# Chapter 9

# Lymphocyte glucocorticoid receptor resistance and depressive symptoms severity: a preliminary report

M.A.C. Tanke, F.J. Bosker, A.V. Gladkevich, H.M. Medema,
J.A. den Boer, J. Korf

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## ABSTRACT

***Objective:*** *Assessment of the temporal interrelationship of neuropsychiatric parameters requires technologies allowing frequent biological measurements. We propose glucocorticoid receptor (GR) function of lymphocytes to assess the temporal relationship between glucocortiocoid resistance and the course of major depressive disorder.*

***Method:*** *Dexamethasone suppression of lymphocyte proliferation was in vitro assessed via 5-bromo-2′ deoxyuridine (BrdU) incorporation in DNA. Optimal conditions were determined using blood of healthy volunteers. Thereafter the relation between depression severity (Hamilton Depression Rating Scale, HDRS, scores), lymphocyte proliferation and morning cortisol levels in blood was studied in thirteen depressed patients, mostly with a history of treatment resistance.*

***Results:*** *Recovery from depression was not directly associated with changes in lymphocyte glucocorticoid resistance. However, a negative correlation was observed between HDRS and BrdU incorporation and a positive correlation between morning cortisol and BrdU incorporation. No significant correlation was found between cortisol and HDRS. A regression analyses showed that HDRS was related to both suppression of BrdU incorporation ( $\beta$ -0.508, $p < 0.001$) and cortisol levels ( $\beta$ 0.364, $p = 0.001$) in a highly significant model ($F_{2,60}$=14,244, $p < 0.001$) Except for one case, such relation could not be found within patients.*

***Conclusion:*** *Our preliminary results suggest a mutual relation between lymphocyte GR function, morning cortisol levels and MDD symptom severity. A direct relation between glucocorticoids resistance and recovery may not exist, but glucocorticoid resistance might attenuate or prevent recovery. It is clear that additional studies using larger and more homogenous groups of MDD patients are required to support our findings.*

## INTRODUCTION

One of the most consistently replicated neurobiological findings in major depressive disorder (MDD) is a disturbed function of the hypothalamic-pituitary-adrenal (HPA)-axis (26). Such HPA-axis dysfunction is believed to be connected with inadequate feedback regulation at the level of adrenocorticotrophic hormone (ACTH) and of cortisol (36). Thus, a considerable percentage of depressed patients appear to be non-suppressors of cortisol after administration of dexamethasone (DEX) alone or in combination with corticotrophin releasing hormone CRH (DEX-CRH) (348;349). Using the DEX-CRH test divergent treatment modalities, including selective serotonin reuptake inhibitors (SSRIs) (350) and electroconvulsive therapy (351) appear to diminish HPA-axis dysregulation (352-354). Arguably, restoration of HPA-axis functioning is necessary for full remission (26;355). Indeed, normalization of HPA-axis function may predict response to antidepressant treatment (356). Conversely, persisting non-suppression in the DEX/CRH test constitutes an enhanced risk for relapse or recurrence (40).

The clinical usefulness of the DEX/CRH challenge is however limited by two factors, first it is labour intensive (at least from a clinical point of view) and second CRH is rather expensive. Non-suppression of cortisol following DEX or DEX/CRH administration is accompanied with a decreased GR density and function of blood lymphocytes (357). Because such changes might also connect with the blunted mitogen-induced lymphocyte proliferation (358) regularly observed in MDD (359), an *in vitro* lymphocyte proliferation test might offer a viable alternative for the DEX or DEX/CRH challenge.

The lymphocyte proliferation test measures the increase of DNA synthesis following the exposure of lymphocytes to a mitogen. The conventional technique is based on the incorporation of [$^3$H]-thymidine into DNA. However, the pyrimidine analogue bromodeoxyuridine (BrdU) is also incorporated into DNA and can be measured via an immuno-enzymatic/ fluorimetric analysis (360) thereby avoiding the problems normally associated with radio-active assays.

