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Glucocorticoid sensitivity of immune cells in severely fatigued adolescent girls: A longitudinal study

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Summary

Fatigue during adolescence is associated with somatic and psychological complaints that resemble the pattern of symptoms described for chronic fatigue syndrome (CFS). Studies in CFS and other stress-related syndromes suggested a dysfunction of the interactions between the hypothalamic–pituitary–adrenal axis (HPA-axis) and the immune system, i.e. a changed glucocorticoid (GC) receptor sensitivity of immune cells, to exist. Here we investigated whether severely fatigued girls from a healthy population have altered cortisol production and immune cell sensitivity for the synthetic GC, dexamethasone (DEX). In a longitudinal design, we examined *ex vivo* DEX sensitivity of monocytes and of T-cell mitogen-induced responses of severely fatigued ($N = 65$) and non-fatigued girls ($N = 60$). Fatigued girls reported more severe comorbid complaints than non-fatigued participants across three measurements during 1 year (T1: spring, T2: autumn, T3: spring) and had higher plasma cortisol levels throughout the study. DEX sensitivity of T-cell mitogen-induced responses showed seasonal variation with increased sensitivity in autumn compared to spring. No systematic variation of monocyte glucocorticoid receptor (GR) sensitivity was observed. Significant rank correlations of DEX sensitivity of T-cell mitogen-induced responses between the three assessments during the year suggest a stable trait of immune function. Groups did not differ in DEX sensitivity on any of the read outs. However, in a *persistently* fatigued subgroup, sensitivity to DEX was significantly reduced on the level of interferon (IFN)- γ production. These results show that although fatigued participants had severe (comorbid) complaints, only in the case when symptoms persisted, altered GC sensitivity of immune cells was observed.

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1. Introduction

Among adolescents, fatigue is a common complaint. In an extensive epidemiological study in adolescent boys and girls, we previously showed strong associations between fatigue on one hand, and depressive symptoms, anxiety, pain, unrefreshing sleep and cognitive difficulties on the other. Based on their symptomatology, severely fatigued participants of the epidemiological study resembled patients diagnosed with chronic fatigue syndrome (CFS) (ter Wolbeek et al., 2006). According to the Centers for Disease Control and Prevention (CDC), CFS is characterized by persistent and disabling fatigue accompanied by symptoms of myalgia, headaches, joint pain, unrefreshing sleep, and memory and concentration disturbances (Fukuda et al., 1994). Symptoms of depression and anxiety are often observed in CFS patients as well (Garralda and Rangel, 2005; van de Putte et al., 2006). Premorbid stress and emotional instability were found to be important predictors of CFS (Salit, 1997; Kato et al., 2006). Furthermore, in adults unexplained fatigue was found to predict the development of CFS later in life (Wessely et al., 1995; Huibers et al., 2004). Severe fatigue among adolescents may reflect an increased burden on stress-physiological systems. In view of early prevention, it is important to identify potential physiological deviations that are associated with fatigue in adolescents while they are still healthy.

The hypothalamic–pituitary–adrenal axis (HPA-axis) is one of the physiological regulatory systems suggested to be involved in the pathogenesis of CFS. Though mainly studied in adults, results, if anything, seem to point to mild hypocortisolism (Cleare, 2003). Viral infections (for example, Lyme disease, Q fever and infectious mononucleosis) and alterations in the immune system function also have been held (at least in part) responsible for the development of CFS. Therefore, a wide range of immune functions in CFS patients has been investigated (Patarca, 2001; Natelson et al., 2002; Lyall et al., 2003).

When the immune system is challenged it releases cytokines, immune cell products which provide communication between components of the immune system and between the immune system and other organ systems, such as the brain (Blalock, 1994; Besedovsky and del Rey, 1996). Subsequently, the HPA-axis is activated which enables cortisol to contribute to the inhibition of further release of pro-inflammatory cytokines. It is important to note that the effectiveness of this process does not solely depend on absolute levels of circulating cortisol but also on the sensitivity of cells for the regulatory effects of glucocorticoids (GCs). It has been suggested that in reaction to either chronic or severe psychological stress such as trauma, or physical stress, like chronic inflammation, sensitivity of GC receptors (GRs) on immune cells can be altered (Kavelaars et al., 2000; Raison and Miller, 2003; Yehuda et al., 2004; de Kloet et al., 2007).

In a few studies with CFS patients the sensitivity of immune cells to a synthetic GC, dexamethasone (DEX), has been investigated. In adults, enhanced sensitivity for inhibition by DEX was observed (Visser et al., 2000; Visser et al., 2001). In the only study in adolescent CFS patients, Kavelaars et al. (2000) found a resistance of the immune system to the regulation by DEX. Alterations in GR sensitivity

of the immune system are not limited to CFS patients but also observed in other stress-related syndromes, such as post-traumatic stress disorder (PTSD), and vital exhaustion (Wirtz et al., 2003; Yehuda et al., 2004; de Kloet et al., 2007). Whether interactions between the HPA-axis and the immune system may be deviant in severely fatigued adolescents from a healthy population remained to be elucidated. In view of the overlap in symptoms with CFS patients, we hypothesized that there could be a potential role for an altered communication between the two systems.

The majority of studies analyzing immune functions in CFS have the limitation that they rely on single point measurements without considering the influence of factors such as seasonal variation in immune reactivity, diurnal variation and exposure to common infections (van Rood et al., 1991; Nelson, 2004; ter Wolbeek et al., 2007b). This may partly explain the contradictions between the results of these studies. When studying subtle differences between groups, between-subject variability due to these confounders should be minimized. Segerstrom et al. (2006) argued, based on a simian study, that even up to 10 assessments of the same animal are required to determine an average immune status or “immune trait”. We observed high within-subject stability in rank scores of cytokine production but clear influences of season in both fatigued and non-fatigued adolescents (ter Wolbeek et al., 2007b). To our knowledge it has not been explored yet whether the communication between the HPA-axis and the immune system is a stable function or “immune trait”.

