Research Article

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Epigenomics

Intergenerational effects of maternal post-traumatic stress disorder on offspring epigenetic patterns and cortisol levels

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Aim: To investigate the association between maternal post-traumatic stress disorder (PTSD) during pregnancy and offspring DNA methylation and cortisol levels. **Materials & methods:** Blood genome-wide DNA methylation and cortisol was measured in the youngest child of 117 women who experienced sexual violence/torture during the Kosovo war. **Results:** Seventy-two percent of women had PTSD symptoms during pregnancy. Their children had higher cortisol levels and differential methylation at candidate genes (*NR3C1*, *HTR3A* and *BNDF*). No methylation differences reached epigenome-wide corrected significance levels. **Conclusion:** Identifying the biological processes whereby the negative effects of trauma are passed across generations and defining groups at high risk is a key step to breaking the intergenerational transmission of the effects of mental disorders.

First draft submitted: 22 January 2021; Accepted for publication: 28 April 2021; Published online: 17 May 2021

Keywords: cortisol • DNA methylation • epigenetics • intergenerational • maternal stress • offspring • PTSD

Background

Post-traumatic stress disorder (PTSD) is a highly debilitating and severe psychiatric condition. It is incident-specific and assumes causation, resulting as a direct consequence of exposure to a single traumatic event or from chronic trauma exposure [1]. Diagnosis of PTSD requires the manifestation of a combination of four clusters of symptoms following the event, which includes reexperiencing, avoidance, hyperarousal and negative alterations in cognition and mood [2]. Although most individuals have the capacity to recover after exposure to trauma [3], certain types of severe trauma are associated with an increased risk of PTSD (NICE Clinical Guidelines) [4] and the incidence is higher in women than men [5].

For many years, Kosovo has suffered from violent conflicts between the Serbian and Albanian peoples. At the end of the 1990s, long-term tensions resulted in armed clashes between Serbian military and Kosovo Albanian civilians. Sexual violence against women and girls was widespread and severe enough to result in long-term trauma [6]. The estimated prevalence of PTSD symptoms in Kosovo Albanian women at the end of conflict in 1999 was 20% [7] and 22.7% in 2005 [8].

The importance of providing support, care and treatment for women who have experienced sexual violence, torture or other major traumatic events during conflict is widely recognized [9]. What remains less clear is how exposure to such trauma could have long-term effects on future generations [10]. Evidence suggests that children born to these traumatized women are more likely to have behavioral and clinical problems, including symptoms of PTSD, as well as other mental and physical health problems [11,12]. Early identification of affected children is

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key to timely interventions and treatment. Furthermore, previous research of war victims in Kosovo has also shown the impacts on health can also result in social and economic influences that can impact entire communities [13,14]. Parental PTSD has been shown to be associated with changes in the hypothalamic-pituitary-adrenal (HPA) axis function, including decreased cortisol levels in the offspring [15]. An unpublished systematic review, which recently looked at intergenerational transmission of PTSD between parents and their offspring, found that ten out of thirteen studies provided some evidence for transmission of PTSD to their offspring [16]. Most of these studies focused on war-related trauma but none included survivors of sexual abuse.

DNA methylation is an epigenetic mechanism that can regulate gene expression. Accumulating evidence suggest that the prenatal environment, including maternal mental health, can result in epigenetic dysregulation of gene functions, and may help explain the long-term effects on disease [17–19]. Interestingly, maternal PTSD has been shown to be associated with alterations in the epigenome of offspring, including decreased blood DNA methylation of the promoter region in the *NR3C1* gene which is involved in HPA-axis signaling, in adult offspring of Holocaust survivors [20]. Furthermore, maternal PTSD is associated with increased glucocorticoid receptor sensitivity [21], which may reflect an increased expression of the *NR3C1* gene.

Epigenetic biomarkers, or 'vulnerability variants,' have a great and as yet insufficiently exploited potential for initial risk stratification in screening and prevention programs. This includes the possibility to not only improve PTSD diagnosis but also, importantly, to establish early intervention for those offspring at high risk. This could help prevent conversion to full PTSD and relevant psychiatric diagnosis, but may also treat the symptoms of impaired cognitive, social and occupational functioning that are already present. As a first step toward breaking the vicious intergenerational cycle of trauma and violence, this study aimed to identify differences in DNA methylation (at the genome-wide level as well as candidate genes) and cortisol levels in offspring born to women exposed to wartime sexual violence, with and without PTSD during pregnancy.

Materials & methods

Study cohort

In total, 415 women survivors of sexual violence during the war in Kosovo were registered in the Kosovo Rehabilitation Centre for Torture Victims (KRCT) database. For inclusion in the present study the women had to be born in Kosovo, be of Albanian ethnicity, have a home address in Kosovo during the war in 1999, had given birth to at least one child after the war (not related to the sexual assault) and, finally, be able to take part in the study examinations at KRCT. Trauma exposure that was considered relevant to this study included: sexual harassment or violence, molestation, rape or insertion of a blunt object into a genital organ and/or the rectum. Excluded were mothers and offspring with mental or growth retardation, significant speech or cognitive impairment, with past or present schizophrenia, major alcoholic or substance abuse problems, blood/injection phobia, having recently undergone chemotherapy/radiotherapy or was to have such therapy within following 6 months to minimize confounding from these variables and/or impede assessments. A total of 200 women were thus eligible for the study. Of these women, we were able to invite 150 to participate given that previous contact had already been made between a KRCT therapist and these women, and they had provided consent for future contact. The therapists invited the 150 women to informative meetings for potential participation in the study where the aim of the study and the research methodology were explained. At this stage, some women declined for various individual or family reasons bringing the number of women included in the study interviews conducted during March–April 2019 to 128. The 128 women had a mean age of 43 years (ranging from 30 to 59 years) and had experienced sexual violence/torture during the Kosovo war when aged 10–37 years. Totally, ten women later declined to provide blood samples from their children, which resulted in 118 mothers bringing 120 children for blood sampling (two sets of twins).