The present study was aimed to address the following questions. First, is the BrdU based lymphocyte proliferation test sensitive to varying concentrations of dexamethasone? Second, is it capable of measuring glucocorticoid resistance in depressed patients? To this end we have investigated the effect of varying dexamethasone concentrations on lymphocyte proliferation *in vitro*, as assessed via BrdU uptake. Next, we have measured morning cortisol levels in blood and GR function by inhibiting BrdU incorporation in proliferating lymphocytes in the presence of dexamethasone in a pilot study involving 13 depressed patients who were followed weekly during the course of treatment.

## MATERIALS AND METHODS

The study was approved by the medical ethical review board of the UMCG. After full explanation of the study, written informed consent was obtained from all subjects

### Dose-effect relation of dexamethasone and BrdU incorporation in vitro

First, optimal conditions for the dexamethasone suppression test were determined in blood of 12 healthy volunteers. Blood was obtained by veni puncture and collected in heparinized glass tubes, which were immediately transported to the laboratory and processed. Fresh heparinized blood was diluted 1:1 in sterile phosphate buffered saline (PBS, pharmacy UMCG), layered on lymfoprep (Lucron Bioproducts B.V., the Netherlands) and centrifuged at 1000 x *g* for 20 minutes. Peripheral Blood Monocytes (PBMC) were recovered from the interface and washed in sterile PBS by centrifugation at 550 x *g* for 10 minutes and then washed with RPMI 1640 (Bio Whittaker, Belgium).by centrifugation at 250 x *g* for 5 minutes. Pellets were resuspended in complete medium: RPMI 1640 supplemented with 1% Pen-Strep (Bio Whittaker) and 10% fetal calf serum (Greiner Hyclone). Cell suspension was diluted with complete media to a final concentration of $4 \times 10^5$ cells/ml ($2 \times 10^4$ cells/well), the optimal concentration for further analyses. The cells were successively cultured in triplicate for 3 days in 96-well plates in the presence of 20 μg/ml mitogen phytohemaglutinin, ensuring maximal stimulation, and increasing concentrations of dexamethasone (0, 0.02, 0.05 and 0.1 μg/ml). BrdU incorporation was measured using the Biotrak ™ cell proliferation ELISA system version 2 (Amersham Bioscience). Briefly, BrdU labeling reagent (final concentration, 10 μM) was added after 48 h of culture. At 72 h, the cells were harvested following the protocol as described by Amersham Biosciences. Optical density was determined at 450 nm using an ELISA reader (Organon Teknika, The Netherlands). Culture medium alone and cells incubated with peroxidase-labeled anti-BrdU in the absence of BrdU were used as controls for non-specific accumulation. The overall analysis intra-assay variability (CV) of dexamethason suppression of BrdU incorporation was 10%.

### Patient characteristics

Thereafter a pilot study in patients was conducted. In this study, thirteen hospitalised depressed patients, eight females (mean age$\pm$ SEM: 47.0$\pm$1.9 yr) and five males (mean age$\pm$ SEM: 49.4$\pm$4.4 yr), at the department of the University Center of Psychiatry (UCP), Groningen, many with a history of treatment resistance, were followed during the course of treatment. Inclusion was according to DSM-IV criteria for MDD, as confirmed by a clinical psychiatric interview with a minimum score of 15 points on the 17-item Hamilton Depression Rating Scale (HDRS). Clinical diagnosis were as follows: one patient (8%) suffered from depression with atypical features, three patients (23%) met the criteria for the melancholic subtype. Two patients (15%) had a MDD episode with psychotic features and

two other patients (15%) a depressive episode within a bipolar disorder. The remaining five patients (38%) suffered from a MDD, which could not otherwise be specified.

### Treatment

All of the patients, except for one, received antidepressant medication ranging from citalopram to tranylcypromine, venlafaxine and imipramine. The majority of patients also received co-medication, mostly anxiolytics such as diazepam, lorazepam, oxazepam and temazepam, but occasionally also typical antipsychotics such as domperidone and pimozide, atypical antipsychotics such as quetiapine and risperidone. In addition to pharmacological treatment patients also underwent cognitive behavioral therapy or electro-convulsive shock therapy.

