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## 4 **Short title:** Early-life adversity and gene expression changes

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6 Idan Shalev<sup>1</sup>, Waylon J. Hastings<sup>1</sup>, Laura Etzel<sup>1</sup>, Salomon Israel<sup>2</sup>, Michael A. Russell<sup>1</sup>, Kelsie A.  
7 Hendrick<sup>1</sup>, Megan Zinobile<sup>1</sup>, Sue Rutherford Siegel<sup>1</sup>

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9 <sup>1</sup>Department of Biobehavioral Health, The Pennsylvania State University, University Park, PA,  
10 USA; <sup>2</sup>Department of Psychology, The Hebrew University of Jerusalem, Jerusalem, Israel.

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12 \*Corresponding author

13 Email: [ius14@psu.edu](mailto:ius14@psu.edu) (IS)

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## 26 Abstract

27 **Objective:** Exposure to early-life adversity (ELA) can result in long-term changes to  
28 physiological systems, which predispose individuals to negative health outcomes. This  
29 biological embedding of stress-responsive systems may operate via dysregulation of  
30 physiological resources in response to common stressors. The present study used a novel  
31 experimental design to test how young adults' exposure to ELA influence neuroendocrine and  
32 inflammatory responses to acute stress. **Materials and methods:** Participants were 12 males  
33 (mean age= 21.25), half of whom endorsed at least three significant adverse events up to age  
34 18 years ('ELA group'), and half who confirmed zero ('controls'). Using a randomized within-  
35 subjects, between-groups experimental design, we induced acute psychosocial stress (Trier  
36 Social Stress Test, TSST), and included a no-stress control condition one week apart. During  
37 these sessions, we obtained repeated measurements of physiological reactivity, gene  
38 expression of *NR3C1*, *FKBP5* and *NFKB1*, and plasma levels of pro-inflammatory cytokines (IL-  
39 1 $\beta$ , IL-6, IL-8 and TNF $\alpha$ ) over a 4-hour window post-test. **Results:** The ELA group evinced  
40 significantly higher cortisol response and lower *NR3C1* gene expression in response to the  
41 TSST compared with controls, while no differences were observed in the no-stress condition.  
42 Cortisol and group status interacted such that increase in cortisol predicted increase in both  
43 *NR3C1* and *NFKB1* expression among controls, but decrease in the ELA group. For pro-  
44 inflammatory cytokines, only IL-6 increased significantly in response to the TSST, with no  
45 differences between the two groups. **Conclusion:** Overall, we provide preliminary findings for  
46 the biological embedding of stress via a dynamic and dysregulated pattern evidenced in  
47 response to acute psychosocial stress. ELA may program physiological systems in a  
48 maladaptive manner more likely to manifest during times of duress, predisposing individuals to  
49 the negative health consequences of everyday stressors. Future studies with larger sample size  
50 including both males and females are needed to replicate these findings.

## 51      **Introduction**

52      An ever-growing body of research suggests that early-life adversity (ELA) can program  
53      biological systems, which predispose individuals to later-life physical and mental-health  
54      problems [1, 2]. Empirical evidence exist for associations between ELA and elevated risk of  
55      depression, cardiovascular disease, diabetes, autoimmune diseases and cancer, to name a few  
56      (reviewed in [3]). Despite the salient role of ELA on disease risk, the biological mechanisms that  
57      play a downstream role in increased disease susceptibility are not well understood.

58      Mechanistic research on the biological embedding of ELA has emphasized maladaptive  
59      programming of the hypothalamic-pituitary-adrenal (HPA) axis with the associate release of  
60      cortisol through processes of allostasis [3, 4]. Specifically, studies have documented a shift in  
61      HPA axis function with hyper- or hypo-secretion of cortisol in depression and post-traumatic  
62      stress disorder (PTSD), respectively [5, 6]. Similar findings have been reported in individuals  
63      exposed to ELA without such diagnoses [7, 8]. This programming, in turn, can result in  
64      mitochondrial dysfunction, failure to down-regulate the inflammatory response and overall  
65      metabolic stress, thereby increasing circulatory levels of lipids, glucose, oxidants, and pro-  
66      inflammatory cytokines [9, 10]. Further mechanistic research on the biological embedding of  
67      ELA suggests physiological dysregulation may be mediated at the genetic level via epigenetic  
68      modifications that can persist over long periods of time [11], including evidence linking ELA and  
69      cortisol responses via methylation levels in the glucocorticoid receptor (*NR3C1*) gene [12, 13].  
70      Other research suggest the involvement of telomere biology in mediating the longer-term link  
71      between ELA and disease risk [14]. What is less clear, however, is how target immune cells  
72      respond to stress *in vivo* as a consequence of ELA, via rapid gene expression regulation [15,  
73      16]. This new knowledge can provide insights into an integrated and dynamic cellular regulatory  
74      system whose signal profiles could forecast disease risk associated with early adversity [17-19].

75 Cells show remarkable flexibility in response to stimuli by regulating gene expression in a  
76 transient manner [16, 20]. In one of the first studies relating peripheral blood mononuclear cells  
77 (PBMC) gene expression to trauma, basal gene expression signatures, both immediately  
78 following trauma and four months later, distinguished survivors who met diagnostic criteria for  
79 PTSD from those who did not [21]. Follow-up studies provided further support for associations  
80 between chronic stress and glucocorticoid signaling, as well as induced or repressed activation  
81 of pro- and anti-inflammatory genes [22-24]. Importantly, several studies have provided  
82 evidence of rapid (e.g., from 30 minutes to 8 hours) gene expression activation in response to *in*  
83 *vitro* stimulation [25], psychological stress [26, 27], physical stress [28] and stress-reduction  
84 methods [29]. Notably, these response patterns were recently dubbed the “conserved  
85 transcriptional response to adversity” [30]. Taken together, theory and evidence suggests that  
86 the programmed immune cells of individuals exposed to early adversity may show compromised  
87 adaptation in response to acute stress, which, if repeated, may play a downstream role in  
88 disease risk.

89 In this study, we focused on the glucocorticoid-immune signaling pathway by measuring  
90 differential expression of glucocorticoid receptor (*NR3C1*), FK506 binding protein 51 (*FKBP5*)  
91 and Nuclear Factor Kappa B Subunit 1 (*NFKB1*) genes [22, 31, 32] in PBMC. The  
92 glucocorticoid-immune signaling pathway has been implicated as a key mechanism in relation to  
93 chronic stress (i.e., caregiving, poverty [23, 31]), through reduced receptor availability, ligand  
94 binding affinity, and functional capacity to regulate gene expression. Specifically, chronic stress,  
95 via extended exposure to cortisol, is associated with reduced *NR3C1* expression, leading to  
96 glucocorticoid resistance and impaired negative feedback inhibition of the HPA axis [33].  
97 Increased glucocorticoid resistance is additionally explained by increased expression levels of  
98 *FKBP5*, an important regulator of the glucocorticoid receptor complex [32]. Reduced levels of  
99 glucocorticoid receptors, in turn, bind less cortisol. This effectively decreases the number of  
100 ligand-bound receptor complexes available to translocate to the nucleus and regulate the

101 expression of genes, including anti-inflammatory genes. Thus, reduced levels of *NR3C1*  
102 expression can lead to impaired immune function. Further, nuclear factor kappa-B (Nf- $\kappa$ B), a  
103 highly conserved transcription factor, can increase levels of pro-inflammatory cytokines, partly  
104 via reduced inhibition by the ligand-bound glucocorticoid receptor complexes [34]. Evidence  
105 exists for elevated levels of pro-inflammatory cytokines in children and adults exposed to ELA  
106 [35, 36]. Dysregulation of the immune system in the context of ELA, as well as the resulting  
107 increase of pro-inflammatory cytokines, can increase risk for a host of diseases, from  
108 autoimmune to atherosclerosis and cancer [37]. Here, in addition to the aforementioned genes,  
109 we focused on four pro-inflammatory cytokines, including interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-  
110 6), interleukin-8 (IL-8), and tumor necrosis factor alpha (TNF- $\alpha$ ).