Ethics statement

This study was approved by the commission for the ethical issues within Kosovo doctors chamber. The study was carried out in accordance with the Ministry of Health Central Ethics Committees in Kosovo, as per Kosovar Government guidelines, and with the Helsinki Declaration.

All participants who agreed to take part provided an informed consent. They were informed that they have the right to withdraw from the study at any time. Any participant suffering from adverse effects of trauma was referred to a psychologist or medical doctor at KRCT.

The information provided by the study participants was treated throughout the process with confidentiality according to the Kosovar law and Declaration of Helsinki II on biomedical research and complied with general data protection regulation.

Clinical assessments

All participants underwent clinical assessments and completed questionnaires. These were conducted by psychologists and psychiatrists at KRCT who had received intensive training using Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) and Clinician-Administered PTSD Scale (CAPS-IV). In addition to basic sociodemographic data (age, education, marital status, place of residence) and lifestyle factors (e.g. cigarette smoking), PTSD diagnosis was based on the DSM-IV criteria [22], using the CAPS-IV. The CAPS-IV is a 30-item semi-structured interview designed to assess current or lifetime diagnosis of PTSD and the severity of symptoms, which reflects a combination of symptom frequency and intensity. Furthermore, the participants were also asked explicitly if PTSD symptoms occurred during the pregnancy to determine PTSD in pregnancy. Questions are focused on the onset and duration of symptoms, the level of distress and the impact these symptoms have on the functioning of the individual. The CAPS-IV was translated and validated in Albanian language [23], was administered by trained clinicians and took about 45–60 mins. Scoring of the CAPS was according to the instruction manual. The CAPS-IV contains frequency and intensity scores (0–5) for each of the three symptom clusters (B, C, D) that are summed to produce a total severity score. To meet the PTSD criteria, a frequency of 1 or more and an intensity of 2 or more is required. A PTSD diagnosis is made if there is at least 1 'B' symptom, 3 'C' symptoms, and 2 'D' symptoms as well as meeting the other diagnostic criteria. The CAPS is highly regarded as the gold standard and valid instrument to assess PTSD, with good inter-rater and test–retest reliability, and internal consistency [24]. The CAPS-IV was used because CAPS-V was not translated and validated in Albanian language. Furthermore, KRCT's therapists had previous intensive training and practice using CAPS-IV for clinical diagnosis.

Blood collection

In May 2019, fasting blood samples were collected at the Tirana Laboratory, Pristina, Kosovo, by experienced laboratory technicians from 120 of the youngest children born to each woman, including two twin pairs. Two blood samples were drawn between 7:30 and 9:30 am, after 20 minutes of rest in a comfortable environment. One blood sample was collected in an 6 ml EDTA plasma tube, and directly stored at -80°C until later extraction of genomic DNA using the DNeasy DNA blood kit (Qiagen, Hilden, Germany). One blood sample was collected in an 6 ml tube (SARSTEDT AG & Co. KG, Nümbrecht, Germany) for direct measurements of morning cortisol concentration, using the electrochemical luminescence immunoassay (COBAS E411, Roche, Basel, Switzerland). We used the reference range for cortisol levels in the laboratory in Pristina (Kosovo) to define low and high cortisol levels.

DNA methylation profiling & bioinformatics

Extracted DNA from 120 offspring was sent to GenomeScan (Leiden, the Netherlands) in June 2019 and measurement of epigenome-wide DNA methylation was performed using the Illumina's Infinium HumanMethylationEPIC BeadChip (Illumina, CA, USA). Using the EZ-DNA Methylation Gold kit (Zymo, CA, USA), 500 ng was bisulfite converted, and then methylation measured according to the Infinium Protocol (Illumina).

DNA methylation data were obtained for two twin pairs; however, one from each pair (the last registered) were excluded from this analysis due to non-independence (sharing the same mother and thus genetics and exposure). Also excluded was one offspring where all covariate data were missing except maternal PTSD status, thus leaving 117 offspring for the following DNA methylation analyses.

Pre-processing and quality control of raw methylation data were undertaken in R (**http://www.bioconductor.org**; **http://cran.r-project.org**, version 3.6.1), using several packages including minfi [25]. Raw array intensity data was normalized using the stratified quantile normalization method (minfi::preprocessQuantile). Some probes were removed prior to analysis, including probes not discernible from background measurement noise (minfi::detection), probes on the X or Y chromosomes and probes that are known to be cross-reactive [26], or which contained a SNP [27]. This left a total of 625,431 probes (called 'CpGs') for analysis.

Following pre-processing, data were converted to β-values (minfi::getBeta) (average probe wise methylation within a sample, denoted between zero (unmethylated) and one (100% methylated) and M-values (minfi::getM) (M = log2[unmethylated signal intensity/methylated signal intensity]) [28]. To determine sources of variation

within the methylation data, principal component analysis was conducted using the WGCNA (v1.68) package. In particular, a number of factors were examined to determine the extent to which explained variation in methylation, which included: batch (ID, chip and well of array), maternal PTSD pregnancy status, current PTSD, maternal age, education level, living situation, smoking and offspring gender, age, estimated cell composition and cortisol level. To estimate the cell counts [29,30], estimateCellCounts2 from the FlowSorted.Blood.EPIC package was used [31].

To identify differentially methylated CpGs between maternal PTSD and control offspring, the limma package was used [32]. All p-values were adjusted for multiple testing using the Benjamini–Hochberg method, with a false discovery rate threshold of 5% [33]. To facilitate biological interpretation, percentage methylation was expressed as β-values (β = Meth/(Unmeth + Meth + 100)). To account for sensitivity of the array technology, we only considered effect sizes that were greater than 1% difference between the two groups. Further, R package DMRcate was used to identify differentially methylated regions (DMRs) across the genome, defined as at least 2 differentially methylated probes within a maximum of 4000 base pairs [34]. The GRCh37 (hg19) genome assembly was used to identify genomic location of the CpGs.

