## Childhood adversity and PTSD in youth

*(Epi-) genetics & treatment effects*

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# DISTINCT SALIVA DNA METHYLATION PROFILES IN RELATION TO TREATMENT OUTCOME IN YOUTH WITH POSTTRAUMATIC STRESS DISORDER

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# ABSTRACT

In youth with posttraumatic stress disorder (PTSD) non-response rates after treatment are often high. Epigenetic mechanisms such as DNA methylation (DNAm) have previously been linked to PTSD pathogenesis, additionally DNAm may affect response to (psychological) therapies. Besides investigating the direct link between DNAm and treatment response, it might be helpful to investigate the link between DNAm and previously associated biological mechanisms with treatment outcome. Thereby gaining a deeper molecular understanding of how psychotherapy (reflecting a change in the environment) relates to epigenetic changes, and the adaptability of individuals. To date, limited research is done in clinical samples, and no studies have been conducted in youth. Therefore we conducted a study in a Dutch cohort of youth with and without PTSD (n= 87, age 8-18 years). We examined the cross-sectional and longitudinal changes of saliva-based genome-wide DNA methylation (DNAm) levels, and salivary cortisol secretion. The last might reflect possible abbreviations on the hypothalamic–pituitary– adrenal (HPA) axis. The HPA-axis is previously linked to DNAm and the development and recovery of PTSD. Youth were treated with 8 sessions of either Eye Movement Reprocessing Therapy (EMDR) or Trauma Focused Cognitive behavioral Therapy (TF-CBT). Our epigenome wide approach showed distinct methylation between treatment responders and non-responders on C18orf63 gene post-treatment. This genomic region is related to the PAX5 gene, involved in neurodevelopment and inflammation response. Additionally, our targeted approach indicated that there were longitudinal DNAm changes in successfully treated youth at the CRHR2 gene. Methylation at this gene was further correlated with cortisol secretion pre- and post-treatment. Awaiting replication, findings of this first study in youth point to molecular pathways involved in stress response and neuroplasticity to be associated with treatment response.

# INTRODUCTION

Posttraumatic stress disorder (PTSD) is a common mental health disorder observed in approximately 16% of youth exposed to traumatic events (Alisic et al., 2014). Youth with PTSD are troubled by frequent re-experiencing of the traumatic event, persistent avoidance, hyper arousal and negative alterations in cognition and mood (Association & Association, 2013). If left untreated, these symptoms can interfere with social functioning and school performance, and have ongoing negative effects on the quality of life of the affected youth (Carrion, Weems, Ray, & Reiss, 2002). Furthermore, they are considered a crucial factor in shaping the vulnerability to depression and suicidality later in life (Molnar, Berkman, & Buka, 2001). This emphasizes the importance of effective treatments for youth with PTSD. Randomized controlled trials (RCTs) have demonstrated the efficacy of trauma-focused psychotherapies in youth with PTSD (Morina, Koerssen, & Pollet, 2016). Nevertheless, response varies considerably among individuals, with high rates of heterogeneity in response and 20-50% of youth not benefiting sufficiently (Bennett, Denne, McGuire, & Hiller, 2020; Leenarts, Diehle, Doreleijers, Jansma, & Lindauer, 2013; Lindebø Knutsen, Sachser, Holt, Goldbeck, & Jensen, 2020; Mavranezouli et al., 2020). Several mechanisms have been associated with differential responses to treatment in youth, amongst them are biological factors, such as; epigenetic, endocrinological and neurological factors (Bryant et al., 2008; Vinkers et al., 2019; Yehuda et al., 2013). Besides the potential role of these biological factors as a predictor for treatment outcome, related studies have shown that symptomatic change is likely mediated by underlying biological mechanisms, such as epigenetic and endocrinological change (Yang et al., 2021). DNA methylation (DNAm) is an important epigenetic mechanism which reflects epigenetic change and affects endocrine functioning (García-Carpizo, Ruiz Llorente, Fernández Fraga, & Aranda, 2011). DNAm represents the transcriptional status of a particular gene and can be influenced by both genetic and environmental factors (Feinberg, 2007; Schübeler, 2015). Provoked by early exposure to traumatic events, DNAm is assumed to be associated with altered hypothalamic-pituitary-adrenal (HPA) axis functioning, and it is related to the development and recovery of PTSD (Labonte et al., 2012; McGowan et al., 2009; Yehuda & Bierer, 2009). It is assumed that DNAm affects glucocorticoid functioning, in particular the release of stress hormones such as cortisol, the end product of HPA-axis activation, which is pivotal in several central mechanisms involved in PTSD, and trauma-focused treatment, such as the primary stress response, (emotional) memory consolidation, memory retrieval, reconsolidation and extinction learning (Dick & Provencal, 2018; Dominique, Aerni, Schelling, & Roozendaal, 2009; Fischer, Schumacher, Knaevelsrud, Ehlert, & Schumacher, 2021; Houtepen et al., 2016; Meir Drexler & Wolf, 2017; Roque et al., 2020). In our prior study, we reported that higher pretreatment basal cortisol secretion was a potential indicator of treatment response in youth with PTSD (Zantvoord et al., 2019). Despite growing evidence showing that DNAm and endocrine

mechanisms are important for a successful adaptation to stressful events, and play an important role in the development and persistence of PTSD, translational studies in clinical practice are still rare. Especially in youth. To the best of our knowledge only four studies (Carleial et al., 2021; Vinkers et al., 2019; Yang et al., 2021; Yehuda et al., 2013) have investigated changes in DNAm in relation to symptomatic response in adults with PTSD. The most recent study (Yang et al., 2021) examined the relation between DNAm and hydrocortisone treatment. This study identified epigenetic markers, previously linked to startle reaction and fear learning and memory processes, predicting both symptom change and PTSD recovery (Carleial et al., 2021; Vinkers et al., 2019; Yang et al., 2021; Yehuda et al., 2013). Confirming the evidence from animal models showing that epigenetic changes can be dynamic and potentially reversible as a consequence of environmental programming (Meaney & Szyf, 2005; Weaver et al., 2006).