111 We delineate a program of research to study ELA-related programming of biological  
112 systems using a within-person, between-groups experimental design. Specifically, in this pilot  
113 study we tested whether ELA leads to dysregulation of physiological, gene expression and pro-  
114 inflammatory cytokines in response to a canonical laboratory stressor, compared with a resting  
115 control condition, and compared with individuals without exposure to ELA. To study the  
116 biological embedding of stress, we used a validated screening instrument [38] and recruited 12  
117 men, 6 of whom who endorsed at least 3 significant adverse events ('ELA group') [39], and 6  
118 who confirmed zero ('controls'). In a randomized within-subjects design, we induced acute  
119 stress in the lab (Trier Social Stress Test, TSST) and included a no-stress control condition  
120 separated by one week. During these sessions, we obtained repeated measurements of  
121 physiological reactivity, plasma levels of pro-inflammatory cytokines, and PBMC gene  
122 expression over a 4-hour window post-test. We examined how young adults' exposure to ELA  
123 influence neuroendocrine and inflammatory responses to acute stress compared with non-  
124 exposed individuals by testing the following: 1) physiological, gene expression, and pro-  
125 inflammatory cytokines response to acute stress compared with a no-stress control condition,  
126 and 2) stress-induced cortisol changes in gene expression and pro-inflammatory cytokines.

127 Based on prior literature, we hypothesized that individuals exposed to ELA will evince  
128 dysregulated physiological, *NR3C1*, *FKBP5*, and *NFKB1* gene expression, and pro-  
129 inflammatory changes to an acute laboratory stressor, a response pattern that may reveal  
130 dynamic biological signatures of early-life programming with implications for life-long health.

131

## 132 **Materials and Methods**

133

## 134 **Participants**

135 Participants were healthy male college students at the Pennsylvania State University,  
136 recruited by word of mouth and advertisements on campus bulletin boards. We focused on men  
137 in this exploratory study due to known sex differences in the stress response [40] and the small  
138 sample size for stratified analyses. To obtain the sample who were exposed to ELA, a trained  
139 clinical interviewer conducted a phone interview to screen over 100 eligible men using the  
140 Stressful Life Events Screening Questionnaire (SLESQ) [38], a 13-item self-report measure that  
141 assesses lifetime exposure to traumatic events. We asked respondents 11 specific and two  
142 general categories of events, such as death of a parent or sibling, life-threatening accident, and  
143 sexual and physical abuse. Based on evidence that three or more traumatic events confers  
144 higher risk for disease [39], and considering the severity of the traumatic events, participants  
145 who responded to at least 3 incidents up to age 18 years (independently reviewed and reached  
146 consensus by MZ and IS) were invited to participate in the ELA group. Respondents' examples  
147 for adverse exposures in this study included (unsubstantiated) child abuse and neglect, severe  
148 violence exposure, parental loss, suicide of a close friend or a family member, severe illness of  
149 an immediate family member or car accidents. In addition, the SLESQ was used to screen  
150 participants without a history of traumatic exposures to serve as the control group. Selection  
151 criteria stipulated that subjects were between 18-25 years, without current medical illness or

152 endocrine illness (for example, asthma, diabetes, thyroid disease or pituitary gland disorders  
153 confirmed by self-report and physical examination), were currently non-smokers and were not  
154 using medication on a regular basis, including psychiatric medication. The final sample included  
155 12 men, 6 of whom experienced early adversity (i.e., 'ELA group') and 6 who did not (i.e.,  
156 'controls') (mean age= 21.25, SD= 2.3). Demographics of the sample are presented in **Table 2**.  
157 The study was approved by the Ethics Committee at the Pennsylvania State University and all  
158 participants provided written informed consent. Participants received a modest monetary  
159 incentive for participation.

160

## 161 **General Procedure**

162 Testing was carried out at the Pennsylvania State's Clinical Research Center (CRC).  
163 Participants made two visits to the CRC during weekdays, one week apart, on the same day.  
164 Testing was scheduled to begin at 11:00am and end by 4:15pm. We used a randomized  
165 counter-balanced order for the two sessions (i.e., TSST and no-stress control conditions) blind  
166 to participants and lab personnel. Lab personnel were also blind to group status. Participants  
167 were given specific instructions to refrain from excessive physical activity on the day of the  
168 testing, consuming alcohol for 12 hours before their arrival, and eating and drinking (besides  
169 water) for 2 hours prior to the testing session. After arrival and consent, trained nurses  
170 completed a physical examination and inserted an IV catheter into the antecubital vein 30  
171 minutes after arrival (30 minutes prior to testing). The TSST session was scheduled to begin at  
172 12:00pm to minimize the effects of circadian changes in cortisol, and was carried out as  
173 described previously [41]. Briefly, the TSST consists of a free speech and a mental arithmetic  
174 task of 10 minutes duration performed in front of a panel of two committee members (mixed  
175 gender) with a camera and microphone situated between the interviewers. Participants were  
176 told that they would play the role of an interviewee for a job and have 5 minutes to make an

177 argument for their candidacy. After 5 minutes, the second task emphasizing cognitive load  
178 commenced. In this task, participants were asked to count backwards from 1,687 in multiples of  
179 13. If a mistake was made, they were instructed to start again from the beginning. In the no-  
180 stress control condition, participants were instructed to sit in a room, read magazines, and to  
181 refrain from any stressful activities (e.g., cell-phone use was restricted). After the second blood  
182 draw, approximately 60 minutes after the TSST session and 90 minutes after the first baseline  
183 measure in the no-stress control condition, participants were administered a set of  
184 questionnaires. These questionnaires were administered in both sessions and the average  
185 score was calculated before analyses (see below for details). Considering the long time-frame  
186 of the study and the repeated collection of multiple blood samples, a standardized low-calorie  
187 meal was provided after the third blood draw (approximately at 1:45pm). Fig 1 outlines the study  
188 design.

189

190 **Fig 1:** Study design for both sessions (TSST and no-stress control condition), separated by one  
191 week

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## 193 **Physiological Reactivity**

194 Salivary cortisol was repeatedly assessed from the 7 saliva samples at the following time-  
195 points: 30 minutes after arrival (30 minutes prior to testing), 1 minutes prior to testing,  
196 immediately after testing (15 minutes after last sample in the control condition), and 15, 30, 60  
197 and 90 minutes post-test. Saliva samples were kept at room temperature throughout the  
198 session, were immediately centrifuged at the end of the session at 3000 rpm at 24°C for 15  
199 minutes, and then stored at -80°C until assayed. Systolic and diastolic blood-pressure were  
200 measured at the same time points as salivary cortisol.

