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ARTICLE

Successful treatment of post-traumatic stress disorder reverses DNA methylation marks

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Abstract

Epigenetic mechanisms play a role in the detrimental effects of traumatic stress and the development of post-traumatic stress disorder (PTSD). However, it is unknown whether successful treatment of PTSD restores these epigenetic marks. This study investigated longitudinal changes of blood-based genome-wide DNA methylation levels in relation to trauma-focused psychotherapy for PTSD in soldiers that obtained remission ($N = 21$), non-remitted PTSD patients ($N = 23$), and trauma-exposed military controls ($N = 23$). In an independent prospective cohort, we then examined whether these DMRs were also relevant for the development of deployment-related PTSD ($N = 85$). Successful treatment of PTSD was accompanied by significant changes in DNA methylation at 12 differentially methylated regions (DMRs) in the genes: *APOB, MUC4, EDN2, ZFP57, GPX6, CFAP45, AFF3, TP73, UBCLP1, RPL13P*, and two intergenic regions (p values < 0.0001 were confirmed using permutation and sensitivity analyses). Of the 12 DMRs related to PTSD symptom reduction, consistent prospective evidence was found for ZFP57 methylation changes related to changing PTSD symptoms ($B = -0.84$, $t = -2.49$, $p = 0.014$). Increasing ZFP57 methylation related to PTSD symptom reduction was present over and above the relation with symptoms, suggesting that psychological treatments exert biological effects independent of symptom reduction. Together, these data provide longitudinal evidence that ZFP57 methylation is involved in both the development and successful treatment of deployment-related PTSD. This study is a first step to disentangle the interaction between psychological and biological systems to identify genomic regions relevant for the etiology and treatment of stress-related disorders such as PTSD.

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Introduction

Evidence has accumulated that epigenetic mechanisms such as DNA methylation are involved in the response to traumatic stress and the subsequent risk for post-traumatic stress disorder (PTSD) [1–3]. In contrast to the extensive attention for epigenetic factors in the relation between trauma and PTSD, considerably less attention has been given to epigenetic mechanisms that drive symptomatic remission from PTSD, either spontaneous recovery or as a result of treatment. Nevertheless, PTSD treatment ultimately will be reflected in biological regulation and, as such, is likely to be accompanied by specific epigenetic changes [4]. Several forms of psychotherapy are effective, such as trauma-focused cognitive behavioral therapy (tf-CBT) and eye movement desensitization and reprocessing (EMDR) [5]. Nevertheless, a significant number of PTSD patients do not reach remission following treatment [6]. To better understand this heterogeneity in treatment response, identification of specific epigenetic changes related to favorable treatment outcomes could shed light on the underlying biological processes. In turn, these epigenetic correlates relevant for PTSD recovery could lead to new treatment targets, for example related to fear learning and extinction, cognitive restructuring, and emotional processing [7, 8]. Moreover, pinpointing specific epigenetic changes related to PTSD recovery could also help unravel the etiology of PTSD and open up avenues to novel treatment and predictive opportunities. We therefore examined genome-wide DNA methylation profiles from blood before and after trauma-focused psychotherapy in both responding and non-responding PTSD patients as well as trauma-exposed controls. Significant DNA methylation findings from this treatment cohort were then related to the development of PTSD in an independent prospective military cohort before and after deployment. We hypothesized that symptomatic remission of PTSD would be accompanied by specific changes in DNA methylation, and that these markers would also be involved in the development of deployment-related PTSD symptomatology.

Materials and methods

Participants

PTSD treatment cohort (BETTER cohort)

Participants were included as part of the observational longitudinal treatment study *BETTER* and carried out between September 2010 and September 2013 (Fig. 1). Data were available for 44 male war veterans with combat-related PTSD (patients) and 23 male war veterans without PTSD (controls). Sample size was chosen to detect effect sizes similar as previous longitudinal studies of PTSD development [3]. Patients were recruited from four outpatient clinics of the Military Mental Healthcare Organization. Trauma-exposed controls were recruited via advertisements in local newspapers. At the time of inclusion, PTSD diagnosis was ascertained using the Clinician-Administered PTSD Scale-IV (CAPS, see below for details) with no current concomitant alcohol or substance dependence or neurological disorder. Trauma-exposed controls had no PTSD symptoms (CAPS score <15), no current psychiatric disorder, no alcohol or substance dependency, no neurological disorder, and no lifetime PTSD. The Structured Clinical Interview for DSM-IV (SCID) was administered to assess (co-existing) psychopathology in all participants. All participants had been deployed at least once for a period of at least 4 months. Participants received monetary compensation for participation. Written informed consent was obtained from all participants in accordance with procedures approved by the University Medical Center Utrecht ethics committee and in accordance with the Declaration of Helsinki.

