## Title

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## Authors

Aschbacher, Kirstin
Derakhshandeh, Ronak
Flores, Abdiel J
et al.

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# Circulating Angiogenic Cell Function is Inhibited by Cortisol in Vitro and Associated with Psychological Stress and Cortisol in Vivo

Kirstin Aschbacher[a,b], Ronak Derakhshandeh[c], Abdiel J. Flores[d], Shilpa Narayan[c], Wendy Berry Mendes[a*], Matthew L. Springer[c,e,f*]

**Affiliations**

[a] Department of Psychiatry, University of California, San Francisco,
3333 California Street, San Francisco, CA 94143.

[b] The Institute for Integrative Health, 1407 Fleet Street, Baltimore, MD 21231.

[c] Cardiovascular Research Institute,
555 Mission Bay Boulevard South, University of California, San Francisco, CA 94143.

[d] Department of Psychology, Columbia University, 406 Schermerhorn Hall,
1190 Amsterdam Avenue, New York, NY 10027.

[e] Division of Cardiology, University of California, San Francisco,
505 Parnassus Avenue, San Francisco, CA 94143.

[f] Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research,
University of California, San Francisco, 35 Medical Center Way, San Francisco, CA 94143.

** Both authors contributed equally*

*Corresponding Author:*

Kirstin Aschbacher, Ph.D.
Assistant Professor
Department of Psychiatry
3333 California Street, Suite 465
San Francisco, CA 94143-0848
Tel: 415-502-7908
Fax: 415-476-7744
Email: kirstin.aschbacher@ucsf.edu

## Abstract

Psychological stress and glucocorticoids are associated with heightened cardiovascular disease risk. We investigated whether stress or cortisol would be associated with reduced circulating angiogenic cell (CAC) function, an index of impaired vascular repair. We hypothesized that minority-race individuals who experience threat in interracial interactions would exhibit reduced CAC function, and that this link might be explained by cortisol. To test this experimentally, we recruited 106 African American participants for a laboratory interracial interaction task, in which they received socially evaluative feedback from Caucasian confederates. On a separate day, a subset of 32 participants (mean age = 26 years, 47% female) enrolled in a separate biological substudy and provided blood samples for CAC isolation and salivary samples to quantify the morning peak in cortisol (the cortisol awakening response, CAR). CAC function was quantified using cell culture assays of migration to vascular endothelial growth factor (VEGF) and secretion of VEGF into the culture medium. Heightened threat in response to an interracial interaction and trait anxiety *in vivo* were both associated with poorer CAC migratory function *in vitro*. Further, threat and poorer sustained attention during the interracial interaction were associated with a higher CAR, which in turn, was related to lower CAC sensitivity to glucocorticoids. *In vitro,* higher doses of cortisol impaired CAC migratory function and VEGF protein secretion. The glucocorticoid receptor antagonist RU486 reversed this functional impairment. These data identify a novel, neuroendocrine pathway by which psychological stress may reduce CAC function, with potential implications for cardiovascular health.


**Keywords:** Angiogenesis, endothelial progenitor cells, circulating hematopoietic progenitor cells, executive function, sustained attention, anxiety

## 1. Introduction

Psychosocial stress constitutes a significant cardiovascular risk factor in large epidemiological studies (Yusuf et al., 2004). The capacity to detect social threats and mobilize robust wound-healing responses may have conferred evolutionary survival advantages. Whereas an "adaptive" acute stress response terminates after the event has passed, chronic stress exposure may impair appropriate resolution, via a mechanism of cellular desensitization to negative feedback (e.g., insufficient inhibition of immune responses by cortisol). This study combines *in vivo* and *in vitro* methods to investigate a neuroendocrine pathway linking threat in interracial interactions with the function of circulating angiogenic cells (CACs). These findings have potential implications for social stress-related deficits in vascular repair.

Peripheral CACs, previously purported to be early outgrowth endothelial progenitor cells (EPCs) (Rehman et al., 2003; Hirschi et al., 2008), are bone marrow-derived immune cell populations involved in vascular regeneration and angiogenesis. Healthy angiogenesis is crucial for vascular regeneration (Toyama et al., 2012) and wound healing (Marrotte et al., 2010), whereas excessive angiogenesis contributes to inflammation (Hirono et al., 2009), atherosclerosis (Holm et al., 2009), and diabetic retinopathy (Titchenell and Antonetti, 2013). We use the term CACs rather than early EPCs, because although CACs exhibit endothelial qualities, CAC cultures consist predominantly of monocytic cells (Heiss et al., 2010), and their therapeutic effects are mediated by paracrine secretion of growth factors and antioxidants (Di Santo et al., 2009; Marrotte et al., 2010), rather than by endothelial differentiation (Hirschi et al., 2008).

A cardinal index of CAC function is the capacity to migrate toward growth factors, such as vascular endothelial growth factor (VEGF), a master regulator of angiogenesis (Gupta and Zhang, 2005). CAC migration *in vitro* reflects the capacity of CACs to migrate toward sites of tissue damage and promote repair via paracrine secretion of growth factors. CAC migration is decreased in patients with coronary artery disease (Vasa et al., 2001), atherosclerosis (Ohtsuka et al., 2013), diabetes (Thum et al., 2007), and older age (Chen et al., 2016). Among healthy individuals without cardiovascular disease or diabetes, reduced CAC migration prospectively predicts greater carotid artery intima-media thickness (Keymel et al., 2008) and correlates with metabolic risk factors (Aschbacher et al., 2012a) and better endothelial function (Van Craenenbroeck et al., 2010). In animals, delivering CACs or CAC-conditioned media to sites of ischemic vascular injury can regenerate damaged tissue (Kalka et al., 2000; Di Santo et al., 2009; Ma et al., 2009; Toyama et al., 2012; O'Loughlin et al., 2013). Hence, CAC function is more than a "biomarker," it is a mechanism of vascular repair.

To date, no published studies have linked psychological stress or stress hormones with CAC function; however, self-reported distress is associated with EPC number (Van Craenenbroeck et al., 2009; Chen et al., 2011). Stressful events could potentially impact CAC function via threat perceptions and secretion of glucocorticoids (GCs), such as cortisol. Cortisol is particularly reactive to social threat (Dickerson and Kemeny, 2004; Aschbacher et al., 2013), and can impair endothelial nitric oxide synthase (eNOS) expression (Liu et al.,

2009), a regulator of CAC migration (Heiss et al., 2010). Hence, the effects of cortisol on CAC function constitute a potentially important CVD risk pathway. The current study investigated this pathway among African Americans because discrimination is a chronic social stressor, and African Americans have higher age-adjusted rates of death from coronary heart disease than European Americans and other major racial/ethnic groups (Gillespie et al., 2013). To elicit social threat, we used an acute interracial interaction paradigm, which permits laboratory manipulation of the social context and quantifies the "live process" of how individuals respond to interracial interactions (Mendes et al., 2007b).

Chronic social stress is associated with decreased leukocyte GC sensitivity (Miller et al., 2008; Bellingrath et al., 2013), and with impaired wound healing (Kiecolt-Glaser et al., 2005). However, to date, no published study has explored whether CACs exhibit stress-associated decreases in GC sensitivity. While decreased GC sensitivity may protect cells from excess GC exposure, it may also impair the restoration of homeostasis after a stressor (Sapolsky et al., 2000). We hypothesized that: 1) cortisol would inhibit CAC function *in vitro*, but 2) stress-reactive participants would have lower CAC-GC sensitivity *in vivo*. To test this hypothesis, we assessed the GC sensitivity of CACs *in vitro* and cortisol *in vivo*, using the cortisol awakening response (CAR). The CAR captures the diurnal cortisol peak in the first 30 minutes post-awakening (Clow et al., 2010), and a higher CAR is associated with life stress (Chida and Steptoe, 2009).

In sum, we hypothesized that cortisol might constitute a pathway by which threat could affect CAC function. Moreover, we defined a "healthy" CAC profile as characterized by robust CAC migration to VEGF *and* sensitivity to inhibition by cortisol (i.e., an *in vitro* profile of reactivity and recovery). To test this idea, we recruited healthy African Americans from the community and investigated relationships among: 1) threat reactivity during an interracial interaction task, 2) the CAR, and 3) CAC function and GC sensitivity *in vitro*.

## 2. Methods & Materials

### *2.1 Participants*

Healthy, young African American men and women (N=106; mean age: 25.31 years, SD: 4.83; 57% female) participated in a lab study that included stressful and cooperative interaction tasks with a same-sex European American stranger (a confederate research assistant). This paradigm has previously been shown to evoke psychological and physiologic stress responses (Mendes et al., 2007a; Mendes et al., 2008). Participants were recruited through craigslist and community advertisements in the Bay Area, and were excluded for depression, smoking, and cardiovascular or steroid medications (see supplemental methods for further details). A convenient subsample of 34 participants was recruited (based on the availability of the clinical research center) to participate in a substudy that collected blood and salivary samples, roughly two months after the initial visit. Blood from two participants could not be analyzed due to an equipment malfunction, leaving a subsample of 32 participants with complete data on CAC migration (mean age: 25.75 years, SD: 5.10; 47% female; cardiovascular risk factors in Table 1). These participants displayed a range of education: high school or less (n=2), some college or currently enrolled college student (n=19), and college graduate (n=6), graduate work or

degrees (MA or MBA; n=4), missing education data (n=1). Because some current college students indicated that they received financial support from parents, income did not hold the same meaning for student versus non-student participants, and was therefore not utilized analytically. This subsample did not significantly differ from the full sample on demographic or cardiovascular risk factors. This study was approved by the Committee for Human Research, at the University of California, San Francisco, and was conducted in accordance with the Declaration of Helsinki. All participants provided written consent to participate in the study.

