Chronic fatigue syndrome and DNA hypomethylation of the glucocorticoid receptor gene promoter 1F region : associations with hypothalamic-pituitary-adrenal axis hypofunction and childhood trauma

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DNA HYPOMETHYLATION OF THE GLUCOCORTICOID RECEPTOR GENE PROMOTER 1F REGION IN CHRONIC FATIGUE SYNDROME

RUNNING TITLE: **"GR HYPOMETHYLATION IN CHRONIC FATIGUE SYNDROME"**

Elise Vangeel, M. Sc.[1 (*)], Filip Van Den Eede, MD, PhD[2,3], Titia Hompes, MD, PhD[1,4], Benedetta Izzi, PhD[5], Jurgen Del Favero, PhD[6], Greta Moorkens, MD, PhD[7], Diether Lambrechts, PhD[8,9], Kathleen Freson, PhD[5], Stephan Claes, MD, PhD[1,4]

1: Genetic Research About Stress and Psychiatry (GRASP), University of Leuven, Leuven, Belgium
2: University Department of Psychiatry, Campus Antwerp University Hospital, Antwerp (Edegem), Belgium
3: Collaborative Antwerp Psychiatric Research Institute (CAPRI), University of Antwerp, Antwerp, Belgium
4: University Psychiatric Center University of Leuven, Leuven, Belgium
5: Department of Cardiovascular Sciences, Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium
6: Department of Molecular Genetics VIB8, Flanders Interuniversity Institute for Biotechnology, University of Antwerp, Antwerp, Belgium
7: Department of Internal Medicine, Antwerp University Hospital, Antwerp (Edegem), Belgium
8: Laboratory of Translational Genetics, Department of Oncology, University of Leuven, Leuven, Belgium
9: Vesalius Research Center (VRC), VIB, Leuven, Belgium

**Corresponding author:*

Elise Vangeel, M. Sc.
elise.vangeel@med.kuleuven.be
Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium
Tel: +32 16 340548

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ABSTRACT

Objective: Chronic fatigue syndrome (CFS) has been associated with hypothalamic-pituitary-adrenal (HPA) axis hypofunction and enhanced glucocorticoid receptor (GR) sensitivity., while In addition, childhood trauma is considered a major risk factor for the syndrome. As epigenetic mechanisms may mediate GR sensitivity and the impact of adverse childhood events, this study examines DNA methylation of the GR gene (*NR3C1*) in CFS and associations with childhood sexual and physical trauma.

Methods: Quantification of DNA methylation within the 1F promoter region of the *NR3C1* gene was performed in 76 female patients (46 with no/mild and 30 with moderate/severe childhood trauma) and 19 healthy controls using Sequenom EpiTYPER. In a secondary analysis, we examined the association of *NR3C1*-1F promoter methylation with the outcomes of the low-dose (0.5 mg) dexamethasone/corticotropin-releasing factor test in a subset of the study population. Mann-Whitney U tests and Spearman correlations were used for statistical analyses.

Results: Overall *NR3C1*-1F DNA methylation was lower in CFS patients than in controls. Following CpG-specific analysis, CpG_1.5 remains significant after Bonferroni correction (adjusted p = 0.0014). Within the CFS group, overall methylation (rho = 0.477, p = 0.016) and selective CpG units (CpG_1.5: rho = 0.538, p = 0.007; CpG_12.13: rho = 0.448, p = 0.025) were positively correlated to salivary cortisol after dexamethasone administration. There was no significant difference in *NR3C1*-1F methylation between traumatized and non-traumatized patients.

Conclusion: We found evidence of *NR3C1* promoter hypomethylation in female CFS patients and the functional relevance of these differences was consistent with the HPA axis hypofunction hypothesis (GR hypersuppression). However, we found no evidence of an additional effect of childhood trauma on CFS via alterations in *NR3C1* methylation.