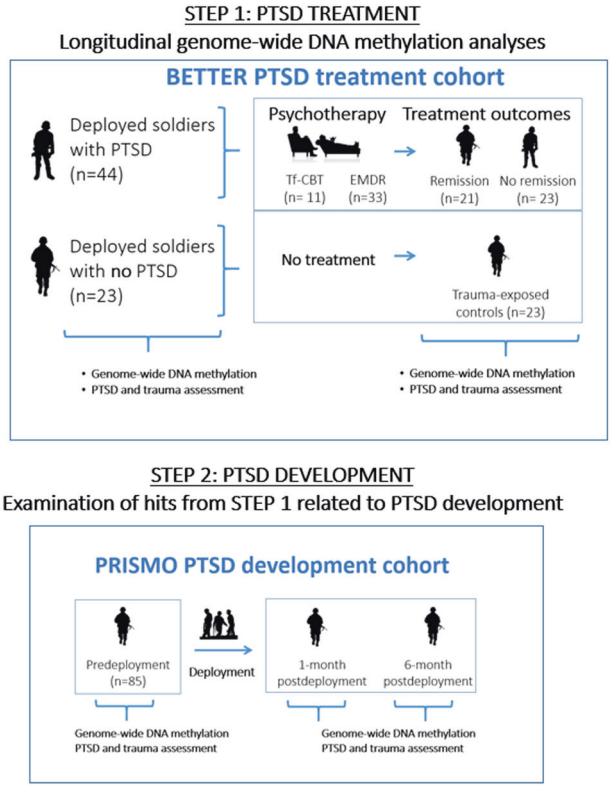

Fig. 1 Design of the longitudinal BETTER treatment cohort used for epigenome-wide analyses related to the successful treatment of PTSD and the PRISMO treatment cohort used to examine the hits in the treatment cohort. *EMDR* eye movement desensitization reprocessing, *CBT* cognitive behavioral therapy

PTSD assessment

All participants were assessed at baseline (T0) and 6–8 months later (T1). A PTSD diagnosis and severity were established using the Clinician-Administered PTSD Scale-IV (CAPS) administered by a clinician or trained researcher [9]. This semi-structured interview is the gold standard PTSD instrument in the field and has good psychometric properties [10]. A PTSD case was identified if subjects endorsed the requisite DSM-IV symptoms at least at a frequency of 1 and at an intensity of 2 [10]. Participants were included in the control group if the total CAPS score was 15 or lower. Depressive symptoms were quantified with the validated 90-item Mood and Anxiety Symptom Questionnaire [11], also validated in the Dutch language [12].

PTSD treatment

All patients initiated trauma-focused psychotherapy after inclusion. Patients received eye movement desensitization reprocessing (EMDR) including trauma-focused cognitive behavioral therapy (tf-CBT) techniques ($N = 32$) or tf-CBT without EMDR ($N = 11$), one patient declined treatment. The number of sessions did not significantly differ between these two groups (EMDR: 10 (SD = 5.5), tf-CBT: 8 (SD = 4.5), $P = 0.19$). Psychotropic medication for PTSD or for comorbid conditions was accepted during treatment on the basis of clinical indications, although it was not the primary mode of treatment. At inclusion 11 patients used SSRIs, two Tricyclic antidepressants, nine were on benzodiazepines, and two on beta-blockers. Symptom reduction was quantified by subtracting the baseline from the follow-up CAPS total score (ΔCAPS) for each participant. Remission of PTSD was defined by CAPS score <45 at follow up [13].