Objective and reliable predictive biological markers of treatment response and/or symptomatic change may have the potential to guide treatment selection and improve treatment efficacy. However, despite the promising results of the studies described above, there remains a considerable knowledge gap. First, it is important to recognize that translational studies in humans are still scarce, and so far have only been conducted in adults. These results cannot be automatically translated to youth, because other biological pathways and mechanisms are possibly involved as both epigenetic and endocrinological regulation undergoes considerable developmental change (Agorastos, Pervanidou, Chrousos, & Baker, 2019; Cisler & Herringa, 2021; Daskalakis, Bagot, Parker, Vinkers, & de Kloet, 2013; Nederhof & Schmidt, 2012). Secondly, it remains insufficiently clear if changes in DNAm related to PTSD, adapt in reaction to trauma-focused psychotherapy, and how these treatment related changes in DNAm are associated with longitudinal changes in the endocrine system, such as cortisol secretion. Third, the predictive value of baseline DNAm for treatment response is still insufficiently clear. Therefore, in this study we aim to address these knowledge gaps by investigating the cross-sectional and longitudinal changes in DNAm and cortisol secretion in relation to the treatment response of youth with and without PTSD. Youth with PTSD received trauma-focused psychotherapy. We used pre-and post-treatment exploratory methylome wide analysis (MWAS) and targeted analysis on differently methylated positions (DMPs) and regions (DMRs). In addition, we measured salivary cortisol to compare changes in cortisol secretion with findings from the methylation analysis.

# METHODS

### *Cohort and study design*

In the present study a MWAS, targeted epigenetic approach, cortisol and clinical assessments were performed in a cohort of youth (N = 87, 54.1% female, aged 8-18), Youth with PTSD (N=46) were matched for age and sex with a control group of trauma exposed controls (TEC) without PTSD (N=41). Youth with PTSD were recruited between April 2011 and September 2018 at the outpatient child psycho-trauma center of the department of child and adolescent psychiatry, de Bascule in Amsterdam, The Netherlands. The participants were part of a larger RCT comparing trauma-focused cognitive behavioral therapy (TF-CBT) and eye movement desensitization and reprocessing (EMDR) (Diehle, Opmeer, Boer, Mannarino, & Lindauer, 2015). They were referred by child welfare services, physicians or their general practitioner. TEC were recruited between June 2011 and September 2018 through local elementary- and high schools by researchers JBZ, RodK and JBME. Exclusion criteria for both groups included imminent suicidality, history of psychotic disorder, substance abuse or dependence; IQ<70; unstable medical condition; recent use of psychotropic medication (past 4 weeks; 6 weeks for fluoxetine); and possibility of pregnancy in females. All participants received a monetary incentive for participation (€5 for each assessment). In both groups written parental and youth assent were obtained for all participants. All procedures were approved by the Medical Ethical Committee of the University Medical Center.



### *Procedures and measures*

Diagnosis for PTSD in the clinical group were established clinically by an experienced child and adolescent psychiatrist or psychologists according to the DSM-IV-TR criteria using both child reports on the Clinician-Administered PTSD Scale for Children and Adolescents (CAPS-CA), which is a reliable semi-structured interview (K. O. Nader et al., 1996). In addition caregiver information was obtained from the PTSD scale of the Anxiety Disorders Interview Schedule – Parent Version (ADIS-P) (Verlinden, van Laar, et al., 2014). (Partial) PTSD diagnosis was determined using joint-child and caregiver reports on individual symptoms. A symptom was established as present, if either child or caregiver reported its presence. All participants were required to have a CAPS-CA total score indicating at least mild PTSD symptom severity (>20 points). Clinical evaluations were performed pre-treatment (T1), post-treatment (8 sessions of psychotherapy) and follow-up (6 months post-treatment). Based on the psychometric properties of the CAPS (-CA) and previous treatment outcome studies using the CAPS-CA, we used ≥30% reduction of CAPS-CA total score as response criterion for clinically meaningful improvement (J. Diehle, C. de Roos, F. Boer, & R. J. Lindauer, 2013; Weathers, Keane, & Davidson, 2001). In the TEC exposure to traumatic events were validated according to A1 and A2 criteria of DSM-IV-TR (American Psychiatric Association, 2000)

using the Children's Revised Impact of Event Scale (CRIES) (Perrin et al., 2005; Verlinden, van Meijel, et al., 2014). Information about additional internalizing and externalizing symptoms were measured using youth and caregiver reports, with the Revised Children's Anxiety and Depression Scale (RCADS) and the Child Behavioral Checklist (CBCL) and Youth Self Report (YSR) (Achenbach, 1991; Achenbach & Edelbrock, 1983; Chorpita, Yim, Moffitt, Umemoto, & Francis, 2000; Kösters, Chinapaw, Zwaanswijk, van der Wal, & Koot, 2015; Verhulst et al., 1997). Clinical characteristics of both groups are shown in Table 1.

### *Treatment*

After study entry, all patients were randomized to receive either protocolled sessions of trauma-focused cognitive behavioral therapy (TF-CBT) or eye movement desensitization and reprocessing (EMDR) by trained and experienced therapists. Supervision by experts on TF-CBT and EMDR was provided throughout the study. Treatment protocols, training and supervision of therapists, as well as treatment fidelity have been described in detail previously (Diehle et al., 2015; Zantvoord et al., 2019).

### *DNA methylation*

Three milliliters of saliva were collected and stored in Oragene DNA sample collection kits (DNA Genotek, Canada). DNA was extracted using a Gentra autopure LS system following manufacturer's protocol. Genomic DNA samples were resolved on a 1% agarose gel to verify that the DNA was of high molecular integrity. Quantification of the DNA was determined using Qubit (Qiagen, U.S.A). Five hundred nanograms of genomic DNA was sodium bisulfite–treated for unmethylated cytosine (C) to thymine (T) conversion using the EZ DNA Methylation-Gold kit (Zymo Research). Prior to DNA methylation profiling, cases and controls were randomized across the 96 well plates. Technical replicates (n = 8) were included for quality control of array, monitoring potential batch effects. Briefly, converted DNA was amplified, fragmented, hybridized, and scanned using the Illumina Methylation EPIC 850k Beadchip, following the manufacturer's guidelines.

