Reduced DNA methylation and psychopathology following endogenous hypercortisolism – a genome-wide study

Camilla A. M. Glad1, Johanna C. Andersson-Assarsson2, Peter Berglund3, Ragnhildur Bergthorsdottir1, Oskar Ragnarsson1,∗ & Gudmundur Johannsson1,∗

Patients with Cushing’s Syndrome (CS) in remission were used as a model to test the hypothesis that long-standing excessive cortisol exposure induces changes in DNA methylation that are associated with persisting neuropsychological consequences. Genome-wide DNA methylation was assessed in 48 women with CS in long-term remission (cases) and 16 controls matched for age, gender and education. The Fatigue impact scale and the comprehensive psychopathological rating scale were used to evaluate fatigue, depression and anxiety. Cases had lower average global DNA methylation than controls (81.2% vs 82.7%; p = 0.002). Four hundred and sixty-one differentially methylated regions, containing 3,246 probes mapping to 337 genes were identified. After adjustment for age and smoking, 731 probes in 236 genes were associated with psychopathology (fatigue, depression and/or anxiety). Twenty-four gene ontology terms were associated with psychopathology; terms related to retinoic acid receptor signalling were the most common (adjusted p = 0.0007). One gene in particular, COL11A2, was associated with fatigue following a false discovery rate correction. Our findings indicate that hypomethylation of FKBP5 and retinoic acid receptor related genes serve a potential mechanistic explanation for long-lasting GC-induced psychopathology.

Hyperactivity of the hypothalamus-pituitary-adrenal (HPA)-axis, with subsequent increase in cortisol exposure at the tissue level1,2, is implicated in neuropsychiatric disorders such as depression, post-traumatic stress disorder and anxiety3–9. Cortisol, the predominant glucocorticoid (GC) in humans, affects the central nervous system through binding to its two receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor, encoded by the NR3C1 and NR3C2 genes, respectively. These receptors are ubiquitously expressed in the brain, particularly in the hippocampus, prefrontal cortex and the parvocellular nucleus of the hypothalamus10.

Early-life adverse events have been associated with long-lasting dysregulation of the HPA-axis11, which may play a pathophysiological role in development of stress-related diseases12,13. This early-life molecular programming of the HPA-axis is thought to be conveyed by epigenetic mechanisms14–20. Several studies have shown that NR3C1 DNA methylation is influenced by both quality of maternal care (rodents)20 and experience of childhood trauma (humans)14,16–19. Furthermore, increased DNA methylation of the NR3C1 gene promoter has been observed in the hippocampus and prefrontal cortex in suicide victims with a history of childhood abuse15. The mechanism behind this change in DNA methylation is not known, however it is plausible that the increased cortisol exposure induced by psychological stress may be involved.

Marked chronic excess and attenuation of the endogenous diurnal variation in cortisol secretion causes Cushing’s syndrome (CS)1, most commonly caused by an ACTH-producing pituitary adenoma (Cushing’s disease; CD) or a cortisol-producing adrenal adenoma. Subjects with CS display a characteristic clinical phenotype including central obesity, muscle and skin atrophy and osteoporosis, as well as marked neuropsychological
complaints such as mental fatigue, anxiety, depression and cognitive impairment\textsuperscript{22}. Following treatment, most features of the syndrome improve; however, despite long-term remission, we and others have shown that fatigue and cognitive dysfunction commonly persists\textsuperscript{22-25}. The mechanism for this persistent cognitive impairment is not known, but the previous excess cortisol exposure is likely to play a mechanistic role\textsuperscript{26}. In fact, in our previous study there were no associations between aetiology, treatment (surgery and/or pituitary radiation therapy) or hormone deficiency and cognitive dysfunction\textsuperscript{21}.

Due to previous observations of associations between epigenetics and psychopathology\textsuperscript{14-20}, we hypothesized that long-standing excessive cortisol exposure induces changes in DNA methylation that are associated with long-lasting fatigue, depression and anxiety. Here, we used patients with CS as a unique human model of endogenous hypercortisolism to assess the impact of cortisol on genome-wide DNA methylation and its relation to psychopathology.

**Patients and Methods**

**Ethical considerations.** Informed written consent was obtained from all patients and controls. The local ethical committee of the University of Gothenburg, Sweden, approved the study. The study was conducted according to the Declaration of Helsinki.

**Design.** This was a cross-sectional, case-controlled, single centre study including 55 patients with CS in remission and 55 controls matched for age, gender and educational level, as previously described\textsuperscript{23}. In this part of the study the association between DNA methylation and fatigue, depression and anxiety in 48 women with CS in remission and 16 controls was analysed. The subjects were studied on three occasions, where medical history was reviewed, physical examination and corticotropin releasing hormone (CRH) stimulation test were performed, blood samples were drawn and psychopathology was evaluated. A 24-h urinary free cortisol (UFC) sampling was performed between the second and last visits, and an overnight dexamethasone suppression test was done following the last visit\textsuperscript{23}.

**Patients.** The mean age of patients was 53 ± 14 years, and the mean age at diagnosis of CS was 37 ± 14 years (Table 1). Thirty-seven (77%) patients had CD and 11 (23%) had a cortisol producing adrenal adenoma. To verify that the initial diagnosis of CD and cortisol producing adrenal adenoma were correct the clinical, biochemical, radiological and histopathological data from the time of diagnosis were reviewed. In patients with CD in remission the primary treatment was transphenoidal pituitary surgery in 25 (68%), radiotherapy in five (14%) and bilateral adrenalectomy in seven (19%). Fifteen patients needed additional treatment. In total, 29 (78%) patients with CD were treated with transphenoidal pituitary surgery, 11 (30%) with radiotherapy and nine (24%) with bilateral adrenalectomy. All patients with cortisol producing adrenal adenoma had been treated with unilateral adrenalectomy. Eighteen (38%) patients had adrenal insufficiency and received replacement therapy with a mean daily hydrocortisone dose of 24 ± 8 mg/day. The mean urinary free cortisol (UFC) excretion was higher in patients compared to controls (Table 1). Seventeen (35%) patients had central (N = 15) or primary (N = 2) hypothyroidism and were receiving a mean L-Thyroxine dose of 104 ± 31 μg/day. Out of 37 patients with CD, 19 (51%) had growth hormone deficiency of whom 15 were on growth hormone replacement therapy. Four out of 20 postmenopausal women ((< 52 years) had hypogonadotropic hypogonadism and were receiving estrogen and progesterone, 2 of 28 postmenopausal women were receiving treatment with oral estrogen. Two women received replacement with dehydroepiandrosterone.