201 Salivette swabs (Sarstedt, Germany) were used to collect saliva. Salivary cortisol was  
202 assessed, in duplicate, through an enzyme immunoassay protocol (Salimetrics) with known  
203 controls. The lower detection limit of the assay is <0.007 ug/dL. Intra-assay CV was 9.88%  
204 across all samples and inter-assay CV 5.79% across four plates. Participants' blood pressure  
205 was measured, while seated, using an automatic monitor (Omron HEM-712C).

206

## 207 **RNA Extraction and Gene Expression Assays**

208 Gene expression changes were measured repeatedly from the four blood samples at each  
209 session at the following time-points: 30 minutes after arrival (30 minutes prior to testing), and at  
210 30 (75 minutes after the first sample in the no-stress condition), 90 and 240 minutes post-test  
211 (Fig 1). Given known changes in immune cell redistribution and composition in response to  
212 acute stress [20], complete blood count with differential was measured within 24 hours by Quest  
213 Diagnostics using additional 4 ml EDTA collection tubes.

214 Whole blood samples were collected in 10 mL EDTA blood tubes via an IV catheter into the  
215 antecubital vein, and immediately centrifuged for 10 minutes at 1500g prior to collection of  
216 plasma. PBMCs were immediately isolated through density-gradient centrifugation using Ficoll.  
217 Immediately following isolation, cells were suspended in RNAlater solution (Ambion) before  
218 being stored at 4 °C overnight. The duration from blood sampling to stabilization of RNA never  
219 exceeded 55 minutes. RNA extraction and cDNA synthesis were performed the following day  
220 using QIAamp RNA Blood Mini Kit and cDNA Synthesis Kit respectively (Qiagen), and then  
221 stored at -80°C until assayed. RNA purity was verified using Nanodrop 2000 spectrophotometer  
222 (Thermo Scientific).

223 All assays were performed on a real-time PCR (Rotor Gene Q, Qiagen). PCR reactions  
224 were set-up using the complementary QIAgility robotic pipettor (Qiagen) to ensure maximum  
225 pipetting accuracy. Samples were assayed in duplicate. All repeated, within-subject samples

226 were run on the same plate. The reaction mix for gene expression assays consists of 5 uL  
227 TaqMan Gene Expression Master Mix (Thermo Fisher Scientific), 1x TaqMan gene expression  
228 primer, UltraPure Water (Rockland), and 100ng DNA in a 10 uL reaction. The cycling profile  
229 consists of an initial denaturing at 95°C for 15 seconds and annealing/extending at 60°C for 1  
230 minute followed by fluorescence reading, 55 cycles. Three hypothesis-driven genes (*NR3C1*:  
231 *Hs00353740\_m1*, *FKBP5*: *Hs01561006\_m1* and *NFKB1*: *Hs00765730\_m1*) were each  
232 normalized to a housekeeping gene (*GADD45A*: *Hs00169255\_m1*). Expression of a given  
233 hypothesis-driven gene and the housekeeping gene were assessed on the same plate in two  
234 independent PCR reactions using cDNA from the same sample aliquot. Each hypothesis-driven  
235 gene was assayed in an independent batch of assays.

236 Sample normalization was done using the  $\Delta\Delta Ct$  method [42]. Briefly, a cycle threshold (Ct) is  
237 defined as the cycle number at which a sample's fluorescence reaches a defined threshold. The  
238 same threshold was used for reactions assessing housekeeping and hypothesis-driven genes.  
239 Thus, each sample on a given plate has two Ct values (e.g.  $Ct_{NR3C1}$  and  $Ct_{GADD45A}$ ). The  $\Delta Ct$  is  
240 calculated as the difference between the Ct of the gene of interest and the Ct of the  
241 housekeeping gene (e.g.  $\Delta Ct = Ct_{FKBP5} - Ct_{GADD45A}$ ). The  $\Delta\Delta Ct$  represents the within-subject  
242 normalization of the three post-test samples to expression levels at baseline. That is,  $\Delta\Delta Ct =$   
243  $\Delta Ct_{POST-TEST} - \Delta Ct_{BASELINE}$ . Thus, the  $\Delta\Delta Ct$  for the baseline sample for each session is always  
244 equal to zero. Lastly, fold change is calculated by exponentiating 2 by  $-\Delta\Delta Ct$  (i.e. Fold Change =  
245  $2^{-\Delta\Delta Ct}$ ). It follows that the fold change for each baseline sample is always equal to one (i.e.  $2^0$ ).

246

## 247 **Pro-Inflammatory Cytokines**

248 Inflammatory assays were performed on plasma isolated from whole blood. Plasma samples  
249 were stored at -80°C prior to use. Plasma levels of IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  were quantified  
250 using Meso Scale Discovery's Multi-Array technology (MSD, V-PLEX Human Proinflammatory

251 Panel II) and analyzed on a Meso QuickPlex SQ 120 instrument (Meso Scale Discovery,  
252 Rockville, MD, USA). Sample concentrations were determined relative to standard curves  
253 generated by fitting electrochemiluminescent signal from stock calibrators with known  
254 concentrations using MSD Discovery Workbench® software. Samples were run in duplicate.  
255 Intra-assay variability was 8.02% across all samples and inter-assay variability was 3.87%  
256 across the three plates. The lower limits of detection for inflammatory markers were 0.646  
257 pg/mL (IL-1 $\beta$ ), 0.633 pg/mL (IL-6), 0.591 pg/mL (IL-8), and 0.690 pg/mL (TNF- $\alpha$ ). Samples with  
258 concentrations below the curve fit range were assigned a value of 0 for analyses considering  
259 those analytes. This occurred for 13 samples (13.5%) for IL-1 $\beta$ . Samples for all other analytes  
260 were within detection ranges.

261

## 262 **Self-Reported Measures and Other Covariates**

263 We administered several questionnaires to assess levels of adverse exposures and mental  
264 health symptoms. Specifically, participants completed the following questionnaires at both  
265 sessions; the Life-Event Stress Scale (LESS) [43], which consists of 42 common events  
266 associated with some degree of disruption of an individual's life and provide a standardized  
267 measure of the impact of a wide range of common stressors; and the Life Events Questionnaire  
268 (LEQ) [44], an 82-item inventory-type questionnaire for the measurement of life changes. The  
269 LEQ consists of items that are designed primarily for use with students. We further assessed  
270 levels of anxiety and depressive symptoms using the Beck anxiety inventory [45], Beck  
271 depression inventory [46], and State-Trait Anxiety Inventory [47]), as well as perceived stress  
272 levels using the 10-item Perceived Stress Scale [48].

273 As noted above, given gene expression changes may depend on specific cell populations  
274 [20], we measured complete blood cell counts during both experimental sessions, as well as  
275 PBMC counts, in duplicate, using a Countess automated cell counter (Invitrogen). Other

276 potential covariates included; age, body mass index, and socioeconomic status (i.e., parental  
277 education and income).

278

## 279 **Data Reduction and Final Measures**

280 Statistical analyses of cortisol data used log transformed cortisol values at 7 time-points and  
281 area under the curve with respect to increase (AUCi) [49]. The variables were examined for  
282 outliers ( $>3$  SD) and none were detected. Blood pressure values were reduced to 4 measures,  
283 from 30 minutes prior to testing to 15 minutes after (samples 1-4) to evaluate the fast  
284 sympathetic response. Moreover, systolic and diastolic blood pressures were combined to  
285 derive a measure of the mean arterial pressure (MAP) to describe the average response in  
286 blood pressure (i.e.,  $MAP = [(2 \times \text{diastolic}) + \text{systolic}] / 3$ ). Raw gene expression data was  
287 analyzed based on the  $2^{-(\Delta\Delta Ct)}$  method, with normalization to a housekeeping gene, and  
288 compared to the first baseline measure in each session [42]. AUCi was computed for each gene  
289 to assess overall responses from baseline. Cortisol slope increase was calculated using the first  
290 three measures for cortisol from baseline to peak levels and dividing by the time between  
291 measures [50].