The aim of the present study was to identify possible deviations in cortisol production and GR sensitivity of immune cells in severely fatigued adolescents as compared to non-fatigued controls. To reliably assess GR sensitivity and to explore the within-subject stability of this parameter in humans, we chose a longitudinal design. In a sample of non-fatigued and severely fatigued adolescents we examined the sensitivity of monocytes to DEX in the production of tumor necrosis factor (TNF)- α and interleukin (IL)-10. Moreover, we investigated DEX sensitivity of T-cell mitogen-induced proliferation and production of interferon (IFN)- γ and IL-10. In addition, we examined GR sensitivity in a selected persistently fatigued subgroup. We performed the measurements on three different time points: T1 (spring), T2 after 6 months (autumn) and T3 after 12 months (spring). Since the prevalence of severe fatigue in our epidemiological study was higher in girls and female predominance was also found in some other studies about fatigue and CFS during adolescence (Wright and Beverley, 1998; Farmer et al., 2004; Ter Wolbeek et al., 2006), only female adolescents were included in the present study.

2. Methods

2.1. Participants

Non-fatigued and severely fatigued participants were selected from a broader epidemiological study on fatigue among adolescents which was conducted at five Dutch high schools. All female participants and their parents (or guardians) received written information about the current study.

Required sample size was determined by power analysis for the group comparison in cytokine production. At least 55 children in each group showed to be sufficient for the present study, based on the magnitude of group differences in a previous study of our group (ter Wolbeek et al., 2007b). A severely fatigued group of 67 girls (age 15.18 ± 1.37 years) with the highest total fatigue scores on the Checklist Individual Strength (CIS) was selected out of 591 girls who were interested in participation (Vercoulen et al., 1999). In addition, a non-fatigued control group ($N = 61$, age 14.74 ± 1.60 years) was selected with the lowest fatigue scores. Participants were included only when the level of fatigue at inclusion was present for at least 1 month. Fatigue duration in the fatigued group was: 22.4% 1–2 months, 17.9% 2–3 months, 10.4% 3–4 months and 49.3% longer than 4 months. Fatigued and non-fatigued participants did not differ in time spent on homework, leisure time activities, nightlife and time spend with friends. Also, an equal proportion of participants of both groups had a job aside from their school work (fatigued 46.3% and non-fatigued 44.3%, $\chi^2 = 8.81$, $p < 0.05$). However, time spent on physical activities was significantly reduced in the fatigued group ($t = 4.52$, $p < 0.001$). Based on self-reports by the participants and telephone interviews with their parents (or guardians), participants with past or current somatic or psychiatric diagnoses were not selected. All non-fatigued and fatigued participants were tested for sub-clinical infections by determination of erythrocyte sedimentation rate. The two groups did not differ in the proportion of participants with elevated sedimentation rates according to a clinical cutoff. Additional information was given by telephone and written informed consent of both parents and adolescents was obtained. This study was approved by the Medical Ethical Committee (IRB) of the University Medical Center Utrecht.

2.2. General procedure

After returning written informed consent, participants were requested to fill out the questionnaires at home without assistance. Within 2 weeks after questionnaire completion, individual appointments were planned at school. During the appointment, questionnaires were collected and participants were asked about their current medication use and flu-like or common cold symptoms at that moment and during the past week. Of all participants, 10 ml of heparinized and 5 ml of EDTA blood was taken between 8.30 and 11.00 a.m. using a Vacutainer® collection system. Participants were included in two enrollment periods, in spring 2002 and spring 2004 and tested on three occasions: in spring (T1), autumn (T2) and the successive spring (T3).

2.3. Questionnaires

Fatigue was assessed using the CIS questionnaire (Vercoulen et al., 1999), which consists of four subscales, namely, *severity of fatigue*, *concentration*, *motivation* and *activity* and was originally developed for adults. Based on the prevalence study, one item was excluded from the subscale *activity* to adjust the questionnaire to the adolescent population (ter Wolbeek et al., 2006). Depressive symptoms

were measured using the Beck Depression Inventory (BDI) (Beck et al., 1961). Trait anxiety was assessed with the Dutch version of the State/Trait Anxiety Inventory for Children (STAIC) (Spielberger, 1973; van der Ploeg, 2000). To assess general sleep quality, the Dutch State and Trait Sleep Assessment Scale was used (GSKS) (Meijman et al., 1990). With the Modified Somatic Perception Questionnaire (MSPQ) somatic and autonomic symptoms were measured using the revised 33-item version (Main, 1983). Self-reports of the CFS-related symptoms unrefreshing sleep, muscle pain, joint pain, headaches, tender lymph nodes, memory and concentration problems in the past 2 weeks were rated using a dichotomous scale (yes/no) (Fukuda et al., 1997).

Single questions were asked about fatigue- and illness-related school absenteeism, smoking, use of oral contraceptives and medication, experience of menarche and age at menarche. Pubertal development was determined according to the Tanner stages of pubertal development (Tanner, 1962). Occasional use of the following medication was reported by non-fatigued (NF) and fatigued (F) participants: antihistamines (F: $N = 1$), beta-blockers (F: $N = 1$), non-systemic corticosteroids (nasal spray or crème) (NF: $N = 3$; F: $N = 6$) and migraine medication or painkillers (NF: $N = 2$; F: $N = 5$). Body Mass Index (BMI) was calculated by dividing body weight by the square of body height (kg/m^2). Self-reported allergic and asthmatic complaints were registered.

2.4. Determination of plasma cortisol

For total cortisol analysis, blood collected in EDTA was kept on ice until centrifugation, and plasma was frozen at -80°C awaiting analysis. A commercially available immunoassay (Centaur, Bayer, Fernwald, Germany) was performed in two runs with samples of non-fatigued and fatigued participants mixed (intra- and inter-assay variability less than 6%).