**Controls.** Controls to patients, matched for age and gender, were recruited from a random population sample obtained from the Swedish Tax Agency. Controls were approached through an invitation letter, responding subjects were interviewed per telephone and those who matched the patient’s educational levels and had no previously known psychiatric or chronic diseases known to affect cognitive function, were included. In this part of the study data from one control (N = 16) per three patients (N = 48) were analysed. The mean age was 54 ± 16 years in controls (Table 1).

**Evaluation of hormone status.** All patients were in remission, defined by an adequate suppression of serum cortisol concentration (≤ 50 nmol/l) following a 1 mg overnight dexamethasone suppression test. The median (interquartile range) duration of remission was 13 (5–19) years. A CRH test was performed in order to evaluate the function of the HPA-axis. Serum cortisol was measured using competitive electrochemiluminescence immunoassay (Cortisol Elecsys, Roche Diagnostics Scandinavia AB). Urinary free cortisol (UFC) was measured using radioimmunoassay (SpectRia Cortisol 125I, Orion Diagnostica Oy, Finland). Thyroid function was evaluated clinically and by measurements of free thyroxin and thyroid stimulating hormone (TSH) in serum. Gonadal function was evaluated by asking for menstruation pattern and/or age at menopause as well as measurements of estrogen and gonadotropins in serum. Growth hormone status was evaluated by review of previously performed stimulation tests and measurement of insulin-like growth factor I.

**Evaluation of fatigue, depression and anxiety.** Fatigue was evaluated using the fatigue impact scale, a 40 item questionnaire where different aspects of fatigue (physical, cognitive and social) are evaluated\textsuperscript{27}. Depression and anxiety were evaluated using the comprehensive psychopathological rating scale\textsuperscript{28}.

**DNA isolation and methylation assessment.** DNA was isolated from whole blood using the QIAamp DNA Blood Maxi kit (QIAGEN, Hilden, GE). DNA methylation was assessed on the Illumina Infinium HumanMethylation450K BeadChip (Illumina, San Diego, CA, USA), which simultaneously interrogates >465,000 CpG sites and covers 99% of RefSeq genes and 96% of CpG islands. Probes are distributed in CpG islands, shelves, shores, promoter regions, 5′ UTRs, first exon, gene body and 3′ UTRs. Methylation assessment
was performed at the Mutation Analysis Facility (MAF) at Karolinska University Hospital. The procedure is briefly described below:

**Bisulfite treatment.** 500ng of genomic DNA (OD260/280 > 1.8) was bisulfite treated using the EZ-96 DNA Methylation Kit (D5004; Zymo Research, Inc., Irvine, CA, USA). The CT conversion reagent was mixed with DNA and incubated in the dark at 50 °C for 16 hours. After desulfonation and washing steps, the samples were purified using spin plates, eluted in 12 μl elution buffer and stored at −20 °C prior to processing.

**Infinium Methylation assay.** The Infinium Methylation Assay was performed according to the manufacturer's instructions. Briefly, 4μl of denatured bisulfite-treated DNA was isothermally amplified over night at 37 °C, followed by an enzymatic fragmentation step. The fragmented DNA was precipitated, resuspended and loaded (using a Tecan EVO robot) on the 12-sample BeadChip, which was then incubated overnight at 48 °C, allowing the fragmented DNA to hybridize to locus-specific 50-mers. Non-specifically hybridized DNA was washed away, followed by a single-base extension reaction using DNP- and Biotin-labeled ddNTPs (with use of a Tecan EVO robot). Subsequently, hybridized DNA was removed from the labeled oligonucleotide and chips were dried under vacuum and imaged using an Illumina iScan scanner.

**Statistical analyses.** **Clinical parameters.** Statistical analyses were performed with IBM SPSS statistics, version 22, or in R version 3.0.3. Data are presented as mean ± standard deviation or median (25–75 percentiles). For comparison between groups we used unpaired t-test for normally distributed data and Mann-Whitney U-test for non-normally distributed data. For proportions, Pearson Chi-square or Fishers exact test were used. Pearson's correlation was used to determine correlation between methylation and clinical parameters. Linear regression (with adjustment for age and smoking habits) was used to analyse the effect of methylation on clinical parameters.

---

|                  | Patients | Controls | p  |
|------------------|----------|----------|----|
| Age at diagnosis (yr) | 37 ± 14  | —        | —  |
| Age at follow-up (yr) | 53 ± 14  | 54 ± 16  | 0.9|
| Duration of remission (yr) | 13 (5–19) | —        | —  |
| Educational level (%) | 1.0      | 0.8      | 0.1|
| Elementary school   | 25       | 25       |    |
| Upper secondary education | 46     | 44       |    |
| University education | 29       | 31       |    |
| Smoking habits (%) | 0.8      | 0.8      | 0.8|
| Non-smoker         | 53       | 44       |    |
| Ex-smoker          | 36       | 44       |    |
| Smoker             | 11       | 13       |    |
| Employment (%)     | 0.1      | 0.1      | 0.1|
| Full-time          | 34       | 63       |    |
| Part-time          | 30       | 13       |    |
| Sick leave/ Disability pension | 11    | —        |    |
| Retirement         | 26       | 25       |    |
| Psychopathology    |          |          |    |
| Fatigue (total score) | 63 (40–88) | 25 (6–37) | <0.01|
| Depression (score) | 4 (3–7)  | 2 (1–3)  | <0.01|
| Anxiety (score)    | 5 (4–7)  | 3 (3–6)  | 0.08|
| Hormone measurements |        |          |    |
| S-cortisol – BL (nmol/l) | 327 ± 129 | 305 ± 119 | 0.6|
| S-cortisol – Peak (nmol/l) | 557 ± 147 | 584 ± 78  | 0.5|
| UFC (nmol/24h)     | 202 ± 158| 131 ± 59 | 0.02|
| FreeT4 (pmol/l)    | 16.7 ± 3.2| 14.8 ± 1.5| < 0.01|
| IGF-I (μg/l)       | 149 ± 78 | 151 ± 84 | 0.9|

Table 1. **Background characteristics, sociodemographic status, psychopathology and hormone measurements in 48 patients with Cushing’s syndrome in remission and 16 controls, matched for age, gender and educational level.** Data is presented as mean ± standard deviation or median (interquartile range). S-cortisol levels were analyzed only in ACTH sufficient patients (N = 30). S-cortisol was measured in the morning, before (baseline; BL) and after administration of CRH; S-cortisol – peak represents the highest level measured after CRH administration. Psychopathology was evaluated through Fatigue impact scale (FIS) and comprehensive psychopathological rating scale (depression and anxiety).
DNA methylation analyses. Data was extracted using GenomeStudio (Illumina, Methylation Module v1.9), which was also used to subtract the background and to normalize staining intensities using internal controls present on the chip. A beta-value was calculated to estimate the methylation level of each CpG locus using the ratio of intensities between methylated and unmethylated alleles (0 = unmethylated, 1 = fully methylated). The performances of the different controls used were evaluated and potential outliers identified. Data quality control and analysis was performed using the ChAMP methylation analysis package (v. 1.4.0)\(^29\) in R. Briefly, intensity data from IDAT files were loaded, normalized using default settings (i.e. BMIQ) and corrected for batch effects using ComBat. Differentially methylated regions (DMR:s) were then identified using the Probe Lasso DMR Hunter function with Benjamini-Hochberg p-value adjustment. Correction for multiple testing was done using the "fdrtool" package (v. 1.2.13) in R.

