children (Tollenaar et al., 2021).

However, the range of influence of stress-related risk factors contributing to accelerated epigenetic ageing is still not fully evaluated and the majority of studies examined psychiatric disorders separately from early-life trauma without integrating these factors in one model for epigenetic ageing prediction, although both factors are known to intertwine in the prediction biological alterations following stress exposure (Carpenter et al., 2004; Danese et al., 2008; Heim et al., 2008; Heim et al., 2001).

A study in adults conducted by Han et al. (2018) addressed additive effects of early-life trauma showing that higher self-reported questionnaire scores for early-life trauma severity augmented acceleration of epigenetic ageing in individuals with major depressive disorder (Han et al., 2018).

Previously, studies have demonstrated the existence of biological subtypes of depression as a function of ELS exposure associating with elevated levels of inflammatory parameters such as C-reactive protein (CRP; Danese et al., 2008) or increased concentrations of stress hormones including cortisol in individuals with depression and ELS exposure, but not in individuals with depression without ELS exposure (Carpenter et al., 2004; Heim et al., 2008; Heim et al., 2001; Heim et al., 2000). Such biological subtypes of depression could therefore exist for stress-related accelerated epigenetic ageing and potentially promote the development of ageing-related diseases and premature mortality. Identification of accelerated epigenetic ageing early in life would potentially increase chances for successful implementation of interventions.

1.5 Development of the PedBE clock for epigenetic age estimation in children

All previous studies in children applying epigenetic age estimation relied on epigenetic clocks that were designed and validated in studies with adults and thus hold a systematic methodological uncertainty. Furthermore, using epigenetic clock validated in adult samples neglects that the rate of DNA methylation changes is up to 4-fold higher in children compared to adults (Alisch et al., 2012). Hence, the application of epigenetic clocks validated in pediatric samples is critically needed for valid results.

The PedBE clock (McEwen et al., 2020) uses information on DNA methylation determined in buccal cells in saliva and was developed in a sample of 1032 children and further validated in an independent sample of 689 children both including preschool-aged children (see Figure 2). McEwen et al. (2020) identified 94 CpG sites to be significantly correlated with chronological age in childhood. Fifty CpG sites showed an increase and 44 CpG sites showed a decrease in DNA methylation with increasing chronological age. They further reported higher reliability of the PedBE clock for epigenetic age estimation in children than the commonly used Horvath's clock. The novel PedBE clock thereby enabled enhanced study methods for ageing research in children.

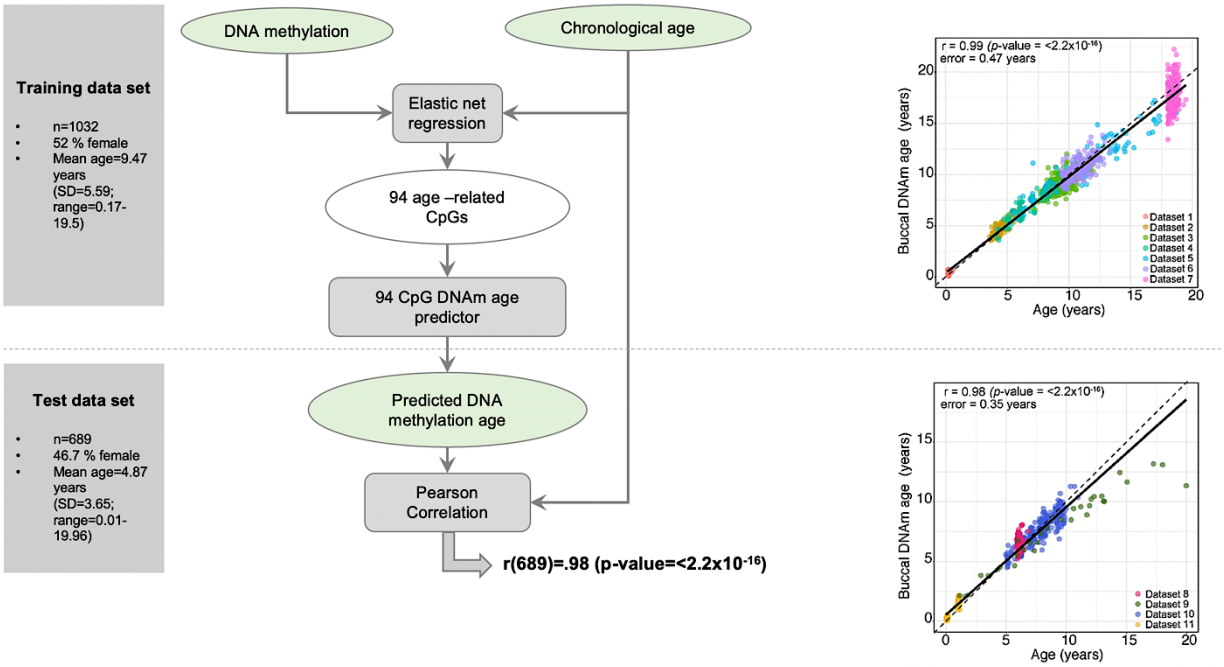

Figure 2. Development and validation of the PedBE clock (Figure modified from Figure 2 in Hannum et al. [2013] and adapted from Figure 1 in McEwen et al. [2020]).

1.6 Study aims

The current study had 5 aims to investigate the relationship between epigenetic ageing and stress in children aged 3 to 5 years. For this purpose,

1) mean epigenetic ageing was compared between children with current and without current internalizing disorder and

2) externalizing disorder was considered as a second main factor and in interaction with internalizing disorder in a further step.

3) Maltreatment exposure was evaluated as moderator in the relationship between epigenetic ageing and internalizing disorder.

4) Biological markers of stress including cortisol and CRP concentrations and their associations with epigenetic ageing were examined within groups of children at differential risk for accelerated epigenetic ageing (i.e., internalizing disorder [yes/no] x maltreatment exposure [yes/no]). Additionally,

5) the set of PedBE clock CpG sites was tested for an enrichment of glucocorticoid-responsive CpG sites in an independent sample and 6) glucocorticoid-responsive CpG sites were lastly compared for differences in DNA methylation between groups of children at differential risk for accelerated epigenetic ageing (internalizing disorder [yes/no] x maltreatment exposure [yes/no]).

2 Methods

The current investigation analyzed cross-sectional data collected from the Berlin Longitudinal Children Study (BerlinLCS) at first study visit (T0). BerlinLCS aimed to examine the immediate biological embedding of maltreatment in children and included an index group of children with verified maltreatment exposure that occurred 6 months prior to the first study visit who were recruited in cooperation with child protection services in Berlin. Study participants from the BerlinLCS control group included children that have not been exposed to any form of maltreatment according to the caretaker. Of all children in the Berlin LCS sample at T0 (N=173) 168 children had complete data for epigenetic age estimation. Due to incomplete data on clinical status in 10 children analyses were carried out in a sample of 158 children. Eighty-five children of the total sample were male, and 73 children were female. Children from the total sample were aged 3 to 5 years and on average 4.25 years old with a standard deviation of 0.8 years.

As outlined in Dammering et al. (2021) chronic medical disease, current medication, neurodevelopmental disorders and disability, psychosis, chronic illness of a caretaker, and parents under the age of 18 years were general exclusion criteria. All procedures adhered to the Declaration of Helsinki and were approved by the ethics committee of Charité − Universitätsmedizin Berlin. Children gave assent and were rewarded with a non-monetary gift for participation while informed consent was obtained from caretakers, who received monetary compensation. Caretakers received psychological and medical reports for their children as well as referrals for psychotherapy or medical treatment, if indicated. Study procedures were conducted at Neurowissenschaftliches Forschungszentrum of the Charité-Universitätsmedizin Berlin (Dammering et al., 2021).

2.1 Operationalization of ELS: Maltreatment Classification

First, each child was screened for signs of physical maltreatment (e.g., bruises, scratches) by a study physician. Maltreatment categories were assessed for each child by applying the Interview for Classification of Maltreatment (Cicchetti et al., 2003; German: Horlich et al., 2014a; Horlich et al., 2014b), a structured interview with the caretaker about the child, and classified with the Maltreatment Classification System

(MCS; Barnett et al., 1993; German: Horlich et al., 2014a; Horlich et al., 2014b). In the current study, 81 children were classified as maltreatment cases.

The complete list of maltreatment categories that were classified in the current study includes sexual maltreatment, emotional and physical maltreatment, physical neglect due to lack of supervision or physical neglect due to failure to provide, educational neglect, and moral-legal neglect. For the current study, a sum score was computed showing the number maltreatment categories each child has been exposed to (Dammering et al., 2021).

2.2 Operationalization of ELS: Psychiatric Assessment

Structured diagnostic interviews were conducted with caretakers to assess psychiatric disorders in children according to DSM-IV. Interview-based raw values were entered into the electronic version of the Preschool Age Psychiatric Assessment (PAPA; Egger & Angold, 2004). The PAPA is tailored for capturing pediatric expression of psychopathology. Internalizing disorders as well as externalizing disorders were coded. Internalizing disorder included dysthymia, major depression, selective mutism, social anxiety disorder, specific phobias, and general anxiety disorder (Dammering et al., 2021). Conduct disorder, attention-deficit-hyperactivity disorder as well as oppositional defiant disorder were coded as externalizing disorders for the current study.

2.3 Biological stress markers: Saliva Samples

Saliva samples constitute a non-invasive alternative for blood samples to measure inflammation and glucocorticoid concentrations in pediatric samples. Several studies have shown that cortisol as well as CRP concentrations in saliva moderately to strongly associate with those in blood plasma (El-Farhan et al., 2017; Ouellet-Morin et al., 2011; Out et al., 2012; Thomasson et al., 2010) and the PedBE clock was validated for buccal samples (McEwen et al., 2020).

Saliva for cortisol and CRP sampling was collected at 9 a.m., 10 a.m. and 11 a.m. using Salimetrics oral swabs suitable for children and were subcooled to −80 °C after sampling. Cortisol concentrations were assessed using a Salimetrics ELISA kit with 0.007 µg/dL sensitivity. Intra-assay variability was 7% and inter-assay variability

was 11%. Cortisol concentrations were reflected by the area under the curve with respect to ground (AUCg).

CRP concentration was measured at 11 a.m. with Salimetrics ELISA kits and with 10 pg/ml sensitivity. Intra-assay variability was 6% and inter-assay variability was 13%. CRP values were log-transformed as assumption of normal distribution was violated and to adjust for outliers.

2.4 Epigenetic age: DNA Methylation in Saliva

[The following section was generated in collaboration with Jade Martins and Elisabeth Binder for Dammering et al. (2021)]: DNA was extracted from saliva samples that were collected with the ORAgene DNA kits (OG500) at 9 a.m. at study visit. PerkinElmer Chemagic360 system was used for a standardized procedure based on magnetic beads to extract DNA from 2 × 400 µl saliva. Methylation status at CpG sites was determined with the Infinium Methylation EPIC BeadChip (Illumina Inc, San Diego, CA, USA). Confounding effects of age, biological sex as well as of maltreatment exposure status were addressed by randomization of samples and hybridization as well as array processing. Normalization of the data was performed with the minfi package (Aryee et al., 2014). Empirical Bayes' method ComBat (Muller et al., 2016) performed with the sva R package (Leek et al., 2012) was applied for identification and removal of batch effects. Further CpG sites located on the X or Y chromosome and probes that were polymorphic or cross-reactive were removed (Chen et al., 2013; Pidsley et al., 2016). Samples with a significance level of detection $p > 0.01$ in over 25% were filtered out. Eventually, 830,206 CpG sites remained after quality check.