### *Cortisol*

Information about Script driven Imagery (SDI) and cortisol collection is published previously (Zantvoord et al., 2019). In short, all participants performed a standardized protocol for SDI (Shalev, Orr, & Pitman, 1992), in combination with the collection of five saliva samples, 10 min and 1 min prior to trauma script imagery as well as 10 min, 20 min, and 30 min after trauma script imagery. Next, for each participant we determined if SDI induced a cortisol stress response

(R. Miller, Plessow, Kirschbaum, & Stalder, 2013). This was defined as an increase of at least 1.5 nmol/l compared to baseline levels. Area under the curve with respect to ground (AUCg) and increase (AUCi) were derived using trapezoidal formulas (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). As the SDI failed to induce a cortisol stress response in all but two participants, we did not use the AUCi as measure of cortisol responsivity in further analyses. Therefore, the AUCg was used (before and after therapy) as a measure of total cortisol output that captures basal cortisol secretion as opposed to stressor reactivity. AUCg was examined for normality of distribution within each group. Non-normally distributed data were log-transformed. See also for more detailed information our previous paper for additional information about the data handling (Zantvoord et al., 2019).

### *Statistics*

The distribution of baseline clinical, trauma and demographic characteristics across responders, non-responders and TEC was examined using $X^2$-tests for categorical variables, independent sample *t*-tests for normally distributed continuous variables and Mann-Whitney tests for non-normally distributed continuous variables. Paired sample *t*-test were used to examine pre- to post-treatment symptom change. These statistical analyses were performed using SPSS version 24.0 (SPSS Inc., Chicago IL, U.S.A). Raw DNA methylation profiles were imported into the statistical programming environment R (v.3.4.2) using the Bioconductor (v3.13) and the Minfi (v1.38.0) package (41). Data quality control was performed using MethylAid. We used the following MethylAid thresholds for quality control; 10.5 for methylated and unmethylated intensities, 12 for overall quality control, 11.75 for bisulphite control, 12.75 for hybridization control, and a detection P-value of 0.95(v1.26.0) (42). At this stage we removed one sample, because it did not meet our quality control criteria (Follow-up measurement). Further statistical analysis were based on M-values calculated as log2 (beta/ (1-beta). Probe expressing infinite values were removed from the dataset. Visualization of associations were based beta-values, i.e. methylation index, which ranges between 0% (methylated) and 100% (methylated). In the next step principal component analysis (PCA) was applied in order to detect any outliers and to evaluate concordant sexes of samples. Moreover, we performed hierarchical clustering analysis using 11 single nucleotide polymorphisms, known to be present on the Illumina EPIC array, to evaluate the concordance of the genotype within the longitudinal sample sets. Next we normalized the dataset applying the function *funnorm* and we removed all probes annotated to the allosomes, susceptible for cross-hybridization, and probes known to confounded by genetic variation with a minor allele frequency >1%.

Subsequently we correlated meta-data, i.e. sex, age and technical potential confounders (slide and array position) with the first eight principal components. These first eight principal

components explained together most of the variance. The qq-plots (of the expected p values versus the observed p values) had a lambda of >.0.85, <1.15 this indicated absence of type-I error inflation and no artificial differences between groups. From this analysis we defined the following statistical models for paired and unpaired analyses respectively:

*Paired analysis* **:** *methylation ~ group*

*Unpaired analyses* **:** *methylation ~ group + sex + age*

Differential methylated positions (DMPs) were obtained using *limma*. For the detection of DMRs we applied the *Bumphunter* function, wherein we used delta beta difference thresholds of 10 % and 5% for paired and unpaired analyses respectively. We analyzed the main (DMPs and DMRs) effects longitudinal and crosssectional between the following groups: (1) PTSD vs TC on the three different time points; T1: baseline level, before treatment, T2: directly after 8 sessions of trauma-focused treatment, T3: follow-up 6 months after treatment. We assumed a false discovery rate (FDR) < .05 for DMPs and a familywise error rate (FWER) of .05 for DMRs as significant. For our targeted approach we selected a limited number of replication loci ($N_{DMP}$ =170, $N_{DMR}$ = 30), which were based on a literature search which included published MWAS data in youth with PTSD, an previous published treatment studies until June 2021. We also added several candidate loci associated with glucocorticoid functioning and previously related to PTSD development. See supplementary Table 1. In our targeted approach we applied Bonferroni correction threshold wherein we assumed a $p < 0.002$ significant for the DMR approach, and a $p < 0.0003$ significant for the DMP approach. The Bonferroni threshold was the product of dividing critical $\alpha = 0.05$ by the number of DMR's and DMPs of interest extracted from previous studies. In total, we tested 170 DMP's and 30 DMR's, which we considered relevant based on previous studies.

Next, we calculated Pearson's correlation in SPSS between DNAm and our cortisol data. We measured the relation between DNA methylation at significant DMP's derived either from our epigenome-wide or from our targeted epigenetic analyses (if considered relevant to the glucocorticoid system) and cortisol secretion.

# RESULTS

### *Participant characteristics and clinical outcomes*

A summary of participant characteristics is shown in Table 1. Responders and non-responders did not differ in baseline sociodemographic, trauma, and clinical characteristics, apart from ethnicity and type of index trauma. At baseline, 93.48% of participants met the full DSM-IV diagnostic criteria for PTSD, the remaining 6.52% met criteria for a partial PTSD diagnosis. The most

common index trauma in the PTSD group was interpersonal violence, followed by sexual abuse. Youth with PTSD did not differ from trauma exposed controls (TEC) (N = 41) in gender and age, but did differ at baseline in ethnicity, and type of index trauma. The average baseline CAPS-CA score in treatment responders was M = 51.14 points, SD = 23.0, and in non-responders M = 47.75 points, SD = 22.6 which is indicative of moderately severe PTSD. Post treatment mean total CAPS-CA scores improved at T2 (M = 32.51 points, SD = 23.04 t (46) = 9.67, $p < .000$), and T3 (M = 23.5 points, SD = 22.59 t (18) = 4.41, $p < .000$). See Table 1.