292 For the four pro-inflammatory cytokines, considering high correlations [51] (Pearson  
293 correlations ranged from .30 to .72), principal component analysis (PCA) indexing *systemic*  
294 *inflammation* of IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  measures was conducted for the four repeated  
295 measures using data from both sessions. In each instance, the first component was extracted  
296 for use in subsequent analyses. The four repeated items mapped to components with  
297 eigenvalues of 2.55 for the first time point, which explained 63.81% of the variance across all  
298 four cytokines, 2.29 for the second time point (57.25% of variance), 2.46 for the third time point  
299 (61.54% of variance), and 2.80 for the fourth time point (69.88% of variance). PCA of AUCi for  
300 all four cytokines yielded two components with eigenvalues 1.93 and 1.01, which explained

301 48.27% and 25.37% of the variance respectively. Closer inspection of the factor loading scores  
302 for the PCA of AUCi revealed that the first component was largely representative of three  
303 cytokines (IL1- $\beta$ , IL-8, TNF- $\alpha$ ) with the second representing IL-6 (Table 1). PCA was also  
304 conducted on the four repeated measures independently within each session (TSST and no-  
305 stress). The four repeated items in the TSST session mapped onto components with  
306 eigenvalues 2.02-2.56, which explained 50.41%-64.01% of variance at each time point. The four  
307 repeated items in the no-stress session mapped onto components with eigenvalues 1.65-2.29,  
308 which explained 41.28%-57.16% of variance at each time point. The components mapped using  
309 data from both sessions were used to investigate within-person differences across sessions,  
310 while the components mapped within each session independently were used to investigate  
311 between-person differences (i.e. ELA status) within each session. Scores in all repeated  
312 questionnaires for both sessions were averaged to increase reliability (Pearson correlations  
313 ranged from .72 to .93). None of the demographics measures differed significantly between the  
314 ELA and control groups (Table 2), and thus were not included as covariates in the analysis.

315

316 **Table 1.** PCA of Pro-Inflammatory Cytokine AUCi: Factor Loading Scores

Cytokine	Factor 1	Factor 2
IL1- $\beta$	0.597	0.224
IL-6	0.174	0.942
IL-8	0.908	-0.080
TNF- $\alpha$	0.849	-0.265

317

## 318 **Statistical Analysis**

319 All statistical tests were carried out using SPSS version 25 (Windows). Repeated measures  
320 general linear models (GLMs), ordinary least squares multiple regression analyses, Pearson  
321 product-moment correlations, and t-tests were carried out as appropriate. Statistical analyses of  
322 changes in gene expression, physiological responses and cytokines levels were subjected to  
323 multivariate GLMs, with salivary cortisol, cytokines and gene expression as the repeated

324 measure, condition (stress/no stress) as a within-subjects factor, and status (risk/control) as  
325 between-subjects factors. In addition to these analyses, univariate tests were applied to  
326 summary cortisol measures (AUCi, [49]) to ascertain reliability of findings, as well as blood  
327 pressure (MAP), gene expression, and pro-inflammatory cytokine measures. Huynh-Feldt  
328 corrections were applied if sphericity (significant differences in variance between groups) was  
329 significant, and only adjusted results are reported.

330

## 331 **Results**

332

### 333 **Sample Characteristics and Self-Reported Measures**

334 The ELA and control group did not differ in demographics measures (i.e., age,  
335 socioeconomic status and body mass index) (Table 2). As expected, the ELA group tended to  
336 report more stressful life events [43] compared with controls (univariate ANOVA between-  
337 subjects effect:  $F=3.55$ ,  $p=0.089$  for LESS;  $F=4.10$ ,  $p=0.070$  for LEQ), as well as higher levels of  
338 anxiety [47] ( $F=2.54$ ,  $p=0.142$ ), and depressive symptoms [46] ( $F=2.73$ ,  $p=0.129$ ). Further, the  
339 ELA group self-reported more perceived stress in the TSST session compared with controls  
340 ( $F=6.07$ ,  $p=0.033$ ), as well as in the no-stress condition ( $F=7.49$ ,  $p=0.021$ ), and tended to report  
341 more stress in response to the TSST (Likert scale from 1-10) ( $F=4.02$ ,  $p=0.080$ ). Overall, these  
342 findings confirm previous studies indicating increased stress and anxiety levels in individuals  
343 exposed to ELA, compared with non-exposed individuals.

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347

**Table 2.** Sample Characteristics

Variable, mean (SD)	Total (N=12)	Control (N=6)	ELA (N=6)	P value diff
Age	21.25 (2.3)	20.83 (1.6)	21.67 (2.9)	0.56
SES (average)	2.83	2.83	2.83	0.23 <sup>1</sup>
1. Working class	1	0	1	
2. Lower middle	2	1	1	
3. Middle	7	5	2	
4. Upper middle	2	0	2	
BMI	25.40 (3.7)	26.26 (3.7)	24.55 (4.0)	0.46
BAI	8.83 (8.2)	6.00 (5.8)	11.67 (9.8)	0.25
BDI	5.79 (6.3)	3.00 (1.3)	8.58 (8.2)	0.13
STAI	70.38 (20.4)	61.58 (9.0)	79.17 (25.5)	0.14
LESS	156.50 (84.9)	114.92 (60.9)	198.08 (89.4)	0.09
LEQ	20.21 (12.5)	13.75 (5.0)	26.67 (14.8)	0.07
PSS- TSST	16.08 (8.6)	11.00 (5.3)	21.67 (8.6)	0.03
PSS- no-stress	15.75 (10.0)	9.50 (4.9)	22.00 (10.1)	0.02

348 <sup>1</sup>p-value from Chi-square

349 SES- socioeconomic status; BMI- body mass index; BAI- Beck anxiety inventory; BDI- Beck  
350 depression inventory; STAI- state-trait anxiety inventory; LESS- life-event stress scale; LEQ- life  
351 events questionnaire; PSS- perceived stress scale.

352

## 353 **Physiological, Gene Expression, and Pro-Inflammatory**

354 **Cytokines Response to Acute Psychosocial Stress**

355 **Compared with a No-Stress Control Condition**

356 ***Physiological***

357 In the whole sample, repeated measures GLMs indicated significant within-subjects effect  
358 for salivary cortisol in response to the TSST, compared with a no-stress condition (Time x  
359 Session,  $F=4.47$ ,  $p=0.003$ , estimated effect size  $\eta^2= 0.17$ ), as well as for mean arterial pressure  
360 (Time x Session,  $F=5.31$ ,  $p=0.003$ ,  $\eta^2= 0.20$ ). Compared with controls, the ELA group exhibited  
361 significantly higher mean arterial pressure response to the TSST (Time x Status,  $F=8.59$ ,  
362  $p<0.001$ ,  $\eta^2= 0.46$ ), and a trend towards a higher cortisol response in the TSST relative to no-

363 stress ( $\Delta$ AUCi:  $F=3.58$ ,  $p=0.088$ ) (Fig 2). Notably, no significant differences were observed  
364 between the ELA and control groups in the no-stress condition (Time x Status,  $F=1.38$ ,  $p=0.257$   
365 for salivary cortisol;  $F=1.01$ ,  $p=0.402$  for MAP). Overall, these findings confirm some [52], but  
366 not all studies [7], indicating increased physiological reactivity to acute stress in young adults  
367 exposed to early adversity, compared with non-exposed individuals.