*2.2 Threat, Anxious Affect & Attention During an Interracial Interaction*

Affective and cognitive factors were assessed before, during, and after participants engaged in a previously standardized interracial interaction task (Mendes et al., 2008), in which the participant gave an impromptu speech about his or her strengths and weaknesses to a trained European American confederate and then completed a cooperative interaction task with the him/her (also see supplemental methods). The Positive and Negative Affect Schedule (PANAS) was used to quantify anxious affect (i.e., feeling anxious as opposed to a clinical diagnosis) and reported attentiveness before and after completing the interaction task (Watson et al., 1988). Trait anxious affect was quantified by averaging two measures of anxious affect, taken at baseline prior to the task, and roughly two months later during the blood draw visit. Threat appraisals were assessed after the speech and prior to the cooperative task, by a measure validated in previous studies (Mendes et al., 2007b; Aschbacher et al., 2012b; Aschbacher et al., 2013). The construct of threat appraisals is quantified as the ratio of two subscales that tap the separate dimensions referred to as *threat* versus *challenge* – i.e., demands (perceived stress, uncertainty, required effort) versus resources (perceptions of individual and social resources to offset task demands).

*2.3 CAC Isolation & Migration*

Blood and salivary samples were taken as part of the second visit, which took place an average of two months after the interaction task. CACs were quantified from a fasting, morning, heparinized blood draw, as in previously published studies (Aschbacher et al., 2012a). Participants were asked to refrain from exercise or caffeine intake on the morning of the draw. Women were tested during the follicular phase of the menstrual cycle. CACs were differentiated from peripheral blood mononuclear cells by removal of initially adherent cells (3 hour preplating) followed by 7 days of culture of non-adherent cells on fibronectin-coated dishes as previously described (Heiss et al., 2010; Aschbacher et al., 2012a). CAC migration was quantified by a transwell chemotaxis assay to using a modified Boyden chamber in triplicate assays. 50 ng/mL VEGF (Sigma) was placed in the lower portion of the chamber to induce a chemotactic gradient (Heiss et al., 2010), whereas cortisol was placed in both chambers (non-gradient). The final unit of analysis was the number of migrated cells per high-power microscope field, as determined using fluorescence microscopy, given that a standardized number of cells (20,000) were placed in the chamber at the start of the migration assay (see Supplementary Methods for details).

*2.4 VEGF Protein in CAC-Conditioned Media*

CACs are believed to exert their therapeutic effects by secreting growth factors, such as VEGF, that stimulate angiogenesis. Hence, we wanted to establish whether cortisol could inhibit this critical therapeutic mechanism. To test whether cortisol inhibits CAC secretion of VEGF, we first conducted a dose and timing experiment to establish the methods. Next, a second experiment was conducted to replicate the significant findings. First, PBMCs from two participants were cultured in standard EBM media with 20% FBS and Single-Quot for 12 days and 14 per standard CAC protocols (Heiss et al., 2010), and non-adherent cells were discarded. 48 hours prior to the end of the experiment, the medium was completely replaced with EBM with no additional growth factors and 5% FBS. During this 48-hour period, we contrasted treatment with and without high dose cortisol (300 and 1000 nM). Thereafter, CAC-conditioned media was collected and frozen for later VEGF ELISA (Human VEGF Quantikine ELISA kit, R&D Systems, Inc.). To replicate the key results, we again cultured PBMCs from three participants for 12 days and compared treatment in wells with versus without 300 nM cortisol (VEGF secreted in CAC-CM expressed as pg/day x $10^6$ cells).

*2.5 Salivary Cortisol Protocol*

Thirty-four participants provided salivary samples (IBL SaliCap devices) on the morning following the blood draw, for calculation of the cortisol awakening response (CAR). Of these, 26 participants provided complete data on protocol adherence. One participant's CAR data was excluded as he/she reported a 170-minute interval between the two samples, when instructed to take them 30 minutes apart. Hence all analyses using the CAR focused on the adherent portion of the sample (n=25). Samples were quantified by a competitive solid phase time-resolved fluorescence immunoassay with fluorometric end point detection (DELFIA) (Dressendorfer et al., 1992).

*2.6 Data Analyses*

Repeated measures ANCOVA and regression analyses were used to analyze the data. Group differences in cell culture experiments are expressed as the mean ± SEM, using a critical alpha of .05. The CAR was quantified as the increase from 0 to 30 minutes post-awakening (Pruessner et al., 2003). CAC glucocorticoid sensitivity (CAC-GS) was quantified as the difference in the number of cells that migrated to VEGF versus to VEGF with 1000 nM cortisol, multiplied by -1, so that higher scores represent greater glucocorticoid sensitivity. CAC functional outcomes and threat appraisals were natural-log transformed to improve the normality of the distribution. Age and gender were included as covariates in all final analyses. Education and income were not significantly related to CAC outcomes in this young sample, and hence these factors were not utilized as covariates.

Samples sizes for the following analyses vary depending on the variables considered. Specifically, task-related changes in psychological factors were analyzed in the full sample of 106, CAC results were analyzed among the 32 participants with blood samples, and CAR results were analyzed focusing on the 25 participants with full adherence data on the timing of their saliva samples and answered questions regarding the prior night's sleep quality. We confirmed that the 32 participants with CAC migration did not differ on task-related changes in anxiety or self-reported attention, threat appraisals, trait anxiety, demographic, or cardiovascular risk factors from those in the larger study of 106.

## 3. Results

## 3.1. Part I: Associations of Threat and Attention During an Interracial Interaction Task with Cortisol and CAC Function

### *3.1.1 Task-Related Change in Attention and Anxiety*

In the larger sample, attention increased significantly from pre- to post-task ($F(1,96)=8.24$, $p=.005$), whereas anxious affect decreased significantly ($F(1,96)=17.961$, $p<.001$), potentially reflecting high anticipatory anxiety pre-task. Participants who perceived the task as more threatening (i.e., situational demands exceeded their coping resources) exhibited significantly poorer sustained attention during the interaction ($r= -.313$, $p=.002$), but no changes in anxious affect ($r= -.048$, $p=.644$).

### *3.1.2 Individual Differences in Threat and Attention are Associated with the CAR*

We hypothesized that greater threat during an interracial interaction would be associated with a higher CAR, a neuroendocrine regulator of arousal, thought to prepare for the anticipated stresses of the day. A higher CAR was significantly associated with higher task-induced threat appraisals (ß=.427, $p=.046$) and poorer sustained attention (ß= -.669, $p=.002$)(Fig. 1), controlling for day-of covariates, gender, and age*, among 25 participants with complete data on CAR adherence factors (e.g., sample timing, and prior night's sleep). These analyses were also significant in the unadjusted analyses of 34 participants with complete cortisol data. Finally, poorer self-reported sleep quality the previous night was an independent predictor of a higher CAR (ß=-.501, $p=.009$).

### *3.1.3 Individual Differences in Threat and Anxiety are Associated with CAC Function*

Trait anxious affect was significantly related to lower CAC migration to VEGF (*ß*=-.545, $p=.003$; Fig. 2), controlling for age and gender*. Greater threat appraisals (demands-resources ratio) during the interracial interaction revealed a marginal association with lower CAC migration to VEGF (*ß*=-.356, $p=.060$) and no relationship to CAC-GS (*ß*=-.258, $p=.169$), controlling for age and gender. Greater perceived demands (expecting the task to be stressful and threatening), was significantly related to poorer CAC migration (*ß*=-.385, $p=.041$) but not CAC-GS (*ß*=-.281, $p=.132$). In contrast, perceived resources (expecting to perform well on the task) were not associated with CAC function. In this young, healthy, non-smoking sample, we did not see significant relationships of age, gender, or BMI with CAC migration to VEGF or CAC-GS.

## 3.2. Part II: Effects of Cortisol on CACs in Cell Culture

### *3.2.1 Cortisol Inhibits CAC Migration to VEGF at High Physiologic and Supraphysiologic Doses*

Cortisol at 1000 nmol/L significantly inhibited CAC migration to VEGF among all 32 participants ($p<.001$). We conducted a dose-response experiment (100, 300, and 1000 nmol/L cortisol) using CACs from 3 participants. The upper end of normal morning plasma total cortisol secretion is roughly 300 nmol/L (Kudielka et al., 2004; Aschbacher et al., 2014), and this level can be provoked by acute psychological stress (Kudielka et al., 2004).

* These results remained significant when additionally controlling for oral contraceptive use or removing those 3 participants from the analyses.

Cortisol impaired CAC migration at 300 and 1000 nmol/L, relative to VEGF alone (*all p's*<.05; Fig. 3a), but did not significantly decrease migration at 100 nmol/L. Possible "priming" glucocorticoid effects (Sapolsky et al., 2000) were explored, in which CACs were exposed to very low dose (30 nmol/L) cortisol for the last 48 hours prior to the migration experiment. No priming effects were observed (data not shown).

*3.2.2 The Glucocorticoid Receptor Antagonist RU486 Reverses Cortisol-Induced CAC Inhibition*

Based on previous literature (Liu et al., 2009) and the fact that lower doses of cortisol did not significantly impair migration (cortisol binds preferentially to mineralcorticoid receptors at low doses (Sapolsky et al., 2000)), we hypothesized that cortisol inhibition of CAC function would be mediated via the glucocorticoid receptor. Indeed, the glucocorticoid antagonist, RU486 (at $10^{-7}$ and $10^{-6}$ mol/L) reversed the inhibition of CAC migration by cortisol at 300 nM (*p's*<.01, Fig. 3b).