**Table 1** Subject characteristics.

| | Responders (n= 22) | Non-responders (n= 24) | Controls (n= 41) | p-value |
|---|---|---|---|---|
| **Sociodemographic characteristics** | | | | |
| Female (%) | 40.9 | 67.7 | 53.7 | .215 |
| Age (years; mean, SD) | 12.09 (3.07) | 12.92 (2.74) | 11.70(3.81) | .379 |
| West European Ethnicity (%) | 73.7 | 47.8 | 85.4 | .006 |
| **Trauma characteristics** | | | | |
| Index trauma (%) | | | | **.000*** |
| Sexual abuse | 22.7 | 20.8 | 0 | |
| Interpersonal violence | 36.4 | 50.0 | 19.5 | |
| Accidents/Medical | 13.6 | 12.5 | 61.0 | |
| Other | 27.3 | 16.7 | 19.5 | |
| Repeated trauma exposure (%) | 59.1 | 58.3 | 22.0 | **.002*** |
| **Clinical characteristics** | | | | |
| CAPS-CA study entry T1 (mean, SD)[b] | 51.14 (23.0) | 47.75 (22.61) | - | .170 |
| CAPS-CA post treatment T2 (mean, SD)[b] | 19.82(16.14) | 45.50 (21.48) | - | **.000*** |
| CAPS-CA Follow-up treatment T3 (mean, SD)[b] | 9.09 (7.80) | 46.14 (19.1) | - | |
| Externalizing problem behavior T1 (%)[c] | 53.3 | 20.0 | 26.7 | **.037*** |
| Internalizing problem behavior T1 (%)[c] | 57.1 | 35.7 | 7.1 | **.002*** |

*Note:* Abbreviations: CAPS-CA, Clinician-Administered PTSD Scale for Children and Adolescents; [a] p-values <0.05 shown in bold. Independent samples t-test for continuous and $x^2$ tests for categorical variables. [b] Range: CAPS-CA total, 0-139; [c] Internalizing and Externalizing behavior are reported if a child scored above clinical cut-off on the RCADS/ YSR and SDQ RCADS, Revised Child Anxiety and Depression Scale;

### *Longitudinal epigenetic trajectories related to treatment outcome in youth with PTSD*

To examine the relation between successful treatment and DNAm we examined the effect of time of measurement (pre- (T1) and post-treatment (T2)) and follow-up treatment (T3) with the diagnosis of PTSD after treatment (responders versus non-responders). The results of the MWAS show no significant differences in treatment responders at T1 vs T2 (see Table 2a and b). Our targeted approach, showed one significant finding in treatment responders (T1 vs T2), at the *CRHR2* gene (*p = 0.0003*). Treatment responders showed increased methylation at T2, see Table 3, Figure 1. A more in depth examination of this gene related to the glucocorticoid system,

showed that increased methylation on the *CRHR2* gene in responders, was further associated with lower levels of basal cortisol at T2 (see Table 4). Additionally, our data indicates, however non-significant that in non-responders *CRHR2* methylation decreases from T1 to T2 (β = -0.013), this points to an opposite relation with cortisol secretion (decrease of methylation vs. increase of cortisol post-treatment), see Table 4. Furthermore, *CRHR2* methylation is correlated with cluster D symptoms (hyperarroU.S.Al) before treatment, in non-responders (see Table 4). We did not detect any longitudinal differences between T1 and T3 in treatment responders. In treatment non-responders, we did not detect any longitudinal differences between the T1 and T2, and T1 and T3.

**Table 2a** EWAS DMPs identified in the treatment responders at T2 compared with T1

| Responders T1 VS T2 | Gene | Probe | Chr | Position | m-value | FDR | Log Beta | Delta Beta |
|---|---|---|---|---|---|---|---|---|
| | *PHF15* | cg07525804 | 5 | 133914473 | 3,83E+07 | 0.2293 | 1,21E+09 | 0.0368 |
| | *HMBS* | cg27472151 | 11 | 118956135 | 5,91E+07 | 0.2293 | 1,63E+09 | 0.0211 |
| | *ARHGEF10* | cg14861020 | 8 | 1772352 | 1,03E+08 | 0.2656 | 3,72E+09 | 0.0258 |
| | | cg18890561 | 10 | 131988419 | 1,69E+07 | 0.3276 | 8,21E+09 | 0.0494 |
| | *RAB27A* | cg24809382 | 15 | 55582033 | 3,33E+08 | 0.3930 | 0.0002 | 0.0070 |

*Note:* Top 5 Responders vs. non-responders at T2 (post-treatment). DMPs: Differently methylated positions Genome build (HG19), Gene: UCSC Reference Gene Name, Chr: chromosome; m-value; FDR: false discovery rate adjusted p-value (Mval); LogBeta and DeltaBeta: delta differences between groups, based on average β-values ¨indicates a significant result.

**Table 2b** EWAS DMRs identified in the treatment responders at T2 compared with T1

| Responders T1 VS T2 | Gene | Chr: start-end | Area | L | Cluster (L) | *p*-value | FWER | Direction |
|---|---|---|---|---|---|---|---|---|
| | *ARSG* | 6: 291687-293285 | 0.557796234341063 | 7 | 7 | 3.15E-4 | 0.48 | T2>T1 |
| | *SOX2OT* | 17:6899207-6899577 | 0.540558928908835 | 8 | 20 | 3.13E-4 | 0.86 | T2>T1 |
| | *BIN2* | 10:12335523-123356041 | 0.514183280465409 | 7 | 14 | 3.98E-4 | 0.91 | T2>T1 |
| | *PARVB* | 3:1954890-195490033 | 0.490337023581752 | 7 | 9 | 4.49E-4 | 0.91 | T2>T1 |
| | *LGR6* | 17:80545175-80545434 | 0.446370934234037 | 5 | 6 | 5.34E-4 | 0.98 | T2>T1 |