Gene ontology analyses. Gene ontology analyses were performed in DAVID Bioinformatics Resources 6.7 (NIADD/NIH) using the Functional Annotation Cluster and Functional Annotation Chart functions\(^30,31\). DAVID provides unadjusted p-values as well as p-values adjusted for multiple testing using both the Bonferroni and the Benjamini methods. Here we present Benjamini-adjusted p-values.

Results

Identification of differentially methylated regions and overall DNA methylation. Initial quality control (QC) analyses of the methylation raw data identified one case sample as a technical outlier due to low levels of detected CpG:s (only 38,007 CpG:s were detected with a detection p-value < 0.01), this sample was removed from further analysis. The final data set consisted of 47 cases and 16 controls. On average, 485,001 CpG:s were detected (484,979 in cases and 485,066 in controls, detection p-value < 0.01).

We first performed DNA methylation analysis in ChAMP, to assess differences between patients with CS in long-term remission and matched controls (Table 2). Overall, patients had lower average percentage of DNA methylation than controls (81.2% vs 82.7%, \(p = 0.002\); Fig. 1a). There were 3,903 probes that lay in differentially methylated regions (DMR:s; \(n = 461\)), the majority (\(n = 3,692\); 94.6%) being hypomethylated. Of the 3,903 probes, 3,246 (83.2%) mapped to a gene (\(n = 337\)). Of the 337 genes, 278 were exclusively hypomethylated, 7 exclusively hypermethylated and 52 genes contained both hypo- and hypermethylated probes (Supplemental Table 1). Of the 3,903 probes, 55.9% (\(n = 2,183\)) had an annotated location; with the most common being gene body (33.3%), 3′-UTR (3.9%) and TSS15 (within 1,500 base pairs upstream or downstream of the transcriptional start site, 2.8%; Fig. 1b).

Identification of probes associated with fatigue, depression and anxiety. To investigate whether the epigenetic status of the CS subjects is associated with persistent fatigue, anxiety or depression a regression analysis adjusted for age and smoking habits was performed. We identified 731 probes in 236 genes that were associated with at least one of the three clinical traits. Of these 731 probes, 434 were associated with fatigue, 374 with depression, and 452 with anxiety. One hundred and sixty five probes in 108 genes were associated with all three traits.

After multiple testing correction using false discovery rate (FDR; 10%), four probes remained significantly associated with fatigue; cg22890571 (qval: 0.052), cg16479323 (qval: 0.052), cg09502339 (qval: 0.073), and cg07889869 (qval: 0.087). These probes are annotated to the following genes: TFDP1, ITPK1, COL11A2, and DAGL. Notably all four probes were also associated with depression and anxiety, however the p-values did not remain significant following FDR testing (qval: 0.13–0.23).

Functional validation of identified probes through gene ontology analyses. To explore the functional relevance of the identified DMRs and clinically associated probes, we next performed gene ontology (GO) analyses using DAVID\(^30,31\). We initially performed GO analysis of all 337 genes with probes that lay in DMRs and found 202 GO terms, of which 18 were significant after Benjamini correction (Fig. 2a). Terms related to retinoic acid, thyroid hormone receptor and hormone/nuclear hormone receptor binding was the most common (Figs 2b and 3a).

Table 2. Summaries from DNA methylation analyses.
When focusing the analysis on the 236 genes that were associated with at least one of the clinical traits fatigue, depression, and anxiety, 184 terms were identified of which 24 terms were significant after Benjamini correction (Table 3). As before, terms related to retinoic acid were among the most common (Fig. 3a).

To explore the potential effect of hypercortisolism on DNA methylation of the \(NR3C1\) gene, we analysed specifically DNA methylation of this gene. Fifteen out of 49 probes annotated to the \(NR3C1\) gene were significantly differentially methylated in CS cases compared to controls. After multiple testing correction using false discovery rate (FDR, 10%), all 15 probes remained significant (qval 0.00019–0.065). The most significant difference was observed for probe cg15645634 (\(p = 8.31 \times 10^{-6}\), qval: 0.00019), located in intron 8 of the \(NR3C1\) gene. Notably, out of these 15 differentially methylated probes, 8 probes were specifically hypermethylated and 7 probes were hypomethylated (Table 4).

Correlation with markers of HPA-axis activity. To validate the functional value of DNA methylation of the \(NR3C1\) gene and the genes involved in retinoic acid signalling (in total, \(n = 672\) probes), we performed correlation analyses with urinary free cortisol (UFC; \(n = 47\)) and the change in serum cortisol concentration (delta cortisol; \(n = 24\)) during a CRH-stimulation test as measures of cortisol exposure and HPA-axis activity, respectively. The CRH-stimulation test was performed in a subgroup of CS patients who had not previously received
pituitary irradiation and who did not receive GC replacement therapy. Thirty-one probes were significantly correlated with UFC (Supplemental Table 2), with the strongest correlation observed for probe cg02319187 annotated to the \( RXRA \) gene \((p = 0.005; \text{Pearson's} \ r = 0.412)\). Twenty-five probes were significantly correlated with change in serum cortisol in response to CRH (Supplemental Table 3). The strongest correlation was observed for probe cg00629244, located in the \( NR3C1 \) gene \((p = 0.002; \text{Pearson's} \ r = 0.598)\). Three probes (cg01367322, cg03058556 and cg03825390) annotated to the \( ZBTB22, ZBTB9 \) and \( RGL2 \) genes (respectively) were significantly associated with both UFC and the change in serum cortisol. None of the correlations remained significant after correction for multiple testing (FDR, 10%).