### Assessments

For logistic reasons only inpatients at the University Center of Psychiatry participated in the study for a maximal period of 16 weeks. Blood sampling for determination of cortisol levels (in EDTA tubes) and lymphocyte proliferation tests (in heparinized tubes) was done weekly between 09.00 a.m. and 09.15 a.m. PBMC (mainly lymphocytes) were isolated and successively cultured in triplicate for 3 days in 96-well plates in the presence of the mitogen phytohemaglutinin (20 μg/ml) and 0.01 μg/ml of dexamethasone, we have chosen a lower concentration to minimize the risk of dexamethason-induced cell death. For further details see first section of materials and methods. Cortisol was determined with a radioimmunoassay (361;362)

Patients were interviewed (HDRS) within four hours after blood sampling. These interviews were assessed by trained research assistants. After the study, these assistant also helped to verify the current and past (pharmacological) treatments.

### Analysis plan

The original idea was to correlate the data of the lymphocyte proliferation tests with remission from depression. However, only two out of 13 patients remitted from depression, as defined by a HDRS score <8 for at least 2 weeks, not allowing to make definitive conclusions regarding a relation between remission and dexamethasone suppression of BrdU incorporation. It was therefore decided to focus on symptom severity according to the HDRS. First, we assessed the correlation between the pooled HDRS scores, BrdU incorporation percentages and plasma cortisol from all patients. Running a logistic regression analysis, we investigated to which extent the combination of BrdU incorporation and morning cortisol correlate with HDRS scores. Finally, we compared the BrdU data at HDRS values $\leq$ 8 with those at HDRS values $\geq$ 20 and looked at the correlation within each patient.

## RESULTS

Dexamethasone suppression was first tested *in vitro* with PBMC from healthy volunteers. At 20,000 cells/well (400,000 cells/ml) after proliferation a clear concentration effect relation was observed ($EC_{50}$ ~ 0.06 µg/ml of dexamethasone, see figure 1).

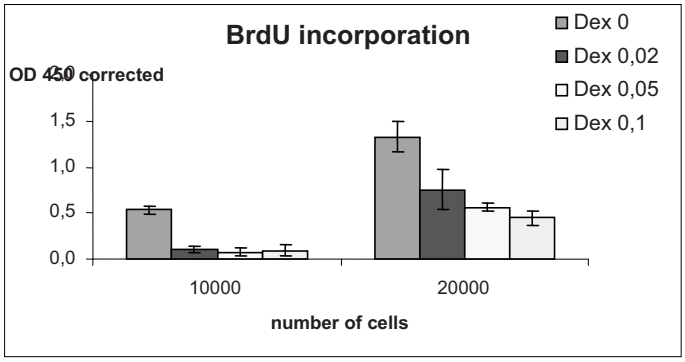


**Figure 1:** Dose effect relation of dexamethasone (µg/ml) and BrdU incorporation *in vitro* measured as optical density at 450 nm, corrected for the non-specific accumulation; data as mean ± SEM, n = 12.

To minimize the risk of premature cell death, we have used an even lower concentration of 0.01 µg/ml of dexamethasone in the patient study. We focused on MDD symptoms severity according to the HDRS. Statistical analysis showed moderate but highly significant Pearson correlations between HDRS and BrdU incorporation of -0.318 (p=0.004) and between cortisol and BrdU incorporation of 0.352 (p=0.005), but no significant correlations were found between cortisol and HDRS (0.185, p=0.154). A regression analysis showed that HDRS was related to both suppression of BrdU incorporation and cortisol levels explaining 30% of the variance ($R^2$ 0.329, adjusted $R^2$ 0.306) in a highly significant model ($F_{2,60}$=14,244, p < 0.001; table 1)