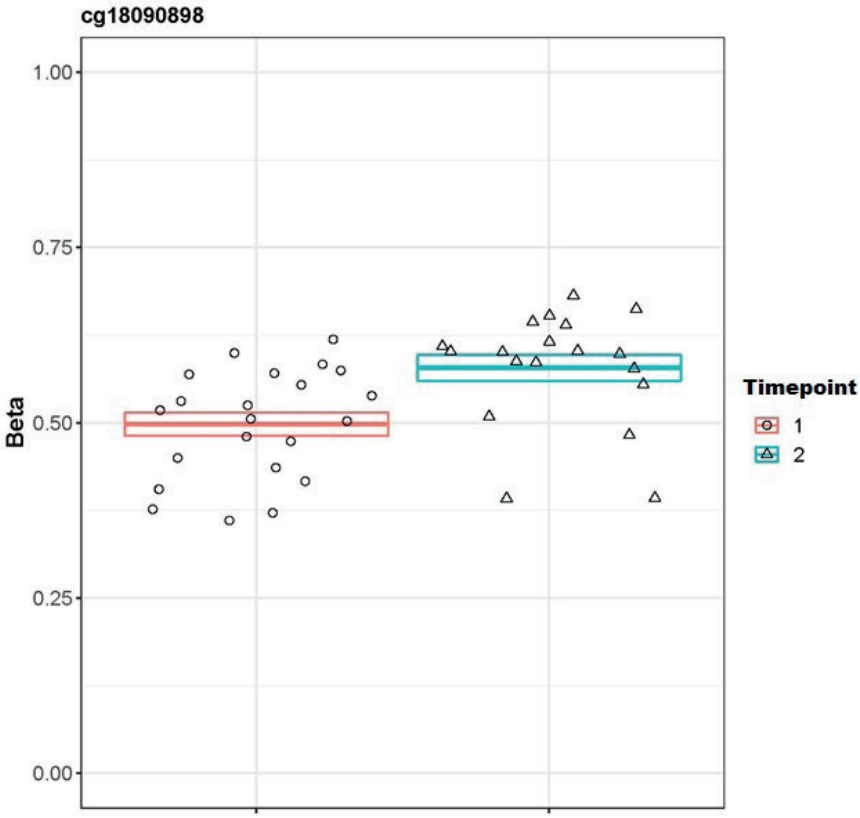
*Note:* Top 5 DMRs of association analyses of 1) Responders vs. non-responders at T2 (post-treatment). DMRs: Differently methylated regions Detected DMRs (L>1) using Bumphunter; Genome build (HG19), Gene: UCSC Reference Gene Name, Chr: start-end: chromosome and position; area: area bump; L: number of probes in DMR; cluster (L): number of probes in cluster; FWER = Family-Wise Error Rate**indicates a significant result.

**Table 3** Targeted search DMPs identified in the treatment responders at T2 compared with T1

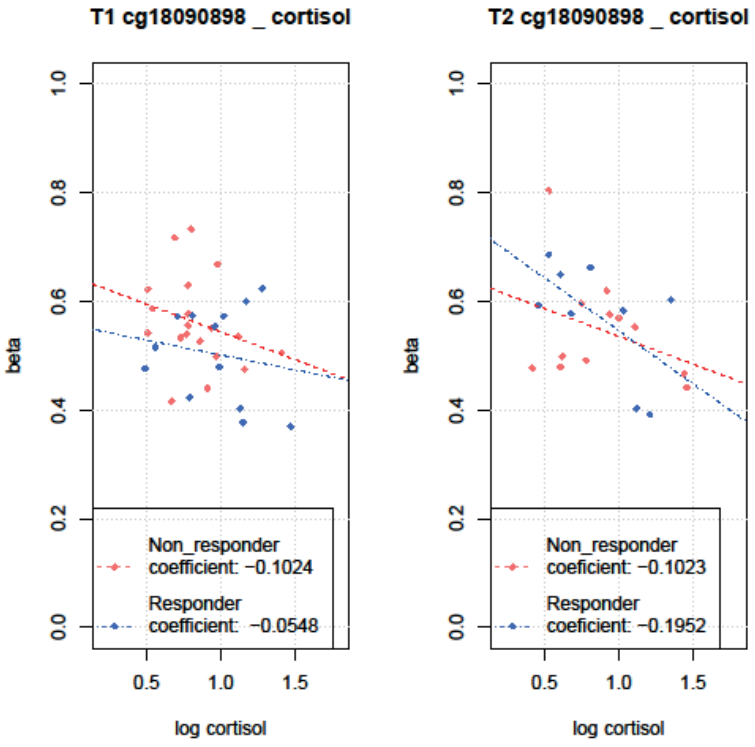
| Responders T1 VS T2 | Probe | Chr | Position | p-value | DeltaBeta | Gene | Gene Feature |
|---|---|---|---|---|---|---|---|
| | cg18090898 | 7 | 30739695 | 0.0003** | 0.0802 | *CRHR2* | 1stExon |
| | cg17700633 | 19 | 13263997 | 0.0018 | 0.0187 | *IER2* | 5'UTR |
| | cg14849556 | 2 | 24991672 | 0.0052 | 0.0382 | *NCOA1* | 3'UTR |
| | cg08550353 | 6 | 1,34E+08 | 0.0227 | -0.0397 | *SGK1* | TSS1500;Body |
| | cg26269677 | 5 | 76251688 | 0.0231 | -0.0546 | *CRHBP* | Body |

*Note:* Top 5 Targeted DMPs analyses of treatment responders at T1 (pre-treatment) vs T2 (post-treatment). DMPs: Differently methylated positions; Chr: chromosome; p-value; DeltaBeta: delta differences between groups, based on average β-value: Genome build (HG19), Gene: UCSC Reference Gene Name; Gene feature: gene feature according Illumina manifest. Bonferonni corrected P-value 0.0003*indicates a significant result.

**Figure 1** Relation between CRHR2 methylation and cortisol before and after treatment




*Note:* Treatment responders at the CRHR2 gene before and after treatment




*Note* Treatment responders and non-responders, cortisol values and CRHR2 gene individual beta's before and after treatment

**Table 4** Correlations between cortisol secretion and CRHR2 methylation

| | cg18090898 (CRHR2) Total sample | cg18090898 (CRHR2) Responders | cg18090898 (CRHR2) Non-Responders |
|---|---|---|---|
| Pretreatment cortisol | **-,395*** | -,173 | ,127 |
| Posttreatment cortisol | **-.458*** | **-,446*** | **,439*** |
| CAPS score Total T1 | **,369*** | **,471*** | -,122 |
| Total score Cluster B T1 | ,323 | ,264 | ,201 |
| Total score Cluster C T1 | ,088 | ,222 | -,071 |
| Total score Cluster D T1 | **,446*** | ,072 | **,534*** |

*Note:* Abbreviations: CAPS-CA, Clinician-Administered PTSD Scale for Children and Adolescents. Pearson's correlations between individual beta's on selected DMP and cortisol secretion data in youth with PTSD and different clusters of PTSD symptoms and cortisol levels before and after trauma-focused psychotherapy. *Correlation is significant at the 0.05 level (2-tailed).

### *Cross sectional epigenetic differences and clinical outcomes in PTSD patients and TEC*

Additionally, we performed a MWAS comparing the treatment responders, non-responders and trauma exposed controls (TEC) across the three different time points. Our DMP analysis revealed significant differences between responders and non-responders at T3, located on the *C18orf63* gene (cg15154763, FDR 0.05), indicating a decrease in *C18orf63* methylation in treatment responders (see Table 5). Our targeted approach yielded no additional significant findings. Despite not surviving our stringent multiple testing correction in our MWAS and targeted approach, of interest are our top five findings from our EWAS DMR analysis. These DMRs identified in the treatment responders vs non responders at T2 and T3 were located at *RNF39, ALOX12, DUSP22, DIP2C* and *HOXA4, MUC4* genes, were all previously related to treatment outcome and/or development of PTSD, see Table 6 (Carleial et al., 2021; Rutten et al., 2018; Vinkers et al., 2019).