Influence of current GC replacement therapy on DNA methylation. To evaluate the effect of current GC replacement therapy on DNA methylation, we performed a subgroup analysis using the entire data set, \( n = 468,149 \) probes, where patients were stratified by occurrence of current GC replacement therapy. 12,128 probes in 6,186 genes were differentially methylated between the two groups. The most significant differentially methylated probe (cg03546163, \( p = 2.99 \times 10^{-6} \); Fig. 3b) was located in the \( FKBP5 \) gene.

In total, there are 34 probes annotated to the \( FKBP5 \) gene on the Illumina 450 K methylation chip and four of these probes showed differential methylation (cg03546163, cg00058684, cg08586216 and cg25114611) in patients with, as compared to without, current GC replacement therapy (Table 5). Most probes annotated to \( FKBP5 \) were hypomethylated in cases receiving GC replacement therapy; (Fig. 3b). No probes remained significantly differentially methylated after multiple testing correction (FDR, 10%). However it is well worth noting that this correction for multiple testing takes into account a very large number of tests, and that in particular the unadjusted \( p \)-value for \( FKBP5 \) probe cg03546163 reached borderline genome-wide significance \((p = 2.99 \times 10^{-6})\). Together, this suggests a true relevance of this finding, despite \( q \text{-val} > 0.1 \).

Discussion

Hyperactivity of the HPA-axis may increase susceptibility to neuropsychiatric disorders such as depression, post-traumatic stress disorder and anxiety\(^2\)\(^\text{9}\). Here we provide evidence for a distinguishable pattern of genome-wide DNA methylation in patients previously treated for CS and propose a mechanism for the long-term
adverse neuropsychological consequences of endogenous hypercortisolism, which is also commonly observed in a number of different psychiatric disorders.

Aberrations in DNA methylation has been associated with neurological and neuropsychiatric disorders such as autism\(^3^2\), schizophrenia\(^3^3\) and Alzheimer’s disease\(^3^4\), as well as early life adverse events such as child maltreatment and parental stress\(^1^7\). Childhood adverse events may influence the life-time set point of the HPA-axis\(^3^5\); one plausible mechanism for the induction of a programmed HPA-axis is through GC-induced epigenetic changes.

### Table 3. DAVID gene ontology analysis with 236 genes with probes significantly associated with at least one clinical trait.

| GO-terms                                      | No of genes | Unadjusted P-value | Benjamini-adjusted P-value |
|-----------------------------------------------|-------------|--------------------|-----------------------------|
| Alternative splicing                          | 135         | 4.91E-07           | 0.0002                      |
| Genomewide Association Study of an AIDS-Nonprogression Cohort Emphasizes the Role Played by HLA Genes (ANRS Genomewide Association Study 02) | 5 | 3.36E-06 | 0.0003 |
| GO:0008134 - transcription factor binding    | 23          | 2.70E-06           | 0.0006                      |
| GO:0004886 - retinoid-X receptor activity    | 5           | 4.36E-06           | 0.0007                      |
| GO:0042974 - retinoic acid receptor binding  | 6           | 2.69E-06           | 0.0012                      |
| GO:0030375 - thyroid hormone receptor coactivator activity | 5 | 1.12E-05 | 0.0013 |
| GO:0010861 - thyroid hormone receptor activator activity | 5 | 1.12E-05 | 0.0013 |
| GO:0003708 - retinoic acid receptor activity | 5 | 1.66E-05 | 0.0015 |
| GO:0051059 - NF-kappaB binding               | 6           | 4.51E-05           | 0.0034                      |
| GO:0030546 - receptor activator activity     | 5           | 7.55E-05           | 0.0042                      |
| GO:0046966 - thyroid hormone receptor binding| 6           | 7.39E-05           | 0.0047                      |
| GO:0006986 - response to unfolded protein     | 9           | 4.56E-06           | 0.0062                      |

| Splice variant                                | 131         | 6.79E-06           | 0.0063                      |
| GO:0030374 - ligand-dependent nuclear receptor transcription coactivator activity | 6 | 1.32E-04 | 0.0066 |
| GO:0042809 - vitamin D receptor binding       | 5           | 2.63E-04           | 0.0117                      |
| GO:0030545 - receptor regulator activity      | 5           | 7.54E-04           | 0.0278                      |
| GO:0035257 - nuclear hormone receptor binding | 7           | 7.06E-04           | 0.0284                      |
| GO:0046978 - TAP1 binding                    | 3           | 1.14E-03           | 0.0386                      |
| GO:0046979 - TAP2 binding                    | 3           | 1.14E-03           | 0.0386                      |
| GO:0046977 - TAP binding                     | 3           | 1.14E-03           | 0.0386                      |
| GO:0042288 - MHC class I protein binding     | 4           | 1.31E-03           | 0.0410                      |
| GO:0046983 - protein dimerization activity   | 18          | 1.51E-03           | 0.0416                      |
| GO:0051427 - hormone receptor binding        | 7           | 1.42E-03           | 0.0417                      |
| GO:0042824 - MHC class I peptide loading complex | 4 | 1.65E-04 | 0.0419 |

### Table 4. Methylation in fifteen significantly differentially methylated probes in **NR3C1**. Methylation in fifteen significantly differentially methylated probes (reported as beta-values) in **NR3C1** on chromosome 5.

| Probe            | Positiona       | Strand | Location       | Intron number | Casesa | Controlsa | Delta-betaa | p                  | q-valoa |
|------------------|-----------------|--------|----------------|----------------|--------|-----------|--------------|--------------------|---------|
| cg15645634       | 142783639       | F      | Intron         | 8              | 0.0356 | 0.0261    | 0.0095       | 8.31E-06           | 0.000189|
| cg14558428       | 142784982       | R      | Intron         | 8              | 0.0396 | 0.0314    | 0.0082       | 0.000363           | 0.004514|
| cg17600381       | 142783569       | R      | Intron         | 8              | 0.0412 | 0.0336    | 0.0076       | 0.003063           | 0.015499|
| cg26464111       | 142784222       | R      | Intron         | 8              | 0.0556 | 0.0465    | 0.0091       | 0.004060           | 0.017017|
| cg18019515       | 142783385       | R      | Intron         | 8              | 0.0235 | 0.0192    | 0.0043       | 0.003479           | 0.017388|
| cg18146873       | 142782827       | F      | Intron         | 8              | 0.0279 | 0.0211    | 0.0068       | 0.005500           | 0.018472|
| cg07733851       | 142781498       | R      | Intron         | 8              | 0.375  | 0.407     | −0.032       | 0.005733           | 0.018648|
| cg25359999       | 142757312       | R      | Intron         | 7              | 0.922  | 0.935     | −0.013       | 0.0110             | 0.030167|
| cg18594054       | 142623446       | R      | Upstream       | 8              | 0.926  | 0.939     | −0.013       | 0.0144             | 0.035689|
| cg04097219       | 142629749       | F      | Upstream       | 8              | 0.972  | 0.978     | −0.006       | 0.0177             | 0.040303|
| cg06770322       | 142851098       | F      | Downstream     | 8              | 0.960  | 0.966     | −0.006       | 0.0279             | 0.051311|
| cg22372527       | 142658828       | R      | 5’UTR          | 8              | 0.977  | 0.982     | −0.005       | 0.0279             | 0.051315|
| cg21702128       | 142784721       | R      | Intron         | 8              | 0.0516 | 0.0473    | 0.0043       | 0.0306             | 0.053550|
| cg25781210       | 142610141       | F      | Upstream       | 8              | 0.959  | 0.967     | −0.008       | 0.0429             | 0.064421|
| cg06521673       | 142782072       | R      | Intron         | 8              | 0.0227 | 0.0201    | 0.0026       | 0.0435             | 0.064851|

