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Epigenetic signatures of intergenerational exposure to violence in three generations of Syrian refugees

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Maternal trauma influences infant and adult health outcomes and may impact future generations through epigenetic modifications such as DNA methylation (DNAm). Research in humans on the intergenerational epigenetic transmission of trauma effects is limited. In this study, we assessed DNAm signatures of war-related violence by comparing germline, prenatal, and direct exposures to violence across three generations of Syrian refugees. We compared families in which a pregnant grandmother versus a pregnant mother was exposed to violence and included a control group with no exposure to war. We collected buccal swab samples and survey data from mothers and 1–2 children in each of 48 families (n = 131 participants). Based on an epigenome-wide association study (EWAS), we identified differentially methylated regions (DMPs): 14 were associated with germline and 21 with direct exposure to violence. Most DMPs showed the same directionality in DNAm change across germline, prenatal, and direct exposures, suggesting a common epigenetic response to violence. Additionally, we identified epigenetic age acceleration in association with prenatal exposure to violence in children, highlighting the critical period of in utero development. This is the first report of an intergenerational epigenetic signature of violence, which has important implications for understanding the inheritance of trauma.

Keywords Maternal trauma, DNA methylation, Germline exposure, Prenatal exposure, Epigenetic age acceleration

Maternal trauma affects the health of the fetus while in utero and throughout adulthood, and may impact future generations through epigenetic modifications such as DNA methylation (DNAm)[1–4]. Maternal stressors known to impact a developing fetus include nutritional deficiencies[5] and exposure to toxins[6], as well as psychosocial stressors such as anxiety[7] and, potentially, exposure to violence and trauma[8,9]. The Developmental Origins of Health and Disease (DOHaD) hypothesis formalized the impact of early life adversity on later life health outcomes by identifying strong associations between early life adversity, such as low birthweight and adverse living conditions, with increased risk of cardiovascular disease in adulthood[10,11]. According to DOHaD tenets, the developing fetus is characterized by high phenotypic plasticity and utilizes environmental cues to determine an optimal phenotype to survive the postnatal environment. However, adaptation based on intrauterine cues might select for a phenotype that is ultimately maladaptive in later life, thus increasing the risk of certain diseases[3,12,13].

The effects of psychosocial stressors and trauma are thought to be transmitted from mother to offspring through cellular changes in the maternal and fetal hypothalamic–pituitary–adrenal (HPA) axes and accompanying changes in glucocorticoid metabolism[3]. However, the underlying molecular mechanism that encodes and preserves trauma information for decades into adulthood is not well understood. Extending the

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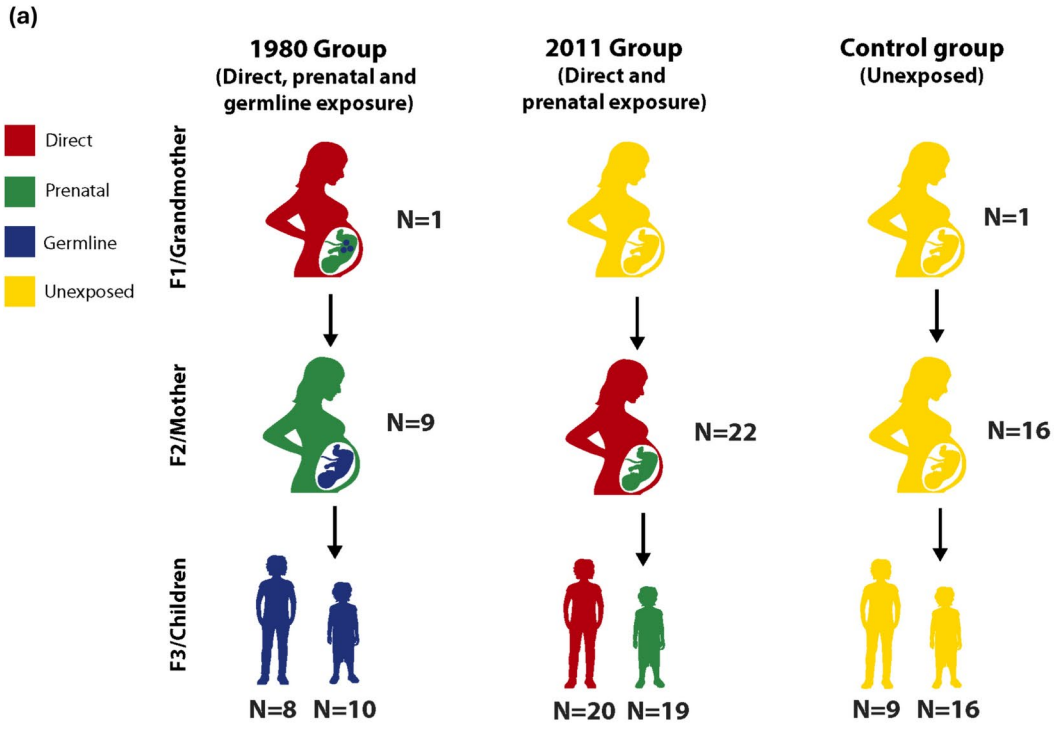