*3.2.3 Long-term, High-dose Cortisol Causes Prolonged but Reversible Inhibition of CAC Migration*

To better approximate a chronic stress exposure *in vitro (Du et al., 2009)*, we investigated whether 1) Inhibition of CAC function would be exacerbated by a longer-term exposure, and whether 2) CAC function would recover after being removed from long-term cortisol exposure. Using a 2x2 design, we contrasted: 1) 48-hour CAC pretreatment with cortisol (1000 nmol/L) versus no pretreatment, followed by, 2) presence or absence of cortisol during CAC migration (lasting 6 hours), using CACs from four participants. Hence, "recovery" was modeled by cortisol pretreatment followed by no cortisol during migration. Long-term exposure (pretreatment without recovery) did not cause greater migratory impairment than shorter-term exposure (cortisol during migration), potentially because short-term exposure already resulted in very low numbers of migrated cells. Cortisol pre-treatment was sufficient to cause a prolonged, but reversible, inhibition of CAC migration. In the recovery condition, CAC migration was significantly higher compared to the No-VEGF negative control (*p*=.020) and to VEGF with cortisol during migration (*p*=.021), whereas it was lower than VEGF alone (*p*=.035; Fig. 3c). In other words, exposing CACs to cortisol only before, but not during, the migration period partially impaired their migration, but not as much as exposure during migration - i.e., CACs partially recovered their migratory capacity when cortisol was no longer present.

*3.2.4 Cortisol significantly reduces CAC secretion of VEGF*

CACs not only respond to VEGF but also secrete VEGF (Heiss et al., 2010). These paracrine effects are considered the primary mechanism of their therapeutic efficacy for vascular repair. To test the hypothesis that cortisol may impair CAC secretion of VEGF, we first compared whether pretreating CACs from two participants with a high dose of cortisol (300 or 1000 nmol/L) for 48 hours would significantly decrease the levels of VEGF protein present in conditioned media. This was tested using ANOVA, including the dose and number of days cells were cultured. Treatment with cortisol at both 300nM and 1000nM significantly reduced the amount of VEGF protein detectable in CAC-conditioned media, relative to treatment with media alone (*p*=.028). Less VEGF was present on day 14 than on day 12. Hence, we conducted a second validation experiment with three participants' CACs, cultured for 12 days with versus without 300 nM cortisol. We counted cells on the final day

and observed that the number of cells in the two conditions did not differ (*ns*). Furthermore, this final experiment verified that cortisol significantly reduced VEGF protein (pg/day x $10^6$ cells) detected in CAC-CM ($p$=.034) (Fig. 3d).

3.3.0 Part III: Diurnal Cortisol *in vivo* is associated with CAC glucocorticoid sensitivity *in vitro*

We investigated whether higher cortisol secretion, indexed by the CAR, would be associated with lower CAC-GS. The mean value of cortisol upon awakening was 7.76 nmol/L (SEM= .97) and at 30 minutes was 9.31 nmol/L (SEM=.96), consistent with previous studies of African Americans (Fuller-Rowell et al., 2012). A higher CAR was significantly associated with lower CAC-GS, both in unadjusted correlations ($r$=-.469, $p$=.032) and in regression analyses (ß=-.524, $p$=.048; Fig. 4), controlling for age, gender, and day-of factors (prior night's sleep quality and salivary sampling protocol adherence), indicating that CAC function was less sensitive to cortisol inhibition among participants with a higher CAR†. Relations of the CAR with CAC migration to VEGF did not reach significance with covariates (ß=-.399, $p$=.126).

## 4. Discussion

These findings implicate CACs as a novel pathway by which psychological stress and cortisol may impact cardiovascular morbidity (Yusuf et al., 2004; Vogelzangs et al., 2010). CACs were previously called early outgrowth endothelial progenitor cells, but their role in vascular repair is now understood to be mediated via responses to and secretion of angiogenic factors like VEGF (Di Santo et al., 2009). We found that trait anxiety and heightened threat during an interracial interaction were both significantly associated with decreased CAC migration among young, non-smoking, African Americans without cardiovascular disease, while controlling for age and gender. CAC function is an early-stage marker of vascular repair and maintenance, detectable even in healthy young individuals. Poor CAC function is associated with early atherosclerotic plaque accumulation in individuals without cardiovascular disease (Keymel et al., 2008), and is impaired in patients with coronary artery disease (Vasa et al., 2001). In sum, the findings of this study suggest the possibility that stress and cortisol could contribute to cardiovascular disease by impairing CAC-mediated vascular repair capacity.

Using cell culture studies, we demonstrate a pattern of findings linking threat and cortisol with CAC function. First, *in vitro*, cortisol inhibits migratory and paracrine functions of CACs. Second, *in vivo,* an elevated CAR (reflecting a higher morning rise in cortisol) is related to decreased CAC glucocorticoid sensitivity. A higher CAR is also related to greater threat in the interracial interaction task. Hence, one possible interpretation is that a sustained cellular state of glucocorticoid insensitivity (e.g., by chronic social stress) may impair the body's ability to terminate angiogenic responses. Whereas acute stress responses are often beneficial, chronic stress leads to toxic health effects. Similarly, angiogenesis is beneficial in the acute context of wound repair, but when not appropriately terminated, it can contribute to inflammation and pathological conditions including

† This association remained significant when controlling for day-of covariates and oral contraceptive use.

atherosclerosis (Frantz et al., 2005; Pober and Sessa, 2007; Holm et al., 2009; Li Calzi et al., 2010). The health implications of decreased CAC sensitivity to glucocorticoids have yet to be revealed. However, we hypothesize that this pathway is most likely to be relevant to chronic disease states where pathological VEGF signaling plays a role, such as atherosclerosis (Holm et al., 2009).

Greater task-induced threat appraisals (the demands subscale) and trait anxious affect were both significant predictors of poorer CAC migratory function. Hence, a key question is whether threat appraisals are attributable: 1) merely to task stressfulness (independent of race), 2) to the interracial context of the interaction, or 3) specifically to perceptions of racial prejudice. These analyses do not investigate perceived discrimination directly, so they cannot address the latter possibility. It is unlikely that threat appraisals merely reflect task stressfulness, because anxious affect did not increase during the task. In contrast, intergroup interactions (as opposed to same-race interactions) have been shown to enhance selective attention to threats (Maner and Miller, 2013). Hence, it is possible that these associations may have particular relevance within the social context of systemic racial bias and discrimination. However, future studies using an European American comparison group are needed to test racial differences in perceived threat, discrimination, or threat-CAC associations. Furthermore, it is worth mentioning that even if threat in interracial interactions does not differ by race, so long as the physiological response to threat is evoked more frequently (e.g., due to cultural contexts of discrimination), that could also result in health disparities. However, to our knowledge, no existing study has compared racial groups on CAC function.

This study utilized parallel *in vitro* and *in vivo* models to investigate whether cortisol is a potential mediator of the effects of psychological stress on CAC function. In cell culture, cortisol impaired CAC migration and secretion of VEGF protein, at doses found *in vivo* in morning plasma cortisol (Kudielka et al., 2004; Aschbacher et al., 2014) and during acute psychological stress (Kudielka et al., 2004). The fact that the glucocorticoid receptor antagonist RU486 reversed these inhibitory effects suggests that they may be mediated via the glucocorticoid receptor. The association of a higher CAR *in vivo* with lower CAC glucocorticoid sensitivity *in vitro* supports the broader relevance of this cell culture index to human health and social stress responses. The CAR captures peak cortisol levels and exhibits relatively high intra-individual stability (Fries et al., 2009). Moreover, a higher CAR prospectively predicts psychiatric disorders (Vrshek-Schallhorn et al., 2013; Adam et al., 2014), slower cutaneous wound healing (Ebrecht et al., 2004), and, among women, faster progression of intima medial thickness (Eller et al., 2005). In sum, neuroendocrine activity is likely one of several pathways by which psychological stress may impact CAC function.

Glucocorticoid sensitivity (or glucocorticoid resistance) of immune cells has been variably defined using different sample types (whole blood, CD14+ monocytes, lymphocytes) and metrics (e.g., transcriptomics, redistribution patterns, and functional tests). Thus far, functional outcomes suppressed by glucocorticoids in human studies have been limited to secretion of a few pro-inflammatory cytokines, such as interleukin-6. In contrast, this study is the first to establish a model of CAC glucocorticoid sensitivity relevant to vascular repair and angiogenesis. Though it is difficult to faithfully model

"chronic stress in a dish," these data extend previous models of glucocorticoid sensitivity, revealing that prolonged CAC exposure to high-dose cortisol in culture can result in delayed functional recovery and impaired VEGF secretion. These data raise the question of whether similar effects occur *in vivo* with prolonged, severe stressors.

### 4.1 Implications for intervention

In the future, stress-management interventions might be optimized not only to reduce biomarkers of "damage" but also to enhance repair and regeneration. The current study provides a foundation to test stress pathways using human-to-animal CAC transplant models of vascular repair (e.g., post-myocardial infarction or leg ischemia)(Sonnenschein et al., 2011; Chen et al., 2016). For example, a previous study of patients with metabolic syndrome demonstrated that exercise improved the ability of these patients' CACs to repair a carotid endothelial injury *in vivo* in a nude mouse model (Sonnenschein et al., 2011). Future studies might therefore test whether CACs from high-stress individuals are less effective at *in vivo* vascular repair than CACs from low-stress individuals, or whether implantation with cortisol-releasing pellets mitigates the therapeutic benefits of CACs.