368

369 **Fig 2:** Normalized change score for physiological, gene expression, and pro-inflammatory  
370 cytokine response to the TSST relative to the no-stress session for ELA group, control group  
371 and full sample. Change scores were calculated by standardizing summary AUCi using data  
372 from both sessions and subtracting participant values from the no-stress session from those in  
373 the TSST session. Error bars represent standard error of the mean. Change scores are  
374 expressed for the full sample (grey), ELA group (red), and control group (green).

375

### 376 *Gene Expression*

377 In the whole sample, there was a significant within-subjects effect of TSST vs. no-stress  
378 condition on *NR3C1* gene expression with increased levels in the TSST (Time x Session,  
379  $F=4.85$ ,  $p=0.006$ , estimated effect size  $\eta^2= 0.19$ ). Group analysis revealed increased levels in  
380 the control group (Time x Session,  $F=5.09$ ,  $p=0.013$ , estimated effect size  $\eta^2= 0.36$ ), but not in  
381 the ELA group, which had a blunted response to the TSST (Time x Session,  $F=1.00$ ,  $p=0.406$ ,  
382 estimated effect size  $\eta^2= 0.09$ ) (Fig 3A), suggestive of *NR3C1* expression resistance and lower  
383 levels to inhibit the HPA axis. Notably, no differences were observed in *NR3C1* expression  
384 between the ELA and control groups in the no-stress condition (Time x Session,  $F=0.78$ ,  
385  $p=0.491$ ) (Fig 3B). *FKBP5* and *NFKB1* expression did not change significantly in response to  
386 the TSST vs. no-stress condition, and responses did not differ by group status.

387

388 **Fig 3:** Fold change in NR3C1 for ELA (dashed lines) and control groups (solid lines) in  
389 response to the TSST (left) and during the no-stress sessions (right). Error bars represent  
390 standard error of the mean.

391

392 ***Pro-Inflammatory Cytokines***

393 In the whole sample, PCA for the four repeated measures of IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  did  
394 not reveal a significant within-subjects effect of TSST vs. no-stress condition using repeated  
395 measures GLM analysis (Time x Session,  $F=0.29$ ,  $p=0.831$ ). Similarly, an analysis of the first  
396 AUCi component did not reveal significant differences in systemic inflammation between the  
397 TSST and no-stress conditions ( $F=2.09$ ,  $p=0.165$ ). However, an analysis of the second AUCi  
398 component (largely representing IL-6) showed significantly greater pro-inflammatory responses  
399 to the TSST relative to the no-stress condition ( $F=5.85$ ,  $p=0.026$ ) (Fig 2). No differences were  
400 observed between the ELA and control groups in response to the TSST relative to no-stress  
401 using either AUCi PCA components.

402 Exploratory analyses of each pro-inflammatory cytokine revealed a significant within-  
403 subjects effect for IL-6 in response to the TSST (Time x Session,  $F=2.97$ ,  $p=0.044$ , AUCi:  
404  $F=7.70$ ,  $p=0.018$ ), but not for the other three cytokines (IL-1 $\beta$ , Time x Session,  $F=0.80$ ,  $p=0.500$ ,  
405 AUCi,  $F=1.37$ ,  $p=0.257$ ; IL-8, Time x Session,  $F=0.85$ ,  $p=0.470$ , AUCi,  $F=0.93$ ,  $p=0.434$ ; TNF- $\alpha$ ,  
406 Time x Session,  $F=0.25$ ,  $p=0.863$ , AUCi,  $F=0.40$ ,  $p=0.537$ ). Again, responses did not differ by  
407 group status.

408

409 **Stress-Induced Cortisol Changes in Gene Expression and**  
410 ***Pro-Inflammatory Cytokines***

411 Ordinary least squares multiple regression analyses tested whether stress-induced cortisol  
412 increase in response to the TSST predicted changes in gene expression and cytokines, and  
413 whether the responses differ between the ELA and control groups. Specifically, we used cortisol  
414 slope increase from baseline to peak levels (from 30 minutes prior to testing to 15 minutes after  
415 stress onset) to predict summary changes in gene expression and cytokines in response to the  
416 TSST (Fig 4).

417

418 **Fig 4:** Scatterplot and fit lines for summary gene expression changes in *NR3C1* (top), *NFKB1*  
419 (middle), and *FKBP5* (bottom) in response to stress induced cortisol increase for ELA group  
420 (red) and control group (green). Cortisol increase calculated as the slope from baseline to peak  
421 levels, and then standardized for figure construction. Gene expression changes expressed as  
422 AUCi summary measure, which was then standardized for ease of comparison across genes.  
423  $R^2$  shown are from models with cortisol slope as the only predictor.

424

425 In the whole sample, cortisol increase did not predict significant changes in gene expression  
426 over time ( $p= 0.728$  for *NR3C1*;  $p= 0.156$  for *NFKB1*,  $p= 0.832$  for *FKBP5*). When group status  
427 was included in the regression analyses, cortisol increase predicted significant changes in  
428 *NR3C1* and *NFKB1* gene expression in response to the TSST ( $\beta= -2.66$ ,  $t= -2.90$ ,  $p=0.023$  for  
429 *NR3C1*;  $\beta= -3.61$ ,  $t= -3.67$ ,  $p=0.008$  for *NFKB1*). Moreover, cortisol increase interacted with  
430 group status such that increase in cortisol predicted increase in both *NR3C1* and *NFKB1*  
431 expression among controls, but decrease in the ELA group (*NR3C1*, Group x Cortisol Increase  
432  $\beta= 2.74$ ,  $t= 3.35$ ,  $p=0.012$ ; *NFKB1*, Group x Cortisol Increase  $\beta= 2.86$ ,  $t= 3.25$ ,  $p=0.014$ ). For  
433 *FKBP5*, group status and cortisol interaction did not reach statistical significance (Group x  
434 Cortisol Increase  $\beta= -1.84$ ,  $t= -1.36$ ,  $p=0.215$ ).

435 For pro-inflammatory cytokines, in the whole sample, cortisol increase did not predict  
436 significant changes in cytokines using a PCA for the repeated measures ( $\beta= -0.12$ ,  $t= -0.36$ ,

437 p=0.730). Further, there was no interaction by group status (Group x Cortisol Increase,  $\beta= 1.00$ ,  
438  $t= 0.67$ ,  $p=0.525$ ) (Fig 4).

439

## 440 Sensitivity Analyses

441 Sensitivity analyses were conducted using the 'leave-one-out' method. Overall, results were  
442 robust to the removal of any individual participant. Differences in sample characteristics and  
443 self-report measures remained consistent upon removal of any given participant, as did  
444 physiological, inflammatory, and gene expression responses to the TSST relative to the no-  
445 stress session. Likewise, the ELA group continued to display increased MAP responses to the  
446 TSST relative to the control group. Differences between ELA and control groups in cortisol  
447 response to the TSST relative to no-stress ( $\Delta AUC_i$ ) were modestly attenuated by removal of  
448 any given participant, but not appear to be driven by a single individual.