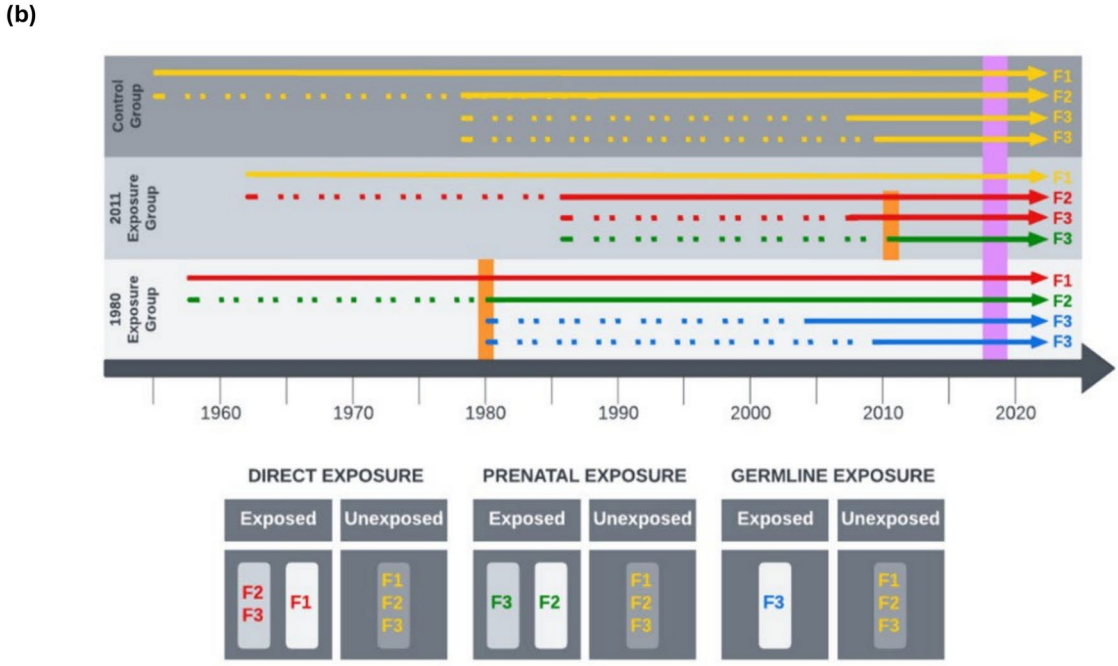

predictive power of the DOHaD hypothesis, epigenetic mechanisms have been proposed to mediate the impact of psychosocial stress and trauma on future generations[14,15]. Specifically, environmentally-sensitive epigenetic modifications may have evolved at select sites in the human epigenome to provide a rapid, short-term response to environmental stressors compared to the more slowly evolving genome. Furthermore, a subset of those epigenetic modifications may have evolved to be heritable to transmit the selective advantages of environmental sensitivity to future generations. A range of epigenetic modifications exist, including DNA methylation (DNAm), histone modification, and noncoding RNAs, which help regulate gene expression. DNAm typically refers to the addition of a methyl group to a cytosine nucleotide base that is followed by guanine base (a CpG site). Changes in DNAm can lead to increases or decreases in gene expression depending on the gene and genome context as well as the developmental stage and replication activity of the genome[16]. DNAm is well-studied because it

◀**Fig. 1**. Three-generation study design. (**a**) Our research strategy was designed to test contrasting exposures to violence (direct, prenatal, germline) for changes in DNAm in three groups of three-generation Syrian families. The violence exposures of three generations (F1, F2, F3) for each group are indicated—the 1980 group was directly, prenatally, and germline exposed in the F1 generation, the 2011 group was directly and prenatally exposed in the F2 generation, and the Control group was unexposed. Exposure types are color coded: red = direct exposure, green = prenatal exposure, blue = germline exposure, and yellow = no exposure. (**b**) Description of exposure groups and primary analytic exposure comparisons. Top panel: Average year of birth, type and time of violence exposure, and time of sample collection are shown for each generation in all three groups. Line color corresponds to exposure type: red = direct exposure, green = prenatal exposure, blue = germline exposure, and yellow = no exposure; dotted line indicates time from oocyte to birth, solid line indicates time from birth to present; vertical orange line indicates year of violence exposure, vertical purple line indicates year of sample collection; righthand labels indicate generation within recruited families: F1 = grandmother, F2 = mother, F3 = child. Bottom panel: participant groups included in each EWAS exposure analysis. Label colors correspond to the color legend in (**a**) and background colors correspond to the background colors in the top panel of (**b**).

is one of the main epigenetic factors that control gene regulation in mammals, plays a critical role in cellular differentiation and reprogramming, and is sensitive to environmental changes[17].

Substantial evidence supports the premise that variation in DNAm may mediate the impact of maternal stress and trauma on a range of offspring health outcomes. Specifically, maternal stress and trauma have been associated with changes in newborn DNAm and epigenetic age acceleration as well as indicators of worsened health outcomes such as diabetes[18–22]. More speculative is the idea that trauma-associated DNAm marks may be transmitted to future generations in humans[4,23,24]. Transmission to the F2 and F3 generations is considered intergenerational epigenetic inheritance, whereas transmission to the unexposed F4 generation and beyond is termed transgenerational epigenetic inheritance[14]. Although the mammalian epigenome is wiped clean of most DNAm marks during gametogenesis and embryogenesis to allow epigenetic reprogramming[25], there is intriguing support from animal models for environmentally-induced epigenetic marks that resist epigenetic reprogramming and are intergenerationally and transgenerationally inherited with phenotypic effects[26,27]. Transgenerational epigenetic inheritance, including in response to environmental stimuli, is well-documented in yeast, plants, fish, *Caenorhabditis elegans* and *Drosophila*[28–33]. There are fewer examples of epigenetic inheritance in animal models, but a recent study reported DNAm inheritance in mice up to four generations[34]. In contrast, only a single study in humans has reported associations of trauma exposure in grandmothers with DNAm changes in grandchildren[35]. The study of epigenetic inheritance in humans is complicated because of the difficulty of conducting multigenerational, experimentation studies as well as the importance of cultural and environmental factors[4]. Overall, the changes in DNAm in humans following fertilization are complex and intricately timed, attesting to the importance of DNAm in early development[36] and supporting the possibility that a small number of DNAm marks may have evolved that are sensitive to the environment and transmissible to future generations.

In this study, we use the DOHaD hypothesis as a framework to investigate epigenetic variation as a mechanism that mediates the impact of psychosocial trauma on future generations. Specifically, we propose the presence of differentially methylated positions (DMPs) in the human epigenome that are sensitive to the psychosocial environment, specifically violence, and are transmissible to future generations. We present results from an epigenome-wide association study (EWAS) in which we test for associations between DNAm and war-related violence in a cohort of Syrian refugee families with contrasting direct, prenatal, and germline exposures to war violence. Our three-generation study cohort with distinct violence exposures is novel among human studies in focusing directly on intergenerational epigenetic signatures. Our hypothesis, namely that exposure to violence may create intergenerational epigenetic marks, has profound implications for our understanding of evolution, such as how a lived experience can become embedded in the genome in an intergenerational manner.

Results

Unique three-generation study design

We used a three-generation study design to identify DNAm signatures of contrasting developmental exposures to violence—*direct* exposure of a living person, *prenatal* exposure of a fetus, and exposure of the *germline*. We included contrasting groups of three-generation Syrian families, currently living in Jordan (Fig. 1). The first two groups were exposed to violence that erupted during regional conflicts: first, the Hama city massacre in Syria that started in 1980, and second, the Syrian uprising and subsequent armed conflicts beginning in 2011. In the 1980 exposure group, maternal grandmothers were pregnant before fleeing Syria, meaning their daughters (F2 generation) were prenatally exposed and their grandchildren (F3 generation) were germline exposed to war violence. In the 2011 exposure group, mothers (F2 generation) were pregnant before fleeing Syria, meaning the developing fetus was prenatally exposed in utero, while older children in the same family (F3 generation) were directly exposed to conflict. In the control group, Syrian grandmothers and mothers lived in Jordan prior to 1980. Survey data and buccal swab samples were collected from mothers (F2 generation) and 1–2 children (F3 generation) in each family (two F1 grandmothers were also included, one each in the 1980 and control groups). The study included 131 participants and 48 families.