### 4.2 Limitations and future directions

As this study focused on African American participants during interracial interactions, it is unclear whether threat appraisals from any type of stressor would be related to CAC function. We also do not know whether other racial groups would show a similar pattern of responses. Education and income were not related to CAC function in this sample; however, this may have been due to the young age, small sample size, and prevalence of college students in whom income was confounded by parental support. This study focused on the CAR rather than task-induced cortisol changes because high levels of cortisol are reliably seen in the morning, whereas the task involved cooperative aspects and therefore was not expected to elicit a high cortisol peak. The sample size was more than adequate to establish the *in vitro* effects of cortisol on CAC migration. However, the associations of psychological factors with the CAR and CAC function, and the study of VEGF secretion, relied on a modest sample size. Alternative stress pathways may also impact CACs, including the autonomic nervous system, sheer stress effects, and oxidative stress. We have defined CAC cultures by their morphologic and adherence properties in culture, rather than by surface phenotype; however, researchers from our team and others have previously characterized CACs using a variety of surface markers (Rehman et al., 2003; Heiss et al., 2010). Due to the number of clinical samples, characterization was outside the scope of this study. Future studies are needed to test whether CAC glucocorticoid sensitivity predicts key clinical outcomes, such as delayed wound healing, unresolved inflammation, and atherosclerotic plaque evolution.

## 5. Conclusion

These data enhance our understanding of how social threat, particularly in interracial interactions, may impact cardiovascular disease. The challenge of chronic disease prevention involves mapping pathways from the psychosocial environment,

through physiology, to cellular function. Novel approaches such as this one, which combine *in vivo* and *in vitro* paradigms, have the potential to advance our understanding of how social stressors impact health outcomes. This study elucidates a novel pathway by which social stress may influence the cellular processes of vascular repair.

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## Author Contributions

K.A., W.B.W., and M.L.S designed research; R.K., S.N., and A.J.F. performed research; K.A. analyzed data; K.A., W.B.M., and M.L.S. wrote the manuscript.

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The authors have nothing to disclose.

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## Figure Legends

Figure 1: Threat and Attention are Associated with the Cortisol Awakening Response (CAR)

$^{*}p \leq .05$, $^{**}p \leq .01$. A higher CAR was significantly associated with higher task-induced threat appraisals (ß=.427, $p$=.046) and poorer sustained attention (ß= -.669, $p$=.002)(Fig. 1), controlling for day-of covariates, gender, and age, among 25 participants with complete data on CAR adherence factors (e.g., sample timing, and prior night's sleep). Scatterplots depict the unadjusted data from 34 participants with available cortisol data (threat: ß=.431, $p$=.011; attention: ß=-.451, $p$=.007). Threat appraisals were quantified as the ratio of demands to resources (log-transformed and normalized), assessed during the interracial interaction task. Sustained Attention was quantified as the residualized change score in self-reported attention from pre to post-task. The CAR is quantified as the increase in salivary cortisol from awakening to 30-minutes post-awakening.

Figure 2. Threat and Trait Anxiety are Associated with Lower CAC Migration

$^{*}p \leq .05$, $^{**}p \leq .01$. Anxiety: $ß$=-.545, $p$=.003$^{**}$, Threat (demands subscale): $ß$=-.385, $p$= .041$^{*}$, controlling for age and gender. CAC migration is quantified as the log-transformed number of cells migrating to VEGF, per high-power microscope field. Trait anxiety is the average of two measures of anxious affect taken roughly two months apart. Threat (demands) is a self-report measure taken after the stressful portion of the interaction task.

Figure 3. Cortisol Inhibits CAC Function *In Vitro*: Dose, Exposure Time, and Antagonism

$^{*}p < .05$, *ns* = not significant. VEGF = vascular endothelial growth factor (50 ng/mL) was placed in the lower chamber to establish a chemotactic gradient. The units for Migrated CACs are standardized per high-power microscope field. Fig. 3A) Varying doses of cortisol (hydrocortisone) were applied to the upper and lower chambers (non-gradient) during migration (CACs: n=3). Fig. 3B) RU486 significantly reversed cortisol inhibition of CAC migration to VEGF (CACs: n=2). Fig. 3C) A 2x2 design was used to test the effects of longer-term cortisol pretreatment ("48hr Cortisol") versus shorter-term exposure of CACs to cortisol during migration ("Cortisol DM"). Longer-term pretreatment did not result in greater CAC impairment than cortisol DM. "Recovery" was modeled as 48hr cortisol pretreatment followed by no cortisol DM (a 6hr period). Recovery values were significantly decreased relative to CAC migration to VEGF alone, but were significantly greater than CAC migration when cortisol was present DM (CACs: n=4). Fig. 3D) CACs were cultured for 12 days (n=3). In the last 48 hours, medium was replaced with growth factor-poor media, with or without 300 nM cortisol. Finally, CAC conditioned media was assayed for VEGF protein.

Figure 4. Diurnal Cortisol *In Vivo* is Associated with CAC Glucocorticoid Sensitivity *in Vitro*

*$p < .05$. A higher CAR (*in vivo*) was significantly associated with lower CAC glucocorticoid sensitivity (*in vitro*): $ß$=-.524, $p$=.048*, n=25. The CAR depicted is residualized for the full model covariates: age, gender, and day-of influences (prior night's sleep and protocol adherence to sample timing).

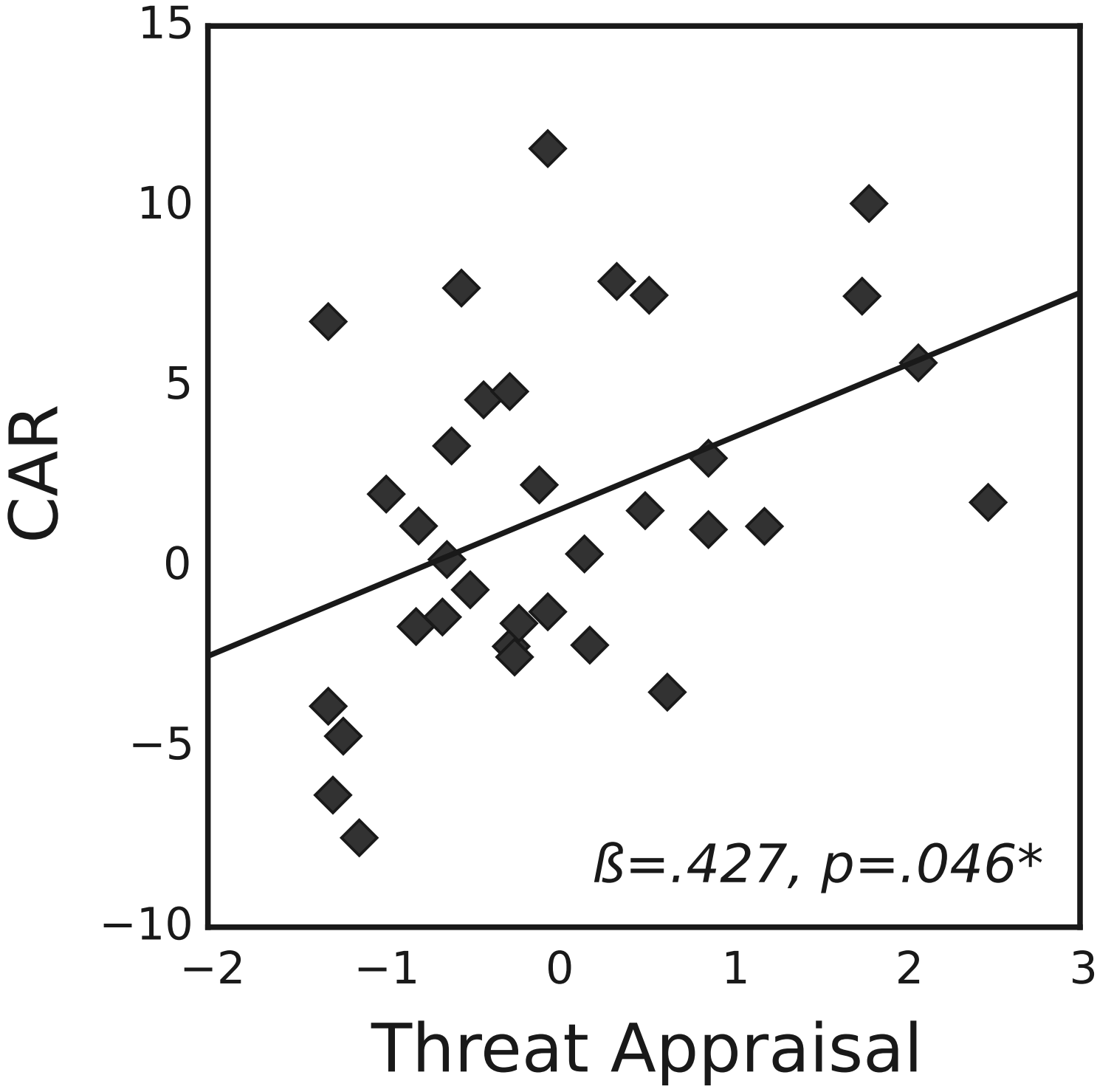
**Table(s)**

**Table 1. Cardiovascular Risk Characterization**

| Cardiovascular Risk Factor | Statistical Estimate |
|---|---|
| Age (years)[a] | 25.75 (5.10) |
| Female Gender[b] | 15 (47%) |
| Body Mass Index[a] | 24.65 (3.58) |
| Triglycerides (mg/dL)[a] | 59.10 (27.09) |
| HDL (mg/dL)[a] | 53.07 (13.80) |
| LDL (mg/dL)[a] | 77.26 (22.02) |
| Fasting Glucose (mg/dL)[a] | 82.23 (6.63) |
| Systolic Blood Pressure (mm Hg)[a] | 128.94 (25.16) |
| Diastolic Blood Pressure (mm Hg)[a] | 88.36 (21.98) |

Note. *a* = Mean (SD), *b* = n (%). *N* = 32: Participants with available CAC migration data. This sample did not significantly differ from the larger sample with task reactivity data (N=106) on these risk factors.