2.5. Ex vivo cell response to dexamethasone

Sensitivity of monocyte cytokine production was measured in whole blood 1:10 diluted with RPMI-1641, supplemented with antibiotics and stimulated with lipopolysaccharide (LPS, *Escherichia coli* 0127: B8, Sigma, final concentration 2 ng/ml) in the presence of 0–300 nM DEX at $37^\circ\text{C}/5\% \text{CO}_2$ in 96 wells flat bottom plates for 24 h. Determination of DEX concentrations was based on our earlier studies and is in line with studies by others (Kavelaars et al., 2000; Yehuda et al., 2004). Supernatants were stored at -80°C and TNF- α and IL-10 production was determined using standard ELISA kits (Sanquin, Amsterdam, the Netherlands). In addition, sensitivity of T-cell mitogen-induced proliferation and production of IL-10 and IFN- γ to DEX was determined in whole blood cultures stimulated with the T-lymphocyte mitogen phytohemagglutinin (PHA). Whole blood diluted 1:10 with RPMI-1641 (Gibco, Grand Island, NY), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 2 mM L-glutamine was stimulated with PHA (Remel Europe Ltd., final concentration 25 $\mu\text{g}/\text{ml}$) at $37^\circ\text{C}/5\% \text{CO}_2$ in 96 wells round-bottom plates in the presence of 0–300 nM DEX. After collection of

supernatants for cytokine analysis, cultures were pulsed after 72 h with 1 μ Ci/well [3 H]-thymidine (Amersham, Buckinghamshire, UK), and 16–18 h later, [3 H]-thymidine incorporation was measured using a liquid scintillation beta-counter.

2.6. Statistics

Analyses were performed using the Statistical Package for the Social Sciences version 12.0 (SPSS 12.0). All variables were tested for normality. Group differences in the normally distributed datasets were assessed by one-way ANOVA, Student's *t*-test, and non-continuous variables by Chi-square or Fisher's Exact test. Repeated measures analysis was performed to analyze the longitudinal patterns of self-reported complaints of non-fatigued and fatigued participants using time as a within-subjects factor and group as the between-subjects factor. Three-way ANOVA was conducted to explore group, dose and time point differences in DEX sensitivity. DEX dose and time point were used as within-subject variables and group as a between-subject variable. As a measure of total DEX inhibition area under the curve (AUC) of proliferation and cytokine production was calculated at all time points. In addition, for each time point the concentration of DEX was determined where 50% of its effect was observed (EC_{50}) as well as the percentage cytokine production or proliferation in the presence of the highest concentration DEX (300 nM) as a measure of maximal inhibition. AUC, EC_{50} and maximal inhibition were used in a repeated measures analysis as dependent variables. Time was used as a within-subjects factor. When sphericity was violated in the repeated measures analyses Greenhouse-Geisser correction was applied in all repeated measures analyses. In addition, in order to determine the within-subject stability of the immunological characteristics, Spearman's rank correlations were calculated between time points. To determine effect sizes, η^2 was calculated which represents the proportion of the total variance that is attributed to an effect and calculated as the ratio of the effect variance (SS_{effect}) to the total variance (SS_{total}), $\eta^2 = SS_{effect}/SS_{total}$.

3. Results

3.1. Demographics

Demographic characteristics and self-reported symptoms of the fatigued and non-fatigued group are presented in Table 1. Fatigued girls were slightly older (fatigued: 15.18 \pm 1.37 years and non-fatigued: 14.74 \pm 1.60, $t = 2.35$, $p < 0.05$). A higher percentage of the fatigued participants had experienced menarche ($\chi^2 = 4.81$, $p < 0.05$) and the average age at menarche was lower in the fatigued group ($t = 2.23$, $p < 0.05$). In the fatigued group 13.5% smoked on a regular basis while none of the non-fatigued girls smoked ($\chi^2 = 8.95$, $p < 0.05$). The fatigued group reported more fatigue- and illness-related school absenteeism in the past month than the non-fatigued group ($\chi^2 = 16.11$, $p < 0.01$). No differences were observed in BMI, medication use, use of oral contraceptives and self-reported allergies.

Table 1 Demographics of fatigued and non-fatigued group at T1.

	Non-fatigued	Fatigued
Age	14.74 (1.60)	15.18 (1.37)
Weight	52.94 (8.92)	54.71 (9.71)
Height	1.67 (0.07)	1.67 (0.07)
BMI	18.80 (2.38)	19.50 (2.80)
Tanner puberty stage		
Breasts		
Stage 1	0.0%	0.0%
Stage 2	16.9%	6.0%
Stage 3	30.5%	23.9%
Stage 4	39.0%	46.3%
Stage 5	13.6%	23.9%
Tanner puberty stage		
Pubic hair		
Stage 1	6.8%	1.5%
Stage 2	28.8%	13.4%
Stage 3	47.5%	58.2%
Stage 4	16.9%	26.9%
Menarche (Yes)	76.7%	91.0%
Age at menarche	12.52 (1.00)	12.07 (1.01)
Oral contraceptives	16.4%	19.4%
Self-reported allergic symptoms	29.3%	31.3%
Self-reported asthmatic symptoms	0.0%	6.0%
Smoking		
1–5	0.0%	6.0%
6–10	0.0%	0.0%
10–20	0.0%	7.5%
>20	0.0%	0.0%
Fatigue- and illness-related absenteeism (past month)		
0 classes	70.5%	39.4%
1–5 classes	14.8%	22.7%
6–10 classes	4.9%	9.1%
11–20 classes	8.2%	10.6%
>20 classes	1.6%	18.2%

Means (S.D.) and percentages presented.