449 Removal of one participant in the ELA group did modify associations between stress-  
450 induced cortisol changes and *NR3C1* and *NFKB1* gene expression. Specifically, cortisol  
451 increase and group status no longer interacted to predict gene expression over time (Group x  
452 Cortisol Increase,  $\beta= 1.637$ ,  $t= 0.626$ ,  $p=0.554$  for *NR3C1*;  $\beta= 1.537$ ,  $t= 0.474$ ,  $p=0.652$  for  
453 *NFKB1*). Instead, removal of this participant increased the contribution of group status in the  
454 model, such that both cortisol slope and group status were independently associated with gene  
455 expression changes, without an interactive effect (Group Status,  $\beta= 3.524$ ,  $t=3.671$ ,  $p=0.008$  for  
456 *NR3C1*;  $\beta= 2.845$ ,  $t=2.418$ ,  $p=0.046$  for *NFKB1*). By contrast, removal of a different participant  
457 from the control group resulted in associations between stress induced cortisol increase and  
458 *FKBP5* gene expression that were previously unobserved. Specifically, models run without this  
459 participant showed a significant association between cortisol increase and *FKBP5* gene  
460 expression ( $\beta= 3.177$ ,  $t=2.534$ ,  $p=0.044$ ) as well as an interactive effect between group status  
461 and cortisol slope (Group x Cortisol Increase,  $\beta= -3.114$ ,  $t= -2.750$ ,  $p=0.033$ ).

462

## 463 Discussion

464 To our knowledge, this is the first investigation of stress-induced gene expression and pro-  
465 inflammatory cytokines changes within-individuals, comparing stratified groups of ELA-exposed  
466 and control individuals. By comparing a validated laboratory-based stressor to a no-stress  
467 condition within the same individuals, we were able to disentangle the effects of acute stress  
468 from noisy measurements in the same individuals. Further, this design allowed us to distinctly  
469 identify if/when differences between ELA-exposed and control individuals were context  
470 dependent (i.e. manifesting only during stress). Results provide preliminary evidence in humans  
471 of a dysregulated pattern of *NR3C1*, *FKBP5* and *NFKB1* gene expression activation as a  
472 consequence of ELA. Importantly, these changes manifest more acutely in the presence of  
473 stress-induced cortisol release as compared to a no-stress resting condition.

474 As predicted by previous research, the ELA group evince higher cortisol response and lower  
475 *NR3C1* gene expression in response to the TSST compared with controls, with no difference  
476 between groups in the no-stress condition. Moreover, cortisol-induced changes in gene  
477 expression revealed a decoupling between the stress-induced cortisol release and nuclear  
478 signaling in the ELA group. Cortisol reactivity was associated with increased *NR3C1* and  
479 *NFKB1* expression in the control group, but in the ELA group these associations were blunted.  
480 Findings for cortisol-induced *FKBP5* expression revealed the hypothesized pattern of increased  
481 activation in the ELA group, and decrease among control individuals, although results did not  
482 reach statistical significance in the full sample. For pro-inflammatory cytokines, only IL-6  
483 increased significantly in response to the laboratory-induced stressor, however, stress-induced  
484 cortisol release did not predict changes in cytokines levels, contrary to hypothesized prediction.  
485 Overall, we provide preliminary findings for the biological embedding of ELA via a dynamic and

486 dysregulated pattern spanning multiple levels of analysis (genomic and physiological), and  
487 which presents more acutely in response to psychosocial stress.

488 Findings concur with the receptor-mediated model of glucocorticoid signaling resistance  
489 [53]. First, ELA was associated with increased cortisol response to the TSST compared with  
490 controls, confirming some [52], but not all studies [7], indicating increased physiological  
491 reactivity in young adults exposed to early adversity. Second, chronic exposure to cortisol, as a  
492 consequence of ELA, can lead to a compensatory response whereby glucocorticoid sensitivity  
493 decreases (e.g. via decreased receptor availability). Here we replicated prior evidence of  
494 reduced *NR3C1* expression levels in ELA-exposed individuals, but only in response to acute  
495 laboratory stress. We also provide preliminary evidence that the reduced *NR3C1* expression is  
496 driven by decreased responsiveness to stress-induced cortisol release into the periphery. Third,  
497 *FKBP5* has been implicated in the glucocorticoid resistance model whereby overexpression of  
498 *FKBP5* reduces cortisol binding affinity to glucocorticoid receptors and further translocation to  
499 the nucleus [54]. Here, we do not confirm previous findings. Future studies employing larger  
500 sample sizes may be required to test for *FKBP5* response as these effects may be more subtle  
501 and/or sensitive to outliers (i.e. sensitivity analyses without a given control participant were in  
502 line with predictions). Fourth, diminished availability of the ligand-bound glucocorticoid receptor  
503 complexes in immune cells is suggested to contribute to reduced inhibition of Nf- $\kappa$ B signaling,  
504 leading to increased pro-inflammatory cytokines [34]. Here, we tested whether ELA is  
505 associated with increased activation of *NFKB1* gene, a DNA binding subunit of the Nf- $\kappa$ B protein  
506 complex. In line with expectations, stress-induced cortisol increase was associated with  
507 increased *NFKB1* expression among controls, suggesting decreased inhibitory action on Nf- $\kappa$ B  
508 signaling [55]. In the ELA group, however, stress-induced cortisol increase was associated with  
509 decrease *NFKB1* expression. Fifth, in vitro studies have established a connection between  
510 glucocorticoid exposure and diminished capacity of immune cells to inhibit pro-inflammatory  
511 cytokines in individuals exposed to psychological stress. Here, only pro-inflammatory cytokine

512 IL-6 increased significantly in response to acute stress, replicating previous studies [56].  
513 However, contrary to expectation, stress-induced cortisol release did not predict increased pro-  
514 inflammatory profile among individuals exposed to ELA.

515 The methodological strengths of this study include a laboratory-based within-subjects  
516 experimental design, which allows stronger causal inferences. We collected repeated  
517 measurements over a relatively long time scale to document changes in gene expression and  
518 pro-inflammatory cytokines. Our within-subjects, between-groups design, combined with four  
519 repeated measurements in each session, reduced biological variability and increased power to  
520 detect true associations. Finally, we tested the moderating effects of ELA, which enables tests  
521 of potential programming of biological systems.

522 We acknowledge limitations. First, this was a pilot study with a small sample size. Although  
523 comparable to similar prior investigations [26, 31, 57], the results from this study still need to be  
524 interpreted with caution. Notwithstanding, the strength of the within-subjects experimental  
525 design combined with the leave-one-out sensitivity analysis alleviate concerns about spurious  
526 findings. We focused on men in this exploratory study due to known sex differences in the  
527 stress response [40] and the small sample size for stratified analyses. Future studies with larger  
528 sample size including both males and females are needed to replicate these findings. Second,  
529 gene expression changes are tissue-specific. As a first test, we isolated PBMC from whole  
530 blood to measure gene expression changes. The exclusion of granulocytes cells provides a  
531 cleaner measure of the more active populations of lymphocytes and monocytes. Nevertheless,  
532 future studies will benefit by measuring gene expression changes in specific sub-populations of  
533 leukocytes. Third, we focused on three hypothesis-driven genes. There are multiple biological  
534 pathways that are activated in response to stress that may play a downstream role in disease  
535 susceptibility, such as the conserved transcriptional response to adversity pathway [30]. Prior  
536 research has investigated multiple genes using microarrays [23, 26-28, 31]. Future studies with  
537 adequate sample size will benefit by testing larger groups of genes/pathways. Fourth, this study

538 did not consider specific types of ELA, or timing of exposure. Here, we focused specifically on  
539 severity of multiple (i.e., minimum of three) ELA exposures up to age 18 years. Future research  
540 can explore specific types of ELA in different populations and settings. Further, our study  
541 included non-Hispanic white males and thus future research need to test whether the  
542 association generalizes to other populations. Finally, although we included a no-stress condition  
543 to control for the higher degree of noise associated with gene expression measurements, the  
544 control session did include the stress of venipuncture. However, this is unavoidable technical  
545 limitation for collecting sufficient immune cells for gene expression research.