**Table 1** Regression model

| Dependent variable | Predictors | *B* (SE) | *β* | Sig. |
|---|---|---|---|---|
| HDRS | Constant | 14.6 (3.1) | | < 0.001 |
| | Cortisol levels | 0.021 (0.007) | 0.364 | 0.004 |
| | Suppression % of BrdU incorporation | − 0.153 (0.036) | − 0.508 | < 0.001 |

A regression model explaining Hamilton score (HDRS) by morning cortisol levels in blood and percent suppression of BrdU incorporation in lymphocytes Model: $R^2$ = 0.329; Adjusted $R^2$ = 0.0.306 Model statistics: F(2,60)=14,244, p< 0.001SE = Standard Error

Thereafter we categorized symptom severities into two groups. ANOVA indicated an almost significant difference (F=3,068; p=0.052), while the Bonferroni post-hoc test indicated that at HDRS values ≤ 8 (n=12) the mean suppression of lymphocyte proliferation (59.3%) tended

(p=0.054) to be more than suppression in the group with values ≥ 20 (43,7%, n=27). As with the DEX or DEX/CRH test there is a glucocorticoid resistance in lymphocytes in the severely depressed condition that is apparently irrespective of the type of therapeutic intervention.

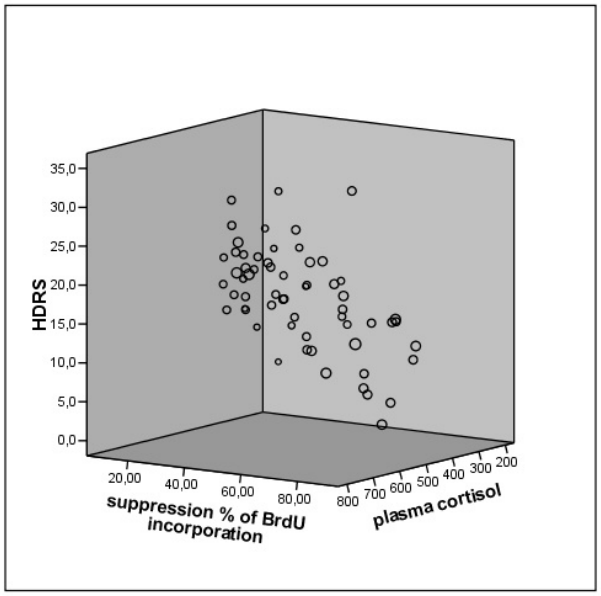


**Figure 2:** Correlation of the pooled data of the Hamilton Depression Rating Scale (HDRS), morning cortisol levels and percent suppression of BrdU incorporation in lymphocytes

Finally we followed individual GR function in 8 patients. Table 2 shows mean HDRS scores and corresponding mean BrdU suppression of these patients and the lacking correlation in the majority of patients.

**Table 2**:.Characteristics of 8 individual patients

| | r | n | HDRS | | %BrdU suppression | |
|---|---|---|---|---|---|---|
| Pat 1 | +0.09 | 11 | 8 ± 3 | (4-13)[#] | 54 ± 9 | (37-68) |
| Pat 2 | +0.03 | 9 | 11 ± 6 | (3-20) | 79 ± 9 | (63-90) |
| Pat 3 | +0.15 | 9 | 15 ± 5 | (6-22) | 34 ± 13 | (19-62) |
| Pat 4 | -0.73* | 8 | 15 ± 4 | (10-21) | 54 ± 19 | (22-80) |
| Pat 5 | +0.02 | 5 | 15 ± 4 | (12-22) | 36 ± 20 | (14-69) |
| Pat 6 | +0.14 | 9 | 19 ± 6 | (11-26) | 65 ± 11 | (42-78) |
| Pat 7 | -0.12 | 9 | 20 ± 3 | (17-26) | 30 ± 8 | (19-45) |
| Pat 8 | +0.45 | 6 | 27 ± 3 | (23-31) | 36 ± 15 | (23-60) |