Directly exposed participants included one grandmother in the 1980 exposure group (F1) and mothers and older children in the 2011 exposure group (F2 and F3, n = 42) (indicated in red in Fig. 1a,b). They endured violent traumatic experiences that included being severely beaten, being persecuted (by the authorities/militia),

seeing a wounded or dead body, and seeing someone else severely beaten, shot or killed; Trauma Events scores were calculated as an unweighted count of affirmative answers to a Traumatic Events Checklist[37,38]. The intergenerational effects of direct violence exposures could be isolated allowing us to compare directly vs prenatally vs germline exposed individuals (see comparison groups in Fig. 1b). Specifically, associations with direct exposure (1980 grandmothers, 2011 mothers, and 2011 older children) vs prenatal exposure (1980 mothers and 2011 younger children) vs germline exposure (1980 younger and older children) were compared to control unexposed participants (Control grandmother, mothers, and children). Study sample characteristics, including demographic and trauma information, for the different comparison groups are presented in Table 1.

Epigenome wide association study

DNAm data were generated using the Illumina EPIC BeadChip that assays over 850,000 CpG sites. We conducted an EWAS to identify differentially methylated positions (DMPs) that were associated with each violence exposure. A two-stage analytic approach was used. In stage one, we performed three separate EWAS of violence trauma using robust linear regression with robust standard errors. For each violence trauma category (direct, prenatal, or germline), site-specific beta-values were modeled as a function of exposure to violence trauma relative to controls (yes/no), adjusting for age at buccal swab collection, sex, and DNAm-based estimates of the proportion of epithelial cells. In stage two, site-specific beta-values were modeled as a function of the same covariates using generalized estimating equations (GEE) to account for clustering by family. This two-stage approach leveraged the ability of robust regression to minimize the influence of DNAm outliers while GEE accounted for the family relatedness that was inherent to the study design. The final set of DMPs was determined by the overlap in DMPs that met strict Bonferroni correction for multiple testing (p value—6.5E−8) in both the robust regression and GEE EWAS.

The final set of 35 DMPs included 14 probes that were associated with germline exposure to violence and 21 probes that were associated with direct violence exposure (Fig. 2, Supplementary Table S1). No DMPs were significantly associated with prenatal exposure to violence. This is the first report of a germline DNAm signature of violence trauma in humans using a cohort study with an unexposed comparison group, which confirms our hypothesis of an intergenerational epigenetic signature of violence. There was no overlap in DMPs associated with germline vs direct violence exposure. However, 32 DMPs showed the same directionality in DNAm differences across all three exposures, suggesting these DMPs may have the same epigenetic response to violence across exposures (Fig. 2).

The largest difference in DNAm relative to unexposed controls was observed at a germline-associated DMP, cg01490163, with lower DNAm among those exposed in germline (Difference: − 0.265, 95% confidence interval (CI) − 0.349, − 0.181) (Fig. 2). The site is approximately 3 kb upstream of Keratin 36 (*KRT36*), which produces keratin and has a potential role in some cancers, and had pronounced inter-individual variability in DNAm. Compared to controls, the highest DNAm was observed at the germline-associated DMP, cg07462448, and two direct-associated DMPs, cg14117527 and cg14832449. Site cg07462448 is annotated to Caspase 7 (*CASP7*), which

	Control (N = 42)	Direct exposure (N = 43)	Prenatal exposure (N = 28)	Germline exposure (N = 18)	Overall (N = 131)
Exposure group					
Control	42 (100%)	0 (0%)	0 (0%)	0 (0%)	42 (32.1%)
2011	0 (0%)	42 (97.7%)	19 (67.9%)	0 (0%)	61 (46.6%)
1980	0 (0%)	1 (2.3%)	9 (32.1%)	18 (100%)	28 (21.4%)
Generation					
F1 Maternal grandmother	1 (2.4%)	1 (2.3%)	0 (0%)	0 (0%)	2 (1.5%)
F2 Mother	16 (38.1%)	22 (51.2%)	9 (32.1%)	0 (0%)	47 (35.9%)
F3 Younger child	16 (38.1%)	0 (0%)	19 (67.9%)	10 (55.6%)	45 (34.4%)
F3 Older Child	9 (21.4%)	20 (46.5%)	0 (0%)	8 (44.4%)	37 (28.2%)
Age					
Median [Min, Max]	13.5 [1.50, 64.0]	26.0 [6.00, 58.0]	4.00 [1.50, 41.0]	7.50 [3.00, 18.0]	12.0 [1.50, 64.0]
Sex					
F	28 (66.7%)	37 (86.0%)	14 (50.0%)	12 (66.7%)	91 (69.5%)
M	14 (33.3%)	6 (14.0%)	14 (50.0%)	6 (33.3%)	40 (30.5%)
Proportion of epithelial cells					
Median [Min, Max]	0.780 [0.310, 0.964]	0.779 [0.0500, 0.975]	0.824 [0.0737, 0.966]	0.824 [0.294, 0.970]	0.787 [0.0500, 0.975]
Trauma events*					
Median [Min, Max]	0 [0, 2.00]	10.5 [6.00, 17.0]	10.5 [3.00, 19.0]	8.50 [3.00, 19.0]	8.00 [0, 19.0]
Missing	0 (0%)	1 (2.3%)	2 (7.1%)	2 (11.1%)	5 (3.8%)

Table 1. Study sample characteristics. *Trauma events is a measure of the exposure of the directly exposed individuals (1980 F1 and 2011 F2 and 2011 F3 Older Child). In Prenatal and Germline exposure groups, the Trauma events score measures the exposure of the directly exposed ancestor of each individual in these groups. The Trauma events score varies according to the directly exposed individuals that were included in each exposure group in the table.