3.2. Longitudinal course of self-reported symptoms

The longitudinal course of symptoms was assessed by self-reports at T1 and after 6 (T2) and 12 (T3) months and has been previously reported by our group (Ter Wolbeek et al., 2007b). In Table 2 questionnaire scores at T1, T2 and T3 are presented. In the fatigued group, the level of fatigue ($CI_{S_{total}}$) decreased somewhat after T1. In the non-fatigued group no obvious changes were observed (time effect: $F_{(2,228)} = 3.32$, $p < 0.05$, $\eta^2 = 0.03$; interaction effect: $F_{(2,228)} = 5.02$, $p < 0.01$, $\eta^2 = 0.04$). Group averages remained substantially different overall ($F_{(1,114)} = 333.62$,

$p < 0.001$, $\eta^2 = 0.75$). The same longitudinal pattern was seen for depression, anxiety, somatic and autonomic symptoms and sleep quality with a small decrease in complaints in the fatigued group, especially between T1 and T2, and a stable pattern in the non-fatigued participants. No longitudinal changes were observed with respect to the number of self-reported CFS-related symptoms in the fatigued girls, while their non-fatigued counterparts reported a little more symptoms at T3 (time effect: $F_{(2,225)} = 0.27$, $p = 0.77$, $\eta^2 = 0.002$; interaction effect: $F_{(2,225)} = 3.43$, $p < 0.05$, $\eta^2 = 0.03$; group effect: $F_{(1,120)} = 147.42$, $p < 0.001$, $\eta^2 = 0.51$).

3.3. Longitudinal analysis of cortisol and glucocorticoid receptor sensitivity

3.3.1. Cortisol in plasma

Cortisol and immunological data were analyzed of 65 fatigued and 60 non-fatigued participants at T1, of 58 and 59 participants at T2 and 60 and 59 participants at T3, respectively. As shown in Figure 1, higher levels of plasma cortisol were present at all time points in fatigued participants compared to non-fatigued participants (group effect: $F_{(1,115)} = 4.42$, $p < 0.05$, $\eta^2 = 0.04$; time effect: $F_{(2,228)} = 0.03$, $p = 0.97$, $\eta^2 = 0.00$).

Table 2 Self-reported complaints of fatigued and non-fatigued participants at T1, T2 and T3.

	Non-fatigued	Fatigued
Fatigue total score (CIS)		
T1	36.14 (12.37)	85.19 (15.81)
T2	36.02 (11.41)	77.97 (18.19)
T3	38.95 (15.11)	79.52 (19.75)
Depression (BDI)		
T1	2.48 (2.65)	12.37 (7.48)
T2	2.54 (2.95)	9.18 (8.47)
T3	2.18 (2.90)	8.77 (8.32)
Anxiety (STAIC)		
T1	26.60 (4.00)	38.13 (6.66)
T2	26.45 (5.07)	35.49 (7.84)
T3	26.20 (5.06)	34.46 (8.02)
Somatic symptoms (MSPQ)		
T1	8.72 (6.58)	22.43 (11.07)
T2	8.11 (6.75)	19.62 (11.75)
T3	9.49 (7.07)	19.18 (11.88)
Sleep quality in general (GSKS)		
T1	2.02 (2.20)	7.75 (2.61)
T2	1.62 (1.94)	6.60 (3.03)
T3	1.89 (2.33)	6.31 (3.58)
Number of CFS-related symptoms		
T1	1.11 (1.00)	3.52 (1.31)
T2	1.23 (1.28)	3.48 (1.39)
T3	1.56 (1.20)	3.28 (1.57)

Means (S.D.) presented.

Since hypersecretion of cortisol is usually observed in depressed patients and hyposcretion in CFS patients (Pariante and Miller, 2001; Cleare, 2003), we corrected the results for BDI depression scores. This resulted in an even more significant difference of cortisol in fatigued participants compared to non-fatigued participants (group effect: $F_{(1,114)} = 10.35$, $p < 0.01$, $\eta^2 = 0.08$; time effect: $F_{(2,226)} = 1.08$, $p = 0.34$, $\eta^2 = 0.01$). In neither the fatigued nor the non-fatigued group cortisol significantly correlated with fatigued scores. Furthermore, no significant correlations were observed after controlling for comorbid symptoms of depression and anxiety or sleep quality. Since the proportion of smokers differed between groups, we also corrected our results for smoking. Smoking did not influence the results at T1 and T3. At T2 the group difference became non-significant after correction for smoking ($F = 2.24$, $p = 0.14$). A direct comparison of smokers and non-smokers within the fatigued and non-fatigued groups did not show significant differences with respect to cortisol levels.

3.3.2. DEX regulation of cytokine production by monocytes

The sensitivity of monocytes to GC regulation was tested in whole blood cultures by adding DEX and examining the regulation of LPS-induced TNF- α and IL-10 release as a monocyte function. Three-way ANOVA with time point and DEX dose as within-subject variables showed that increasing amounts of DEX in the culture resulted in dose-dependent inhibition of TNF- α and biphasic modulation of IL-10 (TNF- α and IL-10 $p < 0.001$). Modulation of TNF- α and IL-10 production by DEX differed between time points but did not differ between fatigued and non-fatigued participants (TNF- α group effect: $F_{(1,106)} = 0.09$, $p = 0.77$, $\eta^2 = 0.00$; time point effect: $F_{(2,198)} = 12.15$, $p < 0.001$, $\eta^2 = 0.10$; IL-10 group effect: $F_{(1,107)} = 0.66$, $p = 0.42$, $\eta^2 = 0.01$; time point effect: $F_{(2,189)} = 2.63$, $p = 0.08$, $\eta^2 = 0.02$). For TNF- α inhibition by DEX the AUC, EC₅₀, and maximal inhibition