2.5 Epigenetic age: Estimation and epigenetic ageing computation

The R code-based algorithm is available online for public use: https://github.com/kobor-lab/Public-Scripts/blob/master/PedBE.Md.

PedBE clock algorithm was applied as described in McEwen et al. (2020). Validity of the PedBE clock algorithm was tested in the current sample, albeit the BerlinLCS sample was specifically recruited for maltreatment vs. no maltreatment exposure and thus not representative for the general population. Pearson's correlation

between epigenetic age, as determined with the PedBE clock, and chronological age yielded a correlation coefficient of r = 0.75 (p < 0.001; see Figure 3).

Regression of epigenetic age against chronological age yielded standardized residuals that were saved as new variable reflecting the deviation between chronological and epigenetic age or in other words epigenetic ageing for each child. Residuals with a value below zero indicated deceleration of epigenetic ageing and residuals with a value above zero indicated acceleration of epigenetic ageing. The current study additionally ran univariate analyses of covariances (ANCOVA) and moderation analysis with unstandardized values for residuals to generate ageing deviation values in months aiding interpretation of results (Dammering et al., 2021).

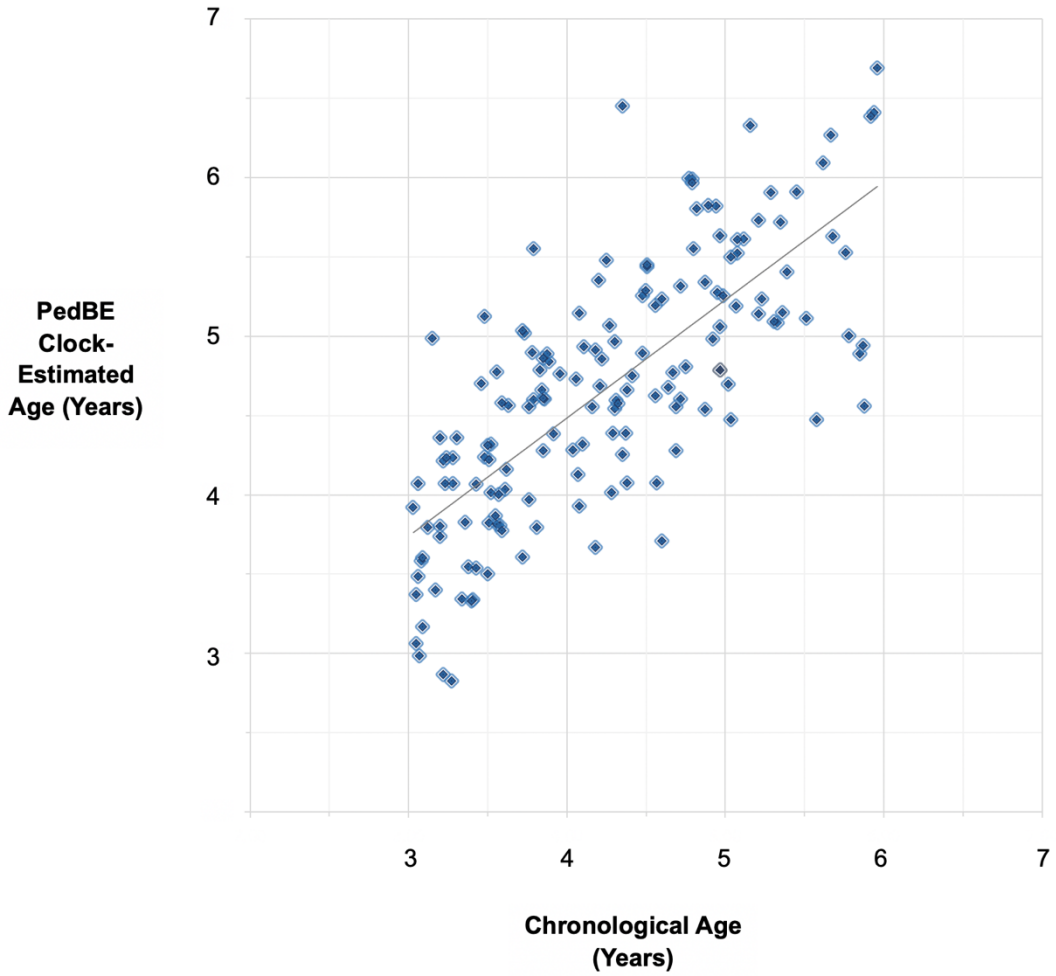

Figure 3. Correlation of PedBE clock-estimated epigenetic age and chronological age (r = 0.75, *p* < 0.001; Figure adapted from Figure 1 in Dammering et al. [2021]).

2.6 Gene set enrichment for glucocorticoid responsiveness of the PedBE clock

[The following section was generated in collaboration with Jade Martins and Elisabeth Binder for Dammering et al. (2021)]: The gene set enrichment analysis in the current study aimed to test whether there are CpG sites within the PedBE clock that show DNA methylation changes after exposure to glucocorticoids and if the number of glucocorticoid responsive CpG sites is higher than would be expected in the gene set. This analysis was performed in an independent sample consisting of 113 adult individuals (Max Planck Institute of Psychiatry [MPIP] cohort). The MPIP cohort is further described in Provencal et al. (2020). In the MPIP cohort, DNA methylation was determined using genome-wide Illumina HumanMethylation450 BeadChips in peripheral blood. Differences in DNA methylation of CpG sites before and three hours after 1.5 mg dexamethasone administration were assessed (FDR-corrected *p* value of 0.10) identifying 23,031 CpG sites with differential methylation after treatment (Provencal et al., 2020).

2.7 Statistical analysis

2.7.1 Epigenetic ageing and ELS

As described in Dammering et al. (2021) mean epigenetic ageing was compared between children with and without internalizing disorder by applying univariate ANCOVA to adjust comparison for covariates. Subsequently, externalizing disorder was included into the model to test for externalizing disorder effects and potential interaction effects of internalizing disorder with externalizing disorder on epigenetic ageing.

Whether maltreatment exposure contributes to the relationship between epigenetic ageing and psychopathology was tested with a moderation model using the PROCESS macro (Hayes, 2017) where the number of maltreatment categories was the moderator and internalizing disorder the predictor of epigenetic ageing. All variables were mean centered for the ordinary least square (OLS) regression in the moderation analysis. This step aided the interpretation of regression coefficients. The moderation model yielded an interaction term that allowed for subsequent simple slope analysis of the moderation effect (Hayes & Rockwood, 2017). The three levels of the moderator

were used to probe the interaction yielding simple slopes for no maltreatment exposure (value = 0), for one to two experienced maltreatment categories (value = 1), and for three or more experienced maltreatment categories (value = 2). To probe the interaction values of the ageing residuals ($\hat{Y}$) at different levels of the interaction (XW) of internalizing disorder (X) and maltreatment severity (W) were computed. Values for X and W were obtained from variable coding (X = 0, X = 1 and W = 0, W = 1, W = 2) while the values of covariates (U_i) are set to their sample means. Representation of the weighted (b_i) effect of X on $\hat{Y}$ as a linear function of the current total model is:

$$\hat{Y} = b_0 + b_1X + b_2W + b_3XW + b_4U_1 + b_5U_2 + b_6U_3 + b_7U_4 + b_8U_5 + b_9U_6 + b_{10}U_7 + b_{11}U_8 + b_{12}U_9$$

The Davidson-MacKinnon heteroscedasticity-consistent standard error estimator was applied for OLS regression to augment validity and power of the model (Davidson & MacKinnon, 1993; Hayes & Cai, 2007).

To examine correlations between epigenetic ageing and stress-related biological markers including cortisol and CRP Pearson's correlational analyses were carried out across all children with complete biological data as well as separately in four groups of children stratified by current internalizing disorder (Yes/No) and maltreatment (Yes/No) status. Total sample sizes differed for cortisol (n = 136) and CRP (n = 147) due to cases with incomplete biological data and 9 cases had to be excluded due to current infection at study visit T0.

2.7.2 Covariates

Factors that have been shown to influence epigenetic ageing were included as covariates in statistical analyses including caregiver-reported sex, body mass index (BMI), and socioeconomic status (SES) as well as cell type composition, and genetic ethnicity (Austin et al., 2018; Horvath et al., 2016; Quach et al., 2017). During the study visit children received a standard medical examination by a study physician to determine health status and BMI. Further, self-reported sex was captured. Values between 1 and 7 for educational level, occupational status, and net income, respectively, were used to calculate a sum score (range 3 to 21) reflecting

SES (Lange et al., 2007). Genetic ethnicity was determined by examining the linkage disequilibrium with plink v1.9 (Chang et al., 2015) to subsequently compute principal component analysis yielding three principal components that reflected genetic ethnicity for each child. Accordingly, these three principal components were included into the analyses as three covariates. To determine cell type composition including buccal cells, CD14, CD34 in saliva the current study applied the deconvolution method (Smith et al., 2015).
SPSS 25.0 for Windows was applied to carry out statistical analyses with an alpha level of significance at $p < 0.05$ (Dammering et al., 2021).

2.7.3 Gene set enrichment analysis with dexamethasone within the PedBE clock

[The following section was generated in collaboration with Jade Martins and Elisabeth Binder for Dammering et al. (2021)]: Enriched glucocorticoid responsive CpG sites within the PedBE clock were assessed by administering dexamethasone and analyzed with Fisher's Exact test. Measurement was performed with the Illumina HumanMethylation450 BeadChip as background. In a last step, mean DNA methylation was compared between four groups of children distributed by current internalizing disorder (Yes/No) and maltreatment (Yes/No) status in all CpG sites that showed significant DNA methylation changes after dexamethasone administration. This last step was performed by applying univariate analysis of variances (Dammering et al., 2021).

3. Results

Descriptive statistics for characterization of the total sample are displayed in Table 1. Additionally, Table 1 contains separate characteristics for children with and without internalizing disorder with t-test comparisons. Chronological age, self-reported ethnicity, sex and BMI (all $p > 0.05$) were not significantly different between both groups. However, they differed significantly in SES with on average lower scores in in children with internalizing disorder ($p < 0.001$).

Chi-squared test revealed that there was a relatively higher number of maltreatment cases in children with internalizing disorder (χ^2 [2, N = 158] = 11.96, $p = 0.003$) indicating maltreatment exposure is associated with internalizing disorder (Dammering et al., 2021).

Table 1. *Statistics and sample characteristics* (Table adapted from Table 1 in Dammering et al. [2021]).

	Total (n=158)	**Children without internalizing disorder (n=109)**	**Children with internalizing disorder (n=49)**	***p***
Chronological age, years	4.25 (0.80)	4.27 (0.79)	4.18 (0.85)	0.507
Female sex	73 (46.20)	52 (47.70)	21 (42.90)	0.572
Self-reported ethnicity				0.296
White-Asian	3 (1.90)	3 (2.80)	0 (0)	
White-Black	11 (6.96)	6 (5.50)	5 (10.20)	
White	144 (91.14)	100 (91.70)	44 (89.80)	
Number of maltreatment categories				0.003
0	77 (48.70)	63 (57.80)	14 (28.60)	
1-2	55 (34.80)	30 (27.50)	25 (51.00)	
≥3	26 (16.50)	16 (14.70)	10 (20.40)	
SES	12.67 (5.21)	13.65 (5.00)	10.49 (5.03)	< 0.001
BMI	15.48 (1.16)	15.42 (1.07)	15.61 (1.35)	0.326

Note. Values are presented as mean (SD) or n (%).