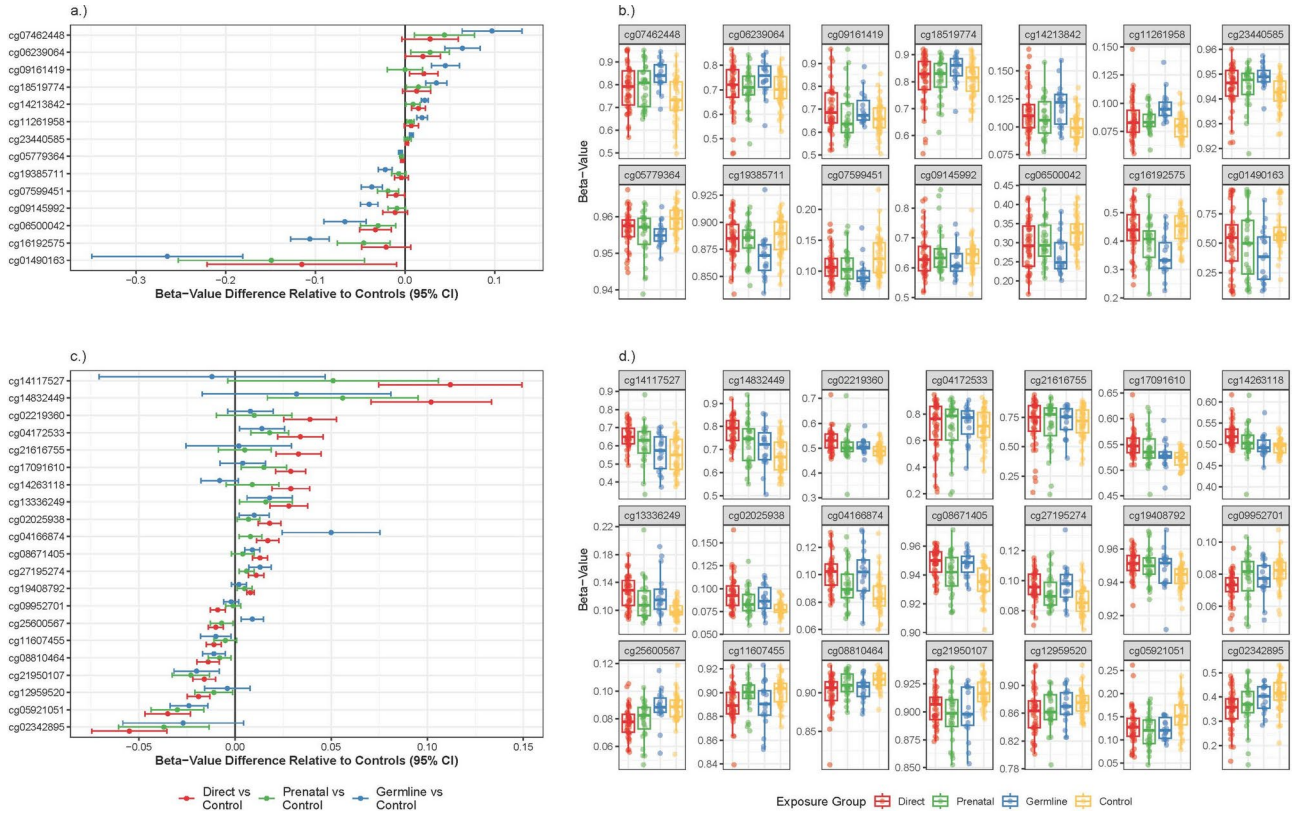

Fig. 2. Genome-wide significant differences in site-specific DNA methylation when comparing violence exposure groups and controls. Forest plots show DNAm levels for loci that reached genome-wide significance (p-value $< 6.5 \times 10^{-8}$) in both robust regression and GEE EWAS when comparing germline exposure to violence (**a,b**) and direct exposure to violence (**c,d**) to controls. Panels (**a**) and (**c**) are forest plots of the beta-value differences between all exposures relative to controls for all significant DMPs. The dots indicate the median difference and bars represent the 95% confidence intervals. Panels (**b**) and (**d**) are boxplots of the distribution of beta-values by violence exposure category; the middle line indicates the median, the box covers the interquartile range, and dots indicate observed beta-values. Exposure types are color coded: red = direct exposure, green = prenatal exposure, blue = germline exposure, and yellow = no exposure.

belongs to a family of proteins that play a central role in cell apoptosis. Site cg14117527 is annotated to *RAB43/ISY1-RAB43* (RAB43 is involved in membrane trafficking pathways and cellular homeostasis and is a member of the RAS oncogene family, and the ISY1-RAB43 readthrough transcript) and cg14832449 is annotated to *RP11-1028N23.3*, which is a long non-coding RNA.

As stated above, 32 of the 35 identified DMPs showed the same directionality in DNAm differences across all three exposures, e.g. the germline-associated cg1490163 showed a statistically significant − 0.265 reduction in DNAm in germline-exposed individuals, but also showed − 0.115 and − 0.149 reductions in DNAm in direct and prenatally exposed individuals, respectively, that were not significant. This result suggests that the 32 DMPs may function similarly across all three exposures, but our small sample size may be insufficient to detect such an effect at statistical significance. In the 1980s, DNAm was proposed to silence genes by adding a methyl group to cytosines in gene promoters that prevented binding of transcription factors, i.e. increased DNAm resulted in decreased gene expression[39]. Subsequent research has shown the effect of DNAm to be more complex as studies have shown that increased DNAm can result in both increases and decreases in gene expression depending on the specific gene, part of the gene, gene context, stage of development, cell and tissue types, and furthermore, that DNAm can induce enhancers to convert to silencers and silencers can act as enhancers[40–42]. Thus, we cannot say if gene expression is increased or decreased as a result of altered methylation at the DMPs we identified, but the fact that the directionality of DNAm changes is similar at 32 DMPs across all three exposures suggests the effect on gene expression is also similar.

Many of the significant DMPs occurred within regions related to regulation of gene expression since these regions are enriched on the EPIC BeadChip. Seven out of 35 DMPs occurred in CpG islands (regions with a high frequency of CpG sites) and seven DMPs occurred in CpG shores (regions that flank CpG islands) (Supplementary Table S1). Twenty-three DMPs occurred in gene bodies or *cis*-regulatory regions including 5′ & 3′ UTR (5′ & 3′ untranslated regions) and TSS200 & TSS1500 regions (200 & 1500 nucleotides upstream of the transcription start site). Although the frequency of DMPs in these regulatory regions was higher than their frequency in the set of assayed CpG sites, the enrichment was not statistically significant. Furthermore, gene

ontology analyses revealed no enrichment for biological processes in the top ~ 5000 DMPs, even when using a relaxed false discovery rate adjusted p value of 0.1.