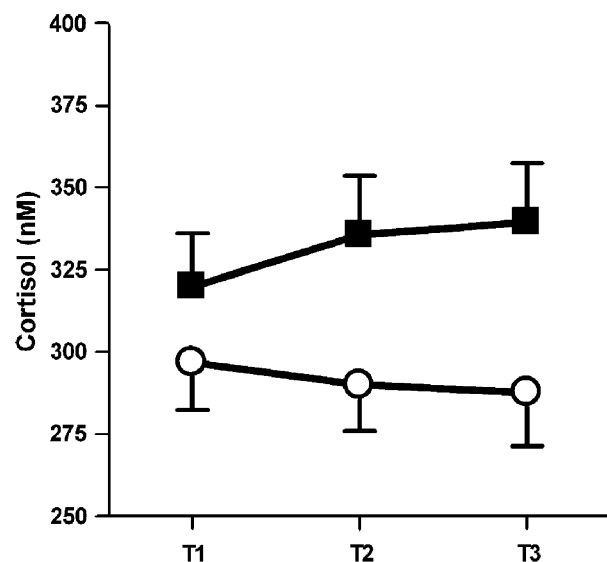


Figure 1 Longitudinal patterns of cortisol production measured in plasma. (-■-) represents samples from the fatigued group and (-○-) represents those from the non-fatigued group. Means and S.E.M.s presented.

were calculated. Figure 2 presents the longitudinal patterns of these parameters. Fatigued and non-fatigued participants did not differ in GR sensitivity of monocytes for DEX (group effect AUC: $F_{(1,106)} = 0.09$, $p = 0.77$, $\eta^2 = 0.00$; group effect EC_{50} : $F_{(1,100)} = 0.28$, $p = 0.60$, $\eta^2 = 0.00$; group effect maximal inhibition: $F_{(1,106)} = 0.46$, $p = 0.50$, $\eta^2 = 0.00$). No significant interaction effects were observed. Inhibition of $TNF-\alpha$ production by DEX was most pronounced at T1 and least at T3 (time effect AUC: $F_{(2,198)} = 12.15$, $p < 0.001$, $\eta^2 = 0.10$; time effect EC_{50} : $F_{(2,178)} = 11.22$, $p < 0.001$, $\eta^2 = 0.10$; time effect maximal inhibition: $F_{(2,196)} = 1.98$, $p = 0.14$, $\eta^2 = 0.02$).

The DEX effect on monocyte IL-10 production is biphasic (de Kloet et al., 2007). Low dose DEX has a stimulating effect on IL-10 secretion, whereas the higher doses inhibit the release. This characteristic of the IL-10 curve does not allow calculation of AUC or EC_{50} . ANOVAs per time point showed that at T1 and T3 the IL-10 curves did not differ between fatigued and non-fatigued participants (group effect T1: $F_{(1,122)} = 0.76$, $p = 0.38$, $\eta^2 = 0.01$; group effect T3: $F_{(1,110)} = 0.23$, $p = 0.64$, $\eta^2 = 0.00$) (Figure 3). At T2, the stimulating effect of DEX was less pronounced in the fatigued group (group effect T2: $F_{(1,116)} = 5.30$, $p < 0.05$, $\eta^2 = 0.04$).

To test whether levels of endogenous cortisol influenced GR sensitivity, the $TNF-\alpha$ and IL-10 dose-response curves were analyzed per time point with cortisol as covariate. This

did not change the results, suggesting that endogenous GC did not mask possible group differences in DEX modulation of cytokine production by monocytes. In addition, fatigue scores did not significantly correlate with any of the monocytes GR sensitivity measures at any of the time points.

3.3.3. DEX inhibition of T-cell mitogen-induced responses

Increasing amounts of DEX in the culture resulted in dose-dependent inhibition of T-lymphocyte proliferation and of IL-10 and $IFN-\gamma$ production after PHA stimulation (all $p < 0.001$). Inhibition of T-lymphocyte proliferation, and IL-10 and $IFN-\gamma$ secretion differed between time points but not between fatigued and non-fatigued participants (T-lymphocyte proliferation group effect: $F_{(1,102)} = 0.69$, $p = 0.41$, $\eta^2 = 0.01$; time point effect: $F_{(2,197)} = 28.05$, $p < 0.001$, $\eta^2 = 0.22$; IL-10 group effect: $F_{(1,94)} = 0.32$, $p = 0.32$, $\eta^2 = 0.01$; time point effect: $F_{(2,169)} = 17.93$, $p < 0.001$, $\eta^2 = 0.16$; $IFN-\gamma$ group effect: $F_{(1,98)} = 1.30$, $p = 0.26$, $\eta^2 = 0.01$; time point effect: $F_{(2,190)} = 15.43$, $p < 0.001$, $\eta^2 = 0.14$).

Figure 4 shows the longitudinal patterns of the AUCs of DEX dose-dependent cytokine production and proliferation after PHA stimulation. Fatigued and non-fatigued participants differed on none of the read outs. Inhibition of T-cell mitogen-induced $IFN-\gamma$ and IL-10 production and proliferation showed comparable fluctuating patterns with enhanced

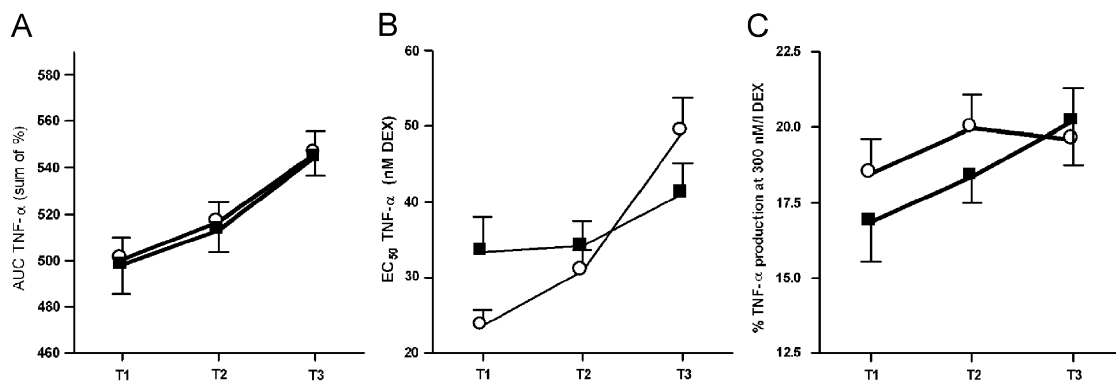


Figure 2 Longitudinal patterns of inhibition of monocyte $TNF-\alpha$: (A) area under the curve (AUC); (B) EC_{50} ; (C) maximal inhibition. (-■-) represents samples from the fatigued group and (-○-) represents samples from the non-fatigued group. Means and S.E.M.s presented.