PTSD development cohort (PRISMO cohort)

To investigate the relevance of treatment-related DNA methylation findings for PTSD development, we used new unpublished longitudinal data from 85 participants in the independent prospective PRISMO military cohort assessed before and at two time points after deployment (1 and 6 months post deployment) to Afghanistan [14]. Participant selection was based on the level of traumatic stress exposure and the presence of PTSD symptoms after deployment to maximize transitions from healthy to PTSD and included only participants not included in previous studies [3]. Mean age at inclusion was 27.3 (SD = 8.7), eight participants (9%) were female. PTSD symptoms over the previous 4 weeks were assessed with the 22-item Self-Report Inventory for PTSD (SRIP), which has good reliability and validity. Epigenetic data were available at three time points: before deployment, at return just after the deployment (average duration of employments 6 months), and after 6 months [3].

Genotyping and methylation quantification

PTSD treatment cohort

DNA was extracted from whole blood using standard protocol, DNA concentration and quality were examined using Picogreen. Genotyping was conducted using Illumina Human OmniExpress 24 v1.1. After treating the DNA with sodium bisulfite (Zymo Research, CA, USA), bisulfited DNA was quantified with the picogreen. Genome-wide DNA methylation was quantified using the Infinium MethylationEPIC BeadChip Kit (Illumina, Inc., San Diego, CA). The dataset was preprocessed in R version 3.3.3 with the *meffil* package [15] using functional normalization [16]. There were no samples with <3 beads in 20% of the probes. All samples matched their genetic identity using SNP profile included on the array for quality control purposes. Four samples were removed because of failed hybridization (outliers on control probes, 5 SD from the mean), and 1673 probes removed (detection p value > 0.01). Non-specific probes and those with SNPs in the probe sequence were removed [17]. After quality control, 865,163 probes were left for further analysis.

PTSD development cohort

Sample processing in the PRISMO cohort was identical to the BETTER cohort. There were no samples with <3 beads in 20% of the probes. All samples were positively identified. The majority by their genetic identity and 13 based on epigenetic age estimate, gender, and smoking status. Nine samples had to be removed because of failed hybridization as indicated by outliers (3 SD from the methylation mean or outside the predefined boundaries of a control probe). 1152 probes with detection p value > 0.01 were removed. Non-specific probes and those with SNPs in the probe sequence were removed [17]. We analysed the identified DMRs in 239 samples of 85 individuals. The level of methylation is expressed as a 'beta' value, ranging from 0 (no cytosine methylation) to 1 (complete cytosine methylation) but analyses were performed using M-values (log2 of beta values), for a better statistical validity [18].

DNA methylation analyses

PTSD treatment cohort

To adjust for technical batch effects, surrogate variables (SVA) were calculated as implemented in Meffil [19]. The

correlation of these SVA with methylation represented as principle components and other potential confounders including genetic principle components has been included in the supplemental material (Supplementary Fig. S1). Cell type composition was estimated using the Houseman algorithm [20]. Optimal fit was obtained based on qq-plotting and inspection of the potential confounding with inclusion of known confounders (genetic ancestry, cell type composition, age, and gender) to avoid type I error inflation. The optimal model included using HC3 robust error estimates to account for differences in distribution. Six SVA were included that effectively accounted for cell type composition and technical batches (see Supplementary Fig. S1) and three genetic principal components. To identify differentially methylated positions, longitudinal analyses were conducted using DNA methylation levels at 6–8 months after treatment as the outcome in a linear regression model with DNA methylation levels at 1 month before deployment as an indicator; i.e., $\text{methylation}_{\text{posttreatment}} \sim \text{methylation}_{\text{pretreatment}} + \text{covariates} +$ change CAPS score. The qq-plot (of the expected p values versus the observed p values) had a lambda of 0,99 (Supplementary Fig. S2) indicated absence of type-I error inflation and no artificial differences between groups. False discovery rate p values were calculated according to the Benjamini–Hochberg method. The assumptions of the linear regression were evaluated by inspecting the distribution of residuals for the identified loci. Differentially methylated regions (DMRs) were calculated based on the p values for each methylation locus using the *DMRcate* package [21]. A DMR consists of a strongly associated locus (p value < 0.0001) and several other significantly associated loci within the proximity of 1.000 base pairs. Using DMRcate, the furthest loci define the borders (start and stop location) of a DMR. *DMRcate* calculates an overall p value for the DMR using the Stouffer procedure. The associations were confirmed using permutation analyses that randomly selected the same number of adjacent loci throughout the genome and calculated the p value of the association of this potential DMR using the Stouffer method. From this distribution of potential DMRs an empirical p value was derived. Moreover, correction for genetic banding was investigated (see Supplementary methods and Supplementary Fig. 3 for details). KEGG and Gene Ontology (GO) pathway analyses were performed for the relevant DMRs (for details see Supplementary Material, code available at request).