We conducted sensitivity analyses to test the robustness of our results to the distribution of age at sample collection. All germline-exposed participants were from the F3 generation, which meant they were younger than the average age of the control F1/F2/F3 participants (Table 1). When we restricted the comparison of germline-exposed participants to F3 controls, 8 of 14 original DMPs maintained genome-wide significance, and all association estimates were consistent between the original and sensitivity analysis (Supplementary Fig. S1). Only one DMP had a much smaller p value (cg14213842, $p = 6.3E{-}3$) that suggested its significance was dependent on the older mothers who were removed in the sensitivity analysis. For our analysis of direct exposure to violence, we evaluated the consistency of our estimates when restricting the analysis to the F2 and F3 generations (removing two F1 participants); all 21 DMPs remained significant values (data not shown).

To determine if there was a linear relationship between the number of violence trauma events and DNAm, we plotted DNAm against the cumulative number of violence trauma events, i.e. Trauma Event score, reported by each participant for each of the 35 DMPs (Fig. 3). A visual inspection of the plots suggests that virtually all DMPs showed a dose–response relationship between DNAm and the number of trauma events (except the flat lines of direct-associated cg04172533 and cg21616755). This result suggests that additional or continuing trauma leads to further shifts in DNAm at the majority of DMPs, rather than a threshold effect of trauma on DNAm.

Epigenetic age acceleration

We also tested for epigenetic age acceleration in association with violence trauma. Epigenetic aging is the deviation between biological and chronological age and there is evidence that specific forms of early life adversity can accelerate epigenetic aging[43–45]. Many epigenetic clocks have been developed to estimate epigenetic age since epigenetic age varies with age and tissue type. The first generation of epigenetic clocks were trained across multiple ages and multiple tissues, but specialized clocks have been developed recently, such as Horvath's Skin and Blood clock[46] and PedBE[47], which is trained on pediatric buccal samples. Given our use of buccal samples, we chose to use the PedBE and Skin and Blood clocks as well as the pan-tissue clock that is trained on chronologic age across many tissues[48].

As expected, there was a very high correlation between epigenetic and chronological age in our study sample ($r = 0.94$–0.99; Supplementary Fig. S2). Compared to the children, mothers showed much higher variation in epigenetic age relative to chronological age. We used GEE to estimate the association between epigenetic age and violence exposure category, adjusting for age, sex, and cell type proportion, and incorporating clustering by family. When we analyzed all the mothers and children in our dataset (F2 + F3; n = 129), there was no significant association between epigenetic age acceleration and trauma exposure using any of the epigenetic clocks (Fig. 4, Supplementary Table S2). However, when we analyzed the children only (n = 82), prenatal exposure to violence trauma was significantly associated with epigenetic age acceleration using the PedBE and Skin and Blood clocks. This is the first report of epigenetic age acceleration with prenatal exposure to violence trauma, highlighting the critical period of in utero development. Consistent with this interpretation, a recent study reported that childhood sexual abuse was associated with both epigenetic age acceleration and accelerated HPA axis trajectory, suggesting epigenetic aging and HPA activity as an integrated mechanism that mediates the impact of trauma on later life health and aging[49].

Discussion

Our study is novel in leveraging a three-generation study design and contrasting developmental exposures to war-related violence among hard-to-reach refugee groups. Results show, for our sample of Syrian refugees, that 14 DMPs were associated with germline exposure to violence and 21 DMPs were associated with direct exposure to violence. Thirty-two of these sites showed a similar directionality of change in DNAm across all three (germline, prenatal and direct) exposures to violence, suggesting the existence of a common epigenetic signature of violence across multiple stages of development. Our results support the existence of an intergenerational epigenetic signature of trauma in humans.

There is strong scientific evidence indicating that impacts of stress and trauma can reverberate far into the future, possibly through epigenetic mechanisms[19,20,24,50–52]. It is not clear if environment-associated DMPs, such as those reported here, are constitutively important for gene regulation and have immediate and causal impacts on phenotype. Instead, these epigenetic marks may enable flexibility or enhanced responses to future stressful experiences, a concept known as epigenetic "priming"[53,54]. Alternatively, some marks may simply serve as biomarkers of stressful exposures that could be used to identify vulnerable individuals who would benefit from intervention programs. A recent study proposes that environment-associated epigenetic marks may be best viewed as a "mixed bag" that includes sites with no regulatory effect plus a smaller number of sites that play a key role in gene regulation, sites that affect gene regulation only under certain conditions, and passive biomarkers[55].

In addition to the germline and direct violence-associated DMPs we report, we identified an association of epigenetic age acceleration with prenatal exposure to violence. In comparing our EWAS and epigenetic age results, it is important to remember that EWAS identifies single CpG sites that associate with violence exposures in contrast to epigenetic age analyses that use hundreds of CpG sites collectively to estimate epigenetic age—thus, we would not necessarily expect both analyses to yield the same results. Previous studies have also shown that early life exposures are more strongly associated with DNAm differences than later life experiences, even if the later experiences occurred closer to the time of sample collection[56,57]. There is also evidence from studies of environmental toxins that future generations may be more affected than those directly exposed[58]. Thus, prenatal and germline exposures and associated changes in DNAm, such as those we report, may be particularly impactful and are consistent with DOHaD tenets. Accelerated epigenetic aging is thought to correlate with accelerated biological aging and may be an underlying mechanism for age-related health outcomes[59]—our results suggest