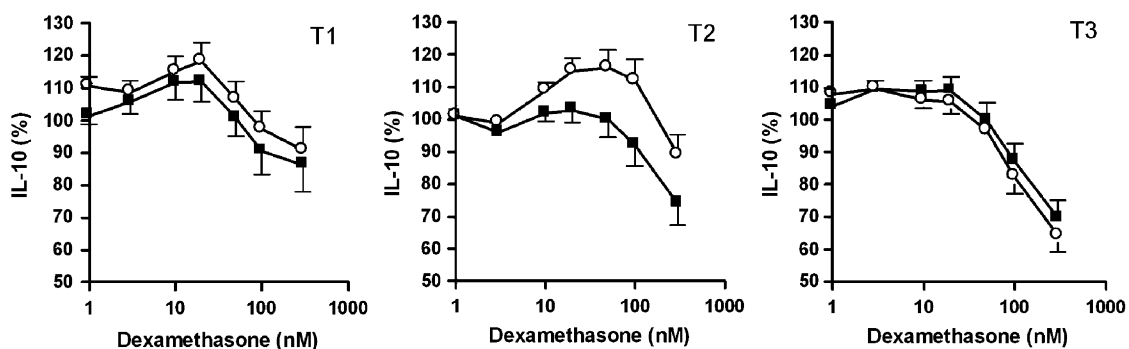


Figure 3 Modulation of IL-10 by DEX in monocytes from fatigued (-■-) and non-fatigued (-○-) participants at T1, T2 and T3. Means and S.E.M.s presented.

inhibition at T2 compared to T1 and T3, indicating enhanced GR sensitivity in autumn compared to spring. This pattern was also seen for the EC₅₀ and maximal inhibition at 300 nM of DEX. No significant interaction effects were observed. Correcting the data for cortisol levels did not alter the results. Within the fatigued and non-fatigued group fatigue scores did not correlate significantly with DEX inhibition of T-cell mitogen-induced responses.

3.3.4. Within-subject stability of GR sensitivity

Inhibition by DEX of PHA-induced proliferation and cytokine production showed seasonal variation in GR sensitivity to DEX (Figure 4). With rank correlation analysis we tested whether, despite this variation, participants' relative position within the group would show stability across time points. With intervals of 6 months, stability of inhibition of LPS-induced TNF- α by DEX showed a mixed picture with low correlations between time points of AUC and EC₅₀ and significant correlations between maximal inhibition at T1, T2 and T3 (Table 3). DEX inhibition of the PHA-induced responses (proliferation, IL-10 and IFN- γ) was fairly stable across time points.

3.3.5. DEX sensitivity and persistent fatigue

A persistently fatigued subgroup ($N = 26$) of the severely fatigued group, who reported high levels of fatigue (CIS_{fatigue severity} ≥ 35) across all time points was compared to the non-fatigued group ($N = 59$) (ter Wolbeek et al., in press). At the initial measurement (T1) more persistently fatigued (PF) than non-fatigued participants had experienced the menarche (PF: 100%; $\chi^2 = 8.46$, $p < 0.01$) and smoked (PF: 11.5% 1–5 cigarettes, 11.5% 10–20 cigarettes; $\chi^2 = 13.19$, $p < 0.01$) (for results non-fatigued group see Tables 1 and 2). School absenteeism was higher among the persistently fatigued group (PF: 30.7% missed more than 20 classes a month; $\chi^2 = 19.89$, $p < 0.01$). Groups did not differ with respect to age, age at menarche, BMI, and medication and oral contraceptives use. As was true for the whole fatigued group, the persistently fatigued group had higher questionnaire scores for depression, anxiety, somatic symptoms, and CFS-related symptoms and reduced sleep quality as compared to the non-fatigued group (all $p < 0.001$). Persistently fatigued participants and the remaining fatigued participants (CIS_{fatigue severity} < 35 at one or more time points) differed with respect to symptom reports

at T1: fatigue (PF: 90.7 ± 16.45), depression (PF: 16.04 ± 7.15) and anxiety (PF: 39.31 ± 6.14) scores were all higher in the persistently fatigued group (all $p < 0.001$).

Cortisol levels and sensitivity of monocytes to DEX did not differ between persistently fatigued and non-fatigued participants, nor between persistently fatigued participants and the remaining part of the fatigued group (all $p > 0.05$). With respect to the T-cell mitogen-induced responses, DEX inhibition of IFN- γ production was reduced in the persistently fatigued group (group effect AUC: $F_{(1,59)} = 4.08$, $p < 0.05$, $\eta^2 = 0.07$). Also, inhibition of T-lymphocyte proliferation by DEX tended to be less pronounced in

Table 3 Rank correlations (r) between time points (T1, T2 and T3) of all participants together (fatigued and non-fatigued) of the area under the curve (AUC), EC₅₀ and maximal inhibition of DEX modulated TNF- α production by LPS-stimulated monocytes and PHA-induced proliferation and IL-10 and IFN- γ production.