Candidate gene analysis

An analysis of key candidate genes was also performed. This was based on the published literature where CpG site specific or gene specific DNA methylation differences in children following early-life exposure to trauma and/or stress had previously been reported. Therefore, a literature search in the Medline database was performed using the following terms: 'DNA methylation,' 'PTSD' and 'child' or 'pregnancy' to identify the genes to be examined further.

Statistical analysis

Continuous variables were tested for normality. Normally distributed data are presented as mean (standard deviation) and compared by Student's t-test. Not normally distributed data are presented as median and interquartile range and compared by the Mann–Whitney U test. χ^2-test was used for analysis of frequencies. Limma models were used as a discovery method for differentially methylated CpGs adjusting for mothers age, the gender and age of the child, batch effects including chip number and chip well location, as well as estimated cell proportions of B cells ($CD19^+$), T lymphocytes ($CD4^+$ and $CD8^+$), monocytes ($CD14^+$), neutrophils and natural killer cells ($CD56^+$). Further multivariate linear regression models for DNA methylation considered adjustment for a number of other potential confounding factors including smoking during pregnancy, current PTSD status and cortisol levels. Current PTSD status was not found to be an important factor influencing children's DNA methylation levels, neither by principal component analysis, nor when adjusting for this factor in the regression models; it was therefore not included in final analysis shown. Effect sizes were reported as β-coefficients and 95% CI. Multinomial regression models were used to examine the association between PTSD during pregnancy and child's cortisol levels (normal, low or high), while adjusting for covariates. Assumptions of variance and normally distributed residuals were visualized in quantile–quantile (QQ) plots and histograms. Statistical analyses were performed using the SAS Enterprise Guide 7.1 statistical software (SAS Institute Inc., NC, USA).

Results

Maternal & offspring characteristics

Characteristics of the 117 mother-dyad pairs included in this study are presented in Table 1. Of the mothers, 72% (n = 84) reported PTSD symptoms during their pregnancy and 64% had current PTSD. Mothers with PTSD during pregnancy were also more likely to have lifetime PTSD, but there was no difference observed between mothers with and without PTSD during pregnancy in either city/rural living locations, educational level, marital status or smoking status. Of the children, there was no difference in the proportion of female and male participants; however, the children exposed to PTSD during pregnancy were on average 4 years older than the controls.

Offspring cortisol measurements

In total, 28 of the children (24%) exhibited abnormal (too high or low) levels of cortisol (Figure 1A). There was no statistical difference between children's cortisol levels (continuous measure) and mothers PTSD during pregnancy (Figure 1B). However, children born to mothers with PTSD during pregnancy were significantly more likely to have high cortisol levels (15.5%) compared with children born to mothers without pregnancy PTSD (3.0%), p

Table 1. Clinical characteristics of the study participants.

	PTSD during pregnancy		p-value
	No (n = 33)	Yes (n = 84)	
Mothers			
Age current, mean (SD)	41.4 (5.1)	43.7 (6.1)	0.06
Age at child's birth	31.9 (4.5)	31.0 (4.9)	0.36
City location, % (n)	27.3 (9)	39.3 (33)	0.22
Secondary or higher education, % (n)	30.3 (10)	23.2 (19)	0.52
Married, % (n)	93.8 (30)	84.5 (71)	0.59
Current PTSD, % (n)	51.5 (17)	69.1 (58)	0.08
Lifetime PTSD, % (n)	90.9 (30)	100 (84)	0.005
Current smoker, % (n)	40.6 (13)	39.8 (33)	0.93
Smoking during pregnancy, % (n)	21.9 (7)	34.9 (29)	0.18
Children			
Female, % (n)	51.5 (17)	42.9 (36)	0.40
Age, median (Interquartile range)	9 (6–13)	13 (9.5–17.5)	0.002
Cortisol, mean (SD)	310.9 (141.8)	349.0 (154.5)	0.22

Values are mean (SD), median (Interquartile range) or n (%). p-values calculated using Student's t-test, Mann–Whitney U test, or χ^2.
n does not equal 117 for all variables, since some variables had missing data: current smoker n = 114, smoking during pregnancy n = 114, education n = 115, marital status n = 116.
PTSD: Post-traumatic stress disorder; SD: Standard deviation.

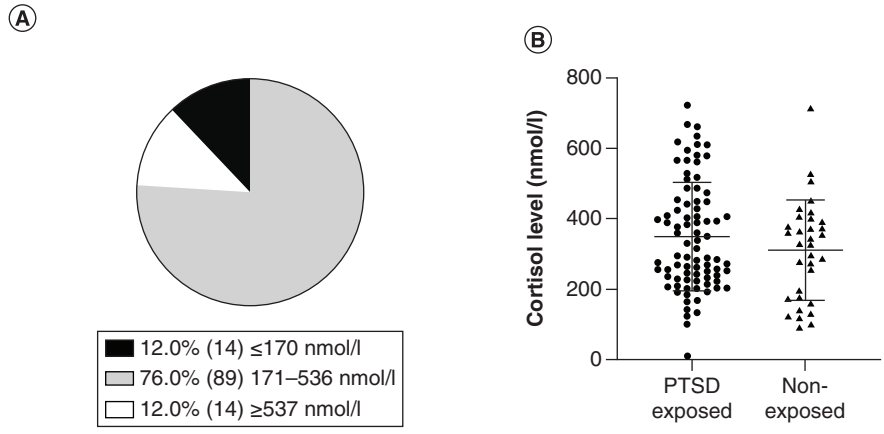

Figure 1. Cortisol levels in post-traumatic stress disorder exposed and non-exposed children. (A) Proportion of offspring with cortisol levels below, within and above reference normal range. **(B)** Cortisol levels in PTSD exposed versus non-exposed offspring.
PTSD: Post-traumatic stress disorder.