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

Key words: cortisol; glucocorticoid receptor; HPA axis; chronic fatigue syndrome; DNA methylation; childhood trauma

ABBREVIATIONS:

AUC	Area under the curve
CFS	Chronic fatigue syndrome
CpG	Cytosine – guanine dinucleotide
CRF	Corticotropin releasing factor
Dex	Dexamethasone
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
NR3C1	Glucocorticoid receptor gene
PTSD	Posttraumatic stress disorder
SCID-1	Structured clinical interview for DSM-IV axis I disorders
STI	Structured trauma interview
TEC	Traumatic experiences checklist

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

INTRODUCTION

Comprising mental as well as physical symptoms, chronic fatigue syndrome (CFS) is defined by idiopathic, disabling fatigue accompanied by at least four additional specific symptoms during at least six months of continuous illness (1).

Although the pathogenesis of CFS remains an enigma, there is evidence of hypothalamic-pituitary-adrenal (HPA) axis hypofunction in a substantial proportion of patients (2). More precisely, the weight of current evidence supports the presence of the following factors related to HPA axis dysfunction in CFS patients: mild hypocortisolism, attenuated diurnal variation of cortisol, enhanced negative feedback of the HPA axis (hypersuppression), and blunted HPA axis responsiveness. Furthermore, HPA axis changes seem clinically relevant because they are associated with more prominent symptoms and disability and poorer outcomes to standard CFS treatments (3).

One of the proposed mechanisms of HPA axis hypofunction in CFS is enhanced glucocorticoid receptor (GR) sensitivity (4), with enhanced suppression after a low dose of dexamethasone (0.5 mg) having been clearly demonstrated in several CFS studies (5–7). However, the underlying mechanism of enhanced GR sensitivity in CFS needs to be further investigated. Hypomethylation of the promoter regions of the GR gene (*NR3C1*) may be one such mechanism. Recently, Yehuda et al. (2014) reported lower *NR3C1*-1F promoter methylation in peripheral blood mononuclear cells in combat veterans suffering from posttraumatic stress disorder (PTSD), a disorder that has also been associated with enhanced negative feedback to the HPA axis (8). Interestingly, in this study *NR3C1*-1F promoter methylation was also associated with functional measures of glucocorticoid activity that have been linked to PTSD, including plasma cortisol concentrations after a low dose (0.5 mg) of dexamethasone (Dex) (8). Furthermore, the involvement of DNA methylation in CFS pathology has been recently suggested in an epigenome-wide study (9). As CFS is also associated with enhanced feedback sensitivity of the HPA axis, it would be interesting to

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

study DNA methylation of the *NR3C1*-1F region in patients suffering from the syndrome (10,11).

Several factors interact to moderate HPA axis changes in CFS (3). One of the best documented predisposing factors for CFS is childhood trauma (12), which has been associated with differential neuroendocrine responses (6,13), although further research on this correlation is required (14).

Considering the importance of early-life environment in the susceptibility to psychiatric disorders, the modification of epigenetic marks has been suggested to mediate this process at least in part (15). Indeed, several studies describe effects of stressful life events and childhood trauma on changes in DNA methylation of important HPA axis genes (16,17). More specifically, studies suggest *NR3C1* promoter hypermethylation to affect GR gene expression, diminishing negative feedback on the HPA axis and causing cortisol levels to rise (17–19). As childhood trauma is an important predisposing factor in CFS, it is important to investigate its impact on DNA methylation of the *NR3C1* promoter.

The primary objective of the current study was to quantify DNA methylation of the *NR3C1* 1F promoter region in CFS and the impact of childhood trauma on this methylation pattern. As a secondary objective we assessed the association of these methylation patterns with the outcomes of a low-dose (0.5 mg) Dex/corticotropin-releasing factor (Dex/CRF) test in a previously described subset of CFS patients (6) in order to determine the functional relevance of *NR3C1* methylation changes.