Adverse neuropsychological consequences of endogenous hypercortisolism, which is also commonly observed in a number of different psychiatric disorders. Aberrations in DNA methylation has been associated with neurological and neuropsychiatric disorders such as autism\(^3^2\), schizophrenia\(^3^3\) and Alzheimer’s disease\(^3^4\), as well as early life adverse events such as child maltreatment and parental stress\(^3^7\). Childhood adverse events may influence the life-time set point of the HPA-axis\(^3^5\); one plausible mechanism for the induction of a programmed HPA-axis is through GC-induced epigenetic changes.

---

\(^{a}\)Human genome build 37. \(^{b}\)CS patients. \(^{c}\)Controls. \(^{d}\)Difference in methylation between cases and controls. \(^{e}\)q-values from multiple correction analysis using a 10% FDR.
We performed a global DNA methylation analysis to assess differences between patients with CS in long-term remission and matched controls. Generally, patients with CS had lower levels of DNA methylation than controls. Four hundred and sixty-one differentially methylated regions were identified, with the majority being hypomethylated in patients. Also, we identified 731 probes in 236 genes that were associated with at least one of three psychopathological traits; fatigue, anxiety and/or depression. Gene ontology analyses revealed an enrichment of genes functioning as retinoic acid receptors, thyroid hormone receptors or hormone/nuclear hormone receptors. These receptors all belong to the nuclear receptor superfamily and serve as ligand-activated regulators of gene transcription and stimulators of intracellular pathways. These genes were hypomethylated in cases as compared to controls, and associated with psychopathology in the patients.

The DNA methylation of the genes belonging to the retinoic acid receptor family was also correlated with UFC and change in cortisol concentrations during a CRH-stimulation test, suggesting a functional link between retinoic acid receptor and HPA-axis activity. The retinoic acid family includes Vitamin A and its derivatives, 13-cis retinoic acid and all-trans retinoic acid. Retinoic acid is a transcriptionally active compound that regulates gene expression via binding to specific nuclear receptors termed RARs or retinoic X receptors (RXRs). Retinoic acid is crucial during development of the central nervous system and for neuronal plasticity in adult brain. Previous data suggests an involvement of the retinoic acid family in the regulation of the HPA-axis. Both chronic (rats) and intermittent (humans) retinoic acid treatment has been shown able to induce HPA-axis hyperactivity and anxiety-like, as well as depressive, behaviour. A plausible mechanism may be that the retinoic acid interrupts the GC receptor induced negative feedback by down-regulating 11β-HSD1 expression and by inhibiting GC receptor transactivation.

| Probe     | Position’ | GCRT - yes’ | GCRT - no’ | Delta_beta’ | p          | qval’ |
|-----------|-----------|-------------|------------|-------------|------------|-------|
| cg03546163 | 35653636  | 0.484       | 0.588      | −0.104      | 2.99E-06   | 1     |
| cg00052684 | 35694245  | 0.514       | 0.550      | −0.036      | 0.00159    | 1     |
| cg08586216 | 35612351  | 0.981       | 0.976      | 0.0050      | 0.0122     | 1     |
| cg25114611 | 35696870  | 0.314       | 0.339      | −0.025      | 0.0218     | 1     |
| cg08915438 | 35697759  | 0.559       | 0.588      | −0.029      | 0.0599     | 1     |
| cg16052510 | 35603143  | 0.809       | 0.783      | 0.026       | 0.0095     | 1     |
| cg20813374 | 35657180  | 0.442       | 0.462      | −0.020      | 0.109      | 1     |
| cg00130530 | 35657202  | 0.690       | 0.710      | −0.020      | 0.115      | 1     |
| cg19226017 | 35697185  | 0.752       | 0.770      | −0.018      | 0.137      | 1     |
| cg10300814 | 35565116  | 0.948       | 0.953      | −0.0050     | 0.175      | 1     |
| cg06807101 | 35551932  | 0.418       | 0.440      | −0.022      | 0.197      | 1     |
| cg14642437 | 35652521  | 0.876       | 0.888      | −0.012      | 0.204      | 1     |
| cg19014730 | 35635985  | 0.681       | 0.695      | −0.014      | 0.226      | 1     |
| cg07843056 | 35656848  | 0.0257      | 0.0229     | 0.0028      | 0.305      | 1     |
| cg07485685 | 35696061  | 0.0394      | 0.0375     | 0.0019      | 0.409      | 1     |
| cg17085721 | 35645341  | 0.945       | 0.949      | −0.004      | 0.442      | 1     |
| cg07061368 | 35631736  | 0.894       | 0.901      | −0.007      | 0.485      | 1     |
| cg17030679 | 35696300  | 0.0215      | 0.0227     | −0.0012     | 0.520      | 1     |
| cg00610228 | 35695934  | 0.0368      | 0.0359     | 0.0009      | 0.582      | 1     |
| cg16012111 | 35656758  | 0.0484      | 0.0500     | −0.0016     | 0.585      | 1     |
| cg23416081 | 35693573  | 0.208       | 0.199      | 0.009       | 0.613      | 1     |
| cg11845071 | 35695859  | 0.0209      | 0.0202     | 0.0007      | 0.616      | 1     |
| cg00140191 | 35656242  | 0.0634      | 0.0615     | 0.0019      | 0.661      | 1     |
| cg08636224 | 35657921  | 0.961       | 0.962      | −0.001      | 0.686      | 1     |
| cg10129490 | 35656906  | 0.0934      | 0.0913     | 0.0021      | 0.687      | 1     |
| cg18726036 | 35543610  | 0.946       | 0.947      | −0.001      | 0.707      | 1     |
| cg03591753 | 35659141  | 0.539       | 0.534      | 0.005       | 0.720      | 1     |
| cg14284211 | 35570224  | 0.139       | 0.134      | 0.005       | 0.738      | 1     |
| cg0937024  | 35695489  | 0.0259      | 0.0264     | −0.0005     | 0.791      | 1     |
| cg09862270 | 35655764  | 0.0256      | 0.0251     | 0.0005      | 0.795      | 1     |
| cg02665568 | 35544468  | 0.921       | 0.923      | −0.002      | 0.799      | 1     |
| cg07633853 | 35569471  | 0.155       | 0.157      | −0.002      | 0.829      | 1     |
| cg15929276 | 35687457  | 0.187       | 0.186      | 0.001       | 0.911      | 1     |
| cg10913456 | 35656590  | 0.0175      | 0.0175     | 0.000       | 0.989      | 1     |