= 0.04. In multinominal regression analysis adjusting for offspring gender (and considering mother and child's age), this association remained significant (β: 2.50 [1.18]; p = 0.03).

Epigenome-wide DNA methylation analysis of blood from PTSD versus control offspring

To investigate whether DNA methylation in blood differed in PTSD exposed versus non-exposed offspring, epigenome-wide DNA methylation was analyzed using the Infinium HumanMethylationEPIC BeadChip. Using multivariate linear regression analysis, with adjustment for mother's age, offspring gender and age, batch effect and cell composition, 12,337 differentially methylated CpG sites ($p < 0.05$) were identified with a difference in DNA methylation of >1.0% between PTSD and non-PTSD exposed groups. However, none remained significant following false discovery rate correction for multiple testing. The largest mean methylation difference between the groups was 12.0%. Approximately half (51.6%) of the differentially methylated CpGs showed a higher DNA methylation level among offspring exposed to PTSD during pregnancy compared with non-exposed offspring.

10.2217/epi-2021-0015

Table 2. Differentially methylated probes between children born to mothers with and without post-traumatic stress disorder during pregnancy.

Gene	Probe	Δ β, %	chr n	Relation_to_Island	UCSC reference gene	p-value
ZNF263	cg03208850	1.84	chr16	Island	TSS200	2.6E-06
AC012314.2	cg22816913	-1.10	chr19	OpenSea	Body	3.6E-06
HELT	cg08178441	-1.06	chr4	N_Shore	TSS1500	5.6E-06
ASF1A	cg26845404	1.62	chr6	S_Shelf	Body	7.0E-06
GAK	cg16950088	-1.61	chr4	Island	Body	8.3E-06
ATE1	cg22055991	-1.29	chr10	OpenSea	Body	8.8E-06
RP11–89K21.1	cg13905258	-4.44	chr2	N_Shore		9.5E-06
<*RGMA*	cg10073794	-1.03	chr15	N_Shelf		9.9E-06
<*FOXL1*	cg05402622	1.75	chr16	OpenSea		9.9E-06
>*NGDN*	cg08406615	-2.14	chr14	OpenSea		1.0E-05
AC099344.2	cg03288148	2.82	chr2	OpenSea		1.2E-05
PDE1C	cg27368417	-2.65	chr7	OpenSea		2.2E-05
GALNTL5	cg20940424	-1.07	chr7	OpenSea		2.4E-05
SP3	cg08848324	-3.42	chr2	Island	TSS1500	2.5E-05
ANK3	cg16134349	2.02	chr10	OpenSea	1st exon; 5′UTR; body	2.7E-05
LINC02702	cg00916185	-1.12	chr11	OpenSea		2.9E-05
CYB5A	cg18274065	-2.70	chr18	S_Shore	TSS1500	2.9E-05
FAM71E1	cg12314945	-1.06	chr19	OpenSea	Body	3.1E-05

< denotes the gene is upstream from the probe and > is downstream.
chr: Chromosome.

Next, we examined DMRs which contain multiple CpGs that show correlative methylation across a region. Using DMRcate (v2.0.0), we identified in total 23 DMRs (comprising 93 CpGs) in the offspring peripheral blood, that where differentially methylated between offspring exposed to PTSD or not. The top 20 ranked list of differentially methylated CpGs and all 23 DMRs are shown in Tables 2 & 3, respectively. Given that no differentially methylated CpGs reached Bonferroni corrected genome-wide significance, we did not proceed with pathway analysis.

DNA methylation of candidate genes differentially methylated in response to early-life stress

Since this data analysis was restrained with power issues due to the relatively small number in a mixed cell sample of blood, compared with the high number of tests conducted when using the EPIC platform, an analysis of key candidate genes available in the EWAS dataset was also performed. This was based on the published literature where CpG site-specific or gene-specific DNA methylation differences in children following early-life exposure to trauma and/or stress had previously been reported. Therefore, a literature search in the Medline database was performed using the following terms: 'DNA methylation,' 'PTSD' and 'child' or 'pregnancy.' Seven genes (*NR3C1*, *NR3C2*, *HTR3A*, *SLC6A4*, *OXTR, FKBP5, BNDF*) that previously had been shown to present differential methylation degree in children exposed to early life stress (i.e. by maternal PTSD) were identified (Table 4). These differences in DNA methylation of these seven genes between offspring exposed to maternal PTSD during pregnancy, and those who were not, are shown in Table 5.

DNA methylation of candidate genes are associated with offspring's cortisol levels

To examine a possible relationship between offspring health and DNA methylation degree, the association between DNA methylation degree of the seven replicated genes (*NR3C1*, *NR3C2*, *HTR3A*, *SLC6A4*, *OXTR, FKBP5, BNDF*) and offspring cortisol levels was examined. Interestingly, when performing multiple regression analysis between single CpG site methylation and cortisol levels, adjusting for offspring gender, age and PTSD group, a significant positive association was observed for cg12612985 (*HTR3A)*, and cg15688670 and cg04481212 (*BNDF*), and a negative association between cortisol levels and methylation degree of cg12612985 (*NR3C1)*, located in the promoter region (Table 5). This is also presented in Figure 2A–C showing simple correlation analyses without adjustments.

Table 3. Differentially methylated gene regions between children born to mothers with and without post-traumatic stress disorder during pregnancy.

