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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,

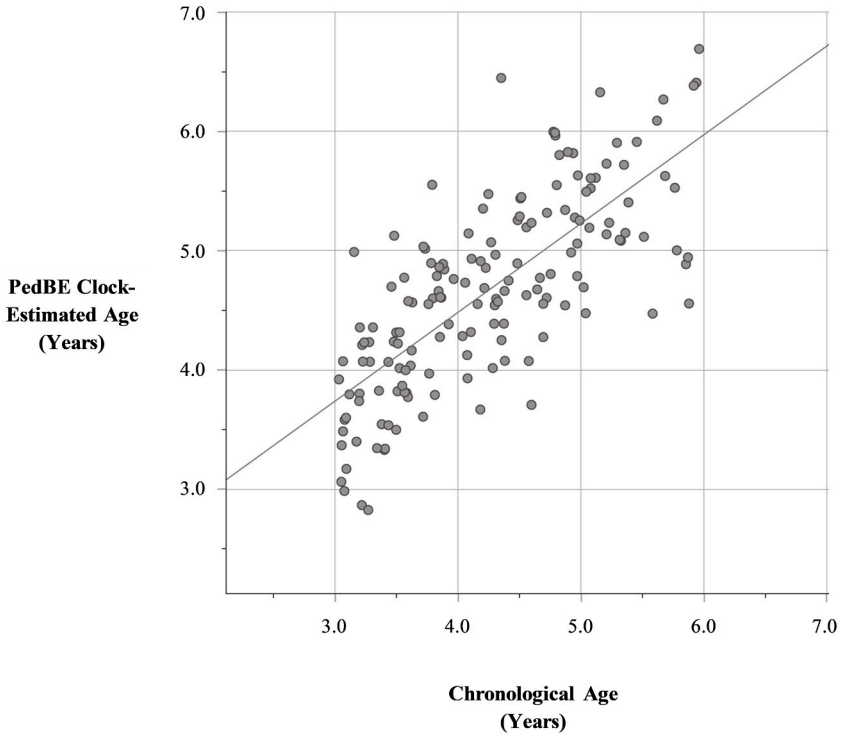

Fig. 1. Correlation of PedBE clock-estimated age and chronological age (r = . 745, p < .001).

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

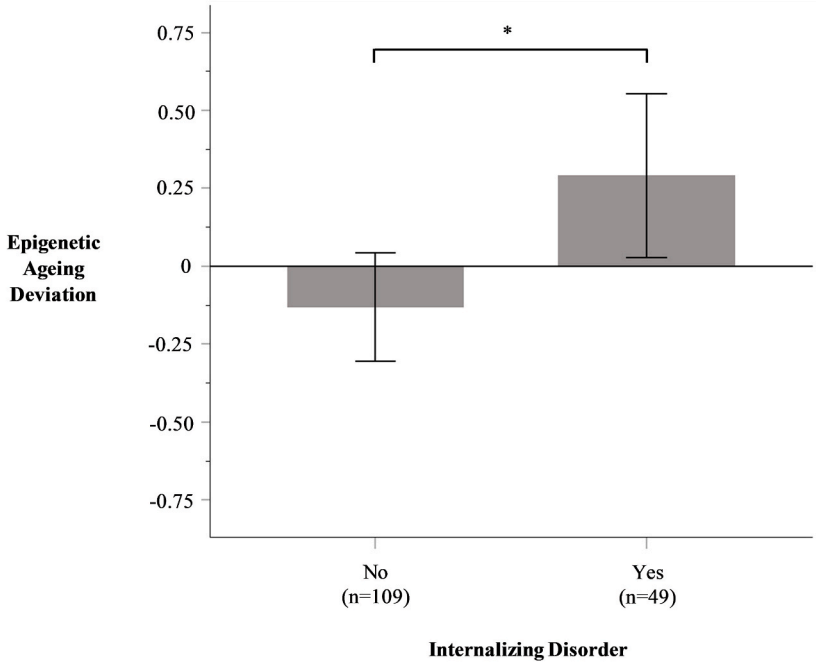

Fig. 2. Analysis of covariance of standardized residuals of PedBE clock-estimated epigenetic age regressed against chronological age in children with (n = 49) and without (n = 109) internalizing disorder (Means and 95% CI error bars). Adjusted for cell composition, sex, genetic ethnicity, BMI, and SES.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

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

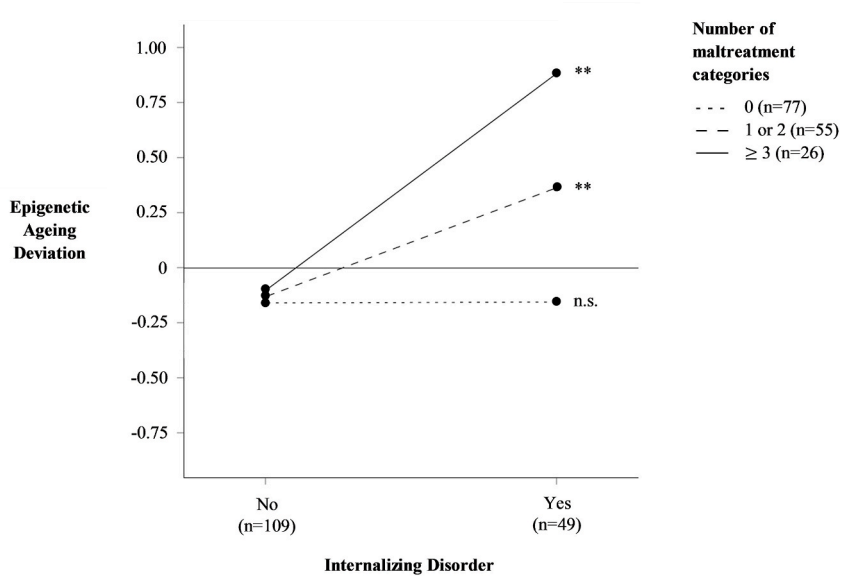

Fig. 3. Simple slope equations of the regression of epigenetic ageing on internalizing disorder at three levels of number of maltreatment categories. Adjusted for cell type composition, sex, genetic ethnicity, BMI and SES. **p < .01.

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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