**Table 5** EWAS DMPs identified in treatment responders vs. non responders at T2 (post-treatment), and T3 (follow-up)

| Responders vs Non Responders T2 | Probe | Chr | Position | FDR | DeltaBeta | Gene | Gene Feature |
|---|---|---|---|---|---|---|---|
| | cg24809382 | 15 | 55582033 | 0.99 | 0.0076 | RAB27A | TSS1500 |
| | cg17251423 | 5 | 139088815 | 0.99 | -0.1560 | CTB-35F21.1 | Body |
| | cg08273874 | 16 | 1060765 | 0.99 | 0.0554 | | - |
| | cg00313642 | 3 | 196014711 | 0.99 | 0.0414 | PCYT1A | Body |
| | cg08313638 | 10 | 12110577 | 0.99 | -0.0443 | DHTKD1 | body |
| **Responders vs Non Responders T3** | cg15154763 | 18 | 71982463 | **0.05**** | 0.2259 | C18orf63 | TSS1500 |
| | cg11198041 | 14 | 35344866 | 0.83 | 0.0801 | BAZ1A | TSS200 |
| | cg17700633 | 19 | 13263997 | 0.83 | -0.0246 | IER2 | 5'UTR |
| | cg07275860 | 12 | 120554537 | 0.83 | 0.0521 | RAB35 | 5'UTR |
| | cg14362428 | 6 | 31116408 | 0.83 | -0.0751 | CCHCR1 | Body |

*Note:* Top 5: EWAS DMPs from our cross sectional analysis between treatment responders vs. non-responders at T2 (post-treatment), and T3 (follow-up). DMPs: Differently methylated positions. Genome build (HG19 Chr: chromosome; FDR: false discovery rate based on bacon adjusted p-value (Mval); DeltaBeta: delta differences between groups, based on average β-value; Gene: UCSC Reference Gene Name; Gene feature: gene feature according Illumina manifest ¨indicates an epi-genome wide significant result.

To control for the effect of trauma exposure and time, we performed an additional analysis between the responders (22) and non-responders (24) and the TEC. We detected no significant DMR's or DMP's between the clinical group and the controls at T1 and T2. At T3, we detected one significant finding between the clinical groups and the control group at one DMR located on chr2: *NUP35* (FWER 0.05) Clinical groups showed hypermethylation compared to controls.

**Table 6** EWAS DMRs identified in treatment responders vs. non responders at T2 (post-treatment), and T3 (follow-up)

| Responders vs | Chr | Start | End | L | Cluster (L) | FWER | Direction | Gene |
|---|---|---|---|---|---|---|---|---|
| **Non Responders T2** | 6 | 30039380 | 30039801 | 10 | 36 | 0.39 | R>NR | *RNF39* |
| | 6 | 291687 | 293285 | 10 | 10 | 0.81 | R>NR | DUSP22 |
| | 17 | 6899207 | 6899577 | 9 | 23 | 0.87 | NR-R | ALOX12 |
| | 10 | 12335523 | 123356041 | 6 | 23 | 0.92 | R>NR | *FGFR2* |
| | 3 | 19548900 | 195490033 | 6 | 11 | 0.93 | NR-R | *MUC4* |
| **Responders vs Non Responders T3** | 6 | 291687 | 293285 | 10 | 10 | 0.51 | R>NR | *DUSP22* |
| | 10 | 530635 | 532357 | 15 | 21 | 0.55 | R>NR | DIP2C |
| | 7 | 2717021 | 27171051 | 14 | 32 | 0.56 | NR-R | HOXA4 |
| | 3 | 1954890 | 195490033 | 11 | 11 | 0.89 | NR-R | *MUC4* |
| | 12 | 47219626 | 47219920 | 8 | 13 | 0.93 | R>NR | *SLC38A4* |

*Note:* Top 5 EWAS DMRs from our cross sectional analysis between treatment responders vs. non-responders at T2 (post-treatment), and T3 (follow-up). DMRs: Differently methylated regions; Detected DMRs (L>1) using Bumphunter; Chr start-end: chromosome and position; L: number of probes in DMR; cluster (L): number of probes in cluster; FWER = Family-Wise Error Rate Direction: R= responders, NR = non-responders **indicates a significant result.

# DISCUSSION

To the best of our knowledge, this is the first study to detect DNAm in youth with PTSD in relation to trauma-focused psychotherapy response.

The results from our epigenome-wide *longitudinal analysis* in treatment responders and non-responders did not indicate any significant effects that survived our multiple testing correction. Only at T3, we detected one significant finding between the clinical groups and the control group at one DMR annotated to the *NUP35* gene. Clinical groups showed hypermethylation compared to controls. Differential methylation on *NUP35* was mentioned before in relation to variation in cognitive function between twins (Wang et al., 2021).

Our epigenome-wide *cross-sectional analysis* showed significant differences between responders and non-responders at T3 (follow-up), located on the *C18orf63* gene. Indicating a decrease in *C18orf63* methylation in treatment responders. A prior epigenome-wide methylation study found a relationship between *C18orf63* methylation and socioeconomic status (SES) in placentas from preterm infants (Santos Jr et al., 2019). We consider these findings relevant to our findings since variance in SES is associated with many health outcomes. Including the development of neurodevelopmental and neurobehavioral disorders in children, as well as poorer adult health status and shorter life expectancy (Chin-Lun Hung et al., 2015; Nelson III, 2017; Santos Jr et al., 2019). The association with DNAm might imply biological embedding of SES adversity within critical developmental periods, which in turn could affect long-term child health outcomes