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

MATERIALS AND METHODS

Study population

We selected the patients for this study from two CFS study populations. In both sample sets CFS had been diagnosed according to the US Centers for Disease Control and Prevention criteria (1) by a qualified internist (GM) in the CFS Reference Centre of Antwerp University Hospital. Alternative medical diagnoses were excluded in all patients following several clinical examinations and laboratory analyses. All participants met with a psychiatrist (FVDE) or a supervised resident in psychiatry for a general psychiatric interview and the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-1, version 2.0).

The first population consisted of 38 CFS patients and 21 controls who had participated in our low-dose Dex/CRF test, reported in 2008 (6), with blood samples being collected between October 2003 and November 2004. In this study we included only female participants since HPA axis research differs by gender (20–22) and since the majority of CFS patients are women (23). Patients who were pregnant, had a lifetime history of organic brain syndrome or a current medical condition, or those that were taking drugs that might interfere with neuroendocrine testing were excluded. The 21 controls were healthy women without a lifetime history of CFS. The patient group was larger in order to allow a secondary analysis of potentially influencing clinical variables.

Because it was one of our objectives to study the impact of childhood trauma on DNA methylation of the *NR3C1* 1F promoter region (*NR3C1*-1F) in CFS, we extended our first study population of 38 patients and 21 controls with an additional 51 CFS patients selected from another study reported elsewhere (24). For the recruitment of this second study, 168 patients originally responded to an invitation to participate and attended 12 test sessions (July 2007 – May 2010). Eight patients did not fully complete the Traumatic Experiences Checklist (TEC) (25) and were excluded, leaving the data of 160 patients for further analysis.

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

To examine the impact of childhood trauma on methylation of *NR3C1*-1F, we created two age-matched subgroups by selecting the 26 patients with the highest TEC scores and another 25 with a zero TEC score from this group of 160 patients. Together with the participants of the first study, the inclusion of these 51 patients makes a total of 89 CFS patients and 21 controls that we could use for DNA methylation analysis. For a schematic overview, see Supplementary Figure S1.

All our studies were approved by the Antwerp University Hospital medical ethics committee (Population 1: EC code 3/29/98, date 19/08/2003; population 2: EC code 7/1/4, date 27/2/2007; Belgian code: B30020072399). Participants were all over 18 years of age at the time of their CFS diagnosis and all provided their written informed consent.

Childhood trauma interviews and questionnaires

For the assessment of childhood trauma in the first CFS sample set, a psychiatrist (FVDE) interviewed the patients under conditions of confidentiality and mutual trust (26) using the Dutch version of the Structured Trauma Interview (STI; for a detailed description, see (27)). In brief, the STI probes for childhood sexual and physical abuse, with sexual abuse being defined as sexual contact with the child against its will or when the child felt that the sexual contact could not be refused due to physical or relational preponderance, emotional pressure, force or violence. The abuse is coded as 'severe' when it was chronical or occurred more than once a month during a particular period in childhood, when it consisted of any form of penetration, when it was associated with violence, or when it occurred in the child's primary support group. Physical abuse was defined as intentional physical aggression by a parent or caregiver towards the child and scored as follows: 'severe' if it happened more than once a month and if it sometimes led to injuries, 'moderate' when the child was hit hard more than once a month without experiencing injuries or when aggression took place less frequently but did cause injuries, and 'mild' when the aggression had a lower frequency and caused no injuries.

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

For the second CFS sample set, childhood trauma was assessed and screened using the TEC, a self-report questionnaire inquiring about 29 types of potential trauma subdivided into 5 subscales: emotional neglect, emotional abuse, physical abuse, sexual harassment, and sexual abuse. For each item the event of concern is briefly described, preceded by the question: 'Did this happen to you?' (25). The severity of childhood trauma is scored using four variables: 1. Presence of the event(s); 2. Duration of the event(s) (less versus more than 12 months); 3. Relationship to the perpetrator (parent/sibling versus others); 4. Subjective response (feeling slightly versus moderately, severely, or extremely traumatized by the event(s)). The variables are scored per age period in which the event(s) occurred, with the TEC composite score being the main outcome variable for childhood trauma. To categorize the respondents into a no/mild childhood trauma versus a moderate/severe trauma group according to the criteria described for our first CFS sample set (Structured Trauma Interview), we evaluated the individual TEC items and the participants' medical files.