3.1 Epigenetic ageing and psychopathology

The ANCOVA revealed a significant main effect of current internalizing disorder on epigenetic ageing indicating acceleration of epigenetic ageing (M = 0.29, SE = 0.13) in relation to children without internalizing disorder (M = −0.13, SE = 0.87, $F_{1,147} = 6.67$, $p = 0.011$; see Figure 4). The identical analysis with unstandardized residuals for epigenetic ageing showed that these results reflect an on average 1.87 months acceleration of epigenetic ageing in relation to chronological ageing. In the absence of current internalizing disorder children exhibited 0.84 months deceleration of epigenetic ageing relative to chronological ageing. Thus, the deviation in epigenetic ageing between both groups was 2.71 months (Dammering et al., 2021).

For the current study, a second ANCOVA model, where the role of externalizing disorder was examined, epigenetic ageing was the criterion and internalizing disorder as well as externalizing disorder were included as factors. Internalizing disorder as main factor remained significant ($F_{1,145} = 4.07$, $p = 0.045$) while neither the main effect of externalizing disorder ($F_{1,145} = 1.75$, $p = 0.189$) nor the interaction effect of externalizing disorder by internalizing disorder reached statistical significance ($F_{1,145} = 0.39$, $p = 0.533$).

Noteworthy, sex had a significant effect on epigenetic ageing in both ANCOVA models reported above ($F_{1,147} = 6.62$, $p = 0.011$ and $F_{1,145} = 7.07$, $p = 0.009$) indicating the female sex was associated with faster epigenetic ageing in the current sample of children. Hence, sex was entered as a second main factor in addition to the internalizing disorder factor within an ANCOVA model. Internalizing disorder ($F_{1,146} = 6.80$, $p = 0.010$) and sex ($F_{1,146} = 6.36$, $p = 0.013$) reached statistical significance as main effect, but the interaction effect of both was not significant ($F_{1,146} = 2.21$, $p = 0.646$). Hence, accelerated epigenetic ageing in children with internalizing disorder appeared to be independent from sex (Dammering et al., 2021).

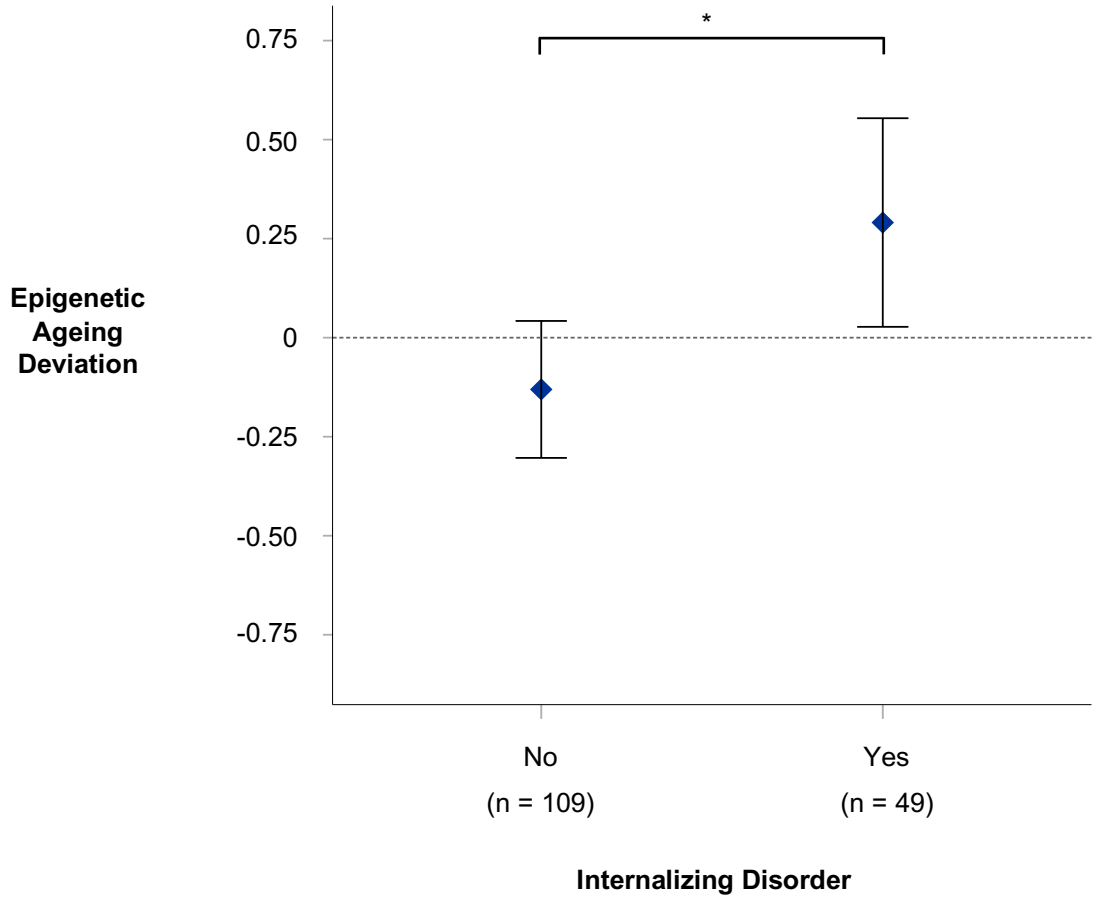

Figure 4. Comparison of mean epigenetic ageing in children with and without internalizing disorder (Means and 95% CI error bars). Adjusted for cell composition, sex, genetic ethnicity, BMI, and SES. * $p < 0.05$ (Figure adapted from Figure 2 in Dammering et al. [2021])

3.2 Epigenetic ageing, psychopathology, and maltreatment

To further elaborate on the relationship between accelerated epigenetic ageing and ELS, a moderation model was applied to test for a potential contribution of maltreatment exposure within the association between epigenetic ageing and internalizing disorder. Severity of maltreatment, operationalized by the number of experienced maltreatment categories, was included as moderator variable into the moderation model where internalizing disorder predicted epigenetic ageing (see Table 2).

Table 2. *Linear model of predictors of epigenetic ageing* (Table adapted from Table 2 in Dammering et al. [2021]).

	b	SE	t	*p*
Constant	-14.82	6.74	-2.20	0.030
Current Internalizing Disorders	0.37	0.16	2.15	0.033
Number of Maltreatment Categories	0.18	0.15	1.23	0.222
Current Internalizing Disorders * Number of Maltreatment Categories	0.49	0.21	2.32	0.022

Note. Model was adjusted for cell type composition (buccal, CD14, CD34), sex, genetic ethnicity, BMI, and SES (Dammering et al., 2021).

The moderation model explained 27.5% of the variance in epigenetic ageing in the current sample (R^2 = .275, $F_{12,145}$ = 4.797, p < 0.001).

OLS regression analysis detected internalizing disorder significantly interact with the number of maltreatment categories in the prediction of epigenetic ageing (b = 0.49, 95% CI [0.073, 0.909], t = 2.322, p = 0.022) indicating that maltreatment severity contributes to accelerated epigenetic ageing in internalizing disorder. As expected, internalizing disorder significantly predicted accelerated epigenetic ageing (b = 0.34, 95% CI [0.027, 0.647], t = 2.149, p = 0.033). Intriguingly, the number of maltreatment categories had no significant effect on epigenetic ageing (b = 0.18, 95% CI [-0.110, 0.471], t = 1.228, p = 0.222). This means that internalizing disorder predicted acceleration of epigenetic ageing depending on the severity of maltreatment exposure and maltreatment exposure alone did not accelerate epigenetic ageing. Again, the covariate sex reached statistical significance in this model (b = −0.32, 95% CI [-0.628, −0.016], t = −2.083, p = 0.039) suggesting female sex predicted acceleration of epigenetic ageing. The moderating effect of maltreatment severity was unaffected by sex.

Simple slope analysis revealed that the interaction is characterized by maltreatment severity facilitating and gradually increasing the effect of internalizing disorder on epigenetic ageing (see Figure 3). On the one hand, in the absence of maltreatment exposure internalizing disorder did not predict accelerated epigenetic ageing and, on the other hand, in the absence current internalizing disorder

maltreatment exposure had no effect on epigenetic ageing (n = 77, b = 0.00, 95% CI [-0.436, 0.444], t = 0.019, *p* = 0.985). Instead, one or two experienced maltreatment categories showed an average of 3.19 months acceleration of epigenetic ageing in relation to chronological ageing in interaction with current internalizing disorder (n = 55, b = 0.50, 95% CI [0.170, 0.821], t = 3.008, *p* = 0.003). Furthermore, children exhibited on average 6.36 months acceleration of epigenetic ageing in relation to chronological ageing, if they had current internalizing disorder and have been exposed to three or more maltreatment categories (n = 26, b = 0.99, 95% CI [0.380, 1.593], t = 3.215, *p* = 0.002). Results mentioned above are also reported in Dammering et al. (2021).

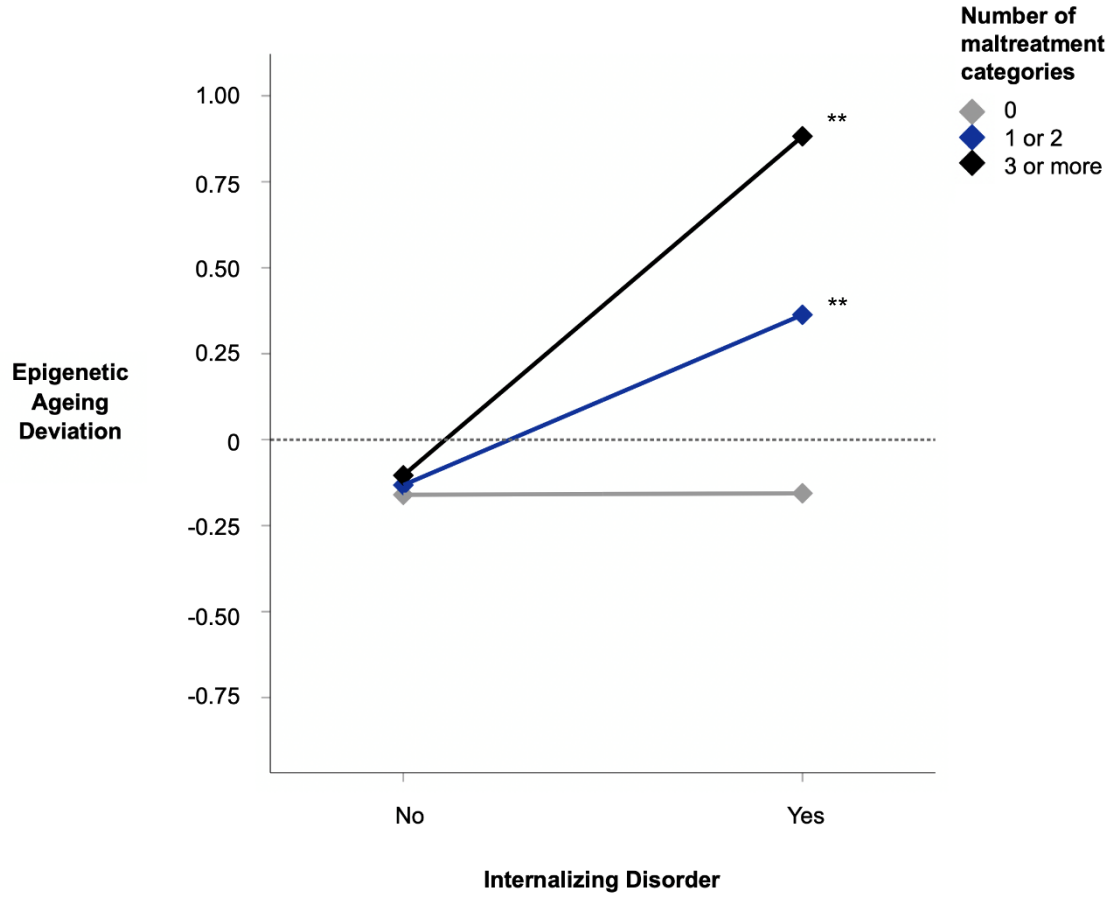

Figure 5. Simple slope equations of interaction effect on epigenetic ageing. Adjusted for cell type composition, sex, genetic ethnicity, BMI, and SES. ** $p < 0.01$ (Figure adapted from Figure 3 in Dammering et al. [2021]).

