Clinical Science

Circulating Lymphocyte Subsets, Natural Killer Cell Cytotoxicity, and Components of Hypothalamic-Pituitary-Adrenal Axis in Croatian War Veterans with Posttraumatic Stress Disorder: Cross-Sectional Study

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Aim To determine peripheral blood lymphocyte subsets – T cells, helper T cells, cytotoxic T cells, B cells, and natural killer cells, natural killer cell cytotoxicity, serum cortisol concentration, and lymphocyte glucocorticoid receptor expression in Croatian combat veterans diagnosed with chronic posttraumatic stress disorder (PTSD); and to examine the relationship between the assessed parameters and the time passed since the traumatic experience.

Methods Well-characterized group of 38 PTSD patients was compared to a group of 24 healthy civilians. Simultaneous determination of lymphocyte subsets and the expression of intracellular glucocorticoid receptor was performed using three-color flow cytometry. Natural killer cell cytotoxicity was measured by ^{51}Cr-release assay and the serum cortisol concentration was determined by radioimmunoassay.

Results We found higher lymphocyte counts in PTSD patients than in healthy controls (2294.7 ± 678.0/µL vs 1817.2 ± 637.0/µL, $P = 0.007$) and a positive correlation between lymphocyte glucocorticoid receptor expression and the number of years that passed from the traumatic experience ($r_s = 0.43$, $P = 0.008$). Lymphocyte glucocorticoid receptor expression positively correlated with serum cortisol concentration both in PTSD patients ($r = 0.46$, $P = 0.006$) and healthy controls ($r = 0.46$, $P = 0.035$).

Conclusion This study confirmed that the immune system was affected in the course of chronic PTSD. Our findings also indicated that the hypothalamic-pituitary-adrenal axis profile in PTSD was associated with the duration of the disorder. Due to the lack of power, greater sample sizes are needed to confirm the results of this study.

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Prolonged or frequently repeated stress response during symptomatic episodes in chronic post-traumatic stress disorder (PTSD) can result in neuroendocrine and immune alterations, posing serious threat to mental and physical health (1,2). Evidence suggests that PTSD is related to increased medical morbidity, particularly from cardiovascular and autoimmune diseases (3). With controversial findings when neurobiology of PTSD is concerned, the patophysiological mechanisms underlying increased susceptibility to disease are not clear (4,5). However, it has been implicated that the sympathetic-adrenal-medullary (SAM) and the hypothalamic-pituitary-adrenal axes are the key mediators in this process (6,7).

The immune system interacts with the hypothalamic-pituitary-adrenal axis in a bidirectional fashion to maintain homeostasis. Being the primary effector of the stress response, cortisol modifies the complex cytokine network and, consequently, leukocyte function and recirculation (8). These effects are achieved through its interaction with the specific intracellular glucocorticoid receptors (9).

Studies of the leukocyte recirculation (10,11), immune cells function (12), and hypothalamic-pituitary-adrenal axis activity (5) in PTSD yielded controversial results. Overall findings support the hypothesis that immune activation in PTSD may be associated with Th2 cytokine shift and alterations in the proinflammatory cytokine system (4). Besides, it is believed that PTSD is linked with low plasma cortisol levels and higher glucocorticoid receptor expression, suggesting enhanced feedback sensitivity to cortisol (13). In contrast to these findings, Gotovac et al (14) showed that Croatian combat veterans with PTSD, approximately 6 years after traumatic event, had lower expression of glucocorticoid receptor in lymphocyte subsets, with higher serum cortisol concentration than healthy subjects. Majority of other studies did not take into account the time passed since the trauma and their sam-

ples mainly included Vietnam veterans (15) or Holocaust survivors (16), who had greater time gap since the traumatic experience than Croatian war veterans.

Considering the strong discrepancies in the results published to date, we performed a cross-sectional study to evaluate the correlation between PTSD in Croatian combat war veterans on and the percentages of circulating lymphocyte subsets, natural killer cell cytotoxicity as a measure of immune function, and the serum cortisol concentration with lymphocyte glucocorticoid receptor expression as components of hypothalamic-pituitary-adrenal axis. The emphasis was put on the relationship between the assessed parameters and the time passed since the traumatic experience.