Combined low-dose Dex/CRF test

For the combined low-dose Dex/CRF test, we adopted the Heuser et al. (28) protocol, but instead of using plasma cortisol, we measured salivary cortisol concentrations using a cotton wool Salivette (Sarstedt, Nümbrecht, Germany). Implementation and results of the procedure are previously described (6). Using a low dose of 0.5 mg Dex and 100 µg CRF and following the Dex/CRF administration protocol, we measured salivary cortisol seven times. Taken into account the results of the previous study by Van Den Eede et al. (6), we defined the following two outcome measures: 'Dex', being the average of free cortisol concentration across three measurements after Dex but before CRF administration, and 'AUCtot,' being the total area under the curve (AUC) of cortisol response at four time points after CRF administration.

DNA methylation analysis

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

DNA (1µg) was isolated from whole blood in all participants and bisulfite treated using the MethylDetector kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions, apart from the conversion reaction, for which we used the incubation program as recently proposed by Izzi et al. (29): one minute at 95°C, 16 cycles of 95°C for 30 seconds and 50°C for one hour, followed by a hold at 4°C.

Primers and specifications for the *NR3C1*-1F amplicon were described previously (30,31). An overview of the 1F amplicon can be found in Figure 1. DNA methylation was analyzed in triplicate by performing PCR on bisulfite-treated DNA followed by reverse transcription, base-specific cleavage, and mass spectrometry according to the Sequenom MassARRAY protocol (Sequenom, San Diego, CA, USA). Using the Sequenom EpiTYPER software (v1.0 build 1.0.5, Sequenom), mass spectrometry signals were presented by values ranging from 0 to 1 representing DNA methylation percentages. DNA fragments containing multiple CpG sites are organized into 'CpG units', e.g. 'CpG_1.5' represents CpG_1 through CpG_5 (see also Figure 1).

DNA methylation values of samples with a standard deviation of more than 5% between replicates were removed. Also, only CpG units with a success rate of 75% or higher were included in the analyses.

Statistical analysis

Since *NR3C1*-1F methylation data were not normally distributed, non-parametric tests were applied, i.e., Mann-Whitney U tests to compare DNA methylation levels between groups and Spearman correlations to assess the relationships between DNA methylation and cortisol outcome measures. Statistical analyses were performed using SPSS Statistics, version 22 (IBM Corp, Armonk, NY, USA).

RESULTS

CFS population characteristics

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

The characteristics of our patient group and those of the controls are presented in Table 1. After quality control testing, the final number of patient and control samples for our DNA methylation quantification analyses was 76 and 19, respectively (Figure S1). All participants were registered as Caucasian, except for one patient of whom the origin of the paternal grandparents was unknown. Patients and controls did not statistically differ in age, educational level (primary, lower secondary, higher secondary or post-secondary education), parenthood, lifetime major depression, anxiety disorder, or STI-based childhood trauma. Six of the patients had been diagnosed with PTSD during their lifetime, while 34 suffered from fibromyalgia according to the US Centers for Disease Control and Prevention 1990 (32).

In the first sample, 10 patients fulfilled the STI criteria for moderate or severe childhood trauma, while 20 patients of the second sample fulfilled these criteria based on their TEC scores. When classifying a duration in excess of 12 months as moderate or severe, six additional patients also met the criteria. Six individuals with an elevated TEC score did not meet the moderate or severe trauma criteria according to the STI and were therefore allocated to the mild childhood trauma group. Thus, a total of 30 patients met the criteria of moderate or severe childhood trauma.