3.3 Epigenetic ageing, Cortisol and CRP

Following up on the finding that accelerated epigenetic ageing was associated with internalizing disorder in dependence of maltreatment, the current study aimed to examine, whether stress-related biomarkers, i.e., cortisol and CRP, are correlated with epigenetic ageing across all children and in four groups of children at differential risk for accelerated epigenetic ageing (internalizing disorder [Yes/No] by maltreatment exposure [Yes/No]). Bivariate Pearson's correlation revealed that across all children epigenetic ageing was significantly associated with cortisol AUCg (r [136] = 0.20, p = 0.022), but not with CRP (r [147] = 0.09, p = 0.274). However, correlational analysis in separate groups showed that the overall correlation between epigenetic ageing and cortisol AUCg was likely driven by significant and higher correlations in children with both internalizing disorder and maltreatment exposure. In this group, accelerated epigenetic ageing was significantly associated with higher cortisol levels (r [32] = 0.36, p = 0.043) as well as with higher CRP levels (r [32] = 0.42, p = 0.016). Correlation coefficients neither reached statistical significance in non-maltreated children with internalizing disorder nor in children without internalizing disorder (Table 3).

Table 3. *Correlations between epigenetic ageing and biological stress markers.*

			Pearson's correlation with Epigenetic Ageing		*p*
Total sample with complete biological data		Cortisol AUCg (n=136)	0.20	*	0.022
		CRP (n=147)	0.09		0.274
Children without Internalizing Disorder	Non-Maltreated	Cortisol AUCg (n=51)	-0.04		0.788
		CRP (n=59)	-0.21		0.107
	Maltreated	Cortisol AUCg (n=41)	0.19		0.237
		CRP (n=44)	0.19		0.216
Children with Internalizing Disorder	Non-Maltreated	Cortisol AUCg (n=12)	0.33		0.293
		CRP (n=12)	-0.26		0.406
	Maltreated	Cortisol AUCg (n=32)	0.36	*	0.043
		CRP (n=32)	0.42	*	0.016

Note. * p < 0.05.

Noteworthy, neither mean cortisol levels ($F_{1,123}$ = 1.14, p = 0.288) nor mean CRP levels ($F_{1,134}$ = 0.02, p = 0.887) differed between the four groups. This means that acceleration epigenetic ageing in maltreated children with internalizing disorder was not explained by generally higher mean cortisol levels or mean CRP levels in this group.

3.4 Enriched dexamethasone responsive CpG sites within the PedBE clock

In a last step, the current study tested for an enrichment of CpG sites within the PedBE clock that show DNA methylation changes after administration of the artificial glucocorticoid dexamethasone. This analysis was carried out in the MPIP cohort, and independent sample of adults (Provencal et al., 2020). There was a significant higher number of CpG sites responsive to dexamethasone as would be expected in relation to the frequency of the total list of CpG sites (Fisher's Exact: $p = 1.65*10^{-6}$, Odds Ratio = 4.36). In detail, enrichment was reflected by 18 out of 94 CpG sites (19%) of the PedBE clock that were differently methylated following dexamethasone exposure. Additionally, there were individual differential methylation levels after adjustment for cell proportions (CD14, C34 and buccal cells) at the CpG site cg16618789 ($F_{1,150}$ = 5.76, p = 0.018) and at the CpG site cg03493146 ($F_{1,150}$ = 6.03, p = 0.015) for children with internalizing disorder and maltreatment exposure (adjusted results from Dammering et al., 2021).

4. Discussion

The current study is the first study that applied the PedBE clock in a clinical sample of children with maltreatment exposure and firstly tested the recently developed PedBE clock for glucocorticoid responsiveness with dexamethasone exposure. This study further expanded research on the relationship between epigenetic ageing and stress in preschool-aged children and integrated environmental and behavioral measures of stress in one prediction model for epigenetic ageing. In addition, this study investigated biological stress markers in relation to epigenetic ageing in groups of children that were observed to be at differential risk for accelerated epigenetic ageing.

4.1 Summary of results and embedding into current state of research

The current investigation demonstrated that internalizing disorder in children can accelerate epigenetic ageing. It further showed that the association between epigenetic ageing and psychopathology was specifically found for internalizing disorder as neither externalizing disorder alone nor in interaction with internalizing disorder was associated with epigenetic ageing in the current study sample. Thereby, these results in children aged 3 to 5 years replicated previous findings in older children aged 6 to 10 years demonstrating that accelerated epigenetic ageing was related to internalizing symptoms, but not externalizing symptoms (Tollenaar et al., 2021). Replication of findings by Tollenaar et al. (2021) was important as in their study epigenetic age in children was estimated with Horvath's epigenetic clock that was designed for adult samples. Further, the current finding is in line with study findings in adults identifying accelerated epigenetic ageing in individuals with depressive and posttraumatic stress symptoms (Fries et al., 2017; Han et al., 2018; Shenk et al., 2021; Wolf et al., 2019), adult psychopathological equivalents of pediatric internalizing disorder.

Maltreatment exposure was found to be a critical moderator in the relationship between epigenetic ageing and internalizing disorder. Depending on maltreatment status of children internalizing disorder had either no effect on epigenetic ageing or was gradually accelerating epigenetic ageing. Han et al. (2018) reported a comparable finding in adults aged on average 41 years showing that childhood trauma augmented epigenetic ageing acceleration in individuals with major depressive disorder. However, childhood trauma was assessed with retrospective questionnaire scores and data on

childhood trauma in individuals without current major depressive disorder was missing making it difficult to disentangle effects of psychopathology and ELS exposure on epigenetic ageing. By including children with maltreatment exposure, but without internalizing disorder, the current study could generate an interaction term with internalizing disorder and maltreatment exposure. The significant interaction demonstrated the existence of distinct biological properties evolving in children who have been exposed to maltreatment and suffer from internalizing psychopathology. Biological subtypes of depression as a function of ELS such as child maltreatment have also been evidenced in previous studies reporting increased cortisol concentrations as well as elevated inflammation levels in individuals with both a history of maltreatment and current depression (Carpenter et al., 2004; Danese et al., 2008; Heim et al., 2008; Heim et al., 2001).

Moreover, at this young age of on average 4.25-year-old children in the current study the number of months of age acceleration is considerably high. Han et al. (2018) showed that adult patients with major depression were on average 7.68 months accelerated in epigenetic ageing reflecting an acceleration of 1.54% compared to healthy controls and based on the average age of 41.5 years of the total sample. The ageing deviation in the current study between children with internalizing disorder and children without internalizing disorder was 2.71 months corresponding to a 5.31% acceleration based on their average age of 4.25 years. The maximum of epigenetic ageing acceleration found in the current study (6.36 months) equals an acceleration of 12.47% based on the average age of the total sample.

Girls were overall epigenetically older than boys in all statistical models. The literature on sex differences regarding epigenetic ageing is divergent and not fully fathomed (Horvath et al., 2016; Simpkin et al., 2016; Tang et al., 2020). Intriguing findings are reported by Yusipov et al. (2020) showing age-by-sex-dependent DNA methylation in adults. For instance, one CpG site was hypomethylated in males compared to females and exhibited hypomethylation with increasing age, while another CpG site was hypomethylated in males compared to females and exhibited hypermethylation with increasing age (Yusipov et al., 2020). However, stress-related accelerated epigenetic ageing as examined in the current study was independent from sex and chronological age effects.

Epigenetic ageing correlated with cortisol across all children with complete biological data. Intriguingly, when calculated in separate groups both higher cortisol and

higher CRP concentrations were associated with acceleration of epigenetic ageing exclusively in children with internalizing disorder and maltreatment exposure, but neither in non-maltreated children with internalizing disorder nor in children without internalizing disorder. Previous studies reported mixed findings showing significant and null associations between glucocorticoids or inflammation and epigenetic ageing. For instance, diurnal cortisol concentration was associated with accelerated epigenetic ageing in 46 adolescent girls (Davis et al., 2017) while a study in 974 adolescent individuals found no association between cortisol and epigenetic ageing (Tang et al., 2020). Inconsistent study results have also been reported for CRP and epigenetic ageing (Irvin et al., 2018; Quach et al., 2017; Stevenson et al., 2018).
In this regard, an important additional finding of the current study demonstrated direct evidence that administration of glucocorticoids in an independent sample led to DNA methylation changes at CpG sites that are included in the PedBE clock. Highly significant enrichment of glucocorticoid responsive CpG sites suggested that pediatric epigenetic ageing is susceptible for glucocorticoids. Moreover, 2 out of 18 dexamethasone responsive CpG sites of the PedBE exhibited different DNA methylation in children with internalizing disorder and maltreatment exposure.

4.2 Interpretation of results

For the current finding that particularly internalizing disorder, but not externalizing disorder, associates with accelerated epigenetic ageing a psychodynamic perspective on results was considered to aid interpretation. Internalizing psychopathology is characterized to be directed towards an individual's 'inside' (i.e., psychological self-punishment, feelings of guilt), while externalizing behavior is directed towards an individual's outside environment reflecting behaviors such as an outburst of anger or physically harmful behavior. It is conceivable that externalizing psychopathology could therefore be used as coping mechanism to relieve anger and fear, or in other words, functions as release for internal stress. However, externalizing behavior often involves psychodynamic defense mechanisms at the expense of others.

Biological alterations as observed in the current study in the form of accelerated epigenetic ageing occurred as prediction by the interaction of maltreatment exposure and concomitant internalizing disorder. Maltreatment exposure means acute or chronic

stress exposure that may have sensitized biological systems potentially leading to reduced stress resilience in children that exhibited accelerated epigenetic ageing. In other words, experiencing maltreatment could have demolished the protective shield in respective children making it easier to exert influence on what is behind the shield. This perspective is supported by the current findings that only in the group of maltreated children with internalizing disorder increasing cortisol levels as well as increasing CRP levels were associated with accelerating epigenetic ageing. Moreover, cortisol and CRP concentrations did not differ between the four groups of children suggesting that acceleration of epigenetic ageing in maltreated children with internalizing disorder was not explained by generally elevated cortisol and CRP concentrations.

However, as the current investigation had a cross-sectional and not a longitudinal study design, an alternative explanation for the observed interaction could be that children with accelerated epigenetic ageing carried a double burden of experiencing maltreatment and suffering from internalizing disorder resulting in excessive stress opening the door for increased biological susceptibility.

Albeit it is conceivable that increasing cortisol and CRP levels in children with maltreatment exposure and internalizing disorder contributed to accelerated epigenetic ageing, little is known about key mechanisms linking stress biology and epigenetic ageing. With respect to glucocorticoids it has been proposed that cortisol acts on epigenetic aging through activated glucocorticoid receptors entailing intracellular processes involving downregulation of DNA methyltransferase 1 (DNMT1) – an enzyme that catalyzes methylation of CpG sites of genomic DNA, as well as upregulation of FK506 – binding protein 51 levels that, in turn, further inhibits DNMT1 activity eventually leading to DNA methylation changes and advanced epigenetic ageing (Gassen et al., 2017). However, other physiological processes involved in cellular stress responses could explain accelerated epigenetic ageing additively.