(Santos Jr et al., 2019). On a molecular level *C18orf63* is related to the PAX5 gene (*C18orf63* Gene - Gene Cards | CR063 Protein | CR063 Antibody). *PAX5* was previously found to be related to PTSD and depression (Erin C Dunn, Wang, & Perlis, 2020; Rasha Hammamieh et al., 2017). Thompson and colleagues suggested that the perceived capacity of *PAX* genes to respond to stress is relative high and PAX genes seem to respond within the central nervous system (CNS) as well as interact with a damaged or regenerating environment (Thompson & Ziman, 2011). In addition, the *PAX5* gene plays an important role in inflammatory responses (by regulating B-cell differentiation). Multiple studies observed the associated between elevated levels of inflammation in PTSD (Kim, Lee, & Yoon, 2020; Speer, Upton, Semple, & McKune, 2018). Additionally, DNA methylation in genes related to the inflammatory responses are observed as well in PTSD (Al Jowf, Snijders, Rutten, de Nijs, & Eijssen, 2021; Katrinli et al., 2022; Alicia K Smith et al., 2020). These findings indicate that alterations of specific inflammatory markers in individuals with PTSD maybe related (or induced by) alterations on specific DNAm sites. In addition to these outcomes our MWAS DMR analysis showed a subsequent amount (nominal significant) of overlap with DMR's reported in the first treatment related studies in adults with PTSD, annotated to *RNF39*, *DUSP22*, *ALOX12, DIP2C* and *HOXA4*, *MUC4* genes (Carleial et al., 2021; Kumsta, 2019; Rutten et al., 2018; Vinkers et al., 2019; Yang et al., 2021). Additionally, we detected one locus of interest regarding responsivity to treatment located on the *ALOX12* genomic region. *ALOX12* is the predominant LOX enzyme in the brain and previously this location was related to cortical thickness in PTSD, responsivity to oxidative stress en elevated inflammatory responses (Løkhammer et al., 2022; G. E. Miller et al., 2008; M. W. Miller et al., 2015). Interestingly, the few structural neuroimaging studies of trauma-focused psychotherapy in adults found evidence for pre-to post treatment changes within several regions of the cortex, including the prefrontal cortex, cingulate cortex and insula (Boukezzi et al., 2017; Helpman et al., 2016; M. W. Miller et al., 2015; Zantvoord, Diehle, & Lindauer, 2013). However, imaging data from the cohort used in this study did not replicate the relationship between treatment response and prefrontal cortex volume change, yet we did show that non-response to trauma-focused psychotherapy was characterized by longitudinal bilateral volume decrease in both the posterior and anterior insula (Zantvoord et al., 2021).

Our targeted approach showed interesting *longitudinal* outcomes. We observed differential DNAm before and after treatment in treatment responders at the *CRHR2* genomic region (cg18090898). In response to acute stress, the hypothalamic corticotrophin releasing hormone (CRH) interacts with CRHR2 (a corticotrophin releasing hormone receptor), and it stimulates the anterior pituitary to release adrenocorticotropic hormone. In turn this hormone stimulates the adrenal cortex to release the hormone cortisol (Bale et al., 2000; Liaw et al., 1996). Interestingly, in our sample substantial correlations between DNAm at this site and cortisol secretion before and after treatment were

observed. Increased methylation on *CRHR2* genomic region in treatment responders was associated with an observed increase of cortisol during trauma-related psychotherapy. The direction of effect of these findings seems in line with the results of previous studies. These studies showed that lower levels of pretreatment cortisol and lower cortisol change during treatment are related to poorer treatment outcome (Castro-Vale & Carvalho, 2020; Zantvoord et al., 2019). Prior studies indicate that enhanced levels of cortisol can modulate memory processes of emotionally arousing experiences. In general, it is found that increased levels of cortisol relate to a better memory consolidation. Since in trauma focused psychotherapy, re-exposure to the traumatic events is important part of the therapy, it might be that the increase of cortisol during treatment facilitates the coping with the emotional load during re-exposure and enhances the extinction of the associated strong emotions during treatment in the responders (Roozendaal, McEwen, & Chattarji, 2009). Despite that we could not confirm other previous found differences in DNAm, in relation to treatment response, in specific glucocorticoid related regions, such as on the *FKBP5* and *NR3C1* genes. Our findings on the *CRHR2* genomic region do support the hypothesis that DNAm at stress-related genes is related to glucocorticoid signaling. This in turn could be related to emotional reactivity during treatment, previously identified as a risk factor for ongoing disbalance after exposure to traumatic events early in life (Agorastos et al., 2019).

This study has several strengths and limitations. The major strengths of our study are that we analysed differential DNAm with use of both a cross-sectional and longitudinal design, and the standardized clinical and biological assessments in a unique sample of youth with PTSD. Furthermore, we used an unbiased methylome-wide approach, extended with a literature based targeted approach, and we included a trauma exposed reference group to control for effect of trauma. We used stringent exclusion criteria and a strict multiple testing correction. However, there are also several limitations that have to be acknowledged. Firstly, the relative small size of the groups included in this study, limited us to detect significant epigenome wide results (P.-C. Tsai & J. T. Bell, 2015). This also limited us in our ability to examine differences between treatment responders and non-responders for both treatment conditions separately. Despite that both are equally effective trauma-focused psychotherapies in youth (Hoogsteder, Ten Thije, Schippers, & Stams, 2021). And both therapies share multiple common elements such as exposure to and reprocessing of traumatic memories (Kooij et al., 2022), we acknowledge that there is need for additional research. Information from cohorts with larger sample-sizes, or combined meta-analyses might provide a deeper insight between possible differential effects of both therapies in relation to epigenetic predictors of treatment response and/or biological changes during treatment. Furthermore, the considerable dropout rate of randomized patients lost at follow-up, restricted us further in our longitudinal analysis. Although, dropout rates in our study reflect routine clinical practice, there is a possibility that drop-out could have influenced our main findings through attrition bias.

Secondly, there are several limitations regarding our post-hoc cortisol measures, such as the reaction on our script driven imagery procedure and the lack of an additional circadian cortisol secretion measures. These limitations are described in more detail in our previous paper (Zantvoord et al., 2019). Furthermore, despite that we have tried to account for most confounding factors, we were not able to include all known factors to influence endocrine function, and for example, we omitted inquiry on menstrual and pubertal stage. Lastly, another possible limitation is the relevance of methylation in saliva to other tissues such as the brain, given that methylation differences across tissues are substantial. Despite that consistent effects of various methylation quantitative trait loci (mQTLs) are found across tissues (Hannon et al., 2018), partial evidence exists on cross-tissue consistent findings (Armstrong, Lesseur, Conradt, Lester, & Marsit, 2014). Therefore, a cautious approach to the interpretation of findings obtained from single tissue analyses is needed. Additionally, for future studies we suggest to link PTSD, related treatment outcome and DNAm with specific brain and endocrine related endophenotypes. Given the expected age and time dependent differences, especially considering HPA-axis and brain plasticity, during critical periods in development, it would be helpful to increase sample sizes and include youth already early in life. Thereby increasing feasibility to differentiate across developmental stages. In addition, the use of continuous outcomes (using symptom dimensions scores instead of a dichotomous diagnosis), reflecting the different symptoms of PTSD and associated emotional and behavioral problems might be considered in future research.