Associations between CFS and NR3C1-1F methylation and childhood trauma

The amplicon of the 1F region used for our methylation analyses, the location of the CpG sites, and an overview of the *NR3C1* gene are schematically represented in Figure 1. Age and smoking were only very weakly correlated to *NR3C1*-1F methylation and therefore we did not include these variables in our analyses. Contrasting the patient and control data, we found a significant difference for the mean of the overall methylation that covers all seven CpG units in the *NR3C1*-1F amplicon (Fig. 2A; 2.4% versus 2.9% methylation, Mann-Whitney U test, $p = 0.016$). We also obtained significant differences in methylation in three out of seven CpG units of the *NR3C1*-1F region (CpG_1.5, CpG_12.13, CpG_19, Table 2, Fig. 2C), with CpG_1.5 as the most significant differentially methylated unit (Fig. 2B; 6.5%

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

versus 8.3%, p = 0.0002). After Bonferroni correction for multiple testing only the latter CpG unit remained significant (adjusted p = 0.0014).

Comparing all *NR3C1*-1F CpG units in the no/mild trauma versus the moderate/severe trauma group, we computed no methylation differences in these patient subgroups (Fig. 3; Mann-Whitney U tests, p > 0.05). Neither did we find any significant differences in *NR3C1*-1F methylation levels between CFS patients with and those without a history of lifetime major depression, lifetime anxiety disorder or fibromyalgia (Mann-Whitney U tests, p >0.05).

Associations between NR3C1-1F methylation and cortisol

One patient was excluded for this analysis due to a panic attack during the Dex/CRF test**,** resulting in extremely high cortisol levels, leaving the data of 25 patients and 19 controls for our analysis.

Within the CFS group, post-Dex cortisol was significantly correlated with the level of overall *NR3C1*-1F DNA methylation (Spearman R = 0.477, p = 0.016; Fig. 4; Table 3). More specifically, significant correlations were also found for unit CpG_1.5 (Spearman R = 0.538, p = 0.007) and CpG_12.13 (Spearman R = 0.448, p = 0.025). Considering all 44 samples, we found unit CpG_1.5 to remain significantly correlated with post-Dex cortisol levels (Spearman R = 0.360, p = 0.018). No significant correlations were obtained for the second outcome measure AUCtot (all CpG units p > 0.05), which represents cortisol measurements following Dex and CRF administration.

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

DISCUSSION

Epigenetics has been extensively described as an important modulator of psychiatric disorders (16,33). Especially *NR3C1,* a complex, epigenetically regulated gene with multiple untranslated alternative first exons (Fig.1) seems promising. Exon 1F is the most studied alternative first exon in the GR gene for DNA methylation analyses in relation to early trauma and its impact on mental health (19,30,34,35). The *NR3C1*-1F promoter region has been reported to contain several canonical and putative nerve growth factor-inducible protein A binding sites (19,36), suggesting a role of methylation in mediating the binding of this transcription factor.

Since folic acid is key in the methyl cycle, the low serum folate levels found in CFS patients and the benefit from folate supplementation therapy suggest a role for DNA methylation in CFS pathology (37). Indeed, a genome-wide epigenetic study recently showed DNA methylation differences in several genes of CFS patients (9).

Comparing patient values with those obtained for the healthy controls, we found a significant overall *NR3C1*-1F hypomethylation in our patient group, as well as, most markedly, in the CpG_1.5 unit, but also in the CpG_12.13 and CpG_19units. Following Bonferroni correction, only the CpG_1.5 unit remains significant. The CpG_1.5 unit is located at the end of the *NR3C1*-1F region (Fig.1), of which the functional relevance has not yet been shown. As DNA methylation has been proposed to play a role in splicing (38), and considering the location of CpG_1.5 (Fig.1), its altered methylation might change splicing efficiency and therefore GR expression.

The functional consequences of DNA hypomethylation in our CFS group are further supported by the hypersuppression of cortisol after a low dose (0.5 mg) of Dex. Overall and CpG-specific methylation values in the CFS group are positively correlated with post-Dex cortisol levels. However, we found no significant association with total cortisol measurements

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

after CRF administration (AUCtot), for which following explanations are possible. First, the power of our study may be too low. Furthermore, the Dex test is a more direct measurement of GR feedback sensitivity than the Dex/CRF test. The Dex/CRF test combines Dex with CRF administration and since CRF passes through the blood-brain barrier, it does not permit assessment of specific components of the axis. Other possible mechanisms include compensatory down-regulation of CRF or arginine vasopressin receptors (35).