Subjects and methods

Subjects

PTSD patients were Croatian combat veterans, all men (Table 1), recruited from the outpatient PTSD program at Vrapče Psychiatric Hospital during the year 2004. Before entering the study, all patients met the International Classification of Disorders (ICD)-10 (17) PTSD criteria, the official classification in Croatian psychiatric practice. For the purposes of this study, the diagnosis of PTSD, as well as the specificities of clinical picture (frequency and intensity of all and selected PTSD symptoms during the period of one month prior to rating), were determined by using the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV (18) based Clinician-Administered PTSD Scale (CAPS) (19) and all 38 patients met the criteria for chronic PTSD. Only the patients without psychiatric premorbidity or comorbidity, including major depression, were selected for the purpose of this study. Each patient had at least one "reexperiencing symptom" within the criterion B, at least 3 "avoidance and numbing symptoms" within the criterion C, and

Table 1. Participants' demographic and clinical characteristics

Variable*	PTSD patients (n = 38)	healthy volunteers (n = 24)	$P^\dagger$
Age (mean±SD)	44.3 ± 9.4	39.0 ± 8.4	0.028
Tobacco use	24	13	0.330
Marital status:			0.443
married	21	12	
unmarried/divorced/widower	17	12	
Lives with the family‡	32	22	0.329
Education:			0.006
elementary school	5	0	
high school	28	13	
university education	5	11	
Work status:			0.012
employed	22	21	
retired	11	0	
unemployed	5	3	
CAPS (mean±SD):			
reexperiencing	16.5 ± 2.2	0	
avoidance/numbing	26.1 ± 3.1	0	
hyperarousal	15.7 ± 3.3	0.5 ± 1.1	
total	58.3 ± 7.0	0.5 ± 1.1	<0.001
Delayed§	20/38		
Years from trauma (median, range)	12 (8-13)		

*Abbreviations: PTSD – posttraumatic stress disorder; SD – standard deviation; CAPS – Clinician-administered PTSD Scale.
†Two-sided values obtained using Fisher exact tests for 2×2 tables, Pearson χ^2 tests for 3×2 tables, and *t* tests; statistically significant if *P*<0.05.
‡Lives with the family.
§The onset of symptoms after the first six months of traumatic incident.

at least 2 "hyperarousal symptoms" within the criterion D. No patients had subsyndromal form of PTSD. In relation to DSM-IV A (traumatic event) criterion, each patient witnessed another person's death and experienced a threat to their physical integrity and/or wounding. On the basis of intrusive thoughts content, the focal traumatic experience was identified and the time passed until assessment was recorded. The PTSD group was relatively homogenous related to the severity of the illness. The average severity of PTSD was qualified as moderate (median = 4; interquartile range = 4 to 5), according to the Clinical Global Impression Severity (CGI-S) scale (20). Control subjects were sex-matched healthy civilians, hospital and laboratory personnel with no combat experience.

Prior to blood drawing, healthy civilians were examined by an experienced physician and their histories were recorded. All controls had negative history of psychiatric disorders.

None of the participants used any psychotropic medication, drug, or reported alcohol abuse for at least one month, and did not suffer from any infectious, allergic, or endocrine disorder. They had no symptoms or signs of acute or chronic physical illness.

The study was approved by the Ethic Committee of the hospital and written informed consent was obtained from all subjects.

Blood samples

Heparinized and unheparinized blood samples (10 mL each) were collected by venipuncture between 8 and 9 AM in Vacutainer tubes (Becton Dickinson Vacutainer System Europe, Grenoble, France). A part of the whole heparinized blood (50 μL per tube) was used for the lymphocyte immunophenotyping and glucocorticoid receptor expression determination by flow cytometry. Peripheral blood mononuclear cells (PBMC) were separated by centrifugation on Ficoll-Hypaque (Pharmacia AB, Uppsala, Sweden) density gradient and used for natural killer cell cytotoxicity determination. The sera for determination of cortisol concentration were stored at -80°C.