PTSD development cohort

In the PTSD development cohort, significant DMR findings from the BETTER cohort were analyzed at three time points using a mixed model for repeated measures using the mean methylation at the DMRs as outcome and time × PTSD symptom level as determinant of interest in a model with baseline DMR methylation and age and gender as covariates. Confirmation was established when treatment-related DMRs would be significantly associated with the development of PTSD, in the opposite direction compared with the treatment effects.

Sensitivity analyses

PTSD treatment cohort

To assess the potential influence of confounding variables in the treatment cohort, the following sensitivity analyses were conducted. First, we rerun the analysis by excluding participants that did not have PTSD and therefore did not receive treatment (trauma-exposed controls). Second, although longitudinal analyses reduce the odds of confounding as participants are their own controls, the statistical relationship of the DNA methylation and main determinants with potential confounders such as medication use, alcohol use and smoking habits was investigated using t-tests. Variables with associations at the $p = 0.1$ level with PTSD and DNA methylation level of the DMR were included as covariate in the subsequent regression models. Third, to investigate whether treatment impacted on methylation changes over and above PTSD symptom change, treatment was added as a factor in the model. Fourth, the effect of type of therapy (tf-CBT or EMDR) was analysed with treatment type as indicator in the subset of participants that received psychotherapy. Outliers were investigated and defined as any observation with more than 3 standard deviations from the mean and a cook's distance larger than 0.5.

Results

PTSD treatment efficacy

PTSD treatment was effective in reducing PTSD symptoms, resulting in symptomatic remission in 21 of the 44 PTSD patients entering treatment (47%, $\chi^2 = 25.7$, df = 1, $p = 3.99 \times 10^{-7}$) (Fig. 2). The mean total CAPS scores were significantly reduced in PTSD patients that received treatment (mean CAPS reduction 22.8, SD = 26.4, $t = 5.55$, $p < 0.001$) (Fig. 2). In these remitted individuals, PTSD symptoms were consequently significantly more reduced ($B = -0.097$, $t = -3.55$, $p < 0.001$) (Fig. 2a).

Genome-wide DNA methylation changes and successful PTSD treatment

Twelve DMRs were significantly associated with change in PTSD symptoms following treatment (Table 1, $P < 0.0001$).

Permutation analysis confirmed the associations with the exception of the DMR in the *RPL13P* promotor (DMR10, empirical p value $p = 0.08$). Changes in medication ($N = 16$), smoking ($N = 6$), and coffee use ($N = 4$) were not associated with changes in PTSD scores (all p values > 0.1), nor were DMR methylation changes related to changes on any of these parameters (all p values > 0.1) and they were therefore not added as covariate. Based on the DMR analyses, pathways associated with PTSD reduction included 'anchoring and adherens junction' as well as 'regulation of cell morphogenesis' (Supplemental Table S1). Single CpG analysis did not identify genome-wide significant results (all p values > 4.2×10^{-7}, see Supplementary Material Table 2 present the full list of nominal significant methylation loci). To assess the specific of the 12 DMRs for PTSD symptoms, we repeated the analyses for depressive symptoms before and after treatment. There was a significant but limited overlap between PTSD symptoms and depressive symptoms ($r = 0.32$, $p < 0.01$). Using the same statistical models, only 1 out of 12 DMRs reached nominal significance (AFF3, $p = 0.030$, other DMRs: all p values > 0.13), but this finding would not survive correction for multiple testing. This sharply contrasts with p values for these DMRs related to PTSD improvement (see Table 1).