Overlapping promoters	DMR n	chr. n	Start	End	Width	n CpGs	Max. Δ β, %	Mean Δ β, %	p-value
NINJ2	13	chr12	678,853	679,287	435	3	-8.0	-5.6	0.0087
IRF6	22	chr1	2.10E + 08	2.10E + 08	669	8	-7.4	-3.7	0.0008
MYADM	20	chr19	54,368,958	54,370,016	1059	15	-7.3	-2.9	0.0001
MLLT1	8	chr19	6,261,277	6,262,175	899	3	-5.8	-3.3	0.0014
AC092295.4, ZNF260	16	chr19	37,018,434	37,018,766	333	3	-4.7	-2.7	0.0084
IGHD1	14	chr14	1.06E + 08	1.06E + 08	152	3	-4.5	-4.1	0.0376
SPTBN4	11	chr19	40,995,921	40,996,118	198	3	-4.1	-2.1	0.0112
HTR2A	21	chr13	47,472,050	47,472,429	380	11	-2.5	-1.5	0.0231
SNORD115	4	chr15	25,432,236	25,432,270	35	2	-1.8	-1.5	0.0177
GAK	3	chr4	899,553	899,775	223	2	-1.5	-1.2	0.0176
SALL3	5	chr18	76,753,238	76,753,357	120	2	-1.2	-0.9	0.0354
>*ANAPC7*	2	chr12	1.11E + 08	1.11E + 08	341	2	1.0	0.0	0.0209
GPER1/C7orf50	7	chr7	1,130,187	1,130,284	98	2	1.3	0.8	0.0376
SH3TC2	1	chr5	1.48E + 08	1.48E + 08	16	2	1.3	1.2	0.0414
PRKAR1B	17	chr7	630,581	630,914	334	3	2.4	1.8	0.0252
ANK3	9	chr10	61,900,413	61,900,940	528	3	2.8	1.9	0.0009
HOXB4, MIR10A, HOXB3	23	chr17	46,657,274	46,658,257	984	7	2.8	1.9	0.0087
SLITRK5	12	chr13	8,832,8471	88,328,813	343	2	3.6	2.5	0.0008
MAPK12	6	chr22	50,701,157	50,701,233	77	2	4.1	2.4	0.0001
C22orf26, FLJ27365, RP6–109B7.3	19	chr22	46,449,430	46,449,752	323	5	4.4	3.4	0.0014
PER3	10	chr1	7,887,199	7,887,560	362	3	4.7	3.3	0.0084
MALRD1	15	chr10	19,777,545	19,777,735	191	3	5.1	4.7	0.0376
TAB1	18	chr22	39,784,598	39,784,982	385	4	13.8	7.7	0.0112

chr: Chromosome; DMR: Differentially methylated region.

Discussion

To our knowledge, this is first DNA methylation study in offspring born to mothers with PTSD resulting from war-related sexual trauma. The results show that children born to mothers with PTSD during pregnancy compared with those without exhibit differential DNA methylation at a number of genes; however, none of these reached significance at epigenome-wide corrected levels. In the nominally significant findings, a number of candidate genes were involved in cortisol, glucocorticoid, serotonin and oxytocin regulation (*NR3C1*, *NR3C2*, *HTR3A*, *SLC6A4*, *OXTR, FKBP5, BNDF*), which have previously been shown to be differential methylated in offspring of mothers exposed to a traumatic event. Interestingly, for a couple of the probes in these functionally relevant candidate genes, we observed significant associations between cortisol level and the degree of DNA methylation. This finding would support our hypothesis that the effects of maternal PTSD during pregnancy may become biologically embedded; however, it needs confirmation in additional larger studies.

DNA methylation changes in children exposed to PTSD in pregnancy

DNA methylation changes in specific genes have been associated with the stress response and regulation of the stress response system, as well as several psychiatric disorders, including PTSD [10]. However, whether PTSD during pregnancy can be passed on to offspring and how this might be mediated is more sparsely described, and no studies have focused on PTSD resulting from war-related sexual trauma. To date, the current literature describing the epigenetic regulation of specific candidate genes in response to early-life maternal PTSD and/or trauma have focused predominantly on the *NR3C1* gene, encoding the glucocorticoid receptor which is a key component of HPA-axis stress signaling. Perroud *et al.* examined the impact of the Tutsi genocide on a total of 50 offspring and reported increased methylation at exon 1F in offspring peripheral blood [35], whereas Yehuda *et al.* in their study of 80 adult offspring of Holocaust survivors found decreased promoter methylation in peripheral blood from offspring whose both parents had PTSD [20]. These studies, however, investigated different genomic locations of *NR3C1*,

Table 4. Overview of candidate gene literature†, showing associations between offspring DNA methylation and early life maternal stress and/or trauma.