546 In conclusion, ELA may program physiological systems in a maladaptive manner more likely  
547 to manifest during times of duress, predisposing individuals to the negative health  
548 consequences of everyday stressors. Although increased activation of the glucocorticoid-  
549 immune signaling in response to acute stress is considered adaptive in the short-term,  
550 persistent activation can increase risk for mental and physical health problems. These results  
551 could potentially identify new targets for therapeutic interventions mitigating the negative effects  
552 of early adversity, such as pharmacological agents acting on the glucocorticoid receptor and  
553 FKBP5 [32]. Further, while previous risk factors and biomarkers of stress contributed to our  
554 understanding of biological embedding processes, these are nevertheless static characteristics  
555 that have not explained health outcomes very well. For example, considering high failure rates  
556 for depression treatments, and in order to tailor individual interventions, identifying objective  
557 changes in stress-induced gene expression may help to predict short-term intervention efficacy  
558 in clinical and non-clinical settings. An example for such an effort could be to utilize models of  
559 dynamic cellular markers as individual-level factors to account for variation in intervention  
560 response and clinical outcomes [17-19]. Thus, future research in this area can have a range of  
561 impacts for basic science, intervention studies and clinical practice that will influence treatments  
562 to match the specific cellular processes operating within an individual.

563

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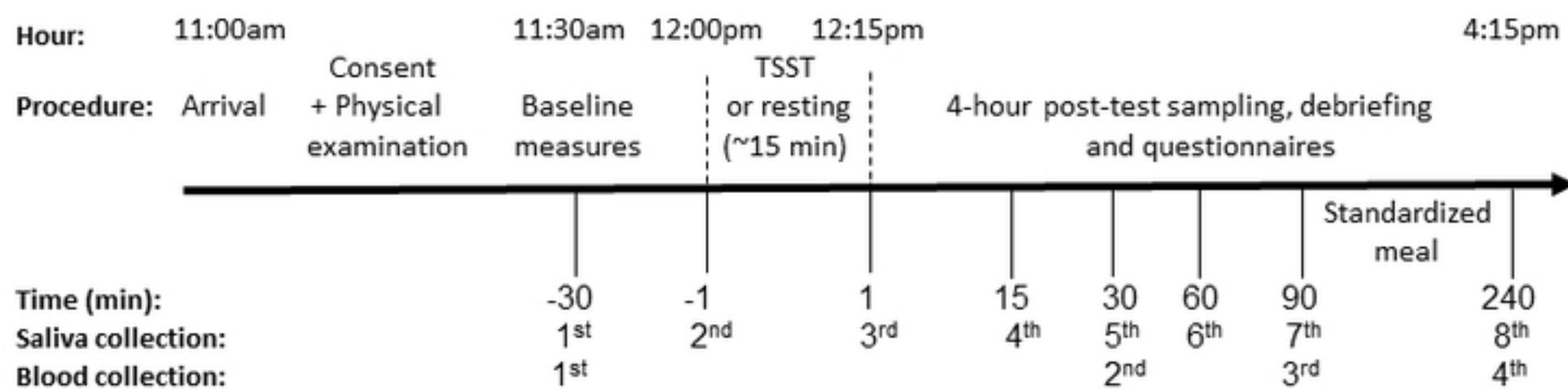