Figure(s)



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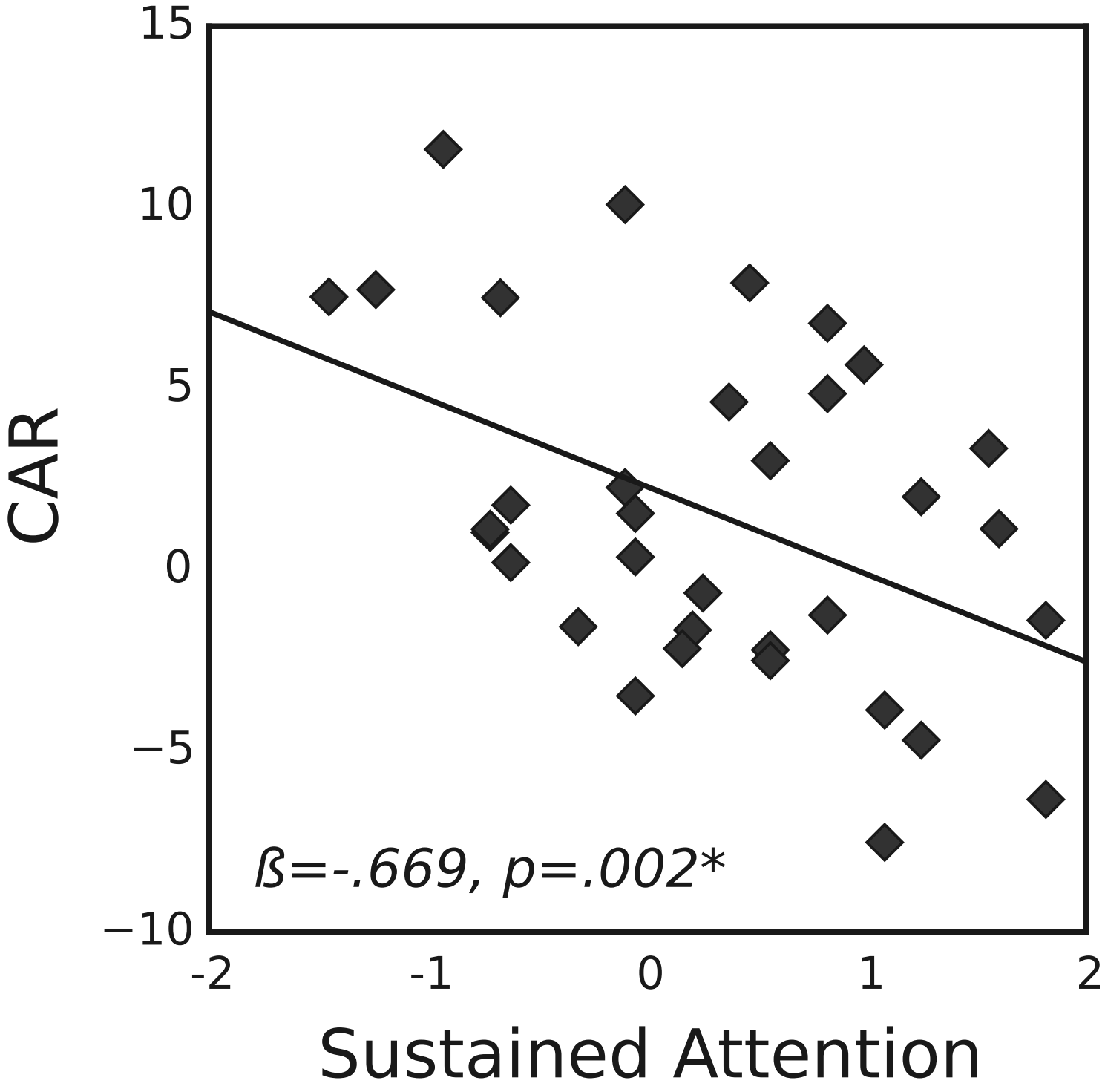

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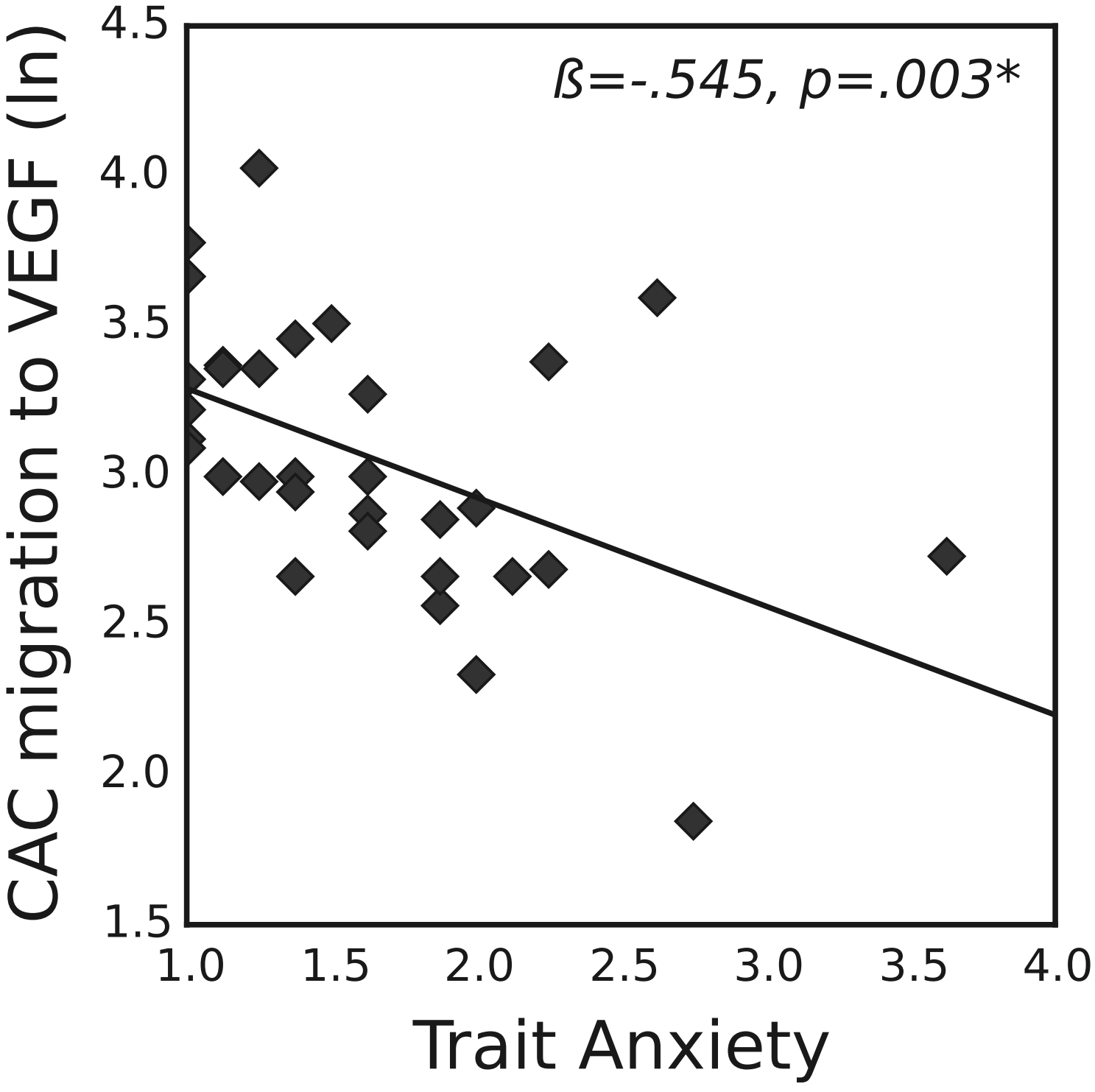
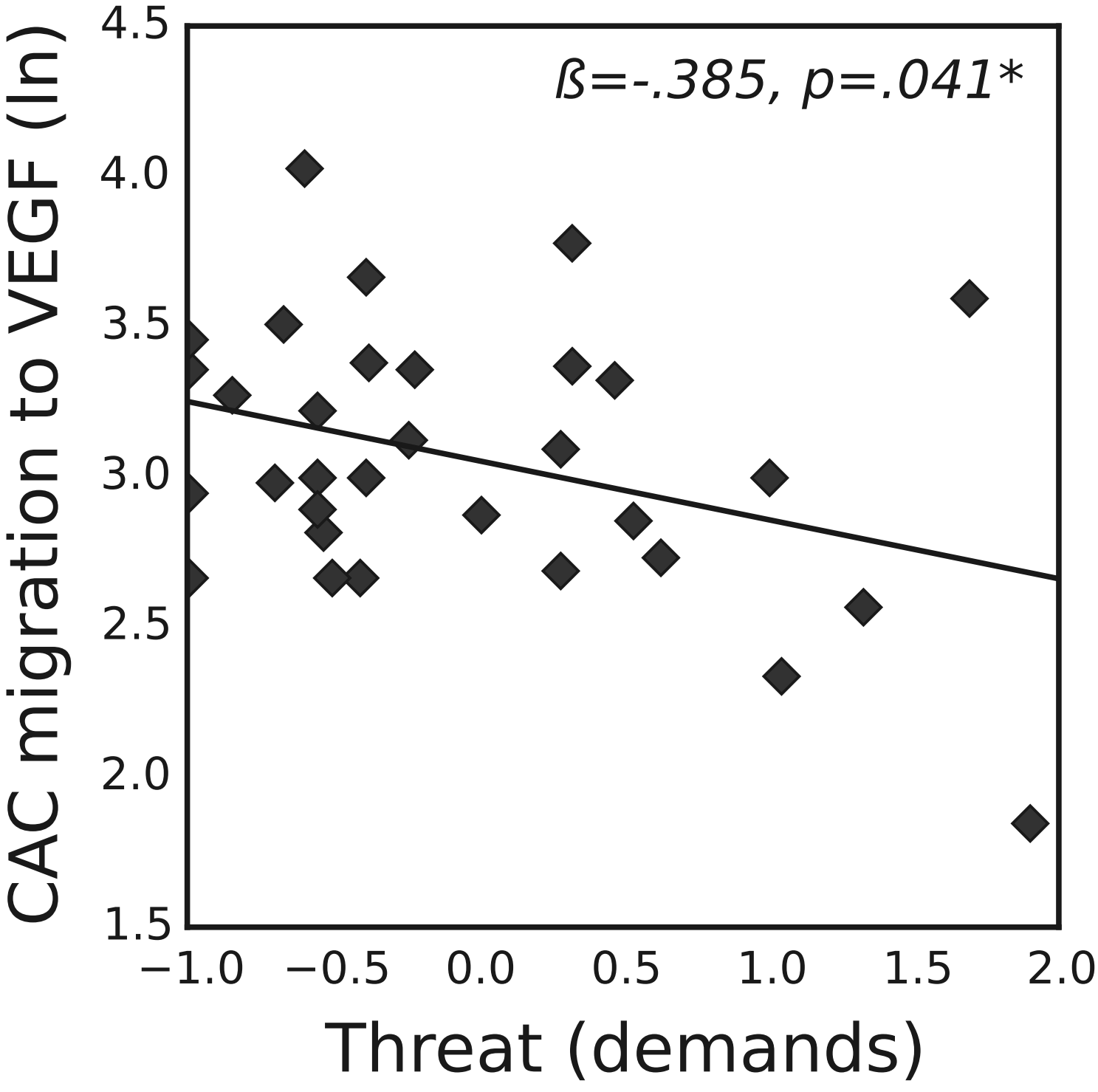
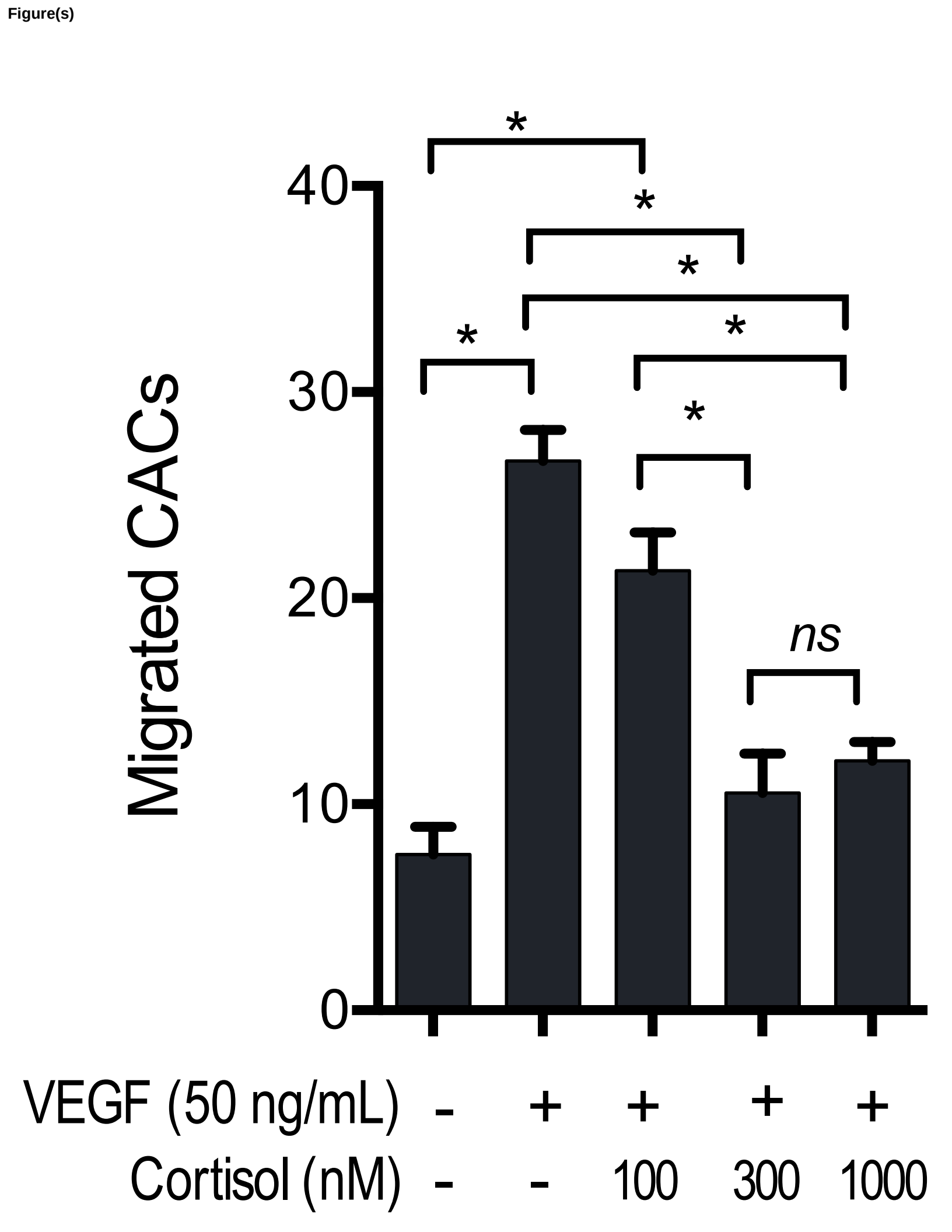