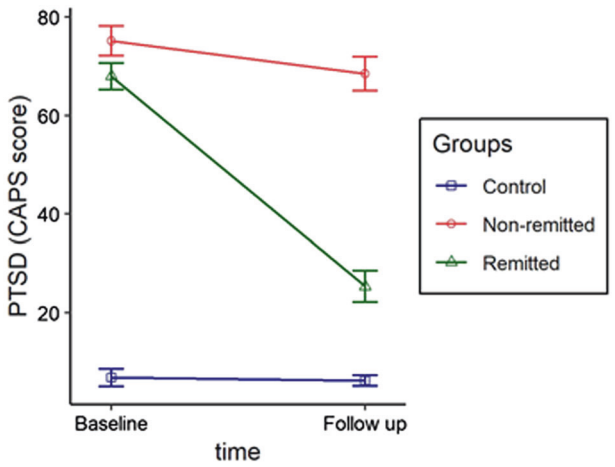

Fig. 2 Efficacy of psychological treatment on PTSD symptomatology (CAPS scores of remitted or non-remitted PTSD patients, green and red line, respectively) compared to trauma-exposed controls with no PTSD (blue line)

Relevance of the treatment-related DMRs for PTSD development

The relevance of the 12 identified DMRs for the development of PTSD symptoms was examined using data from a new independent sample of the longitudinal PRISMO cohort with epigenetic data from three waves. Table 2 shows the results of the analyses in the PTSD development cohort. Of these 12 DMRs, methylation of one DMR significantly decreased related to increased PTSD symptoms: *ZFP57* ($B = -0.85$, $t = -2.49$, $p = 0.014$). Another DMR was also significantly associated with increased PTSD symptoms but DNA methylation increased both for PTSD development (*UBCLP1*: $B = 2.09$, $t = 2.09$, $p = 0.038$) and PTSD remission and therefore does not pose a true replication. Visualization of *ZFP57* methylation over time shows an increase in *ZFP57* methylation following symptomatic remission and a significant association of reduced PTSD symptoms with changes in ZFP57 methylation ($B = 1.3$, $t = 3.46$, $P = 0.001$) (Fig. 2b, c).

ZFP57 as an epigenetic locus for both the development and remission of PTSD

Sensitivity analyses

As *ZFP57* methylation was the only DMR consistently related to both PTSD development and treatment-related remission, this DMR was further examined. Restricting *ZFP57* methylation analyses to participants with PTSD (excluding the trauma-exposed controls) increased the strength of the association ($B = -0.016$, $t = -2.82$, $p = 0.008$). Also, restricting the *ZFP57* DMR to previously identified CpGs (cg20228636, cg11383134, and cg03198009) [3] yielded similar results ($B = -0.005$, $t = -2.26$, $p = 0.027$). No outliers were identified.

PTSD treatment types

Compared with no treatment, the effects of PTSD treatment on increased ZFP57 methylation were over and above the effect of symptom reduction alone ($B = 0.623$, $t = -2.298$, $p = 0.025$). Of the two treatment modalities, this effect was

Table 1 Characteristics of the PTSD treatment cohort

	All	Controls	Remitted	Not remitted
N	67	23	21	23
Mean age (sd)	37.9 (9.9)	38.03 (10.7)	34.7 (9.3)	40.8 (9.1)
PTSD score baseline Mean CAPS (sd)	49.4 (33.4)	5.0 (4.4)	66.9 (13.0)	75.1 (14.4)
PTSD score follow up Mean CAPS (sd)	33.6 (29.4)	5.7 (4.5)	25.0 (14.2)	68.4 (18.3)
Therapy type: EMDR (%)	18 (26.8%)	0	7 (33%)	11 (47%)

Table 2 Differentially methylated regions (DMRs) significantly associated with reduced PTSD symptoms following treatment and the changes of these DMRs in an independent PTSD development cohort