[The following conclusion was developed in collaboration with Sonja Entringer and Christine Heim for Dammering et al. (2021)]: The current finding that girls were generally epigenetically older than boys is difficult to interpret as previous findings in animals reported that sex differences in epigenetic ageing may in part be mediated by sex hormones (Sugrue et al., 2021). In the current study, however, children were pre-pubertal and circulating concentrations of sex hormones are very low. A recent study in the BerlinLCS sample found that particularly maltreated girls, but not boys or non-

maltreated girls showed increased levels of CRP (Entringer et al., 2020). Increased inflammation in girls may have contributed to the observed sex difference, although maltreatment in interaction with sex did not predict epigenetic ageing in the current study.

[The following conclusion was developed in collaboration with Christine Heim for Dammering et al. (2021)]: In general, a faster pace of an individual's epigenetic clock during childhood could imply faster maturation following early adversity and threat experiences to evoke an earlier onset of puberty ensuring reproduction and survival in an unsafe environment (Ellis & Del Giudice, 2019). Rapid developmental pace may be beneficial for survival, but may also hamper qualitative aspects of maturation potentially leading to 'maturation gaps' and thereby promote increased morbidity later in life (Belsky et al., 2015)

4.3 Outlook

Future research on epigenetic ageing in children as well as in adults may benefit from considering differential vulnerabilities for adverse effects following stress exposure. For instance, stress-related biological correlates of epigenetic ageing should be examined by considering various forms of ELS such as maltreatment and pediatric psychopathology. On that note, there is a need for longitudinal observations of children at increased risk for accelerated epigenetic ageing.

To further elucidate sex differences in epigenetic ageing before puberty, it may be of interest to examine pre-pubertal sex steroid concentrations. For that, studies should apply sensitive methods to measure low concentrations of circulating estrogenic and androgenic hormones as, for instance, outlined by Courant et al. (2010).

On the flipside, it is important to examine factors that can buffer epigenetic age acceleration to not only identify indicators for increased risk but for increased resilience as well. In fact, a small number of studies found factors that were associated with deceleration of epigenetic ageing. For instance, nutritional factors such as fish intake or higher education levels were associated with decelerated epigenetic ageing in adults (Quach et al., 2017). In view of ELS, decelerated epigenetic ageing was found in juveniles receiving family support services after racial discrimination compared to racially discriminated juveniles without help (Brody et al., 2016). Further, a study in

meditators showed that increasing years of meditation practice associated with a slower pace of the epigenetic clock (Chaix et al., 2017). Researchers should build up on these promising findings of ageing decelerating effects by including psychotherapeutic interventions in future studies and progressively contrast epigenetic ageing in individuals receiving psychotherapeutic treatment with epigenetic ageing in individuals who do not receive psychotherapy.

4.4 Strengths and weaknesses of the study

This is the first study applying the PedBE clock for epigenetic age estimation in a clinical sample of children strengthening reliability and validity of the current study results. Another strength is the in-depth assessment of psychopathology and maltreatment experiences in children aged 3 to 5 years. Psychiatric disorders were assessed with profound clinician-administered diagnostic interviews suitable for expression of psychopathology in preschool-aged children. Due to recruitment strategies maltreatment exposure was highly prevalent in the current sample facilitating stratified analyses as well as examination of biological correlates of epigenetic ageing of different risk groups.

Factors limiting the validity of the current study results include the relatively small sample size and the cross-sectional design. Thus, mediation models including longitudinal study designs that would have made causal conclusions possible were not applicable in the current study. With regard on the correlation between epigenetic ageing and cortisol as well as with CRP, the current findings must be interpreted with caution as this analysis was carried out in subsamples with low sample sizes. Longitudinal multi-modal study approaches with bigger sample sizes are needed to tackle these limitations.

5. Conclusions

In conclusion, the current study supported and extended previous work of researchers demonstrating that there is a subtype of psychiatric disorders that manifests in relation to severe stress exposure such as maltreatment and has distinct biological features. Importantly, this biological subtype, as reflected by accelerated epigenetic ageing, is identifiable as early as at the age of 3 to 5 years. Furthermore, stress exposure appears to have a greater impact on epigenetic ageing in preschool-aged children than in adults as outlined in section 4.1.

Additionally, the current findings consolidated the association of epigenetic ageing to environmental, behavioral, and biological stress. Accelerated epigenetic ageing may therefore depict a relevant indicator for stress exposure and disease risk and may as well function as measure of success for stress-targeting treatment such as psychotherapy.

The article “The battered child syndrome” (Kempe et al., 1962) first sparked research on the somatic and psychiatric consequences of child maltreatment.
Child maltreatment is a highly prevalent symptom of our society. However, detection of cases is difficult as children rarely attempt to gain a hearing due to feelings of loyalty towards caretakers or feelings of shame.
In their article, Kempe et al. (1962) postulated that there is

> *“a duty and responsibility to the child to require a full evaluation of the problem and to guarantee that no expected repetition of trauma will be permitted to occur.”* (Kempe et al., 1962).

The current study aimed to do so while underscoring the need for targeted health care interventions that prevent disease associated with stress as well as promote health throughout life eventually leading to healthy ageing.

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Statutory Declaration

“I, Felix Dammering, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic Early-Life Stress and Epigenetic Ageing in Preschool-Aged Children (German: Lebensgeschichtlich Frühe Stresserfahrungen und Epigenetische Alterung bei Kindern im Vorschulalter) independently and without the support of third parties, and that I used no other sources and aids than those stated.All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (regarding practical work, laboratory regulations, statistical processing) and results (regarding figures, charts, and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

Declaration of your own contribution to the publications

I conducted diagnostic tests in the BerlinLCS study that provided the data for the current dissertation project. I collected saliva samples from children and preprocessed biological samples in the laboratory. Cortisol and CRP levels were determined by the lab technician, Heiko Klawitter, and by Dr. Karin de Punder. I further curated and processed the data used for the current study and calculated epigenetic ageing scores for each child. I performed statistical analyses including t-tests, chi-squared tests, regression models, univariate analyses of covariance, moderation and simple slope analyses as well as correlations analyses. I wrote the dissertation and marked the text that was originally generated in collaboration with Dr. Christine Heim and Dr. Sonja Entringer as well as with Jade Martins and Dr. Elisabeth Binder for the published article. Jade Martins, Dr. Elisabeth Binder and other colleagues from the Max Planck Institute of Psychiatry assessed DNA methylation status and ran the DNA methylation-based PedBE clock algorithm for each child as well as the gene set enrichment analysis. Regarding the published article, I wrote the first draft, and edited and reviewed the manuscript, in collaboration with Dr. Christine Heim. Jade Martins and Dr. Elisabeth Binder contributed to the manuscript in Section 2.2 as well as in Section 2.5. They further provided the results of the gene set enrichment analysis. Except for the latter, all inference statistical analyses and descriptive statistics were carried out by me. I generated all visualizations including Figures 1, 2, and 3 as well as Tables 1 and 2 in the published article. The current study includes additional analyses compared to the published article including correlational analyses as well as analyses of variance assessing the effects of externalizing disorder as factor and cortisol and CRP levels as criteria, all of which were carried out by me.

Felix Dammering contributed the following to the below listed publication:

Dammering, F., Martins, J., Dittrich, K., Czamara, D., Rex-Haffner, M., Overfeld, J., de Punder, K., Buss, C., Entringer, S., Winter, S.M., Binder, E.B., Heim, C. (2021). The Pediatric Buccal Epigenetic Clock Identifies Significant Ageing Acceleration in Children with Internalizing Disorder and Maltreatment Exposure. Neurobiology of Stress, 15.

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Signature, date, and stamp of first supervising university professor / lecturer

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Signature of doctoral candidate

Excerpt from Journal Summary List

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3	BEHAVIORAL AND BRAIN SCIENCES	9,395	17.333	0.008170
4	TRENDS IN COGNITIVE SCIENCES	27,705	15.218	0.036050
5	JOURNAL OF PINEAL RESEARCH	10,537	14.528	0.009430
6	NEURON	95,056	14.415	0.199640
7	ACTA NEUROPATHOLOGICA	21,908	14.251	0.040740
8	TRENDS IN NEUROSCIENCES	20,011	12.891	0.021220
9	Annual Review of Neuroscience	13,215	12.547	0.012740
10	MOLECULAR PSYCHIATRY	22,227	12.384	0.054730
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17	FRONTIERS IN NEUROENDOCRINOLOGY	4,491	9.059	0.007050
18	ANNALS OF NEUROLOGY	37,304	9.037	0.044120
19	NEUROSCIENCE AND BIOBEHAVIORAL REVIEWS	28,873	8.330	0.051900
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21	NEUROPATHOLOGY AND APPLIED NEUROBIOLOGY	3,992	7.500	0.005960

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
22	Neurobiology of Stress	1,055	7.197	0.003840
23	NEUROPSYCHOPHARMACOLOGY	26,281	6.751	0.040680
24	npj Parkinsons Disease	662	6.750	0.002500
25	BRAIN BEHAVIOR AND IMMUNITY	16,285	6.633	0.028560
26	Brain Stimulation	6,537	6.565	0.015580
27	NEUROSCIENTIST	5,188	6.500	0.007220
28	Acta Neuropathologica Communications	4,070	6.270	0.014730
29	CURRENT OPINION IN NEUROBIOLOGY	14,959	6.267	0.028730
30	Alzheimers Research & Therapy	3,876	6.116	0.011650
31	Neurotherapeutics	4,998	6.035	0.009520
32	GLIA	14,220	5.984	0.017250
33	NEUROIMAGE	102,632	5.902	0.125360
34	Annual Review of Vision Science	601	5.897	0.003700
35	Molecular Autism	2,510	5.869	0.007450
36	Journal of Neuroinflammation	13,709	5.793	0.025870
37	Translational Stroke Research	2,274	5.780	0.004520
38	JOURNAL OF CEREBRAL BLOOD FLOW AND METABOLISM	19,492	5.681	0.024230
39	JOURNAL OF NEUROSCIENCE	167,114	5.673	0.181170
40	BRAIN PATHOLOGY	5,308	5.568	0.007020
41	Translational Neurodegeneration	1,030	5.551	0.002790
42	NEURAL NETWORKS	14,065	5.535	0.018910
43	PAIN	37,753	5.483	0.035730

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The pediatric buccal epigenetic clock identifies significant ageing acceleration in children with internalizing disorder and maltreatment exposure

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ABSTRACT

Background: Studies reporting accelerated ageing in children with affective disorders or maltreatment exposure have relied on algorithms for estimating epigenetic age derived from adult samples. These algorithms have limited validity for epigenetic age estimation during early development. We here use a pediatric buccal epigenetic (PedBE) clock to predict DNA methylation-based ageing deviation in children with and without internalizing disorder and assess the moderating effect of maltreatment exposure. We further conduct a gene set enrichment analysis to assess the contribution of glucocorticoid signaling to PedBE clock-based results.
Method: DNA was isolated from saliva of 158 children [73 girls, 85 boys; mean age (SD) = 4.25 (0.8) years] including children with internalizing disorder and maltreatment exposure. Epigenetic age was estimated based on DNA methylation across 94 CpGs of the PedBE clock. Residuals of epigenetic age regressed against chronological age were contrasted between children with and without internalizing disorder. Maltreatment was coded in 3 severity levels and entered in a moderation model. Genome-wide dexamethasone-responsive CpGs were derived from an independent sample and enrichment of these CpGs within the PedBE clock was identified.
Results: Children with internalizing disorder exhibited significant acceleration of epigenetic ageing as compared to children without internalizing disorder ($F_{1,147} = 6.67$, $p = .011$). This association was significantly moderated by maltreatment severity (b = 0.49, 95% CI [0.073, 0.909], t = 2.322, $p = .022$). Children with internalizing disorder who had experienced maltreatment exhibited ageing acceleration relative to children with no internalizing disorder (1–2 categories: b = 0.50, 95% CI [0.170, 0.821], t = 3.008, $p = .003$; 3 or more categories: b = 0.99, 95% CI [0.380, 1.593], t = 3.215, p = .002). Children with internalizing disorder who were not exposed to maltreatment did not show epigenetic ageing acceleration. There was significant enrichment of dexamethasone-responsive CpGs within the PedBE clock (OR = 4.36, $p = 1.65$*10–6). Among the 94 CpGs of the PedBE clock, 18 (19%) were responsive to dexamethasone.
Conclusion: Using the novel PedBE clock, we show that internalizing disorder is associated with accelerated epigenetic ageing in early childhood. This association is moderated by maltreatment severity and may, in part, be driven by glucocorticoids. Identifying developmental drivers of accelerated epigenetic ageing after maltreatment will be critical to devise early targeted interventions.