Table 5. Methylation in FKBP5, grouped based on GC replacement therapy. Summary of methylation (reported as beta-values) in FKBP5 on chromosome 6. GCRT = glucocorticoid replacement therapy. Human genome build 37. Patients receiving GCRT. Patients not receiving GCRT. Difference in methylation between patients receiving GCRT and those not receiving such therapy. q-values from multiple correction analysis using a 10% FDR.
Early adverse events and poor maternal care have been linked to changes in the GC receptor (NR3C1) DNA methylation. To explore the potential effect of hypercortisolism on NR3C1 DNA methylation specifically, we analysed methylation in this gene and found that 15 out of 49 probes annotated to the NR3C1 gene were differentially methylated in cases as compared to controls. Previously, increased levels of NR3C1 methylation have been observed in post-mortem hippocampal brain tissue from suicide victims who had endured childhood abuse15, and in peripheral blood from subjects with a history of perinatal stress16-18 and neglect or abuse during childhood19,20. Recently, common stressful life events were found to be associated with higher blood DNA methylation of the NR3C1 gene in adolescents21, suggesting that the NR3C1 DNA methylation is subject to change not only during childhood. In accordance, herein we report that adults who have endured long-term endogenous hypercortisolism have a differential pattern of NR3C1 DNA methylation than matched controls, lending further support for the importance of excess cortisol exposure as a possible cause in the programming of the HPA-axis and its psychological consequences.

To evaluate the possibly confounding effect of current GC replacement therapy on our results, we performed a subgroup analysis dividing the cases into groups of patients receiving GC replacement or not. These analyses revealed that the DNA methylation of 12,128 probes in 6,186 genes was influenced by current GC replacement therapy. One of the genes that were specifically hypomethylated in cases compared to controls, with an additional reduction in patients currently receiving GC replacement therapy, was the FK506 binding protein 5 (FKBP5). FKBP5 binds to and negatively regulates GR function, which subsequently reduces affinity of the GR to corticosteroids. Common genetic variants in the FKBP5 gene have been associated with a relative GR resistance, and found to interact with childhood abuse to predict post-traumatic stress disorder33. Previously, studies in mice have reported that long-lasting exposure to GC decreases FKBP5 DNA methylation in the hippocampus, hypothalamus and blood, and that this demethylation is associated with anxiol-like behavior19,20 and reflect previous GC load33. Consistent with these findings, herein we show that FKBP5 is indeed hypomethylated in CS patients as compared to controls, and that the methylation is further reduced in a sub-group of CS patients receiving GC replacement. These findings validate the suitability of CS as study model for GC exposure and further enlighten the strong effect of GC on DNA methylation.

Despite the rigorous study protocol and adequate study model this project is not without limitations. Firstly, the DNA methylation was assessed in whole blood and not in brain or any other isolated GC target tissue. A recent study, however, provided evidence that DNA methylation variation observed in the brain is in fact reflected in the blood. Secondly, the findings herein remain to be validated and further explored as for whether the observed changes in DNA methylation are indeed associated with subsequent changes in mRNA and protein expression. Lastly, these findings remain to be validated in studies of patients with specific psychiatric disorders with HPA-axis hyperactivity.

In conclusion, our findings suggest that long-standing hypercortisolism reduces global DNA methylation, specifically in genes that are known to attenuate the sensitivity of the GC receptor and therefore may induce hyperactivity of the HPA-axis. Consequently, this may be of importance for the action of cortisol on the central nervous system, and by such contribute to the frequent psychopathology observed in our patients. The mechanism proposed might also apply to other disorders with transient or chronic hyperactivation of the HPA-axis that affects a considerable part of the general population; such as depression, generalised anxiety and post-traumatic stress.