							PTSD treatment cohort[a]		PTSD development cohort[b]	
Name	loc	Start	End	Length	Nr CpGs	Gen ID	Direction	*p* value	Direction	*p* value
DMR1	chr3	195489306	195490309	1004	9	*MUC4*	−	6,85E−16	+	0.865
DMR2	chr2	21266500	21267212	713	12	*APOB*	+	8,54E−15	−	0.846
DMR3	chr1	41950237	41950392	156	4	*EDN2*	+	3,97E−09	−	0.979
DMR4	**chr6**	**29648271**	**29648623**	**353**	**15**	***ZFP57***	+	**8,32E−09**	−	**0.014**
DMR5	chr6	28478268	28478579	312	4	*GPX6*	+	2,38E−08	+	0.540
DMR6	chr1	159869902	159870134	233	7	*CFAP45*	−	6,12E−08	−	0.106
DMR7	chr 2	100720526	100720529	4	2	*AFF3*	−	2,53E−07	+	0.573
DMR8	chr 1	3600735	3600879	145	3	*TP73*	−	9,94E−07	−	0.280
DMR9	chr 5	158689508	158689629	122	3	*UBLCP1*	+	3,96E−06	+	0.038
DMR10	chr 6	28829171	28829433	263	14	*RPL13P*	−	5,06E−06	−	0.982
DMR11	chr 19	11784955	11785188	234	4	–	−	1,16E−05	−	0.380
DMR12	chr 17	6558365	6558440	76	2	–	−	1,01E−04	−	0.546

Loc = DMR chromosome, Start/end: start and end position of DMR (hg19), Length: number of base pairs, Nr CpG: the number of CpGs measured within the DMR, Gen ID the gene symbol. *p* value: Stouffer *p* value of the DMR. Direction: direction of the relation between PTSD symptoms and DNA methylation levels

[a]+: increased methylation with PTSD remission

[b]+: increased methylation with PTSD development

stronger for EMDR ($B = -0.61$, $t = -2.19$, $p = 0.033$) than for tf-CBT ($B = -0.67$, $t = -1.70$, $p = 0.095$), even though the effects of tf-CBT and EMDR were not different in a direct comparison ($p = 0.836$).

Banding and mQTL analysis

For *ZFP57*, a single SNP on chromosome 9 (Hg19, chr 9:23576726) was strongly associated with DNA methylation at the *ZFP57* DMR ($p = 2.6 \times 10^{-9}$). Multilevel analysis using the derived genetic background as random factor in a mixed effect showed no confounding by genetic background and a similar association of *ZFP57* methylation with PTSD reduction ($B = -0.010$, $t = -2.23$, $p = 0.026$).

Discussion

In this study, we demonstrate that recovery from PTSD following trauma-related psychotherapy is accompanied by specific DNA methylation changes. Based on longitudinal genome-wide DNA methylation analyses in both war veterans with PTSD and trauma-exposed controls, 12 differentially methylated genomic regions were identified that appeared to be specific for PTSD improvement but not related to changes in depressive symptoms. Of these regions, increased DNA methylation in the *ZFP57* (zinc-finger protein 57) genomic region was the most consistent finding. The effects of PTSD treatment on *ZFP57* methylation were robust, and remained significant after correction for confounders and the effects of genetic banding. Whereas *ZFP57* methylation increased following trauma-related psychological PTSD treatment, *ZFP57* methylation decreases when PTSD develops over three time points before and after deployment. This is a new and fully independent confirmation and extension of recent epigenome-wide evidence for involvement of this genomic region in PTSD from another samples of the PRISMO cohort and a sample from the Marine Resilience Study (MRS) [3]. *ZFP57* is a transcriptional regulator of genomic imprinting. A central role for *ZFP57* in the etiology and treatment of PTSD is consistent with evidence showing that removal of the *ZFP57*-dependent co-factor KAP1 in the rodent hippocampus increased stress vulnerability with associated local epigenetic changes [22]. Interestingly, the effects of both trauma-focused CBT and EMDR on *ZFP57* methylation levels were over and above the effect of symptom reduction alone. This suggests that psychotherapy may have direct biological effects that are not mediated via symptom reduction per se. Altered DNA methylation of the ubiquitin like domain containing CTD phosphatase 1 gene (*UBLCP1*) was also related to the development and treatment of PTSD. Nevertheless, the interpretation of this finding is equivocal since DNA methylation increased both related to PTSD development and remission.