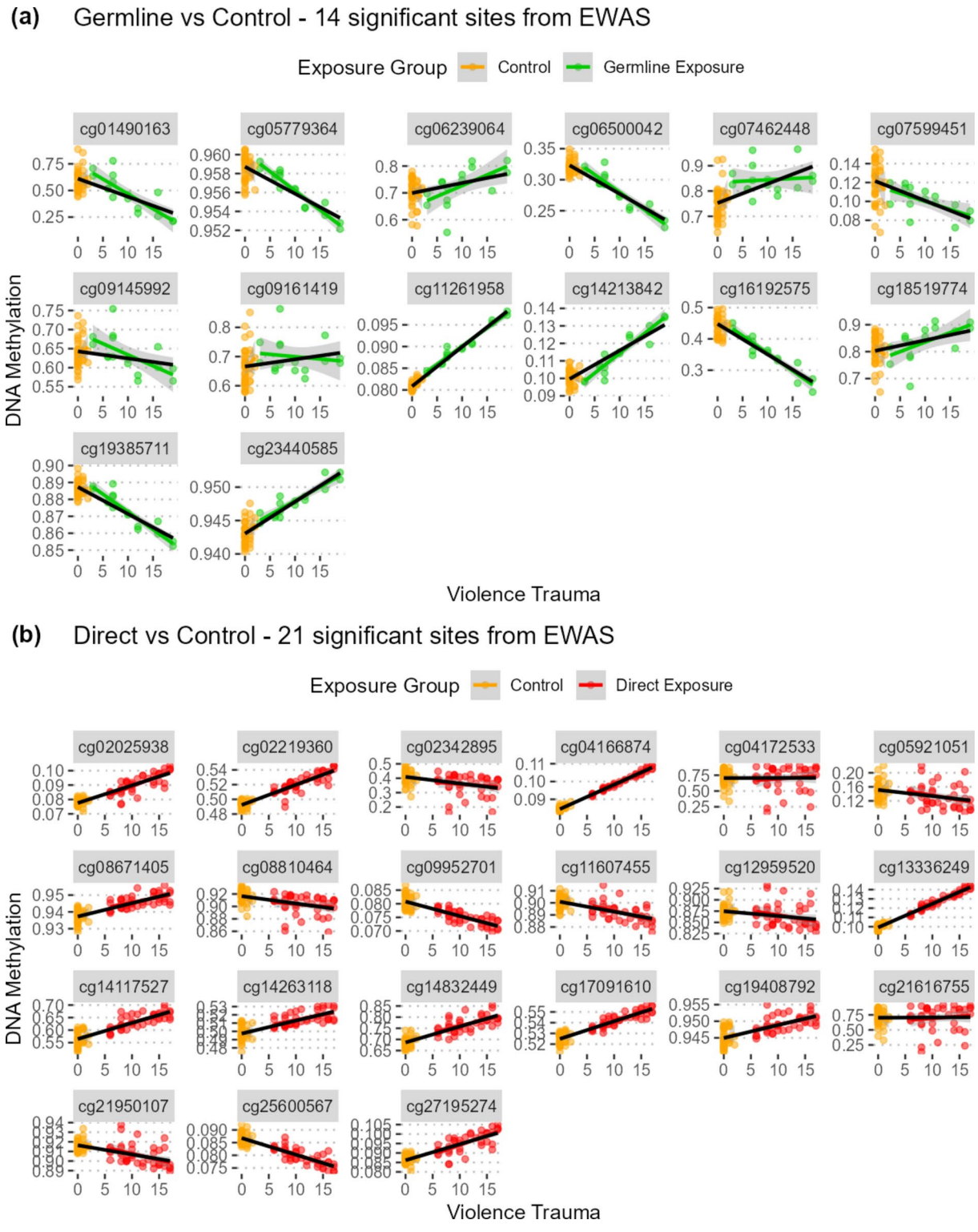

Fig. 3.. DNAm levels and violence trauma exposure scores. Plots show DNAm levels (Y axis) for individual Trauma Event scores (X axis) for (**a**) 14 germline exposure DMPs and (**b**) 21 direct exposure DMPs. Black lines are regression lines for all points and gray shading corresponds to 95% confidence intervals.

that the impact of violence may accelerate the aging trajectory and compound the tragedy of the initial violence exposure.

Our study has both strengths and limitations. The research design—namely, a multi-generational study of Syrian refugees with direct, prenatal, and germline exposures compared to Syrians with no direct exposure of war—is a major strength. We were able to approximate the kind of experimental, intergenerational study

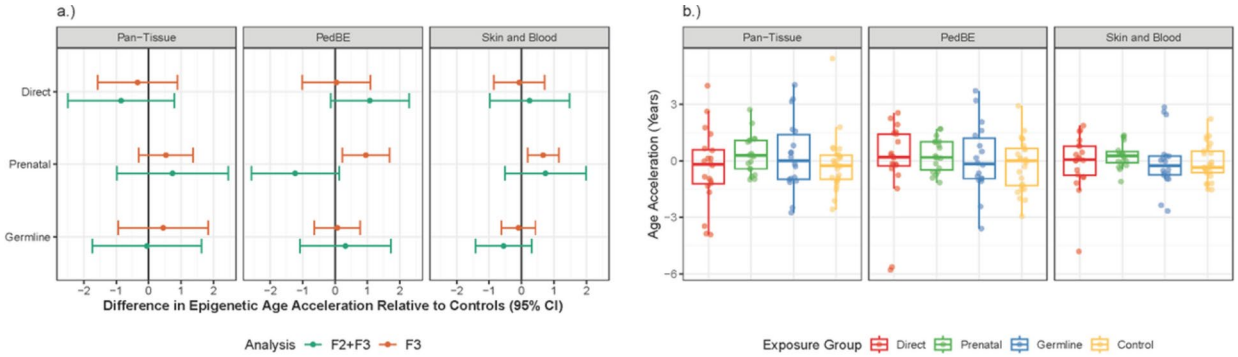

Fig. 4. Epigenetic age acceleration and violence exposure. (**A**) Forest plots show differences in epigenetic age acceleration among those exposed to violence relative to controls, comparing the analyses of F2 + F3 (mothers and children) and F3 (children). (**B**) Boxplots of the distribution of epigenetic age acceleration (residual variation independent of chronological age) by violence exposure category in F3/children. The middle line indicates the median, the box covers the interquartile range, and dots indicate observed Age Acceleration values.

design typically only possible in animal models with the following three limitations. First, we collected buccal samples, rather than blood, to minimize invasiveness of data collection for refugee women and children. The choice of study tissue is critically important in DNAm studies, since tissue-dependent differences in DNAm are one way in which gene expression is regulated to differentiate cell types. Human studies have primarily focused on peripheral tissues, such as blood samples, since tissues that may be more likely to show phenotypic effects, e.g. brain tissue to study cognitive effects, are not easily accessible in humans. Although buccal cells are understudied, there is evidence to suggest they may be more informative for DNAm studies than blood samples since buccal cells contain more hypomethylated sites that overlap in other tissues, and these sites are enriched for regulatory regions such as DNase I hotspots and genetic variants associated with phenotypic outcomes[60]. Furthermore, buccal cells are derived from the same embryonic tissue as the brain and, thus, may be informative for studies on the impact of psychosocial stressors such as violence. Second, our findings are based on relatively small sample sizes, although this limitation is typical of genetic studies in human refugee populations. Finally, it is possible that other epigenetic modifications, such as histone modifications or non-coding RNAs, may be transmitted intergenerationally and could also play a role in mediating the impact of war violence on future generations.