ß=-.545, p=.003*
CAC migration to VEGF (ln)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Trait Anxiety

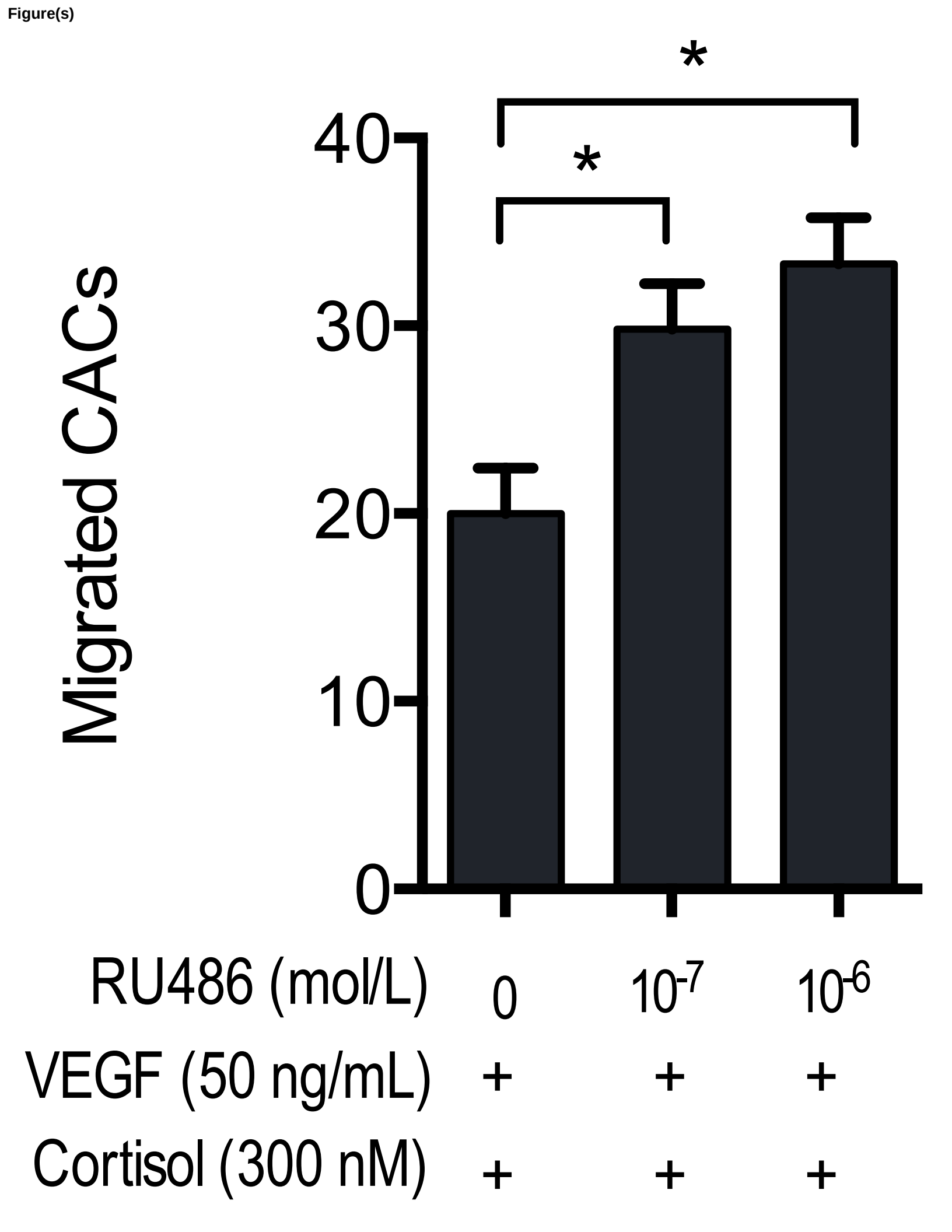
Figure(s)


ß=-.385, p=.041*
CAC migration to VEGF (ln)
Threat (demands)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
−1.0
−0.5
0.0
0.5
1.0
1.5
2.0