Correlation between HDRS score and BrdU suppression and mean HDRS and BrdU values per patient **r** = correlation between HDRS and BrdU suppression within each patient. **n** = number of observations per patient; 5 female patients were excluded because the number of observations was considered to be too low (n<5). Data are expressed as mean ± SD and range. * p=0.01; [#] HDRS > 15 at inclusion

## DISCUSSION

The present study shows that dexamethasone dose-dependently suppresses the incorporation of BrdU into lymphocytes after stimulation with a mitogen. This dose effect relation suggests a GR mediated event, indicating that BrdU incorporation might be a viable alternative for the currently used radioactive assay with thymidine. BrdU has been used in various *in vitro* assays, for instance to assess proliferation of dentate gyrus neuronal stem cells in stress models (363). BrdU uptake for measuring lymphocyte proliferation has been used by others (364). The present study, however, is the first that combines a BrdU ELISA assay with dexamethasone-induced suppression of PBMC proliferation. It is clear that this novel implementation of the DST has the advantage of lower costs and less environmental pollution than the conventional $^{3}$H-thymidine based assay. Moreover, our test enables measuring ligand-induced plasticity of GR regulation, which according to Rupprecht is more relevant in MD than the assessment of GR binding and/or GR gene polymorphisms per se (363;365).

The main findings in the present patient study are that the severity of depressive symptoms is associated with GR function, and that this effect is apparently modified by the patient's cortisolemic status at the time of blood sampling. GR function is known to be dependent on cortisol levels, but the positive correlation observed in our study does not support the traditional view that high cortisol coincides with low GR function. For example, Calfa et al. (357) state that GR density is decreased in patients with high-normal basal cortisol levels. (366) reported that cell sensitivity to glucocorticoids is highest in the morning when plasma cortisol levels are high. It is however difficult to compare these studies with ours, because Gratsias et al. used whole blood samples, including cortisol and Calfa et al. measured GR density, but not function.

Despite the highly significant correlation between symptom severity and BrdU incorporation in the overall analysis, this was not found in the individual patient, indicating that the diagnostic potential of this test may be limited. Possible confounding factors are the heterogeneity of the patient group with regard to HPA-axis function, and the CV of 10% which, although respectable, may not permit detection of subtle but relevant changes in the individual patient.

The observation that HDRS scores negatively correlate with suppression of BrdU incorporation is consistent with other studies showing that full remission coincides with restoration of GR function e.g. (357;367-369). Most referred studies on the possible relation between the depressive state and GR function are based on cross-sectional studies and do not provide information how close changes in depressive state relate to GR resistance. However, one longitudinal study has shown that the dexamethasone suppression test already normalizes in the 3-4 weeks prior to full resolution of clinical symptomatology in patients that had an abnormal DST response at admission (41). The present preliminary longitudinal data emphasize that the condition of increased GR function coincides with less

symptom severity, but that there is no obvious relationship in the individual patient, suggesting that lymphocyte GR resistance is conditional rather than causal in the recovery from depression. Model fitting of epidemiological data on the time course of recovery from depression suggested that depression has to be considered as a randomly fluctuating mood, rather than as a disorder with a progressive time course (370;371). Combining the latter idea and the current preliminary data it is tempting to speculate that GR resistance attenuates random fluctuations of mood and, conversely, maximal GR flexibility facilitates recovery. It is clear, however, that the present study has several limitations. First, the number of patients is relatively small. Second, the number of time points per subject is limited. Third, the patient population is far from homogenous and treatment modalities are very divergent. Conversely, the latter indicates that our observation of a robust relation between severity of depressive symptoms and lymphocyte GR function may be largely independent of symptom profile, gender and kind of treatment.

In conclusion, our preliminary results indicate a relation between lymphocyte GR function and MDD symptom severity. Clearly, additional studies using larger and more homogenous cohorts of MDD patients are required to investigate the full potential of our variant of the dexamethasone suppression test in both clinical research and clinical practice.

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