Conclusions

Much work remains to be done, including more intergenerational data collection on diverse population groups and causal validation of mechanistic pathways. Skinner and colleagues' 2023 proposal that future generations many be more impacted than those directly exposed reinforces their call for more transgenerational epigenetic studies to address issues of transgenerational environmental justice[58]. Our study responds to this call with support for the intergenerational epigenetic transmission of violence trauma in humans through the identification of novel DNAm sites and evidence for epigenetic age acceleration. These results indicate important directions for future research.

Our findings are relevant to research and intervention efforts focused on the plight of violence-affected populations. One recent paper called for a stronger evidence base for humanitarian action and proposed that "epigenetic research can be potentially beneficial to address some of the issues associated with refugees and asylum seekers"[61]. We propose that a better understanding of the nature and long-term effects of intergenerational trauma may encourage policymakers and humanitarian agencies to provide targeted resources to vulnerable populations, including healthcare access, special lodging, sanitation, and nutrition. We note the scale of the global refugee crisis: by the end of 2022, there were 108.4 million forcibly displaced people worldwide, including 62.5 million internally displaced people, 35.3 million refugees, and 5.4 million asylum seekers[62]. Our results also have clear relevance for societies such as the U.S. given high levels of interpersonal violence, particularly against women[63,64]. The presence of a heritable epigenetic signature of violence has important implications for addressing some of society's most vexing problems, including multigenerational cycles of violence, abuse, and poverty. Specifically, the possibility that the impacts of these traumas may be mediated by epigenetic mechanisms and passed on to future generations may change the scope of prevention efforts, discourage "victim-blaming" in instances of intergenerational trauma, and spur policymakers to dedicate more resources to programs to alleviate violence, abuse, and poverty.

Finally, we recognize the resilience of traumatized and marginalized populations around the world who have survived and flourished in the face of adversity[65]. The role of epigenetics in explaining individual differences in psychosocial resilience has been under-studied. A better understanding of epigenetic mechanisms, with data from hard-to-reach groups in cross-cultural contexts, would enrich empirical knowledge and theoretical understanding of human development. We propose that future research adopt a positive framework to investigate

epigenetic signatures of adaptive mechanisms that underly the tenacity and successful expansion of human populations throughout evolution[66].

Methods

Study design

Three groups of three-generation Syrian families with contrasting exposures to violence were sampled (Fig. 1). Two regional conflicts were used to define the exposure groups—the Hama city massacre in 1980 and the Syrian uprising and subsequent armed conflicts beginning in 2011. Survey data and buccal swab samples were collected from mothers and their children in all exposure groups (and from two grandmothers, one each in the 1980 and Control groups). Details on the families and family members included in the study are provided in Supplementary Table S3.

The study received ethical approval from The Hashemite University, Amman, Jordan (IRB ID 171.99E approved on 3/9/2014) and Yale University (IRB ID 1502015359 approved on 4/24/2015). Written informed consent was obtained in Arabic from all participants or their guardians prior to the start of the study. This study was conducted in accordance with the principles of the Declaration of Helsinki.

Study population

Participants, recruited in Jordan via snowball sampling, were Syrian women who had experienced war and been pregnant during the 1980 or 2011 conflicts, then fled to Jordan, as well as Syrian families who moved to Jordan before 1980. Inclusion criteria were: (1) Syrian families who fled armed conflict (all but two fled to Jordan; these two were recruited through their Syrian relatives living in Jordan and had fled to Saudi Arabia or Lebanon); (2) for exposure groups, exposure to either the 1980 or 2011 conflict, i.e. no double exposures; (3) for the 1980 group, the grandmother was pregnant with a daughter during the 1980 conflict and the daughter subsequently gave birth to two children, (4) for the 2011 group, the mother was pregnant during the 2011 conflict and also had an older child; we refer to the older and younger child to distinguish who had a direct and prenatal war exposure, respectively. The 1980 conflict began in June 1979 and continued through 1980 while the 2011 conflict began in March 2011 and lasted through June 2012; most mothers and grandmothers in our study experienced violence exposure during multiple trimesters, or their entire pregnancies, so we cannot distinguish exposure during specific trimesters.

Families were recruited between November 2015 and December 2020. Survey data and buccal samples were collected from the mother and children for 10 families in the 1980 exposure group (two families did not have an older child, one family did not include a sample from the mother, and one family included a sample from the maternal grandmother), for 22 families in the 2011 exposure group (two families did not have an older child and three gave samples of insufficient quality for the younger child), and for 16 families in the Control group (seven families did not have an older child and one family included a sample from the maternal grandmother). The final sample size of buccal samples was 48 families with 131 individuals, including 45 younger children, 37 older children, 47 mothers, and two grandmothers.

Participant recruitment began with the informed consent process. Women were interviewed in Arabic by DH, whose family are Syrian refugees and part of the refugee community in Jordan. Interviews were conducted in person at which point the study was explained and participants were screened for exposure to only one of the violent conflicts in 1980 or 2011, written informed consent was obtained, demographic data were collected, the Traumatic Events Checklist[37,38] was administered, and buccal swabs were collected. The Traumatic Events Checklist is a yes/no checklist that asks participants if they had experienced a particular traumatic event. Trauma Events scores were calculated as an unweighted count of affirmative answers. Mothers were interviewed about their experiences during the 2011 conflict as well as the experiences of the older child who was too young to accurately report on the conflict. Grandmothers were interviewed about their experiences during the 1980 conflict unless they were deceased, in which case the mother was interviewed. Interviews and buccal swab collection generally occurred during a single visit.

Study sample characteristics

Study sample characteristics are shown in Table 1. Information on age, sex, and birth order of study participants was collected during the interview. Proportions of epithelial cells were calculated from DNAm data using robust partial correlation with the EpiDISH package[67]. A Trauma events score was calculated as an unweighted count of affirmative answers to the Traumatic Events Checklist[37,38].

Buccal sample collection and DNA extraction

Buccal samples were collected using Transport Swabs (APCO Laboratory Consumable Plastic, Jordan) or DNA Buccal Swabs (Isohelix, United Kingdom). Participants rinsed their mouths with water and then brushed both sides of their mouth with the collection swab for up to 30 s. DNA was extracted from the buccal swabs using either the Qiagen DNA Investigator Kit (Qiagen, USA) or Xtreme DNA Isolation Kit (Isohelix, United Kingdom). DNA extractions were performed according to manufacturer's recommendations with the exception that the AW2 wash was performed twice for swabs extracted using the Qiagen kit.