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1. Introduction

Individuals with psychiatric disorders die on average over ten years earlier as compared to the general population and the main causes of death are common ageing-related diseases (Walker et al., 2015). It is believed that accelerated biological ageing may contribute to premature morbidity and mortality in individuals with psychiatric disorders (Danese and McEwen, 2012; Gassen et al., 2017). One common indicator for cellular ageing is DNA telomere shortening that occurs with each cell division. Telomere shortening has been reported for nearly all of the major psychiatric disorders (Darrow et al., 2016). Another indicator of biological ageing is derived from epigenetic assessments. So-called epigenetic clocks consider time-dependent changes in DNA methylation at specific cytosine-guanine dinucleotide sites (CpGs), resulting in estimates of epigenetic age (Hannum et al., 2013; Horvath, 2013). The gap between epigenetic age and chronological age indicates deviation of biological ageing, i.e. acceleration or deceleration.

A number of studies document accelerated epigenetic ageing in individuals with psychiatric disorders, including major depression (Han et al., 2018), bipolar disorder (Fries et al., 2017), posttraumatic stress disorder (Shenk et al., 2021; Wolf et al., 2019), and internalizing symptoms in children (Tollenaar et al., 2021). Accelerated epigenetic ageing has also been reported for cardiovascular disease, obesity, diabetes, and cancer, as well as for all-cause mortality (Fransquet et al., 2019; Horvath et al., 2014; Nevalainen et al., 2017; Perna et al., 2016). Little is known, however, as to whether risk factors that drive both psychiatric and physical morbidity, such as early-life stress (ELS), contribute to accelerated ageing in these disorders. Notably, within psychiatric disorders, there are important subtypes with distinct biological features that occur as a function of ELS. E.g., glucocorticoid dysregulation and inflammation, both drivers of ageing, have been shown to occur in depressed individuals with ELS, but not in depressed individuals without ELS (Heim et al., 2004; Danese et al., 2008; Teicher and Samson, 2013). It is conceivable that ELS also contributes to accelerated ageing in these disorders.

Several studies provide evidence for increased telomere shortening in adults exposed to ELS (Kananen et al., 2010; O'Donovan et al., 2011; Rentscher et al., 2020; Surtees et al., 2011; Tyrka et al., 2010) as well as children with ELS (Drury et al., 2012; Mitchell et al., 2014; Shalev et al., 2013). Furthermore, epigenetic ageing indicators provide evidence for accelerated ageing in adults and children exposed to poverty, trauma, abuse, threat, and neighborhood violence early in life (Austin et al., 2018; Hamlat et al., 2021; Jovanovic et al., 2017; Marini et al., 2020; Sumner et al., 2019; Wolf et al., 2018). One study in adults reported that epigenetic ageing acceleration within a group of depressed individuals was accentuated by the severity of ELS (Han et al., 2018).

However, there is an important methodological caveat of the above studies that estimated epigenetic ageing in children. These studies uniformly applied epigenetic ageing estimates that were validated for adults. The applied algorithms are likely not suitable to estimate epigenetic ageing in children. Importantly, specific CpGs and methylation patterns associated with maturation during early development likely differ from those that mark ageing in later life. Moreover, DNA methylation changes during childhood occur at a 3 to 4-fold higher rate compared to adults (Alisch et al., 2012). Hence, epigenetic clocks must be developed for specific age ranges. To that end, a novel epigenetic clock has recently been developed for application in pediatric samples (McEwen et al., 2020). The pediatric buccal epigenetic (PedBE) clock was generated from a training dataset of 1032 children aged 0–19 years and evaluated in an independent test dataset of 689 children of the same age range. The PedBE clock estimates epigenetic age based on methylation patterns across 94 CpGs and has been shown to demonstrate higher accuracy than Horvath's clock to estimate epigenetic age in healthy children.

The objectives of the current study were 4-fold: ***1)*** We use the PedBE clock in a clinical study of young children aged 3–5 years. ***2)*** We assess epigenetic ageing deviation in children with internalizing disorder as compared to children without internalizing disorder. ***3)*** We assess the contribution of ELS exposure to epigenetic ageing in children with internalizing disorder. ***4)*** We determine the contribution of glucocorticoid signaling to PedBE clock results by identifying specific CpGs that are responsive to the glucocorticoid receptor agonist dexamethasone, derived from an independent sample, within the PedBE clock and we assess differential methylation in these stress-associated CpGs as a function of internalizing disorder and ELS.

2. Methods

This study is part of the larger Berlin Longitudinal Children Study (BMBF 01K1301). A sample of 173 children was recruited to include children with maltreatment exposure within 6 months and non-maltreated children. Maltreated children were recruited via child protection services in the Berlin area. Non-maltreated children were recruited from the community. Data on epigenetic age was available for 168 children. Of those, 10 children were excluded due to missing data on clinical status, resulting in a final sample of 158 children, including 73 girls and 85 boys, with a mean age of 4.25 years (SD = 0.8, range 3–5 years). A total of 81 children were classified as maltreatment cases according to the Maltreatment Classification System (MCS; Barnett et al., 1993). The MCS codes the occurrence, onset, duration, severity, and frequency of 7 types of maltreatment. We used severity cutoff scores for entry in the maltreatment group (emotional maltreatment $\geq$2, physical maltreatment $\geq$1, and/or neglect $\geq$1). For assignment to the group of non-maltreated children, any maltreatment or other trauma was excluded. Exclusion criteria for all children included parents under the age of 18 years, severe chronic medical disease, psychosis, neurodevelopmental disorders, disability, current medication, and chronic illness of a caretaker. All procedures adhered to the Declaration of Helsinki and were approved by the ethics committee of Charité – Universitätsmedizin Berlin. Informed consent was obtained from caretakers and assent was obtained from children. Caregivers received monetary compensation and children received a small gift for participation. Caregivers received diagnostic results and referrals for psychosocial or medical follow-up.

2.1. Demographic and clinical assessments

Study procedures were implemented during a clinic visit. Children underwent a standard medical examination to exclude health problems and monitor physical signs of maltreatment. Trained clinicians administered structured interviews based on caretaker report to assess psychiatric disorders and maltreatment features. Psychiatric disorders were assessed according to DSM-IV using the electronic Preschool Age Psychiatric Assessment (Egger and Angold, 2004). Presence or absence of current internalizing disorder was coded, including dysthymia, major depression, social anxiety disorder, selective mutism, specific and social phobia, and general anxiety disorder. Maltreatment features were assessed using the Maternal Interview for the Classification of Maltreatment (Cicchetti et al., 2003; German: Horlich et al., 2014a,b) and coded according to the MCS (Barnett et al., 1993; German: Horlich et al., 2014a,b). MCS categories include sexual abuse, physical, and emotional abuse, neglect due to lack of supervision or failure to provide, educational neglect, and moral-legal neglect. A sum score of experienced maltreatment categories was computed for each child (range 0–7). Sex, body mass index (BMI), and socioeconomic status (SES) according to Winkler and Stoltenberg (1999) were recorded.

2.2. DNA methylation

Saliva for genomic DNA extraction was collected using ORAgene DNA kits (OG500) at 9 a.m. during the clinic visit. DNA extraction was performed with a standardized and automated procedure based on

magnetic beads for 2× 400 μl saliva samples using the PerkinElmer Chemagic360 system. The Infinium Methylation EPIC BeadChip (Illumina Inc, San Diego, CA, USA) was used to measure DNA methylation (DNAm). Samples were randomized with regards to maltreatment status, age, and sex to avoid confounding. Hybridization and array processing were performed as specified by the manufacturer. Functional normalization implemented by the minfi package (Aryee et al., 2014) was used to normalize the data. Batch effects were identified and removed with the Empirical Bayes' method ComBat (Müller et al., 2016) included in the R package sva (Leek et al., 2012). CpGs located on the X or Y chromosome, cross-reactive and polymorphic probes were removed (Chen et al., 2013; Pidsley et al., 2016), and probes with detection p > .01 in more than 25% of the samples were filtered out. A total of 830,206 CpGs remained after batch correction and quality control. Cell composition of the buccal swab samples was estimated using the deconvolution method described by (Smith et al., 2015) and was corrected for in all statistical models.

2.3. Epigenetic age estimate and ageing deviation

We used the PedBE clock algorithm that has been developed for epigenetic age estimation in individuals aged 0–20 years (McEwen et al., 2020). This algorithm uses information on methylation status at 94 empirically selected CpGs that either show an increase or decrease in methylation with time. The algorithm was applied as previously described (McEwen et al., 2020). To test the validity of the PedBE clock for age estimation in our sample, we used Pearson's correlation coefficient between PedBE clock-estimated age and chronological age and found a correlation of r = .745 ($p < .001$; see Fig. 1). We next computed a linear regression model of estimated epigenetic age regressed against chronological age and standardized residuals were used to compute indices for each individual ageing deviation. Residuals with negative values indicate ageing deceleration and residuals with positive values indicate ageing acceleration. These indices were used in all statistical models. Unstandardized residuals were used to compute ageing acceleration in months to aid interpretation of results.

2.4. Statistical analysis of ageing deviation

The total sample of 158 children was stratified into those with (n = 49) and without (n = 109) current internalizing disorder. We used univariate analysis of covariance to test for differences in mean epigenetic ageing deviation scores, i.e. residuals of epigenetic age regressed against chronological age, between children with and without current internalizing disorder. Covariates with known impact on epigenetic ageing were entered in the model (see below). Next, we examined whether the number of maltreatment categories moderates the association between the presence or absence of internalizing disorder and epigenetic ageing deviation using the PROCESS macro (Hayes, 2017). The moderation hypothesis is first tested with a regression analysis, in which the effect of the predictor on the outcome variable is not under constraint to be fixed, but can vary as a function of the moderator variable, yielding an interaction term. In a second step, simple slope analysis "probes" the nature of the established interaction, while simultaneously considering data points and covariance in the total model and based on the entire sample. We computed the model as follows: We coded a moderator variable based on the number of maltreatment types that was coded at 3 severity levels (Table 1): ***1)*** No maltreatment (value = 0, n = 77), ***2)*** Mild to moderate maltreatment: 1 to 2 maltreatment categories (value = 1, n = 55), and ***3)*** Severe maltreatment: 3 or more maltreatment categories (value = 2, n = 26). For the ordinary least square (OLS) regression in the moderation analysis, variables were mean-centered to enhance interpretation of effects. We applied the Davidson-MacKinnon heteroscedasticity-consistent standard error estimator for ordinary least square regressions to enhance validity and power (Davidson and MacKinnon, 1993; Hayes and Cai,

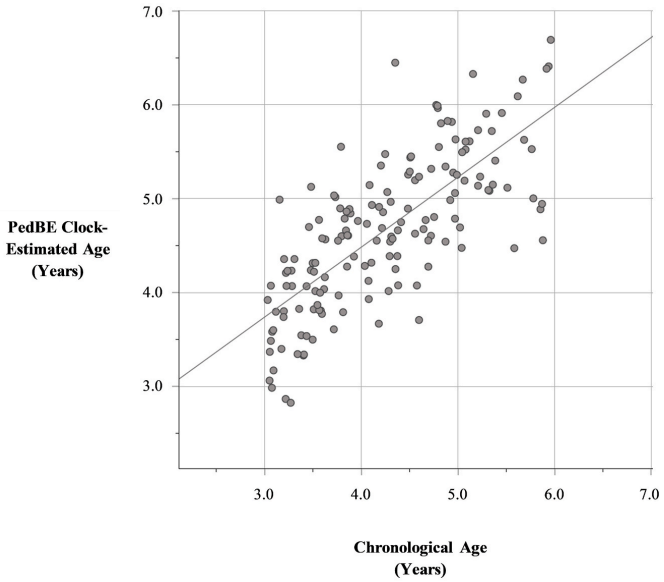

Fig. 1. Correlation of PedBE clock-estimated age and chronological age (r = . 745, p < .001).