	T1–T2	T1–T3	T2–T3
TNF- α (LPS-induced)			
AUC	0.19*	-0.12	0.10
EC ₅₀	0.18	-0.22	-0.07
Maximal inhibition	0.33***	0.20*	0.33***
Proliferation (PHA-induced)			
AUC	0.61***	0.66***	0.51***
EC ₅₀	0.52***	0.54***	0.40***
Maximal inhibition	0.46***	0.48***	0.20*
IL-10 (PHA-induced)			
AUC	0.44***	0.40***	0.40***
EC ₅₀	0.32***	0.40***	0.24*
Maximal inhibition	0.40***	0.37***	0.56***
IFN- γ (PHA-induced)			
AUC	0.30**	0.46***	0.34***
EC ₅₀	0.30**	0.40***	0.23*
Maximal inhibition	0.42***	0.34***	0.18

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

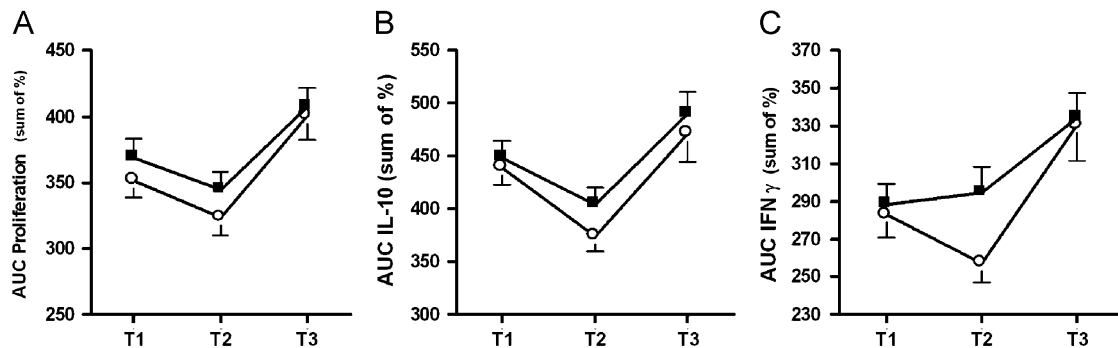


Figure 4 Longitudinal patterns of inhibition (area under the curve, AUC) of PHA-induced proliferation (A), IL-10 (B), and IFN- γ (C). (-■-) represents samples from the fatigued group and (-○-) represents those from the non-fatigued group. Means and S.E.M.s presented.

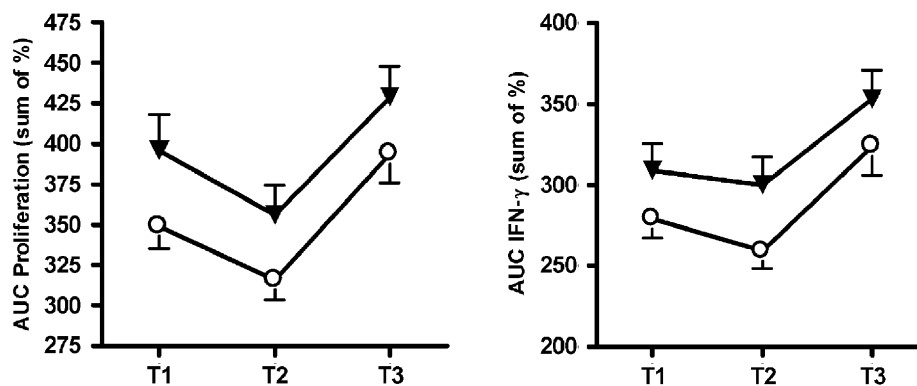


Figure 5 Longitudinal patterns of inhibition (area under the curve) of PHA-induced proliferation (left) and IFN- γ (right) production. (-▼-) represents samples from the persistently fatigued group and (-○-) represents those from the non-fatigued group. Means and S.E.M.s presented.

persistently fatigued participants as compared to non-fatigued individuals (group effect AUC: $F_{(1,65)} = 3.94$, $p = 0.05$, $\eta^2 = 0.06$) (Figure 5). The persistently fatigued group and the temporarily fatigued group did not differ in read outs for DEX sensitivity.

4. Discussion

In the present study we investigated whether severely fatigued adolescent girls, with high resemblance of symptomatology to patients with CFS, would show alterations in plasma cortisol level and GR sensitivity of LPS-induced and T-cell mitogen-induced responses as compared to non-fatigued adolescents. Higher levels of cortisol were present in the fatigued group. GR sensitivity of T-cell mitogen-induced responses was fairly stable across time points, though in both groups sensitivity to DEX was higher in autumn than in spring. Fatigued and non-fatigued participants did not differ in sensitivity to DEX. However, in the subgroup of persistently fatigued girls we observed reduced GR sensitivity on the level of PHA-induced IFN- γ production.

The longitudinal design of our study enabled us to examine the stability of immunological profiles in the participants as recommended by Segerstrom et al. (2006). In line with our previous report on the stability of cytokine production and leukocyte cell subsets (ter Wolbeek et al., 2007b), in the same group of participants we showed long-term consistency of the participants' relative position compared to the rest of the group with respect to GR sensitivity of T-cell mitogen-induced responses. To our knowledge, we are the first to demonstrate that GR function is also relatively stable over a long period of time. However, the magnitude of stability of GR sensitivity was smaller than of cytokine production and cell subsets (ter Wolbeek et al., 2007b). We showed increased DEX sensitivity in samples drawn at T2 compared to T1 and T3. We are confident that these differences were not caused by differences in methodology or storage since participants were recruited in two 'waves' with a 2-year interval and seasonal effects were observed in both experimental series. Thus, there is no reason to assume that differences in personnel or storage

duration account for the fluctuations and all stimulants came from the same batch. Therefore, we conclude that the observed differences are due to seasonal variation. We suggest that in future research in humans, multiple measurements of (neuro)-immune parameters are necessary to increase reliability. In any case, taking into account the time of year of the assessment is important. In contrast to our finding of stability of GR sensitivity of PHA-induced proliferation and cytokine production, DEX inhibition of TNF- α production by monocytes was variable across time points. In line with this observation, group differences in DEX modulation of monocyte IL-10 production differed between time points. DeRijk et al. (1996) already showed that monocyte sensitivity to DEX is dynamic and is rapidly affected by physical activity. We reckon that the latter measure therefore is not suitable for establishing immune traits, but rather reflects the actual situation. These data are in line with the fact that the innate immune response does not reflect an immune trait, but is more reactive to the day-to-day threats of the environment, whereas the adaptive T-lymphocyte response is a more stable phenomenon (Roitt et al., 2006).