Candidate gene	Cohorts	Tissues	Results	Study	Ref.
NR3C1/*NC3C2* glucocorticoid receptors	Rwandan mothers and children, exposed to the Tutsi genocide, n = 50	Peripheral blood leukocytes	Increased methylation at exon 1F promoter, in both mothers and children	Perroud *et al.*, World J. Biol. Psychiatry (2014)	[28]
	Adult offspring of Holocaust survivors, n = 80	Peripheral blood mononuclear cells	Decreased promoter methylation in maternal + paternal post-traumatic stress disorder. Greater cortisol suppression was associated with lower methylation degree	Yehuda *et al.*, Am J Psychiatry (2014)	[15]
	Congolese newborns, of mothers exposed to sexual violence during wartime, n = 25	Cord blood	Increased promoter methylation	Mulligan *et al.*, Epigenetics (2012)	[31]
	10–19-year-old offspring of East-European mothers, exposed to partner violence during pregnancy, n = 25	Peripheral blood leukocytes	Increased promoter methylation	Radtke *et al.*, Transl. Psychiatry (2011)	[32]
	EWAS (HM450K arrays) in 8–18-year-old children from Brazil, exposed to partner violence during pregnancy, n = 120	Saliva	*NR3C1* and *FKBP5* were among the most differentially methylated	Serpeloni *et al.*, Front Genet (2019)	[33]
	EWAS (HM450K arrays) in neonates exposed to maternal depression and anxiety during pregnancy, n = 13	Cord blood	Decreased methylation of the *NR3C1* CpG site: cg00629244	Non *et al.*, Epigenetics (2014)	[29]
FKBP5, repressor of glucocorticoid activity	Maternal Holocaust survivors, n = 32, and their offspring, n = 22	Peripheral blood mononuclear cells	Increased (mothers) and decreased (offspring) intron 7 methylation	Yehuda *et al.*, Biol. Psychiatry (2016)	[34]
HTR3A, serotonin receptor	Mothers and children aged 12–42 months, n = 35	Saliva	Increased maternal promotor methylation associates with child aggression	Schechter *et al.*, Behav Brain Res (2017)	[35]
SLC6A4, (5-HTT), serotonin transporter	Newborns exposed to maternal depression in pregnancy, n = 82	Cord blood	Decreased promotor methylation	Devlin *et al.*, PLoS ONE (2010)	[36]
BNDF, neurotrophin, involved in neuron growth	Adult offspring exposed to poor maternal care in early life, n = 85	Peripheral blood leukocytes	Increased exon 6 methylation	Unternaehrer *et al.*, Stress (2015)	[37]
OXTR, oxytocin receptor	Adult offspring exposed to poor maternal care in early life, n = 85	Peripheral blood leukocytes	Increased exon 3 methylation	Unternaehrer *et al.*, Stress (2015)	[37]

†Where available, results from studies focused on war trauma and/or sexual violence are shown for comparative purposes with the current study.

Table 5. Differential methylation of candidate genes between the two groups.

Gene	Probe	Δ β, %	chr n	Position	Strand	UCSC reference gene	p-value (association with post-traumatic stress disorder)	p-value (association with cortisol)
HTR3A	cg12612985	-0.78	chr11	113847121	-	TSS1500; body	0.008	0.01
SLC6A4	cg0199110	-0.55	chr17	28555935	-	5′UTR	0.049	-
OXTR	cg17036624	-2.00	chr3	8811601	-	TSS1500	0.020	-
OXTR	cg03710862	-0.73	chr3	8811728	-	TSS1500	0.029	-
OXTR	cg14483142	-0.98	chr3	8811758	+	TSS1500	0.036	-
NR3C1	cg07715663	-0.79	chr5	142721796	+	Body	0.017	-
NR3C1	cg21209684	-1.88	chr5	142783848	-	5UTR; TSS1500	0.036	0.019
NR3C1	cg26464411	-3.42	chr5	142784222	-	TSS1500; 5′UTR	0.048	-
NR3C2	cg1315799	-2.11	chr4	149191991	-	Body	0.008	-
NR3C2	cg13373360	1.33	chr4	149364881	+	TSS1500	0.032	-
FKBP5	cg09268536	1.21	chr6	35611576	+	5′UTR	0.049	-
BDNF	cg18595174	-1.69	chr11	27701991	+	Body; 5′UTR	0.039	-
BDNF	cg20340655	0.68	chr11	27721661	+	TSS1500	0.002	-
BDNF	cg15688670	1.86	chr11	27723190	+	TSS1500; Body	0.014	0.035
BDNF	cg04481212	0.69	chr11	27740495	+	Body; 5′UTR	0.029	0.025
BDNF	cg04106006	2.31	chr11	27742454	-	Body; TSS1500; TSS200	0.033	-
BDNF	cg10022526	0.83	chr11	27744557	-	TSS1500	0.049	-