Immunophenotyping and intracellular glucocorticoid receptor determination

Lymphocyte counts were determined by flow cytometry, using Flow-Count™ Fluorospheres (Coulter Corporation, Miami, FL, USA) according to the manufacturer's protocol. Flow cytometric, three-color staining method was used for the simultaneous labeling of surface lymphocyte markers and intracellular lymphocyte glucocorticoid receptor expression. The method was previously described in detail (14). Briefly, surface staining was achieved by phycoerytrin (PE) conjugated anti-CD3, anti-CD16,56, anti-IgG2a, and peridinin chlorophyll protein (PerCP) conjugated anti-CD4, anti-CD8, anti-CD20, anti-IgG1 monoclonal antibodies (MoAb) (Becton Dickinson, Heidelberg, Germany), 5 μL per 50 μL of whole blood each. After incubation in the dark for 15 minutes, cells were washed and fixed, followed by erythrocytes lysis. The cells were
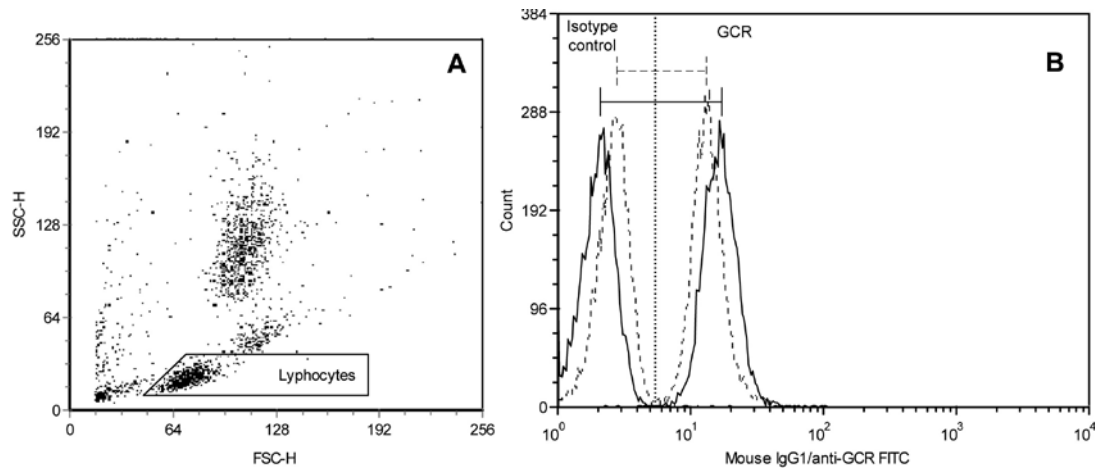
Figure 1. Representative flow cytometric analysis of glucocorticoid receptor expression in lymphocytes of the patient with posttraumatic stress disorder (PTSD). Lymphocytes were gated on light scatter dot plot (**A**), and the relative quantity of glucocorticoid receptor was calculated as the difference between mean fluorescence intensity of glucocorticoid receptor and isotype control (**B**). Overlaying histograms show that the glucocorticoid receptor expression is higher in PTSD patient. SSC-H – side scatter-hight; FSC-H – forward scatter-hight; GCR – glucocorticoid receptor; FITC – fluorescein isothiocyanate; full line – patient with posttraumatic stress disorder, dashed line – healthy volunteer.

then washed in the permeabilization buffer, containing predetermined optimal concentration of fluorescein isothiocyanate (FITC) conjugated anti-glucocorticoid receptor MoAb (21) or 5 µL of isotype control, left for 20 minutes in the dark, washed with permeabilization buffer again, and resuspended in 500 µL of fixation buffer. Prepared samples were run on FACSCalibur flow cytometer and analyzed by CELLQuest software (Becton Dickinson, Heidelberg, Germany). At least 5000 events in the light-scatter lymphocyte gate were acquired and the percentages of surface marker positive cells were determined in PE vs PerCP dot plots. The relative quantity of lymphocyte glucocorticoid receptor (mean glucocorticoid receptor fluorescence), expressed as mean fluorescence intensity, was calculated as the difference between mean values of glucocorticoid receptor and isotype control (Figure 1). The instrument calibration was performed on a daily basis.

Natural killer cell cytotoxicity

The in vitro natural killer cell cytotoxicity toward ^{51}Cr-labeled K562 target cells (185 MBq/mL, Amersham, Little Chalfont, UK) was performed according to the previously described technique

(22). We represented natural killer cell activity as the mean percentage of lysis across three effector-target cell ratios (25:1, 50:1, 100:1) (Table 2). Lytic units were not calculated because not all of our data fitted the assumptions required for lytic unit transformation. To avoid missing data and minimize the number of comparisons and consequent type I error, only the mean percentage of lysis was included in the overall analyses.