References

1. Juruena, M. F. et al. Different responses to dexamethasone and prednisolone in the same depressed patients. Psychopharmacology 189, 225–235, doi: 10.1007/s00213-006-0555-4 (2006).
2. Pariente, C. M. Risk factors for development of depression and psychosis. Glucocorticoid receptors and pituitary implications for treatment with antidepressant and glucocorticoids. Annals of the New York Academy of Sciences 1179, 144–152, doi: 10.1111/j.1749-6632.2009.04978.x (2009).
3. de Kloet, C. S. et al. Assessment of HPA axis function in posttraumatic stress disorder: pharmacological and non-pharmacological challenge tests, a review. Journal of psychiatric research 40, 550–567, doi: 10.1016/j.jpsychires.2005.08.002 (2006).
4. Ehert, U., Gaab, J. & Heinrichs, M. Psychoneuroendocrinological contributions to the etiology of depression, posttraumatic stress disorder, and stress-related bodily disorders: the role of the hypothalamus-pituitary-adrenal axis. Biological psychology 57, 141–152 (2001).
5. Gotlib, I. H., Joormann, J., Minor, K. L. & Hallmayer, J. HPA axis reactivity: a mechanism underlying the associations among 5-HTTLPR, stress, and depression. Biological psychiatry 63, 847–851, doi: 10.1016/j.biopsych.2007.10.008 (2008).
6. Holsboer, F. The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology 23, 477–501, doi: 10.1016/S0893-133X(00)00159-7 (2000).
7. Pariente, C. M. & Lightman, S. L. The HPA axis in major depression: classical theories and new developments. Trends in endocrinology 31, 464–468, doi: 10.1016/j.tiendocrin.2008.06.006 (2008).
8. Shea, A., Walsh, C., Macmillan, H. & Steiner, M. Child maltreatment and HPA axis dysregulation: relationship to major depressive disorder and post traumatic stress disorder in females. Psychoneuroendocrinology 30, 162–178, doi: 10.1016/j.psyneuen.2004.07.001 (2005).
9. Veehuberg, S. A. et al. Major depressive disorder and hypothalamic-pituitary-adrenal axis activity: results from a large cohort study. Archives of general psychiatry 66, 617–626, doi: 10.1001/archgenpsychiatry.2009.50 (2009).
10. Reul, J. M. & de Kloet, E. R. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117, 2505–2511, doi: 10.1210/endo-117-6-2505 (1985).
11. Murgatroyd, C. & Spengler, D. Epigenetic programming of the HPA axis: early life decides. Stress 14, 581–589, doi: 10.3109/10253890.2011.602146 (2011).
12. Hein, C. & Binder, E. B. Current research trends in early life stress and depression: review of human studies on sensitive periods, gene–environment interactions, and epigenetics. Experimental neurology 235, 102–111, doi: 10.1016/j.expneurol.2011.10.032 (2012).
13. Lupien, S. J., McEwen, B. S., Gunnar, M. R. & Heim, C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nature reviews. Neuroscience 10, 434–445, doi: 10.1038/nrn2639 (2009).
14. Hompes, T. et al. Investigating the influence of maternal cortisol and emotional state during pregnancy on the DNA methylation status of the glucocorticoid receptor gene (NR3C1) promoter region in cord blood. Journal of psychiatric research 47, 880–891, doi: 10.1016/j.jpsychires.2013.03.009 (2013).
15. McGowan, P. O. et al. Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nature neuroscience 12, 342–348, doi: 10.1038/nn2270 (2009).
16. Mulligan, C. J., D’Errico, N. C., Stees, J. & Hughes, D. A. Methylation changes at NR3C1 in newborns associate with maternal prenatal stress exposure and newborn birth weight. Epigenetics: official journal of the DNA Methylation Society 7, 853–857, doi: 10.4161/epi.21180 (2012).
17. Oberlander, T. F. et al. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics: official journal of the DNA Methylation Society 3, 97–106 (2008).
18. Radlje, K. M. et al. Transgenerational impact of intimate partner violence on methylation in the promoter of the glucocorticoid receptor. Translational psychiatry 1, e21, doi: 10.1038/tp.2011.21 (2011).
19. Romens, S. E., McDonal, J., Saven, J. & Pollak, S. D. Associations between early life stress and gene methylation in children. Child development 86, 303–309, doi: 10.1111/cdev.12270 (2015).
20. Weger, I. C. et al. Epigenetic programming by maternal behavior. Nature neuroscience 7, 847–854, doi: 10.1038/nn1276 (2004).
21. Newell-Price, J., Bertagna, X., Grossman, A. B. & Nieman, L. K. Cushing’s syndrome. Lancet 367, 1605–1617, doi: 10.1016/S0140-6736(06)68699-6 (2006).
22. Vala, E. et al. The European Registry on Cushing’s syndrome: 2-year experience. Baseline demographic and clinical characteristics. European journal of endocrinology / European Federation of Endocrine Societies 165, 383–392, doi: 10.1530/EJE-11-0277 (2011).
23. Ragnarsson, O., Berglund, P., Eder, D. N. & Johannsson, G. Long-term cognitive impairments and attentional deficits in patients with Cushing’s disease and cortisol-producing adrenal adenoma in remission. The journal of clinical endocrinology and metabolism 97, E1640–1648, doi: 10.1210/jc.2012-1945 (2012).
24. Resmini, E. et al. Verbal and visual memory performance and hippocampal volumes, measured by 3-Tesla magnetic resonance imaging, in patients with Cushing’s syndrome. The journal of clinical endocrinology and metabolism 97, 667–671, doi: 10.1210/jc.2011-2231 (2012).
25. Tiemensma, J. et al. Subtle cognitive impairments in patients with long-term cure of Cushing’s disease. The journal of clinical endocrinology and metabolism 95, 2699–2714, doi: 10.1210/jc.2009-2032 (2010).
26. Tata, D. A. & Anderson, B. J. The effects of chronic glucocorticoid exposure on dendritic length, synapse numbers and glial volume in animal models: implications for hippocampal volume reductions in depression. Physiology & behavior 99, 186–193, doi: 10.1016/j.physbeh.2009.09.010 (2010).
27. Fisk, J. D. et al. Measuring the functional impact of fatigue: initial validation of the fatigue impact scale. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 18 Suppl 1, S79–83 (1994).
28. Asberg, M., Montgomery, S. A., Perris, C., Schalling, D. & Sedvall, G. A comprehensive psychopathological rating scale. Acta psychiatraca Scandinavica. Supplementum 5–27 (1978).
29. Morris, T. J. et al. ChAMP: 450k Chip Analysis Methylation Pipeline. Bioinformatics 30, 428–430, doi: 10.1093/bioinformatics/btt684 (2014).
30. Huang da, W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols 4, 44–57, doi: 10.1038/nprot.2008.211 (2009).
31. Huang da, W., Sherman, B. T. & Lempicki, R. A. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic acids research 37, 1–13, doi: 10.1093/nar/gkn293 (2009).
32. Nagarajan, R. P., Hogart, A. R., Meng, Y., Martin, M. R. & LaSalle, J. M. Reduced MeCP2 expression is frequent in autism frontal cortex and correlates with aberrant MECP2 promoter methylation. Epigenetics: official journal of the DNA Methylation Society 1, e1–11 (2006).
33. Mill, J. et al. Epigenomic profiling reveals DNA-methylation changes associated with major psychosis. American journal of human genetics 82, 696–711, doi: 10.1016/j.ajhg.2008.01.008 (2008).
34. Mastroeni, D. et al. Epigenetic changes in Alzheimer’s disease: decrements in DNA methylation. Neurobiology of aging 31, 2025–2037, doi: 10.1016/j.neurobiolaging.2008.12.005 (2010).
35. Plotsky, P. M. et al. Long-term consequences of neonatal rearing on central corticotropin-releasing factor systems in adult male rat offspring. Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology 30, 2192–2204, doi: 10.1038/sj.npp.1300769 (2005).
36. Durand, B. et al. Activation function 2 (AF-2) of retinoic acid receptor and 9-cis retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity. The EMBO journal 13, 5370–5382 (1994).
37. Tsukada, M. et al. 13-cis retinoic acid exerts its specific activity on human sebocytes through selective intracellular isomerization to all-trans retinoic acid and binding to retinoid acid receptors. The Journal of investigative dermatology 115, 321–327, doi: 10.1046/j.1523-1747.2000.00066.x (2000).
38. Marshall, J., Iredes, N., Capron, C. C., Nguyen, E. & Chabot, G. G. Retinoid acid metabolism and mechanism of action: a review. Current drug metabolism 4, 1–10 (2003).
39. Durston, A. J. et al. Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature 340, 140–144, doi: 10.1038/340140a0 (1989).
40. Maden, M. Retinoid acid in the development, regeneration and maintenance of the nervous system. Nature reviews. Neuroscience 8, 755–765, doi: 10.1038/nrn2212 (2007).
41. Maden, M. & Holder, N. The involvement of retinoic acid in the development of the vertebral central nervous system. Development Suppl 2, 87–94 (1991).
42. Aoto, J., Nam, C. I., Poor, M. M., Ting, P. & Chen, L. Synaptic signaling by all-trans retinoic acid and binding to retinoic acid receptors. The Journal of neuroscience: the official journal of the Society for Neuroscience 34, 308–320, doi: 10.1016/j.neuroscience.2008.08.012 (2008).
43. Jacob, S. et al. Retinoic acid is required early during adult neurogenesis in the dentate gyrus. Proceedings of the National Academy of Sciences of the United States of America 103, 3902–3907, doi: 10.1073/pnas.0511294103 (2006).
44. Wang, H. L., Zhang, Z., Hintre, M. & Chen, L. Decrease in calcium concentration triggers neuronal retinoid acid synthesis during homeostatic synaptic plasticity. The Journal of neuroscience: the official journal of the Society for Neuroscience 31, 17764–17771, doi: 10.1523/JNEUROSCI.3964-11.2011 (2011).
45. Cai, L., Yan, X. B., Chen, X. N., Meng, Q. Y. & Zhou, J. N. Chronic all-trans retinoic acid administration induced hyperactivity of HPA axis and behavioral changes in young rats. European neuropsychopharmacology: the journal of the European College of Neuropsychopharmacology 20, 839–847, doi: 10.1016/j.euroneuro.2010.06.019 (2010).
46. McCance-Katz, E. F. & Price, L. H. Depression associated with vitamin A intoxication. Psychosomatics 33, 117–118, doi: 10.1016/S0033-3182(92)72003-7 (1992).
47. Hu, P. et al. All-trans retinoic acid-induced hypothalamic-pituitary-adrenal hyperactivity involves glucocorticoid receptor dysregulation. Translational psychiatry 3, e336, doi: 10.1038/tp.2013.98 (2013).
48. Aubry, E. M. & Odermatt, A. Retinoic acid reduces glucocorticoid sensitivity in C2C12 myotubes by decreasing 11beta-hydroxysteroid dehydrogenase type 1 and glucocorticoid receptor activities. Endocrinology 150, 2700–2708, doi: 10.1210/en.2008-1618 (2009).
49. Perroud, N. et al. Increased methylation of glucocorticoid receptor gene (NR3C1) in adults with a history of childhood maltreatment: a link with the severity and type of trauma. *Translational psychiatry* 1, e59, doi: 10.1038/tp.2011.60 (2011).