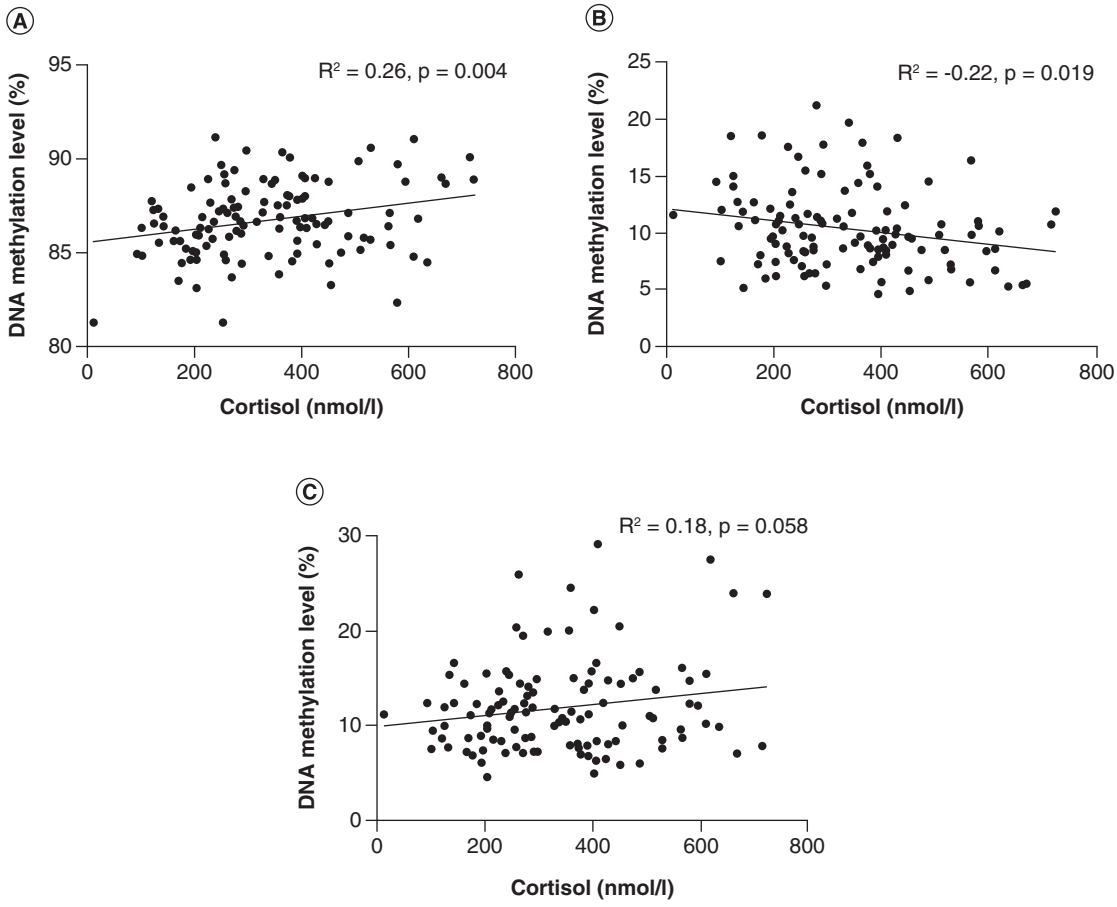

Figure 2. Association between cortisol level and DNA methylation levels at candidate gene CpG sites. (A) *HTR3A* (cg12612985). **(B)** *NR3C1* (cg21209684). **(C)** *BNDF* (cg15688670). Analyses represents Pearson correlation test. The scales on each graph are different, so directly comparing across CpG sites is not possible.

which may explain the discrepant findings. Our results support the study by Yehuda *et al.* showing that exposure to PTSD in pregnancy is associated with decreased methylation in the promoter region of *NR3C1*. Interestingly Non *et al.* also performed a discovery genome-wide methylation study in cord blood from 58 offspring including some exposed to maternal depression and anxiety in pregnancy. In their candidate gene analysis, they also showed a decreased methylation of the *NR3C1* promoter at a specific site (cg00629244) aligning with the methodology and findings of the present study [36].

Our findings concerning the serotonin transporter gene, *SLC6A4*, also align with and extend existing literature, where maternal stress has been shown to influence *SLC6A4* expression in the placenta [27] and a study of 82 mother–infant dyads found decreased DNA methylation in offspring exposed to maternal depression and anxiety [37]. Indeed, disruption of serotonergic signaling is thought to be implicated in the pathophysiology of PTSD [38] and genetic variants in *SLC6A4* gene have been associated with an increased risk of PTSD [39]. Early-life trauma and *SLC6A4* genetic variation has also been shown to influence risk of brain development [40] and psychiatric outcomes [41], and gene–DNA methylation interactions are likely to be involved [42]. The *OXTR* gene is expressed in different regions of the brain and is involved in social behavior and bonding, in the mechanisms of birth and lactation, but also in mother–child bonding [43]. Interestingly, it has been shown that adults exposed to low maternal care during childhood had elevated *OXTR* DNA methylation levels in one of the exons of the gene [43]. In our study, we observed decreased *OXTR* promoter methylation levels; however, this region is far from the exon region, and can, therefore, not be directly compared with the previous results.

Finally, the DMR analysis identified *NINJ2* as a DMR between the PTSD and control offspring. The *NINJ2* DMR was previously reported as one of the top DMPs and DMRs by Rutten *et al.* in an investigation of PTSD in military personnel [44].

Cortisol regulation in offspring exposed to PTSD during pregnancy

Cortisol is the key glucocorticoid stress hormone which is triggered in response to major stress or trauma. Chronic or severe stress may disrupt functioning of the HPA-axis, and this is commonly observed in depression and PTSD [45]. Prior studies have shown that an increase in cortisol level is observed in children reporting early life adversity and is a marker of heightened stress reactivity [46]. High cortisol levels in children are associated with anxiety symptoms and with other longer term behavioral and cognitive effects [47]. Here, we report that the children exposed to PTSD during pregnancy had significant 2.5-fold risk of having cortisol levels (above normal reference) compared with unexposed children. It may be speculated whether a transmission of PTSD to the offspring is associated with a biological change of the HPA axis, resulting in the increased cortisol levels. Interestingly, the negative association between offspring cortisol levels and offspring methylation levels of the *NR3C1* CpG site, located in the promoter region, aligns with PTSD resulting in increased cortisol levels in the offspring. This would lend some support behind the hypothesis that the effects of maternal PTSD during pregnancy could become biologically embedded, and dysregulation of stress signaling would thus be one possible mechanism that could explain the longer-term negative effects on offspring health. Although it should be remembered that the candidate genes investigated are not specific markers of PTSD but rather genes involved in stress signaling, and thus may not be directly related to maternal PTSD.

Diagnostic challenges in assessing intergenerational PTSD

Criteria used to diagnose PTSD may not be sufficient to capture symptom-related functional impairment [4], thus resulting in a underestimating of PTSD in both children and adults. It is more challenging to make proper diagnosis of potential 'transgenerational' PTSD in offspring whose mothers were exposed to war or related traumatic events while the offspring did not have a direct exposure. A number of PTSD symptoms overlap with those of other psychiatric disorders or children/adolescent behavior problems and share common pathways involved in the deregulation of stress-responsive endocrine systems and neurotransmitters and neuromodulators involved in arousal, depression and anxiety [4]. In addition, self-reporting of one's own feelings can be biased, ill-defined and vary across culture, resulting in improper diagnosis and often leads to treatment inefficacy. Misclassification and misdiagnosis of PTSD cases is common in children and adolescents in the postwar or resource-limited settings where qualified mental health professionals are absent [48]. Therefore, a reliable early screening or diagnosis of transgenerational PTSD in the offspring would be of considerable value, because severe adverse consequences develop progressively if the after-effects in offspring are unrecognized and untreated, which will affect the long-term development and mental health of offspring.