Serum cortisol determination

Serum cortisol concentration was determined by the radioimmunoassay kit CORT-CT2 (Schering S.A. Cis bio international, Gif-sur-Yvette, France). All samples were analyzed in duplicates following manufacturer's protocol. The sensitivity of the assay was 0.17 µg/100 mL, and the intra-assay and inter-assay coefficients of variation were less than 6% and 8%, respectively.

Statistical analysis

Categorical data were analyzed using Fisher exact or Pearson χ^2 tests. Since not all continuous variables were normally distributed, as confirmed with Shapiro-Wilk W test, transformations were applied to meet parametric tests assumptions of normality and homogeneity of variance (assessed

Table 2. Comparison of the immune and endocrine parameters between PTSD patients and healthy volunteers*

Variable[†]	Findings in participants		Statistics		
	PTSD patients (n = 38)	healthy volunteers (n = 24)	t	P	d[‡]
Lymphocytes (count/μL)	2294.7 (2077.8-2511.5)	1817.2 (1544.3-2090.1)	2.763	0.007	0.006
T cells (%)	70.7 (67.5-73.8)	67.9 (63.6-71.9)	0.812	0.419	0.028
Helper T cells (%)	44.8 (42.0-47.5)	42.2 (38.7-45.6)	1.023	0.310	0.017
Cytotoxic T cells (%)	22.8 (20.3-25.6)	20.8 (17.9-24.1)	0.768	0.444	0.039
B cells (%)	11.2 (9.8-12.8)	11.5 (9.7-13.5)	-0.237	0.813	0.050
Natural killer cells (%)	16.2 (13.7-18.8)	17.9 (14.7-21.5)	-0.276	0.783	0.044
Natural killer cell cytotoxicity (mean)[§]	45.5 (39.2-51.7)	47.4 (39.4-55.4)	-0.778	0.439	0.033
(25:1)	31.6 (25.5-37.8)	35.0 (27.5-42.5)	-0.711	0.480	
(50:1)	46.5 (40.1-52.4)	49.5 (41.7-51.4)	-0.629	0.532	
(100:1)	56.5 (50.8-62.2)	59.6 (52.2-67.1)	-0.684	0.497	
Lymphocyte glucocorticoid receptor (m.f.i.)	42.5 (38.5-46.6)	39.2 (34.5-44.3)	0.856	0.394	0.022
Cortisol (μg/100 mL)	13.7 (11.1-16.5)	12.0 (9.1-15.4)	1.140	0.258	0.011

*Abbreviations: PTSD – posttraumatic stress disorder; m.f.i. – mean fluorescence intensity.
†Values are means or backtransformed means with 95% confidence intervals in the parentheses. To meet t test assumptions variables were transformed as follows: square for T cells (CD3+), logarithm for cytotoxic T cells (CD3+CD8+), square root for B cells (CD20+), natural killer cells (CD56+CD16+), glucocorticoid receptor, and cortisol.
‡Threshold value calculated using the false discovery rate method. If the P value exceeds corresponding d, performed test is not statistically significant at 0.05 level.
§Percentage of lysis represented as the mean of three effector:target cell ratios (25:1, 50:1, 100:1), separate effector:target cell ratios were not included in the calculation of d values.

by Levene test). The group differences were tested with analysis of covariance (ANCOVA), using age as covariate, and two-tailed *t* tests. Data could not be modeled to meet multivariate test assumptions, leaving the greater possibility of type I error because of the multiple comparisons performed. Therefore, we used the false discovery rate method (23) for 9 group comparisons and 15 planned correlations. Partial correlations controlling for age and smoking in both groups, including "delayed" factor only in PTSD group, were performed. Correlations with the number of years since the trauma were examined using nonparametric Spearman rank order correlations because this variable could not be normalized. All statistical analyses were performed with Statistica, version 6 (StatSoft, Inc., Tulsa, OK, USA).