Figure 1

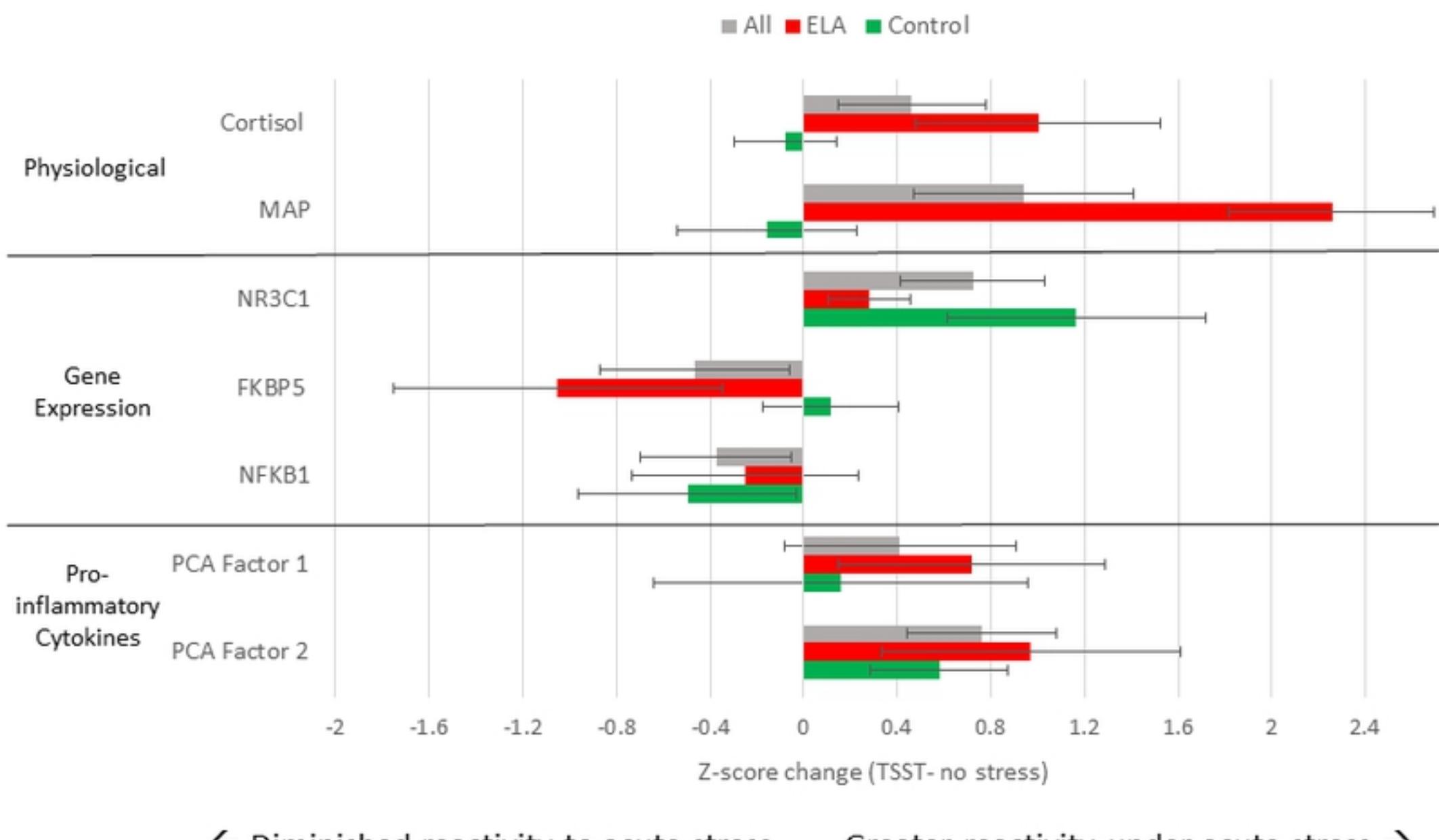


Figure 2

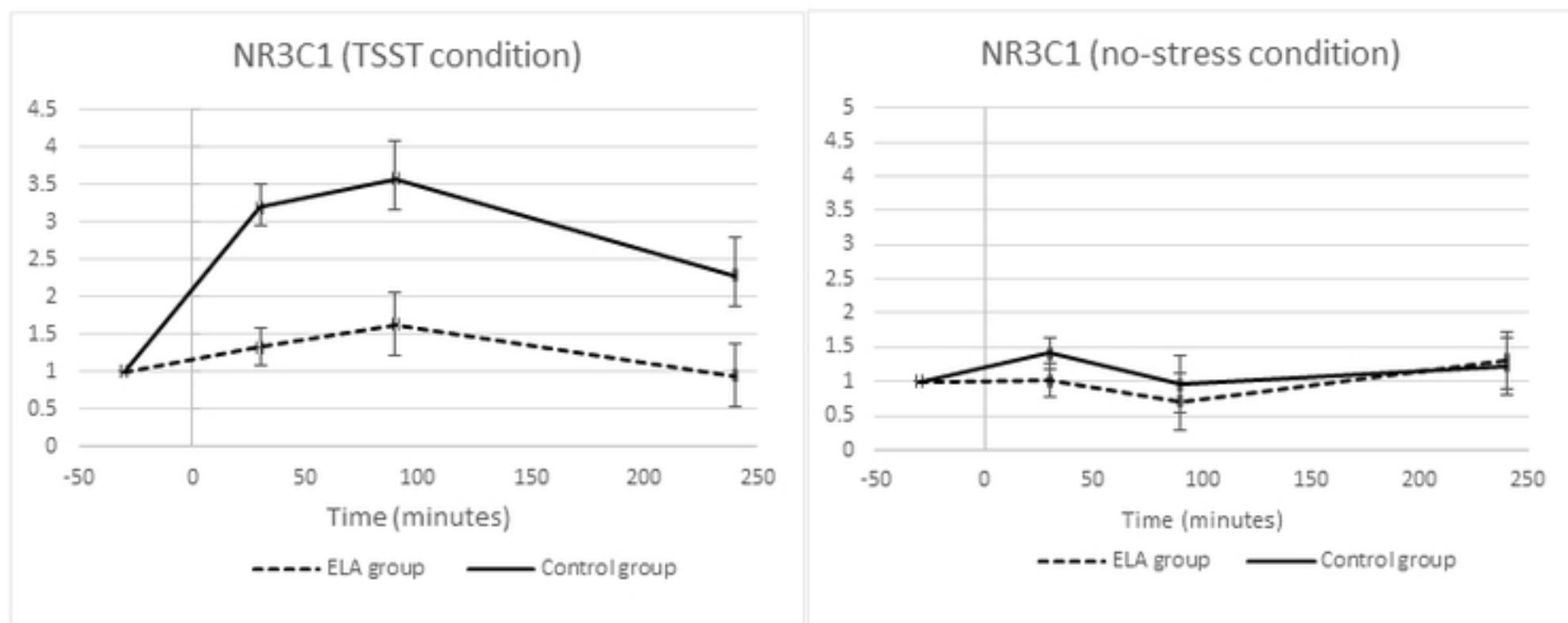


Figure 3

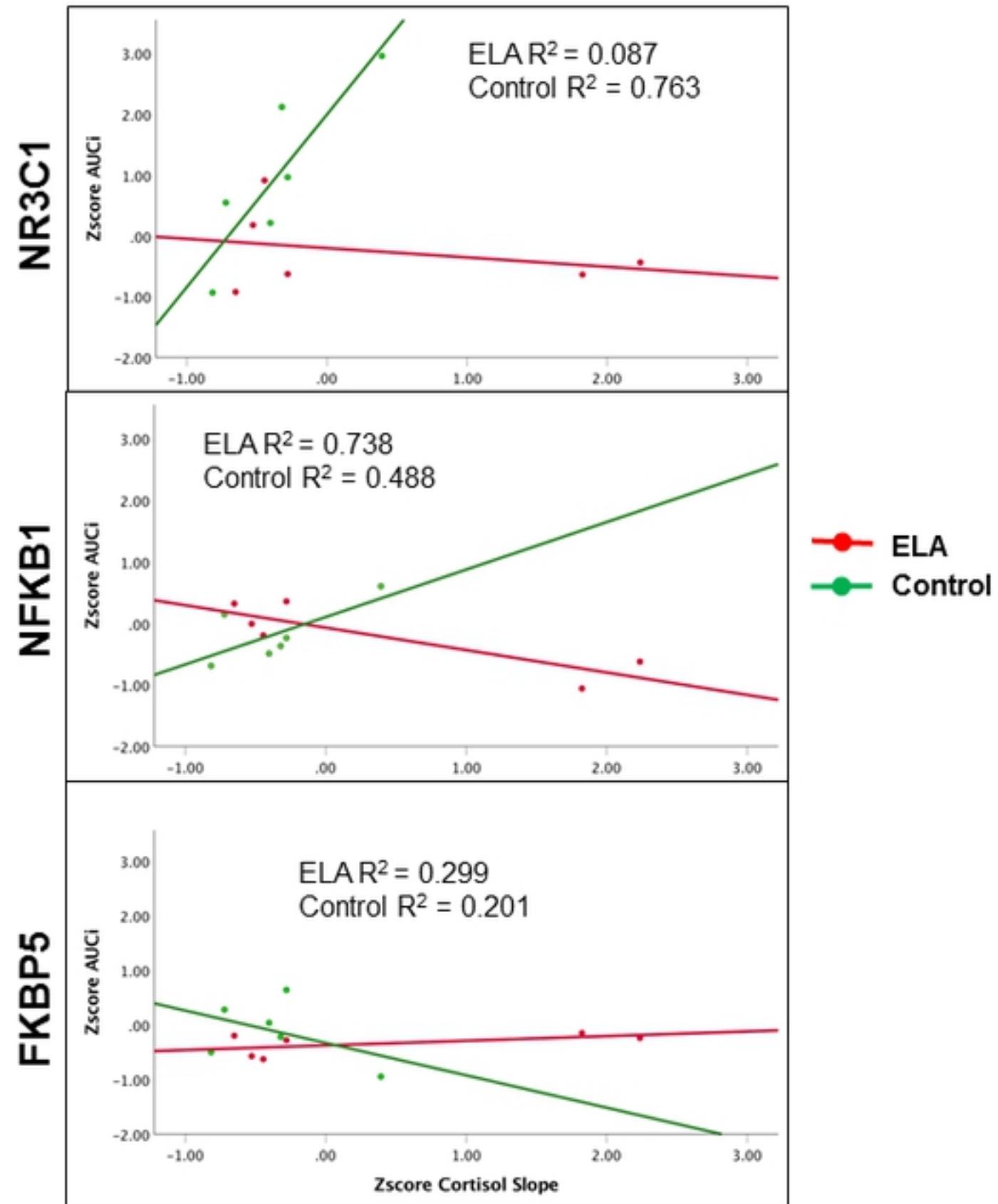


Figure 4