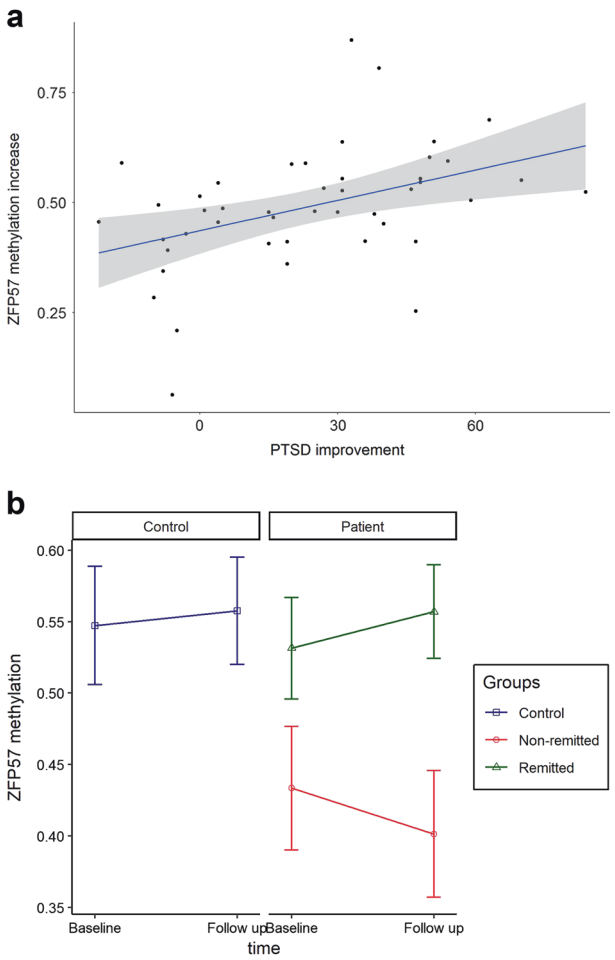

Fig. 3 **a** Scatterplot ZFP57 methylation increase and PTSD symptom reduction in PTSD patients. **b** PTSD treatment efficacy and ZFP57 methylation over time

Overall, our results support and extend previous studies presenting DNA methylation changes in specific genes related to psychological treatments for psychiatric disorders including PTSD [4, 23–25]. Using an unbiased approach, this study finds specific genes associated with treatment response, and underscores the potential of epigenetic markers related to successful treatment and symptomatic remission of stress-related disorders such as PTSD. New is that our results indicate that trauma-focused psychotherapy does indeed change biology and that these changes can be detected by analysis of the epigenetic state across the genome. Therefore, this study also provides biological evidence for the clinical notion that psychological and biological systems interact when it comes to both development of, and recovery from PTSD.

This study has several strengths and limitations. The major strengths of our study are the longitudinal sampling and standardized PTSD assessments before and after treatment, an unbiased epigenome-wide approach and confirmation of relevance for PTSD development in an independent longitudinal sample over three waves. Moreover, we extensively corrected for possible confounders which included genetic variants that influence DNA methylation levels, even though the prospective study design reduces the risk of (genetic) confounding. Nevertheless, we cannot exclude residual confounding. Sample size was relatively small and predominantly from male and Caucasian origin. Figure 3b suggest that there may be baseline albeit non-significant differences in ZFP57 methylation with the non-remitted group showing less methylation at both time points compared to the remitted group and controls. However it is important to note that the division: remitted, non-remitted control is only done to provide additional insight and is for graphical purposes only. The main analyses are driven by continuous measures of PTSD symptom levels. Nevertheless, it is apparent that there is ZFP57 hypomethylation in treatment-resistant PTSD patients. Another possible limitation is the relevance of peripheral blood methylation to the brain. Methylation differences across tissues are substantial, even though consistent effects of various methylation quantitative trait loci (mQTLs) are found across tissues [26].

In conclusion, this study demonstrates that successful psychotherapeutic treatment of PTSD is associated with specific DNA methylation changes. Of these epigenetic changes, the finding of *ZFP57* methylation is the most consistent, as DNA methylation in this region decreases during the development of PTSD but increases following its successful treatment. This study is the first step to identify the epigenetic mechanisms underlying a successful treatment of PTSD. Insight into the epigenetic determinants of successful psychotherapy for PTSD may help to better understand how psychological and biological systems interact in order to improve and individualize treatment outcomes.

Acknowledgements MB and EG had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. MB and DN conducted the analyses.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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