Results

Group comparisons

Groups were not matched by age, education, and work status. PTSD patients were slightly older, less educated, and substantial proportion of them was retired (Table 1). The age difference was considered as possible confounding factor, but ANCOVA, using age as a covariate, showed no significant effect of age on the investigated

variables (Table 2). Results based on raw *P* value from *t* test indicated higher lymphocyte count in PTSD patients and the calculated power yielded 80% for group sample sizes of 38 and 24. Calculation for two-sided two-sample *t* test was based on the observed difference of 477.4/μL, with group standard deviations of 678.0 and 637.0, and with significance level (α) set to 0.05. However, with α set to 0.006, as indicated by false discovery rate threshold value (d), calculated power yielded only 56%, with a sample size of 49 participants per group needed to achieve the power of 80%. No other measured parameter significantly different between the PTSD patients and healthy volunteers (Table 2).

Correlational analyses

No biological parameter significantly correlated with total CAPS scores, but the number of years after trauma positively correlated with lymphocyte glucocorticoid receptor expression, as indicated by the raw *P* value (Figure 2). Positive correlation was also found between lymphocyte glucocorticoid receptor expression and serum cortisol concentration in the PTSD patients (r = 0.46, P = 0.006, d = 0.003) and healthy volunteers (r = 0.46, P = 0.035, d = 0.010). However, false discovery rate analysis indicated type I error since all *P* values exceeded corresponding d
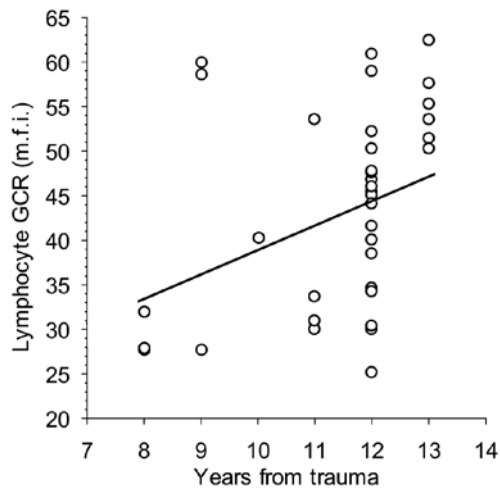
Figure 2. Correlation between the glucocorticoid receptor (GCR) expression in lymphocytes and number of years passed since the trauma. Spearman rank order correlation controlled by the false discovery rate at $\alpha = 0.05$ for total number of 15 correlations performed in this study: $r_s = 0.43$, $P = 0.008$, d = 0.007; m.f.i. – mean fluorescence intensity.

values (Table 2). The other planned correlations of cortisol concentration, lymphocyte count, and natural killer cell cytotoxicity with the number of years from trauma, were not statistically significant.

Discussion

Our study showed increased lymphocyte count in PTSD patients and positive correlation of the lymphocyte glucocorticoid receptor expression with the time passed since traumatic experience. The effect of PTSD on the peripheral blood lymphocyte subsets, the immune cell function measured by natural killer cell cytotoxicity, and the components of hypothalamic-pituitary-adrenal axis was not observed.

Generally, few studies on the immunity in PTSD have been published to date and even fewer addressed well-characterized fully developed PTSD according to the DSM-IV criteria. Our study could not confirm either the previous findings of higher peripheral blood percentages of helper T cells (CD4) and natural killer cells (CD16,56) in Croatian PTSD patients (24),

or higher percentages of T cells (CD3), CD4, and cytotoxic T cells (CD8) in Vietnam veterans (11). However, other studies also reported the lack of differences in the peripheral blood lymphocyte phenotype in combat-related (12), or childhood sexual abuse-related (10) chronic PTSD. Recently, Boscarino et al (3) showed that Vietnam veterans with PTSD had higher lymphocyte counts, consistent with the higher prevalence of autoimmune diseases. We noticed that PTSD patients had higher lymphocyte counts in this group, as well as in other examined PTSD groups (our unpublished data), although this rise could not be contributed to any particular lymphocyte subpopulation, as seen from the lack of difference between percentages of subpopulations. However, observed difference in lymphocyte count must be interpreted with caution because of the possibility of type I error, as indicated by false discovery rate analysis. Our future study will address the same question by examining a larger group of patients (minimally 49 per group, as shown by the sample size analysis).