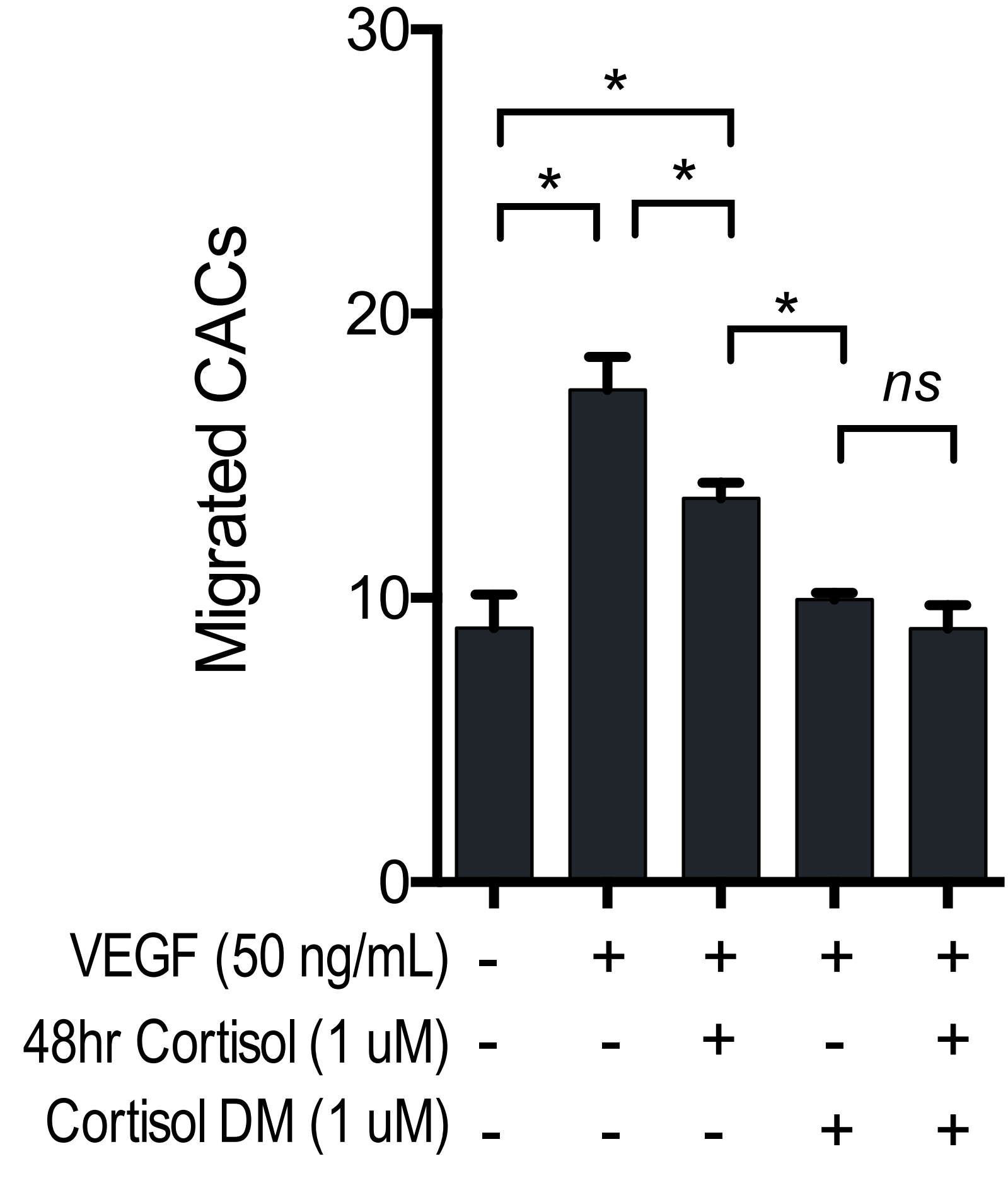
Figure(s)


Migrated CACs
40
30
20
10
0
*
*
*
*
*
*
ns
VEGF (50 ng/mL) - + + + +
Cortisol (nM) - - 100 300 1000

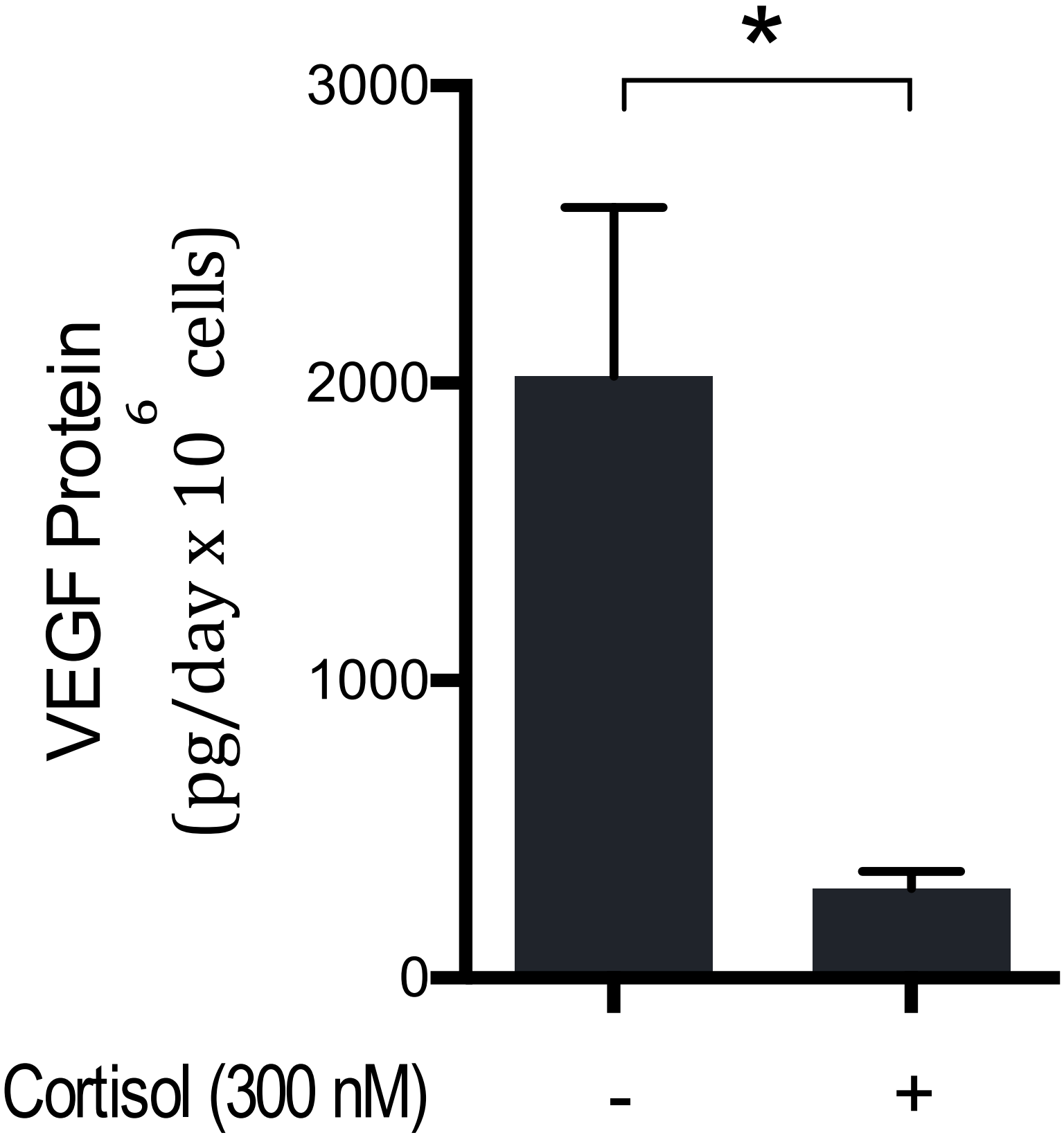
Figure(s)


*
*
40
30
20
10
0
Migrated CACs
RU486 (mol/L)
0
10-7
10-6
VEGF (50 ng/mL)
+
+
+
Cortisol (300 nM)
+
+
+