## Conclusion

In conclusion, this is the first study in youth with PTSD that shows the association between successful trauma-focused psychotherapy with specific DNAm changes. Overall, our results do support and extend previous outcomes presenting DNAm change in relation to treatment response in PTSD. Presenting DNAm change in specific genes related to exposure to trauma, the development of PTSD and its related psychological treatments (Hoye et al., 2020; Vinkers et al., 2019; Yehuda et al., 2013). This study provides further insight in underlying biological mechanisms, and indicates how biological mechanisms might interact with symptomatic change in youth with PTSD. These results provide novel insights that may contribute to the discovery of the epigenetic mechanisms underlying a successful treatment of PTSD, especially related to HPA-axis related endophenotypes. We expect that these findings may help to better understand how psychological and biological systems interact on a molecular level in order to improve and individualize treatment outcomes. Since ideally we aim to prevent ongoing PTSD symptoms in youth, and its severe consequences, by improving interventions that are better tailored to each individual patient.

# SUPPLEMENTARY INFORMATION

**STable 1** Selected DMPs and DMRs for targeted approach

| |
|---|
| cg17700633 cg21972431 cg26196496 cg00862770 cg03591753 cg06937024 cg07485685 cg07843056 cg11845071 cg15929276 cg19226017 cg25114611 cg03546163 cg06087101 cg07485685 cg08423118 cg16052510 cg16586394 cg17860381 cg19645279 cg20728768 cg21702128 cg25579735 cg01170198 cg16562342 cg20012601 cg05616442 cg00130530 cg00629244 cg01277438 cg01294490 cg01312837 cg01967637 cg02521996 cg02564102 cg02665568 cg02842899 cg03857453 cg03883275 cg04444450 cg04444450 cg05121010 cg05790989 cg05790989 cg06521673 cg06613263 cg06937024 cg07061368 cg07528216 cg07733851 cg08586216 cg08845721 cg09566021 cg10300814 cg10847032 cg10913456 cg10913456 cg11152298 cg11321922 cg11540119 cg11916669 cg13103915 cg13135255 cg13344434 cg13648501 cg13986355 cg14284211 cg14558428 cg14642437 cg14825287 cg14849556 cg15115787 cg15910486 cg15912732 cg16005389 cg16012111 cg16012111 cg16182267 cg16224829 cg16335926 cg16569373 cg17030679 cg17085721 cg17406386 cg17617527 cg18019515 cg18068240 cg18071894 cg18146873 cg18484679 cg18849621 cg19014730 cg19261497 cg19457823 cg20090430 cg20509117 cg20509117 cg20730067 cg20813374 cg21979215 cg22237988 cg23273257 cg23416081 cg23462257 cg23523922 cg23624957 cg23751680 cg24026230 cg24295963 cg24307117 cg25368824 cg25535999 cg26049684 cg26464411 cg26495008 cg26560981 cg27345592 cg22046703 cg24738082 cg01049782 cg01819552 cg04923928 cg23185751 cg03667083 cg09516959 cg04922810 cg07658503 cg21773872 cg01972879 cg13094036 cg02712145 cg01718447 cg15615793 cg01972879 cg23185751 cg18090898 cg26269677 cg05620787 cg13777717 cg17238830 cg26196496 cg16545105 cg21199406 cg01071966 cg21842274 cg17448335 cg05183646 cg05966641 cg25661219 cg03146155 cg08550353 cg21834463 cg03762694 cg21676440 cg14905466 cg21078322 cg09404376 cg08647910 cg13307058 cg07340870 cg25025235 cg11856561 cg06642177 cg25150212 cg12871835 cg17284168 cg12009778 cg24688636 cg02904344 cg21064939 |

*Note:* Selected DMPs (Differentially methylated positions)

**STable 2**

| Name | loc | Start | End |
|---|---|---|---|
| TNXB | chr 6 | 32064573 | 32064660 |
| PM20D1 | chr 1 | 205818956 | 205819609 |
| TNXB | chr6 | 32063901 | 32064258 |
| DUSP22 | chr6 | 291687 | 293285 |
| GDF7 | chr2 | 20870087 | 20871401 |
| SLC1A4 | chr2 | 65217211 | 65217623 |
| KLHL35 | chr11 | 75139390 | 75139680 |
| ZNF714 | chr19 | 21264896 | 21265421 |
| OLFM3 | chr1 | 102312608 | 102312671 |
| NR3C1_1 | chr5 | 142782046 | 142782472 |
| NR3C1_2 | chr5 | 142783585 | 142783906 |
| NR3C1_3 | chr5 | 142784559 | 142784950 |
| SLC6A4_1 | chr 17 | 28562939 | 28563283 |
| SLC6A4_2 | chr 17 | 28562574 | 28562952 |
| SLC6A4_3 | chr 17 | 28562328 | 28562682 |
| OXTR-1 | chr 3 | 8799262 | 8799615 |
| OXTR-2 | chr 3 | 8800371 | 8800739 |
| FKBP5 | chr 6 | 35.558.322 | 35558593 |
| MUC4 | chr3 | 195489306 | 195490309 |
| APOB | chr2 | 21266500 | 21267212 |
| EDN2 | chr1 | 41950237 | 41950392 |
| ZFP57 | chr6 | 29648271 | 29648623 |
| GPX6 | chr6 | 28478268 | 28478579 |
| CFAP45 | chr1 | 159869902 | 159870134 |
| AFF3 | chr 2 | 100720526 | 100720529 |
| TP73 | chr 1 | 3600735 | 3600879 |
| UBCLP1 | chr 5 | 158689508 | 158689629 |
| RPL13P | chr 6 | 28829171 | 28829433 |
| DMR11_BOKS | chr 19 | 11784955 | 11785188 |
| DMR12_BOKS | chr 17 | 6558365 | 6558440 |

*Note:* Selected DMRs (Differentially methylated positions)