Research on the immune function in PTSD showed generally an increased delayed-type hypersensitivity (3,25), higher percentage of perforin positive natural killer cells (24), and inconclusive natural killer cell cytotoxicity measurements (12,26,27). According to the results from chronic stress studies of decreased natural killer cell cytotoxicity (28), the same would be expected in chronic PTSD. Nevertheless, it was shown that Croatian professional soldiers released from concentration camp had higher natural killer cytotoxicity (29). Moreover, our preliminary results of a six years follow-up indicate that natural killer cell cytotoxicity declines over time in chronic PTSD, after the initial increase within eight years from the trauma, compared with healthy individuals (30). In this study, we were not able to show the group difference in natural killer cell cytotoxicity, as well as expected negative correlation with the time passed since the trauma. Prospec-

tive studies are needed to elucidate the dynamics of the immune function in chronic PTSD.

In contrast to immunological studies, extensive research has been done regarding the components of hypothalamic-pituitary-adrenal axis in PTSD (5). In fact, low cortisol level with enhanced negative feedback sensitivity to cortisol, ie, higher number of glucocorticoid receptors, is considered to be a distinctive biological feature of PTSD that separates this disorder from other psychiatric conditions such as depression (31). Simultaneous determination of cortisol and its receptor enables better characterization of the hypothalamic-pituitary-adrenal axis than cortisol alone (32). Using this approach, we found that Croatian combat veterans had higher cortisol concentration with lower lymphocyte glucocorticoid receptor expression (14). This finding was attributed to the limited duration of PTSD compared with other studies, and thus shorter duration of the stress response with preserved negative feedback by higher cortisol levels. During the repeated activation of stress response, as seen in PTSD, the stress system re-establishes stability at a higher set point by the process called allostasis that, over time, becomes a burden for the living system (allostatic load) (33). Thus, we expect that PTSD patients will adopt distinctive hypothalamic-pituitary-adrenal axis profile if the disorder persists. Present study investigated a group of PTSD patients with longer duration of the disorder (8 to 13 in this study vs 6 to 8 years in the previous study), so the lack of difference between the groups was not surprising. Moreover, Yehuda et al (34) showed a positive correlation between the amount of cortisol suppression after a low dose of dexamethasone (indirect glucocorticoid receptor sensitivity measure) and the number of years passed since the traumatic event. The hypothesis that the duration of allostatic load is the key factor in the modulation of hypothalamic-pituitary-adrenal axis activity is further supported by the positive correlation between lymphocyte glucocorticoid receptor expression and

the number of years from the trauma found in this study (Figure 1). Preliminary results from our ongoing follow-up study, which show significant decrease of the glucocorticoid receptor expression in PTSD patients after six years follow up period, also support this hypothesis (30).

Another positive correlation between cortisol concentration and lymphocyte glucocorticoid receptor observed in this study suggests that factors other than endogenous glucocorticoids are involved in the mutual modulation of glucocorticoid receptor expression and cortisol secretion. A possible candidate is interleukin (IL)-6, which directly stimulates production of glucocorticoids (35) and also up-regulates glucocorticoid receptor and expression of glucocorticoid receptor mRNA in peripheral blood mononuclear cells (36). Although some studies reported increased IL-6 in cerebrospinal fluid (37) and sera (38) of PTSD patients, our future studies will further investigate the proposed relationship.

We should also point out the limitations of this study. The age difference could have confounded the results because aging is associated with the immunological changes that resemble those following chronic stress (39). Since this difference was very small and statistically controlled for by ANCOVA, it should have not interfered with the results. Another possible limitation is that 9 healthy controls were chosen from psychiatry hospital personnel (psychiatrists and technicians) who are believed to experience higher amount of stress than laboratory personnel or other healthy civilians not working in the hospital. Careful examination of the biological data from these individuals did not show any deviations from their counterparts in the control group. Although the examined group of patients was well-defined, relatively homogeneous, and without psychiatric comorbidities, depressive symptoms were not controlled for, neither in the PTSD nor in the control group.

Cross-sectional research of PTSD is connected with many obstacles, and inconsistency of the

results in literature may be due to differences in gender, age, stressor type, diagnostic instrument chosen, and timing of the assessment in relation to the trauma onset. Moreover, biological changes in PTSD are very small and large sample sizes are needed to obtain enough statistical power to confirm them. Because of the lack of power, results from this study are more indicative than conclusive. More controlled laboratory challenges could reveal group differences in biological parameters in relation to acute individualized traumatic stimuli (40) and long term prospective studies (41) could confirm the dynamics of endocrine and immune parameters in PTSD implicated in this study.

Acknowledgments

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