The results generated from the present study provide some support for the hypothesis that maternal PTSD could result in DNA methylation changes in children, although whether this relationship is causal, can't be ascertained. Furthermore, further work is now required in larger studies that ideally follow women from the time of pregnancy, where retrospective recall bias of PTSD is minimized, and which measure DNA methylation in neonates. Such studies would strengthen evidence for the intergenerational transmission of trauma effects, and the involvement of epigenetic mechanisms. Differentially methylation has potential utility as a biomarker for screening to identify high-risk individuals, and thus permitting a targeted intervention approach to improve the offspring's health and well-being. Indeed, recommendations for future practices to address the early detection and prevention of transgenerational trauma for the victims of sexual violence is needed in postwar settings.

Limitations

There are limitations to this study. The first concerns the assessment of PTSD which is subject to recall bias, as women reported on their symptoms, level of distress and impact of these symptoms during pregnancy. Thus, it is possible that some women have been misclassified as having PTSD during pregnancy. Power is a general issue in all studies referred to as well as our present study despite being the largest study performed to date. The high number of probes tested for in an epigenome-wide discovery study (625,413) makes it very unlikely to yield statistically significant results when performing cross-sectional studies at a single timepoint. Furthermore, blood DNA is a mixture of multiple cell types, and while we controlled for estimated cell composition in our analysis, the findings may still reflect in part, variations in the proportions of different cells.

We did not have information of the children's current trauma exposure, since questions related to the children's mental health were not part of the current study. Indeed, this information could influence the results, and it would be highly valuable to include interview questions on the children's mental health and exposure in a future study.

However, we did control for other postnatal factors, including maternal current PTSD. Although the data on the children's current psychological health was not collected, the current study will now form the basis of a subsequent study where we also will assess the potential beneficial effects of an intervention. Indeed, the interaction of genetic vulnerability and environmental factors results in vast individual differences in the development and expression of diseases, as well as in the response to intervention. Indeed, target interventions for PTSD is highly important in post-war settings, as in Kosovo, to prevent trauma in generations. However, longitudinal epigenetic studies focusing family therapy intervention is also needed to examine if its effectively possible to reverse the epigenetic modifications with PTSD.

Conclusion

In conclusion, this is the first study of women experiencing war-related sexual trauma resulting in PTSD during pregnancy. We provide some preliminary evidence for the relationship between maternal PTSD during pregnancy and differential DNA methylation in the offspring; however, further larger studies are now required.

Future perspective

Identifying the underlying biological processes whereby the negative effects of trauma are passed across generations is a key step to breaking the intergenerational transmission of the effects of mental disorders and physical health problems. Our research highlights the importance of early screening of children born to women who experienced sexual violence/torture, and particularly those with PTSD symptoms during pregnancy. The findings from this research may enable us to identify the children at risk for both short and long-term negative effects on their health and well-being and to develop the most appropriate early intervention strategies to promote resilience in children. Healthy childhood development determines the basis for every child's future health, well-being and learning and economic potential. This is essential for post conflict societies to break the vicious cycle of violence in order to build sustainable peace and conflict prevention. Further research is now needed to determine if the epigenetic changes are associated with long-term effects in the child and whether family therapy intervention may alter the child´s epigenetic profile and related health changes.

Summary points

- Evidence suggests that children born to women with post-traumatic stress disorder (PTSD) are more likely to have behavioral and clinical problems, including symptoms of PTSD, as well as other mental and physical health problems.
- This study aimed to determine if maternal PTSD is associated with epigenetic changes in the offspring, since early identification of affected children is key to timely interventions and treatment.
- In total, 117 women were recruited for the study who had experienced sexual violence/torture during the Kosovo war, and were registered in the Kosovo Rehabilitation Centre for Torture Victims database.
- Blood samples collected from the youngest children born to each woman were used to measure genome-wide DNA methylation with the Illumina EPIC platform, as well as cortisol levels.
- Offspring of mothers with PTSD during pregnancy (n = 84), had higher cortisol levels and differential methylation at a number of genes, but none of the methylation differences reached epigenome-wide corrected significance levels.
- Maternal PTSD was associated with differential methylation at a number of stress-related candidate genes, including *NR3C1*, *HTR3A* and *BNDF*, and DNA methylation at a number of genes was associated with cortisol levels.
- These findings support the hypothesis that the effects of maternal PTSD during pregnancy may become biologically embedded, resulting in epigenetic changes in the offspring.
- However, more work is needed in large studies to determine whether these findings are explained by the potential intergenerational transmission of trauma.
- Given the long-term negative effects of major trauma, more research is also needed to determine which interventions can support the long-term well-being of both mothers and their children in the post-war setting.

Acknowledgments

The authors greatly appreciate all the children and their mothers who participated in the study. The authors would like to thank K Bjerre for her extensive and prompt input on database development and troubleshooting, and B Zamroni for assisting with initial data analysis. Special thanks go to IC Bygbjerg and J Modvig who helped with the initial conceptualization of the study. The authors

also thank to PRG Christensen and MH Thøgersen for administrative support and guidance. Finally, authors are honored by the encouragement and generous support of HRH Countess of Wessex to continue our commitment to 'Preventing Sexual Violence in Conflict Initiative.'

Financial & competing interests disclosure

This study was funded by the Foreign, Commonwealth and Development Office through the British Embassy in Pristina and the Danish Institute against Torture (Denmark). L Hjort is funded by the Danish Diabetes Academy supported by the Novo Nordisk Foundation. J Ryan is supported by a National Health and Medical Research Council Research Leader Fellowship (grant no.: 1135727). The contents of this publication are the sole responsibility of the authors and do not necessarily reflect the views of the Foreign, Commonwealth and Development Office. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Data sharing statement

Data are accessible from the first and senior authors on reasonable request.

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