As previously mentioned, enhanced negative feedback to the HPA axis (GR hypersuppression) has been reported in both CFS and PTSD. Interestingly and consistent with our findings in CFS, in their recent PTSD study, Yehuda et al. (8) showed that lower *NR3C1*-1F methylation was associated with lower cortisol levels after a low dose of dexamethasone. They further provided the 'missing link' by showing that these findings are associated with an increased *NR3C1* gene expression and consequently a higher function and hypersuppression of the HPA axis. Similarly, Labonté et al. (39) describe lower methylation levels of two other *NR3C1* alternative exons (1B and 1C) in PTSD, correspondingly higher gene expression, and low HPA axis activity as expressed by cortisol values. Therefore, methylation of exon 1B and 1C may also be informative in CFS, in addition to the 1F region. ~~Also, we recognize that the methylation differences observed between CFS patients and controls in our study are relatively small. Although these changes relate to HPA axis function, we agree that several mechanisms may play a role in CFS pathology.~~

In contrast to our expectations, *NR3C1*-1F DNA methylation was not associated with childhood trauma in our CFS patients. This finding appears to conflict with previous studies that did report significant correlations of this region with different types of early-life stress, including perinatal stress (17,30). However, the lack of association with trauma may be due to following reasons. First, with respect to our tool to assess childhood trauma, use of a continuous scale would be more powerful. Second, the criteria that we used to categorize

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

patients into no/mild trauma or moderate/severe trauma groups strongly focus on sexual and physical trauma, whereas recent research shows the importance of psychological trauma in CFS (40). Third, lower *NR3C1*-1F methylation might serve as a biomarker for enhanced HPA axis negative feedback (as seen in CFS and PTSD), independent of a predisposing influence of childhood trauma, as suggested by Yehuda et al. (8). Regarding this, it would be interesting to examine methylation patterns of *NR3C1*-1F in other disorders that have been associated with enhanced HPA axis negative feedback and are characterized by long-term exhaustion, such as burnout and major depression with atypical features (41,42).

Some limitations have to be considered when interpreting our data. First, since this is not a longitudinal study, we cannot state whether the differential methylation is a predisposing factor for CFS or if it is a consequence of the pathology. Secondly, due to their small number, we could not determine the effect of trauma in our healthy controls, although the sample size was sufficient for our primary aim. Moreover, the standard deviation for methylation values within the control and the patient groups was relatively small. Also, we recognize that the methylation differences observed between CFS patients and controls in our study are relatively small. However, our results show that DNA methylation levels are associated to HPA axis function. ~~Although these changes relate to HPA axis function, we agree that several mechanisms may play a role in CFS pathology.~~

Differences in DNA methylation may reflect a shift in blood cell composition, which we, unfortunately, were not able to determine from the collected blood samples. Although DNA methylation has been shown to be tissue-specific and despite the fact that the use of blood as a surrogate tissue to study non-immune phenotypes has been the subject of recent debate (43), we measured DNA methylation in DNA derived from whole blood samples. However, several studies published in recent years have identified similar methylation patterns in blood and brain tissue in a number of psychiatric disorders (44,45). In addition, the consistency of our results and the findings of other papers, and the correlations with HPA

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

axis functional tests are supportive of epigenetic studies using easily accessible blood samples.

Some caution is also warranted when reporting on patients suffering from CFS since there is a high comorbidity with PTSD and major depressive disorder (10,46). Then again, we found no significant methylation differences between CFS patients with a lifetime major depression or anxiety disorder (i.e., PTSD) and those without. Also, although CFS is more common in women, we cannot make any statements about the male CFS population given that all our patients were female. Finally, while we can assume that the causal link between hypomethylation of *NR3C1*-1F and lower cortisol in response to dexamethasone lies in increased expression of the glucocorticoid receptor, we have not actually assessed GR expression levels.