50. Tyrka, A. R., Price, L. H., Marist, C., Walters, O. C. & Carpenter, L. L. Childhood adversity and epigenetic modulation of the leukocyte glucocorticoid receptor: preliminary findings in healthy adults. *PloS one* 7, e30148, doi: 10.1371/journal.pone.0030148 (2012).

51. van der Knaap, L. J. et al. Glucocorticoid receptor gene (NR3C1) methylation following stressful events between birth and adolescence. The TRAILS study. *Translational psychiatry* 4, e381, doi: 10.1038/tp.2014.22 (2014).

52. Wochnik, G. M. et al. FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells. *The Journal of biological chemistry* 280, 4609–4616, doi: 10.1074/jbc.M407498200 (2005).

53. Binder, E. B. et al. Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. *Jama* 299, 1291–1305, doi: 10.1001/jama.299.11.1291 (2008).

54. Lee, R. S. et al. Chronic corticosterone exposure increases expression and decreases deoxyribonucleic acid methylation of Fkbp5 in mice. *Endocrinology* 151, 4332–4343, doi: 10.1210/en.2010-0225 (2010).

55. Lee, R. S. et al. A measure of glucocorticoid load provided by DNA methylation of Fkbp5 in mice. *Psychopharmacology* 218, 303–312, doi: 10.1007/s00213-010-2307-3 (2011).

56. Davies, M. N. et al. Functional annotation of the human brain methylome identifies tissue-specific epigenetic variation across brain and blood. *Genome biology* 13, R43, doi: 10.1186/gb-2012-13-6-r43 (2012).

Acknowledgements

We would like to extend our gratitude to all of those who aided in performing this study, especially Ann-Charlotte Olofsson and Jenny Tiberg at the Centre for Endocrinology and Metabolism at Sahlgrenska University Hospital, for their skilful technical support and to Jessica Lindvall, Kristina Duvefelt and Gunnar Falk at the Mutation Analysis Facility at Karolinska University Hospital for excellence in planning, running and correspondence related to the DNA methylation analyses. Last but not least, we are indebted to the patients and controls that participated in this study. This project has received financial support from the Swedish federal government under the LUA/ALF agreement (Drs Ragnarsson and Johannsson), The Health & Medical Care Committee of the Regional Executive Board, Region Västra Götaland (Dr Ragnarsson), The Swedish Society of Medicine (Dr Ragnarsson) and The Swedish Society of Endocrinology (Dr Ragnarsson).

Author Contributions

Dr Johannsson 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. Study concept and design: Glad, Berglund, Bergthorsdottir, Ragnarsson, Johannsson, Acquisition, analysis, or interpretation of data: All authors. Drafting of the manuscript: Glad, Ragnarsson. Critical revision of the manuscript for important intellectual content: All authors. Statistical analysis: Glad, Anderssson-Assarsson. Obtained funding: Ragnarsson, Johannsson. Administrative, technical, or material support: Glad, Anderssson-Assarsson, Berglund. Study supervision: Ragnarsson, Johannsson.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing Interests: CG, JAA, RB and OR have no disclosures. PB has received lecture fees from Boehringer Ingelheim and Lundbeck. GJ has received lecture fees from NovoNordisk, Pfizer, Otsuka and Shire, and has been a consultant for Viropharma/Shire and Astra Zeneca.

How to cite this article: Glad, C. A. M. et al. Reduced DNA methylation and psychopathology following endogenous hypercortisolism – a genome-wide study. *Sci. Rep.* 7, 44445; doi: 10.1038/srep44445 (2017).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2017