Table 1
Demographic and Clinical Characteristics of the Sample. Values are presented as mean (SD) or n (%).

	No Internalizing Disorder (n = 109)	Internalizing Disorder (n = 49)	*P* Value
Chronological Age in Years	4.27 (0.79)	4.18 (0.85)	.507
Female Sex	52 (47.70)	21 (42.90)	.572
Self-Reported Ethnicity			
White	100 (91.70)	44 (89.80)	.296
White-Black	6 (5.50)	5 (10.20)	
White-Asian	3 (2.80)	0 (0)	
Maltreatment Categories			.003
0	63 (57.80)	14 (28.60)	
1-2	30 (27.50)	25 (51.00)	
≥3	16 (14.70)	10 (20.40)	
SES	13.65 (5.00)	10.49 (5.03)	<.001
BMI	15.42 (1.07)	15.61 (1.35)	.326

2007). In a second step, we computed simple slopes at the three levels of maltreatment severity categories from the regression equation of the significant interaction term in the moderation model (Hayes and Rockwood, 2017). To probe the interaction, the integrated model tests whether each of the 3 slopes shows a significant change in ageing acceleration residuals. This means that we tested whether each slope shows epigenetic ageing acceleration as a function of internalizing disorder under the conditional effect of a given level of maltreatment severity. A post-hoc power analysis was conducted for the OLS regression in the moderation model using G*Power (Faul et al., 2009). All analyses were adjusted for confounders with known impact on epigenetic ageing, including ***1)*** cell type composition (buccal cells, CD14, CD34), ***2)*** population structure and relatedness (3 variables) as described in Martins et al. (2021), and ***3)*** caregiver-reported sex, BMI, and SES. Analyses were performed using SPSS 25.0 for Windows. Alpha level of significance was set at $p < .05$.

2.5. Gene set enrichment analysis

To elucidate the contribution of glucocorticoid signaling to epigenetic ageing estimates in the PedBE clock, we tested whether dexamethasone-responsive CpGs are enriched within the 94 CpG sites of the PedBE clock. We obtained data on dexamethasone-responsive CpGs from an independent cohort (n = 113), which is described by Provençal et al. (2020) in detail. In this cohort, DNA was extracted from peripheral blood taken before and 3 h after ingestion of 1.5 mg dexamethasone. DNA methylation was measured using the Illumina HumanMethylation450 BeadChip and differentially methylated CpGs after treatment with dexamethasone were assessed (FDR-corrected *p* value of .1). The analysis identified 23,031 CpGs that were responsive to dexamethasone as indicated by differential methylation post-treatment. For the current study, enrichment of dexamethasone-responsive CpGs within the 94 CpGs of the PedBE clock was tested using Fisher's Exact test with all CpGs measured on the Illumina HumanMethylation450 BeadChip as background. Methylation levels of dexamethasone-responsive CpGs within the PedBE clock were contrasted between groups stratified by internalizing disorder and maltreatment exposure using analysis of variance.

3. Results

Demographic and clinical features of the sample are presented in Table 1. Children with and without internalizing disorder did not differ in age, ethnicity, sex or BMI (all $p > .05$). However, mean SES was lower in children with internalizing disorder compared to those without internalizing disorder ($p < .001$). As expected, the proportion of maltreated children was higher among children with internalizing disorder compared to children without internalizing disorder (χ^2 [2, N = 158] = 11.96, $p = .003$).

Univariate analysis of covariance revealed a significant main effect of internalizing disorder on epigenetic ageing deviation: Children with current internalizing disorder exhibited significant acceleration of epigenetic ageing (M = 0.29, SE = 0.13) compared to children without internalizing disorder (M = −0.13, SE = 0.87, $F_{1,147} = 6.67$, $p = .011$; see Fig. 2). Children with internalizing disorder on average were 1.87

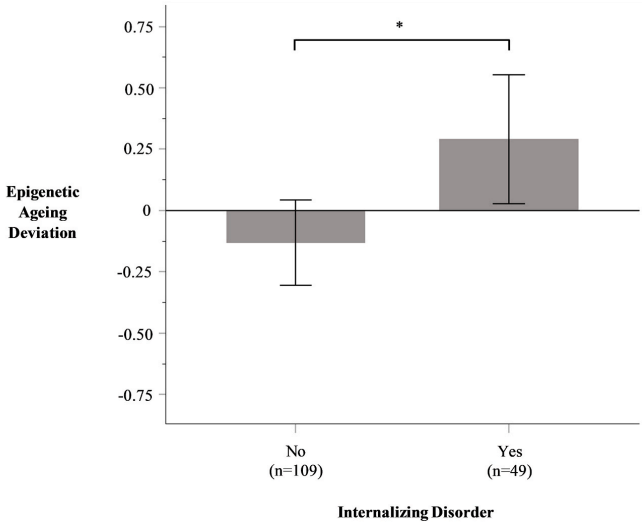

Fig. 2. Analysis of covariance of standardized residuals of PedBE clock-estimated epigenetic age regressed against chronological age in children with (n = 49) and without (n = 109) internalizing disorder (Means and 95% CI error bars). Adjusted for cell composition, sex, genetic ethnicity, BMI, and SES. *$p < .05$.

months epigenetically older than their chronological age, whereas children without internalizing disorder on average were 0.84 months younger than their chronological age. This means that children with internalizing disorder exhibited on average 2.71 months age acceleration compared to children without internalizing disorder.

Of note, there was a significant effect of sex on epigenetic ageing acceleration ($F_{1,147} = 6.62$, $p = .011$) indicating higher epigenetic ageing in girls. To follow up on this finding, we tested a second model that included an interaction term of internalizing disorder by sex. The main effects of both internalizing disorder ($F_{1,146} = 6.80$, $p = .010$) and sex ($F_{1,146} = 6.36$, $p = .013$) remained significant and there was no interaction effect of internalizing disorder by sex ($F_{1,146} = 2.21$, $p = .646$), suggesting that the main effect of internalizing disorder on epigenetic ageing is unaffected by sex.

We next examined whether severity of maltreatment moderates the relationship between internalizing disorder and epigenetic ageing acceleration (see Table 2). First, OLS regression revealed that there was no significant main effect of number of maltreatment categories on epigenetic ageing (b = 0.18, 95% CI [-0.110, 0.471], t = 1.228, $p = .222$), whereas the effect of internalizing disorder remained significant in this model (b = 0.34, 95% CI [0.027, 0.647], t = 2.149, $p = .033$). Moreover, we found a significant interaction effect between internalizing disorder and the number of maltreatment categories (b = 0.49, 95% CI [0.073, 0.909], t = 2.322, $p = .022$), suggesting that the severity of maltreatment significantly moderates the association between internalizing disorder and epigenetic ageing acceleration. In other words, children without internalizing disorder did not show ageing acceleration, regardless of maltreatment status, whereas children with internalizing disorder exhibited graded epigenetic ageing acceleration as a function of maltreatment severity. Again, there was a significant effect of sex in this model (b = −0.32, 95% CI [-0.628, −0.016], t = −2.083, $p = .039$) with girls demonstrating greater ageing acceleration than boys. However, the interaction effect between internalizing disorder and number of maltreatment categories predicting epigenetic ageing acceleration was unaffected by sex.

Post-hoc simple slope analysis of the conditional effects of internalizing disorder at three levels of the moderator variable revealed a graded effect on epigenetic ageing acceleration (see Fig. 3). Internalizing disorder in children who had experienced 1 or 2 maltreatment categories was associated with significant epigenetic ageing acceleration relative to their chronological age (n = 55, b = 0.50, 95% CI [0.170, 0.821], t = 3.008, $p = .003$), equivalent to an average of 3.19 months ageing acceleration. Internalizing disorder in children who experienced 3 or more maltreatment categories was significantly associated with epigenetic ageing acceleration relative to their chronological age (n = 26, b = 0.99, 95% CI [0.380, 1.593], t = 3.215, $p = .002$), equivalent to an average of 6.36 months ageing acceleration. In the absence of maltreatment exposure, internalizing disorder was associated with congruency between chronological age and epigenetic age (n = 77, b = 0.00, 95% CI [-0.436, 0.444], t = 0.019, $p = .985$).

The total moderation model accounted for a significant amount of variance in epigenetic ageing acceleration ($R^2 = .275$, $F_{12, 145} = 4.797$, $p < .001$). A post-hoc power analysis for the OLS regression model with 158 children, 12 predictors (internalizing disorder, maltreatment severity categories, interaction term, and 9 covariates) with an alpha of .05 and the observed effect size of $f^2 = .38$ revealed a statistical power of > .99.

Using data from an independent sample that identified dexamethasone-responsive CpGs on a genome-wide level (Provençal et al., 2020), we observed a highly significant enrichment of dexamethasone-responsive CpGs within the PedBE clock (Fisher's Exact: $p = 1.65*10–6$, Odds Ratio = 4.36). Specifically, of the 94 CpGs that compose the PedBE clock, 18 were found to show significant differences in DNA methylation following dexamethasone exposure, suggesting that a substantial proportion of the CpGs in the PedBE clock is susceptible to glucocorticoid-induced DNA methylation changes. Among these CpGs, we found individual differential methylation at cg16618789 (F = 3.80, p = .005) and at cg03493146 (F = 2.92, p = .023) in children with maltreatment exposure and internalizing disorder.

Table 2
Linear Model of Predictors of PedBE clock-based Estimates of Epigenetic Ageing as a Function of Internalizing Disorder and Maltreatment Severity Categories Adjusted for cell type composition, sex, genetic ethnicity, BMI, and SES.

	b	SE	t	*p*
Constant	−14.82	6.74	−2.20	.030
Internalizing Disorder	0.37	0.16	2.15	.033
Number of Maltreatment Categories	0.18	0.15	1.23	.222
Internalizing Disorder * Number of Maltreatment Categories	0.49	0.21	2.32	.022

4. Discussion

This is the first clinical study that applies the PedBE clock for the estimation of epigenetic age to a pediatric sample of children with internalizing disorder or maltreatment exposure, or both. Because epigenetic ageing in early life as compared to older age likely involves different DNA methylation patterns and follows a different temporal pace, adult clocks are less suitable to estimate epigenetic ageing deviation in young children. The PedBE clock provides a highly accurate molecular measure of biological age and was specifically developed and validated for the age range of children included in our sample (McEwen et al., 2020). Thus, our study represents a significant methodological advance over prior studies that reported on biological ageing deviation in children with affective disorders or maltreatment exposure using adult epigenetic clocks.