DNA methylation

DNA was hybridized to the EPIC BeadChip (Illumina, San Diego) at the Hussman Institute for Human Genomics, University of Miami. Raw .idat files were processed using R version 4.2.0 (R Core Team 2021). The SeSAMe package was used for non-linear dye-bias correction, detection p-value calculation, and noob background correction[68]. ComBat was used to adjust for bisulfite conversion plate[69,70]. The EpiDISH package was used to estimate proportions of epithelial cells, immune cells, and fibroblasts[67].

Probe-level quality control was performed as follows. Probes with zero intensity or cross-reactivity with genomic regions, non CpG probes, probes with fewer than three beads, and sex chromosome probes were all masked. In addition, probes that failed to reach a significant detection p-value ($p < 0.05$) for more than 10% of the samples were masked. There were 768,625 probes available for analysis, corresponding to a Bonferroni-corrected p-value of 6.505×10^{-8}.

Sample-level quality control was performed using the R packages ewastools[71] and meffil[72] to check for sample contamination and sample identification confirmation, respectively. An epigenetic predictor of age[46] was also used to confirm sample identity. Finally, a custom script comparing genotyping results from the EPIC array across all samples was used to test for duplicated samples. Poorly performing samples were identified using the ratio of methylated to unmethylated probes in the meffil package[72].

For all analyses of DNAm data, see the figshare files listed under the Code Availability statement for relevant R scripts and associated data files.

Epigenome-wide association of violence trauma

A two-stage analytic approach was used to identify associations between violence trauma and DMPs. In stage one, robust linear regression with robust standard errors was used to conduct three separate epigenome-wide association studies (EWAS) of violence trauma. For each violence trauma category (direct, prenatal, or germline), site-specific beta-values were modeled as a function of exposure to violence trauma relative to controls (yes/no), adjusting for age at time of sample collection, participant sex, and proportion of epithelial cells. For each exposure group, different generations were combined, such that we controlled for age at time of collection in all analyses. Participant sex and cell type proportion was also controlled. There was no sustained smoking among participants, so smoking was not included as a covariate. Parity (or birth order) and socioeconomic status were each considered as possible covariates, but missing data prevented their incorporation into analyses. A Bonferroni-corrected *p*-value of 6.5E−8 was used to identify significantly associated DMPs.

In stage two analyses, generalized estimating equations (GEE) were used to specify clustering by family relatedness inherent in the study design (with an exchangeable correlation structure). The same EWAS was conducted as in stage one, with the same covariates, but with the addition of clustering by family. The final set of DMPs was determined by the overlap in DMPs that met Bonferroni correction in both the robust regression and GEE EWAS. Significant DMPs were annotated using a published annotation[73].

Sensitivity analyses

The robustness of results was tested by comparing subsets of comparison groups to better control for age differences. For the direct versus control comparison, the two grandmothers were removed to restrict the comparison to two generations (F2 + F3) instead of three (F1 + F2 + F3). In the germline versus control comparison, the comparison was restricted to 1980 and Control children. In a second set of sensitivity analyses, a dose–response relationship between the cumulative number of reported traumatic events and DNAm was tested at the significant DMPs.

Enrichment analyses

GOmeth[74] was used to test for enrichment of biological processes among the top DMPs. We first filtered the GO terms tested for biological processes with 20–200 DMPs annotated to each process. Then, for each of our three comparisons, a *p*-value cutoff was selected for the top DMPs to yield ~ 5000 DMPs, specifically 5193 DMPs for direct versus control ($p < 0.00045$), 5070 DMPs for prenatal versus control ($p < 0.0047$), and 5111 DMPs for germline versus control ($p < 0.0015$). A relaxed false discovery rate adjusted *p*-value cutoff of 0.1 was used for each enrichment analysis.

Epigenetic age estimation

Based on prior studies[75], we estimated epigenetic age based on: the pediatric buccal clock (PedBE)[47], the Horvath Skin and Blood Clock (Skin and Blood)[46], as well as a foundational Pan-Tissue clock that has been used in most prior studies of exposure to violence and epigenetic aging in children[48]. These clocks are known to perform particularly well with pediatric buccal swab DNA, making them well-suited for the current study.

Raw methylation data were noob normalized[76] using the SeSAMe package[68] prior to epigenetic age calculation using the algorithms corresponding to the PedBE, Skin and Blood, and Pan-Tissue clocks. The methylCIPHER[77] package was used to compute epigenetic age for the PedBE, Skin and Blood, and Pan-Tissue clocks.

Epigenetic age acceleration was estimated as the residual variation in each clock independent of chronological age, modeled linearly. GEE were used to model epigenetic age acceleration as a function of violence trauma category (direct, prenatal, germline; referent: controls), adjusting for chronological age at time of sample collection, participant sex, and epithelial cell proportion.

Data availability

Raw methylation data (.idat files) are available at gene expression omnibus under record number GSE226085.

Code availability

R scripts and associated data files for the following analyses are available at figshare: methylation data quality control—https://figshare.com/s/8c0ea5088435801782d2. epigenome-wide analysis—https://figshare.com/s/e62913140c6128fef796. epigenetic age quality control—https://figshare.com/s/804208ad194319ff20ff. epigenetic age acceleration analysis—https://figshare.com/s/48782e37381065963581

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Author contributions
C.J. Mulligan, C. Panter-Brick and R. Dajani designed the study; D Hamadmad and R.D. identified eligible families and collected buccal swab samples and demographic, trauma, and socioeconomic data; E.B. Quinn, C.L. Dutton, and L. Nevell generated DNAm data and performed data analyses; A.M. Binder performed or supervised DNAm analyses; E.B.Q and A.M.B. created tables and figures; C.J.M. wrote the manuscript with input from all other authors.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
The study received ethical approval from The Hashemite University, Amman, Jordan (IRB ID 171.99E approved on 3/9/2014) and Yale University (IRB ID 1502015359 approved on 4/24/2015) and the University of Florida approved the project as exempt (IRB201901822 approved on 7/1/2019). Written informed consent was obtained in Arabic from all participants or their legal guardians prior to the start of the study. This study was conducted in accordance with the principles of the Declaration of Helsinki.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-89818-z.

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