The observation of higher cortisol levels in the severely fatigued group contrasted with our expectations. Previously we showed that the cortisol response to awakening, as determined in saliva samples, did not differ between fatigued and non-fatigued participants (ter Wolbeek et al., 2007a). If anything, we expected to find indications for hypocortisolism in line with the majority of previous findings in adult CFS patients (Cleare, 2003). Hypercortisolism has been consistently found in depressive disorders and is related to a reduced negative feedback sensitivity of the HPA-axis (Pariante and Miller, 2001). However, in a CFS patient group in which participants with comorbid depression were excluded, high levels of saliva cortisol levels were observed as well (Wood et al., 1998). To get insight in the influence of depressive comorbidity in the analyses of cortisol production, we corrected our analyses for BDI scores and observed an even stronger difference in cortisol levels between fatigued and non-fatigued participants, suggesting that the cortisol elevation in our fatigued participants cannot be attributed to higher levels of depressive symptoms. We previously reported that severely

fatigued girls experienced more negative life events (ter Wolbeek et al., 2007a). Possibly exposure to these stressful events has contributed to the observed alteration in HPA-axis output. It should be noted, however, that measurement of cortisol at one specific time point in the diurnal cycle does not adequately reflect HPA-axis activity. Although at T2 there appeared to be a contribution of the stimulating effect of smoking, the fact that we observed a consistent difference over a period of 1 year despite using a single measurement at each time point nevertheless suggests that these differences are real. It should also be mentioned that in saliva free cortisol is measured whereas in plasma cortisol levels reflect total cortisol, which includes the fraction bound to cortisol binding globulin, which could be the basis of the differences in our findings.

We separately analyzed data of a persistently fatigued subgroup and observed no differences in cortisol between non-fatigued and persistently fatigued participants. This is likely due to loss of power since the persistently fatigued participants and the remaining part of the fatigued group also did not differ with respect to cortisol levels. In addition, we hypothesize that the levels of the persistently fatigued group may gradually decrease and that along with further dysregulation and possible de-conditioning this group eventually will be characterized by hypocortisolism, just as in adult CFS patients (Cleare, 2003). Interestingly, our data indicated that persistently fatigued individuals have alterations in GR sensitivity on the level of PHA-induced cytokine production and tended to be different on the level of proliferation. In line with findings in an adolescent CFS patient group and in PTSD patients (Kavelaars et al., 2000; de Kloet et al., 2007), participants with persistent fatigue had reduced DEX sensitivity of T-lymphocytes as compared to non-fatigued participants. The fact that the persistently fatigued group did not differ from the transiently fatigued group may be related to the small group sizes and therefore again may be a matter of reduced power. GC resistance in persistently fatigued girls does not seem to be associated with the duration of the complaints, since altered sensitivity was already present at T1. Our results suggest that the persistently fatigued participants may form a clinically important subgroup which is at higher risk for development of physiological dysfunction and perhaps more severe symptoms of fatigue and comorbidities. In our future studies, we will try follow up this group to observe whether these girls are more prone to develop CFS.

What may be the origin of reduced DEX sensitivity as observed in the persistently fatigued subgroup? Immune system activation is controlled by GCs that bind to GRs in immune cells and via intracellular processes that inhibit production of cytokines. Insufficient GC signaling can be a consequence of low levels of GCs or decreased receptor-mediated signal transduction (Raison and Miller, 2003; Lewis-Tuffin and Cidlowski, 2006). The latter may either result from downregulation of the number of receptors, altered receptor affinity, or reduced intracellular effects of agonist-occupied receptors. Physical or psychological stress can contribute to GC resistance of immune cells since excessive or enduring HPA-axis activation can desensitize receptors and/or downregulate the number of receptors (Raison and Miller, 2003; Yehuda et al., 2004; Lewis-Tuffin

and Cidlowski, 2006). If this process was involved, one would expect to find associations between current cortisol levels and GC sensitivity of cells. However, correcting the sensitivity measures for plasma cortisol levels did not affect our results. Although in our sample cortisol levels did not differ between persistently fatigued participants and controls, and therefore could not explain altered GR sensitivity, one can speculate that HPA-axis over-activation may possibly have occurred in the history of these girls, which may have resulted in the observed reduction in DEX sensitivity of monocytes.

Since reduced GC sensitivity of target tissue has previously been shown in depressive patients (Pariente and Miller, 2001; Raison and Miller, 2003), this may imply that our persistently fatigued group is more similar to depressive patients than to CFS patients. On the other hand, Kavelaars et al. (2000) excluded CFS patients with high depression scores from their study and also observed reduced DEX sensitivity. Furthermore, de Kloet et al. (2007) did not observe DEX sensitivity differences between PTSD patients with or without comorbid depression. Moreover, in our persistently fatigued participants no elevation in cortisol levels was observed, which is generally considered to be a characteristic of depression (Holsboer, 2001). Although we used self-reports to identify participants with psychiatric or somatic illnesses and therefore do not know whether participants fulfill the definition of major depression or PTSD and cannot completely rule out the possibility that we accidentally included diseased participants, we are dealing with a normal school population and not with a patient group, and therefore we feel that the relative contribution of diseased participants will be small.

In conclusion, severely fatigued participants reported significantly more CFS-related symptoms, depression, anxiety, somatic symptoms and reduced sleep quality than non-fatigued participants and their complaints resembled those of CFS patients. Fatigued participants had higher cortisol levels but the expected GR resistance could not be demonstrated. The longitudinal stability of GR sensitivity of T-cell mitogen-induced responses (but not monocyte sensitivity) strengthens the conclusion that this function in severely fatigued healthy adolescents is not altered. Only in the subgroup of persistently fatigued participants GR sensitivity of PHA-induced responses was reduced, which paralleled the results found previously by our group in adolescent CFS patients and PTSD patients. The significance of immune dysregulation associated with persistent fatigue during adolescence as a possible risk factor for development of CFS in later life should be further elucidated in a larger sample and in a follow-up study of the present sample.

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Conflict of interest

None declared.

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