Using residuals from the regression of PedBE clock-based epigenetic age estimates against chronological age, we found that internalizing disorder in children is associated with markedly accelerated epigenetic ageing as compared to children without internalizing disorder. We further demonstrate that the association between internalizing disorder and epigenetic ageing is moderated by maltreatment severity. This means that epigenetic ageing acceleration occurs in children with internalizing disorder who also experienced maltreatment, but not in children with internalizing disorder alone and not in children with maltreatment alone. This apparent moderation supports a subtype of internalizing disorder that manifests in relation to stress and has distinct biological features and pathophysiological pathways (Heim et al., 2004; Teicher and Samson, 2013). Using the more accurate and developmentally-sensitive methodology of the PedBE clock, our results provide validation for previous studies results that reported accelerated epigenetic ageing in children with affective disorders or maltreatment exposure based on adult epigenetic clocks (Austin et al., 2018; Hamlat et al., 2021; Han et al., 2018; Jovanovic et al., 2017; Marini et al., 2020; Sumner et al., 2019; Shenk et al., 2021). We extend these findings by providing the stratified moderation analysis identifying a subtype of internalizing disorder and maltreatment exposure that demonstrates accelerated epigenetic ageing. It is noteworthy that a biological subtype of internalizing disorder related to ELS is already distinguishable at the early age of 3–5 years, further underscoring the potential for defining early drivers of pathology and the need for designing novel early interventions that mitigate these drivers. Importantly and in line with findings in adults (Han et al., 2018), our results suggest that the severity of ELS might be critical for the link between internalizing disorder and epigenetic ageing acceleration. While, among children with internalizing disorder, significant epigenetic ageing acceleration was detectable in those exposed to 1 to 2 categories of maltreatment exposure, the pace of acceleration appeared to be faster in those with 3 or more forms of maltreatment exposure.

Stratified effects as a function of the co-occurrence of major depression and ELS have been observed for glucocorticoid signaling and

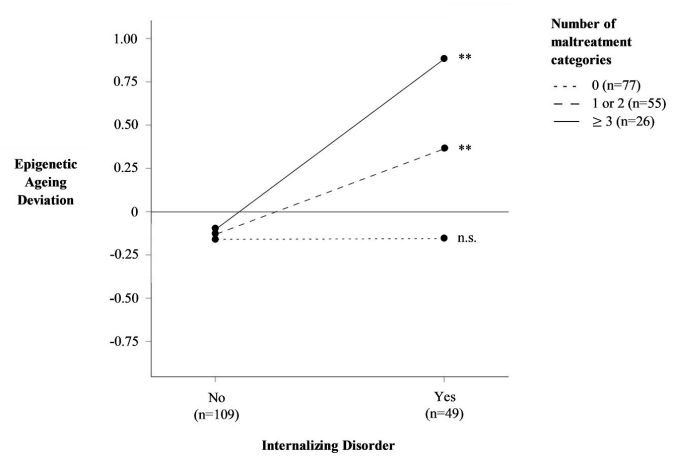

Fig. 3. Simple slope equations of the regression of epigenetic ageing on internalizing disorder at three levels of number of maltreatment categories. Adjusted for cell type composition, sex, genetic ethnicity, BMI and SES. **p < .01.

systemic inflammation in adults (Heim et al., 2008; Danese et al., 2008). Systemic inflammation, as evidenced by elevated levels of C-reactive protein, has been reported for children as young as 3–5 years of age as a correlate of maltreatment (Danese et al., 2011; Entringer et al., 2020). There is evidence that glucocorticoids and immune mediators are drivers of epigenetic ageing (Horvath and Raj, 2018; Quach et al., 2017). We previously reported dynamic methylation change in CpGs composing the Horvath clock and altered transcription of genes neighboring these CpGs in adults 3 h after oral intake of dexamethasone (Zannas et al., 2015). One study reported that diurnal cortisol secretion associates with epigenetic ageing acceleration in adolescent girls (Davis et al., 2017). It is, hence, conceivable that glucocorticoid signaling contributes to the effects observed in the current study.

Importantly, we here demonstrate direct evidence that the CpGs composing the PedBE clock are at least in part regulated by glucocorticoids, which we consider a highly relevant finding. Based on a genome-wide identification of dexamethasone-responsive CpGs in blood cells obtained from an independent sample (Provençal et al., 2020), we were able to conduct an enrichment analysis by comparing observed frequency of dexamethasone-responsive CpGs within the PedBE clock against the background of the genome-wide list of dexamethasone-responsive GpGs. We found a highly significant enrichment indicating that significantly more CpGs within PedBE are responsive to glucocorticoids than would be expected based on the frequency in the total list. This means that during early development, epigenetic ageing could be regulated and influenced by glucocorticoids, which is in line with a sensitive period for stress effects on biological ageing. This finding suggests that the observed effects in our study are in part mediated by glucocorticoid exposure in these young children. Accordingly, within the 18 dexamethasone-responsive CpGs of the PedBE, two sites were differentially-methylated in children with internalizing disorder and maltreatment exposure. The mechanism by which glucocorticoids regulate DNA methylation and, hence, epigenetic ageing likely involves local glucocorticoid receptor-induced genomic processes, such as DNA-excision repair mechanisms (Kress et al., 2006; Thomassin et al., 2001).

The observation of accelerated epigenetic ageing as a function of internalizing disorder and maltreatment in early childhood together with the observation of high susceptibility of the PedBE clock for stress signaling raises important theoretical implications: Accelerated DNA methylation-based ageing in early childhood, as observed in our study, could either reflect accelerated maturational pace, i.e. more rapid developmental change, versus more rapid ageing-related decline. On the basis of evolutionary theory, it has been suggested that early adversity and threat experiences may indeed lead to a more rapid development, leading to earlier onset of puberty, to ensure reproduction and survival in an unsafe environment (Ellis & del Giudice, 2019). In this framework, it also makes sense that the CpGs that compose the PedBE clock are particularly sensitive to stress signaling. While rapid developmental pace may be beneficial for survival, it may result in failure to reach full potential and may promote increased morbidity and rapid ageing-related decline over time (Belsky et al., 2015). While our data are compatible with this theoretical framework, prospective studies are needed to scrutinize the trade-off between adaptation and vulnerability in relation to early accelerated DNA methylation ageing as a response to stress in early life.

Interesting in this regard is the finding that girls exhibited overall greater epigenetic ageing than boys, although there was no interaction of sex and internalizing disorder in the prediction of epigenetic ageing acceleration and the moderation effect of internalizing disorder and maltreatment exposure on epigenetic ageing acceleration was unaffected by sex. The literature on sex differences in epigenetic ageing is inconsistent. Greater epigenetic ageing has been reported for adult and adolescent males compared to females, but not for pre-pubertal children (Horvath et al., 2016; Simpkin et al., 2016). One study reported accelerated epigenetic ageing as a function of ELS in adolescent girls, but not in boys (Tang et al., 2020). It should be noted that pubertal stage is critical to such studies, as sex differences may be driven by sex hormones. In our study, children were pre-pubertal and, therefore, the main effect of sex cannot be attributed to sex hormones. Of note, we recently observed sex differences in levels of inflammation as a function of maltreatment in our current cohort, as evidenced by elevated levels of C-reactive protein over 24 months among maltreated girls as compared to non-maltreated girls and maltreated and non-maltreated boys (Entringer et al., 2020). Increased inflammatory signaling in girls may contribute to sex differences in epigenetic ageing (Quach et al., 2017); however, we did not see an interaction effect of sex and maltreatment in our study in the prediction of epigenetic ageing. Interestingly, elevated levels of inflammation in pre-pubertal children have been reported to affect the onset of menarche (Michels et al., 2020). Early menarche onset

has been shown to associate with accelerated epigenetic ageing based on DNA methylation in the GrimAge epigenetic clock (Lu et al., 2019) that has validity for adults and predicts mortality risk (Hamlat et al., 2021), in line with the above theoretical considerations of a trade-off between rapid maturation and increased morbidity or mortality. Future studies should scrutinize the role of sex differences in accelerated epigenetic ageing and pubertal pacing in response to stress.

Unique strengths of our study include the application of the PedBE clock in a clinical study of very young children combined with an in-depth clinician-administered diagnostic assessment of children from a sample that was enriched for maltreatment exposure as well as access to an independent sample to assess the role of glucocorticoid signaling in our clinical results. Limitations of the study include a small sample size as well as the cross-sectional design hampering causal interpretations. In addition, we did not relate our results to mediators, such as inflammation or stress hormone levels in children. Longitudinal multi-system studies are needed to address these limitations.

In conclusion, we provide a valid estimate of epigenetic ageing in early childhood using the novel PedBE clock. Using this advanced methodology, we provide evidence for accelerated epigenetic ageing in children with internalizing disorder, but only in those who were exposed to maltreatment, reflecting a moderation effect and supporting the existence of a distinct biological subtype as a function internalizing disorder and concomitant maltreatment exposure that is already identifiable at the age of 3–5 years. We finally provide compelling evidence that the PedBE clock is enriched for CpGs that are responsive to glucocorticoid signaling, suggesting that epigenetic ageing in childhood might be sensitive to stress. Stress-regulation of epigenetic ageing during early childhood may enable a trade-off between more rapid maturation to enhance survival with the toll of increasing morbidity. Our findings underscore the need for the development of early interventions that may mitigate adverse outcomes of ELS and promote healthy trajectories across the lifespan.

CRediT authorship contribution statement

Felix Dammering: Investigation, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Jade Martins:** Investigation, Methodology, Formal analysis, Data curation, Writing – review & editing. **Katja Dittrich:** Investigation, Resources. **Darina Czamara:** Methodology, Formal analysis, Data curation. **Monika Rex-Haffner:** Investigation, Resources. **Judith Overfeld:** Investigation, Resources. **Karin de Punder:** Investigation, Resources. **Claudia Buss:** Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing. **Sonja Entringer:** Supervision, Formal analysis, Analysis, Resources, Writing – review & editing. **Sibylle M. Winter:** Conceptualization, (clinical part), Funding acquisition, Investigation, Resources, Supervision. **Elisabeth B. Binder:** Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing. **Christine Heim:** Conceptualization, Funding acquisition, Project administration, Investigation, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None.

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Curriculum Vitae

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Publication list

Publication 1: Katthagen, T., Dammering, F., Kathmann, N., Kaminski, J., Walter, H., Heinz, A., & Schlagenhauf, F. (2016). Validating the construct of aberrant salience in schizophrenia - Behavioral evidence for an automatic process. Schizophrenia Research: Cognition, 6, 22-27. IF: 0.79

Publication 2: Heim, C., Dammering, F., & Entringer, S. (2020). Frühe Programmierung von Gesundheit und Krankheit. In U. Egle, C. Heim, B. Strauß, & R. von Känel, Psychosomatik - Neurobiologisch fundiert und evidenzbasiert. pp. 185-191. Stuttgart: Kohlhammer.

Publication 3: Martins J., Czamara, D., Sauer, S., Rex-Haffner, M., Dittrich, K., Dörr, K., de Punder, K., Overfeld, J., Knop, A., Dammering, F., Entringer, S., Winter, S. M., Buss, C., Heim, C., Binder, E. B. (2021). Childhood adversity correlates with stable changes in DNA methylation trajectories in children and converges with epigenetic signatures of prenatal stress. Neurobiology of Stress, 15. IF: 7.142

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