In conclusion, we found a significant overall and CpG-specific hypomethylation of the *NR3C1*-1F region in women diagnosed with CFS. Furthermore, the hypomethylation was associated with lower post-Dex cortisol levels, indicating a potential functional role for this specific CpG unit. Our findings are consistent with previous evidence of HPA axis dysregulation in CFS patients and indicate a role for DNA methylation in this complex illness. As this is the first study linking CFS with *NR3C1* epigenetics, further investigations in larger, biologically similar cohorts are needed to confirm our findings.

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

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Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

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Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

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Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

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Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

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Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

TABLE AND FIGURE LEGENDS

Table 1: Characteristics of the chronic fatigue syndrome (CFS) patients and the healthy controls~~. SCID-1, Structured Clinical Interview for DSM disorders~~

Table 2: Mean DNA methylation percentages of *NR3C1*-1F for the controls ~~(n = 19)~~ and chronic fatigue syndrome ~~(CFS)~~ patients ~~(n = 76) based on Mann-Whitney U tests. The number of available valid methylation data is indicated for each CpG unit. Significant p-values are shown in bold.~~

Table 3: Spearman correlations of *NR3C1*-1F DNA methylation with post-Dex and post-Dex/CRF ~~(dexamethasone)~~ cortisol levels for chronic fatigue syndrome ~~(CFS)~~ patients ~~(n = 25)~~ and all participants ~~(patients plus controls; n = 44).~~

Figure 1: Glucocorticoid receptor gene (*NR3C1*) structure and the 1F amplicon analyzed with Sequenom EpiTyper: Representation of *NR3C1* exon 2 through 9 (▭) and its alternative first exons (⬠). Indicated in the text box is the genomic sequence of the 1F region as used in our methylation analyses and all CpG sites are specified by their respective numbers (1 through 47). CpG units noted in the text represent combined CpG sites, e.g. 'CpG_1.5' represents CpG_1 through CpG_5. The 1F exonic region is underlined. The figure is not to scale and based on Turner et al. (47) and Hompes et al. (30,31).

Figure 2: *NR3C1*-1F DNA methylation in chronic fatigue syndrome (CFS) patients (n = 76) and controls (n = 19)

A, Average DNA methylation across the entire *NR3C1*-1F amplicon. **B,** Mean DNA methylation values of the *NR3C1*-1F CpG_1.5 unit in the control (left) and CFS group (right). Each dot represents the methylation value of one individual and the median is indicated by a horizontal line. **C,** Mean DNA methylation values for controls (light grey) and CFS patients (dark grey) for all CpG units within the *NR3C1*-1F amplicon (represented on the x-axis).

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium

Significantly different CpG units are indicated with their respective p-values. For exact numbers of successful samples per CpG unit, we refer to Table 2. CI, confidence interval

Figure 3: DNA methylation values for all chronic fatigue syndrome (CFS) patients with a moderate or severe childhood trauma history (dark grey; n = 30) and those with a mild or no trauma history (light grey; n = 46), for all CpG units within the *NR3C1*-1F amplicon (represented on the x-axis). There are no significantly different CpG units. CI, confidence interval

Figure 4: Correlation between DNA methylation at CpG_1.5 and post-Dex cortisol measurements (in ng/100 mL), in the chronic fatigue syndrome patients only. Spearman R^2 = 0.289, p = 0.007

Supplementary Figure S1: Schematic overview of control and CFS patient population compositions. Subjects from Population 1 were previously described in (6) and include 21 controls and 38 patients. From a population previously described in (24), we selected 51 CFS patients. Samples that were lost due to technical reasons are indicated with dotted arrows. Altogether, 19 control and 76 CFS patient samples remain of which we have *NR3C1*-1F DNA methylation data. Numbers between brackets indicate a subgroup of CFS patients with moderate/severe trauma.

Elise Vangeel, Research Group Psychiatry
Kapucijnenvoer 33 blok g – bus 7001
3000 Leuven, Belgium