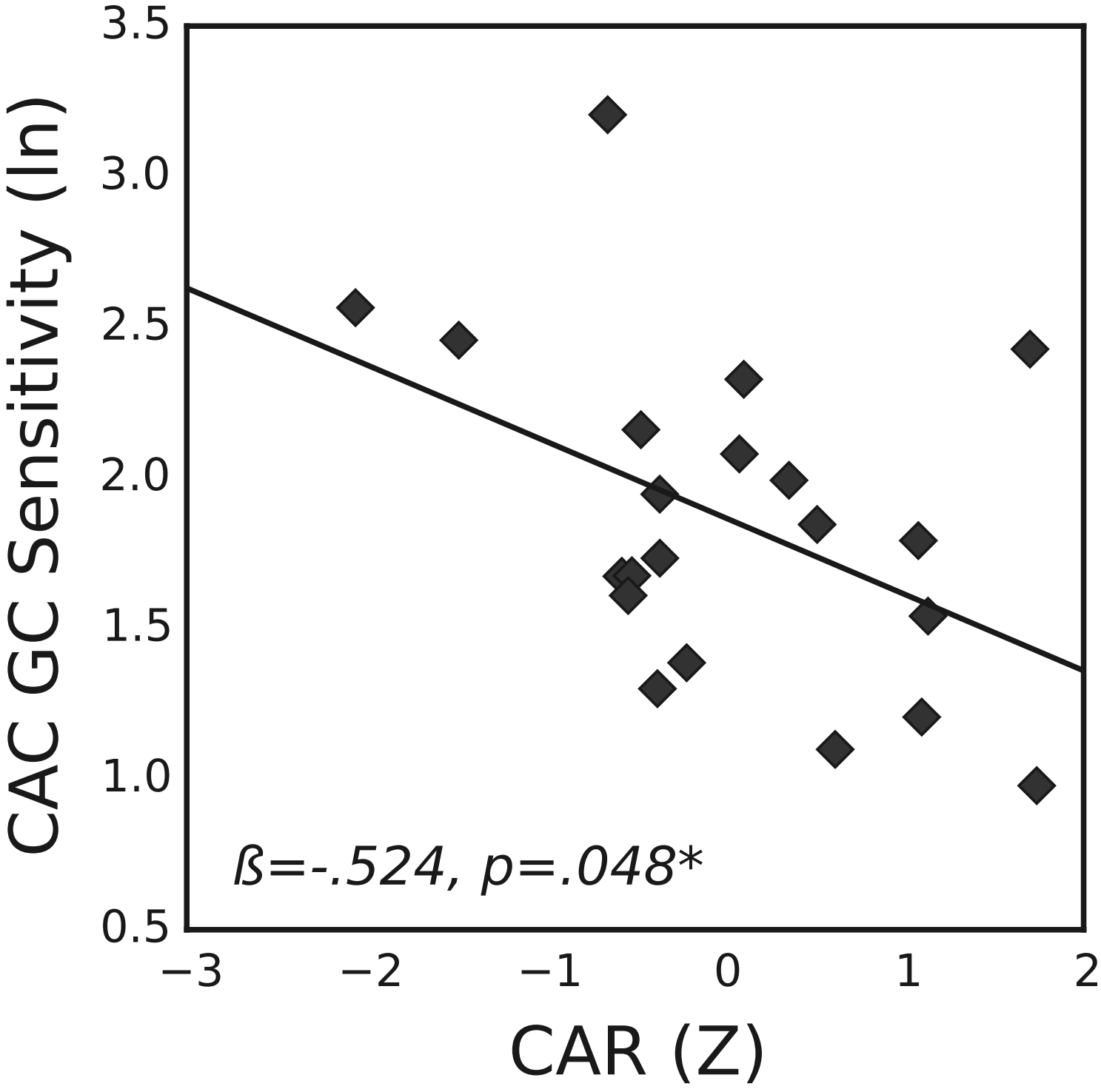
Figure(s)


30
20
10
0
Migrated CACs
*
*
*
*
ns
VEGF (50 ng/mL) - + + + +
48hr Cortisol (1 uM) - - + - +
Cortisol DM (1 uM) - - - + +

Figure(s)


*
3000
2000
1000
0
VEGF Protein
(pg/day x 10^6 cells)
Cortisol (300 nM)
-
+

Figure(s)


CAC GC Sensitivity (ln)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
−3
−2
−1
0
1
2
CAR (Z)
ß=-.524, p=.048*

**Optional e-only supplementary files**

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## Highlights

- We model social threat in interracial interactions among African Americans.
- We assayed circulating angiogenic cell (CAC) function, a marker of vascular repair.
- In vivo, threat was associated with higher cortisol and decreased CAC function.
- In vitro, cortisol inhibited CAC function.
- Social threat may impact vascular repair via a cortisol-CAC pathway.

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*Contributors

## Author Contributions

K.A., W.B.W., and M.L.S designed research; R.K., S.N., and A.J.F. performed research; K.A. analyzed data; K.A., W.B.M., and M.L.S. wrote the manuscript.

## Conflict of Interest

The authors have nothing to disclose.

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# Supplemental Methods

*Participant Exclusion/Inclusion Criteria*

Exclusion criteria included current smoking; a report of a physician's diagnosis of hypertension, a heart murmur, CAD, diabetes, renal disease, dyslipidemia, cancer, autoimmune disease; having a pacemaker; pregnancy or breastfeeding; a mood disorder; blood draw phobia; or use of steroid medications (oral or topical) or cardiovascular medications, such as anti-hypertensives, anti-coagulants, statins, beta-blockers. Three women were taking oral contraceptives; hence, this was statistically controlled for in all final analyses of CACs.

*The Interracial Interaction Task*

In the larger sample (N=106), a previously published, laboratory-based, interracial interaction task was conducted to elicit psychological reactivity (1). African American participants provided initial consent and then rested to establish basal responses. Next, they were introduced over a television monitor with a live feed to a White, same-sex member of the research team posing as a participant (i.e., confederate). The dyad was instructed that one of them would be giving a speech about their strengths and weaknesses, while the other one would evaluate the person giving the speech. After the speech, the dyad was brought together in the same room, where they received evaluative feedback and completed cooperative tasks. The evaluations, previously shown to elicit a physiologic stress response (1), were standardized and delivered by a written form.

*Psychological Measures*

Self-reported affect and cognitive appraisals were assessed before, during and after participants engaged in the task (1). Based on our previous research demonstrating that threat reactivity is a key psychological factor associated with heightened cortisol (2), we hypothesized that threat would also be related to poorer CAC function. Moreover, as threat activates neural circuits (connecting the amygdala with the prefrontal cortex) associated with anxiety and poorer sustained attention (3), we adapted the Positive and Negative Affect Schedule (PANAS) to construct brief measures of self-reported anxious affect and attention before and after the task. Anxious affect was assessed as the mean of four items: "scared, nervous, afraid, jittery." Attention was assessed using the mean of the items: "attentive" and "alert." Responses were scored by Likert scale (1= Not at all, 5 = A great deal). Cronbach's alphas for anxiety and attention were .77 and .78 respectively, including pre and post task items.

Cognitive threat appraisals during the interaction task were assessed as in previous studies (1). The appraisal questionnaire includes items assessing the amount of perceived demands of the situation (e.g., "The upcoming task is very stressful") and perceived resources (e.g., "I have the abilities to perform the upcoming task successfully.") Demands and resources were assessed on a 7-point scale (1=Strongly Disagree to 7=Strongly Agree) with four items each (demands: $\alpha$=.76; resources: $\alpha$=.83). Threat appraisal was quantified as the ratio of demands to resources, with higher ratios indicting greater perceived demands relative to resources, and hence, a psychological state of *threat.* Five participants did not answer all post-task questions and were not included in the analyses of change. In the larger study, half the participants were administered intranasal oxytocin, and our analyses established that this did not impact the psychological factors examined herein (e.g., changes in anxiety, attention); hence, it was

included as a covariate in all analyses of these factors. Biological outcomes were measured during a separate visit, and anxious affect was reassessed prior to the blood draw on that same day.

*Visit #2: Saliva and Blood Sampling*

In a second visit, roughly two months after the laboratory task, a convenient subsample of 34 participants returned and provided blood and saliva samples. Blood from two participants could not be assayed due to technical problems, yielding 32 participants with CAC assays (mean age: 25.75 years, SD: 5.10; 47% female). This subsample did not significantly differ from the full sample on demographic or cardiovascular risk factors.

*Saliva Collection Protocol*

Research staff instructed participants in how to use IBL SaliCap tubes to take saliva samples, following a standard, home-sampling protocol (4). Participants practiced collecting a sample in front of research staff to verify their understanding. Participants were instructed to provide samples on the morning following the blood draw, immediately upon awakening and 30-minutes after awakening. To assess protocol adherence, participants were asked to record the exact time each sample was gathered, and research staff sent reminders to participants via text messages or phone calls. Participants also answered questions about the previous night's sleep quality, which is known to impact the CAR (4). Thirty-four participants returned salivary samples for cortisol assays, and 26 of those provided data on potential confounds and adherence. CAR data for one participant were excluded because the second sample was taken 170 minutes after the first sample (i.e., CAR-time). The mean CAR-time in the remaining 25 participants with adherence data on the timing of their salivary samples was 33 minutes (SD = 9.18). Poor sleep and protocol adherence (CAR-time) were controlled for in all subsequent analyses of the CAR.

*Cortisol Assay*

Salivary samples were frozen at -80° until analysis. After thawing, samples were centrifuged at 2000g for 10 minutes to obtain a clear supernatant. 100 µL of saliva was used for duplicate assays using a competitive solid phase time-resolved fluorescence immunoassay with fluorometric end point detection (DELFIA)(5). 96-well-Maxisorb microtiter plates were coated with polyclonal swine anti-rabbit immunoglobulin, incubated for 24 h at 4°C, and washed three times with wash buffer (pH=7.4). Next, plates were coated with a rabbit anti-cortisol antibody and incubated for 48h at 4°C. Standards were formed by mixing synthetic saliva mixed with cortisol in a range from 0-100 nmol/L. 50 µL of biotin-conjugated cortisol was added, and after 30 min of incubation, the non-binding cortisol / biotin-conjugated cortisol was removed by washing (3x). 200 µl europium-streptavidin (Perkin Elmer, Life Science Turku, Finland) was added to each well for 30 minutes, and washed 6 times. Next, 200 µl enhancement solution was added (Pharmacia, Freiburg, Germany), and within 15 min on a shaker, it induced fluorescence detected using a VICTOR™ *X*4 Multilabel Plate Reader (Perkin Elmer, Massachusetts, USA). A standard curve was generated, and the cortisol concentration of the samples was calculated. The intra-assay coefficient of variation was between 4.0% and 6.7%, and the corresponding inter-assay coefficients of variation were between 7.1% -9.0%. Cortisol values are given in nmol/L.

*Blood Draw Protocol*

Participants came into the Clinical Research Center at University of California, San Francisco for a fasting blood draw between 9-11 am. Women were tested during the follicular phase of the menstrual cycle when estrogen is at the lowest point. Participants were asked to refrain from exercise or caffeine intake on the morning of the draw. After participants had rested for 15 minutes, a trained research nurse drew blood. Whole blood collected into sodium heparin tubes was processed within 30 minutes, to isolate serum, plasma, and peripheral blood mononuclear cells (PBMCs).

*CAC isolation*

PBMCs were isolated by density centrifugation in Accuspin™ System-Histopaque-1077 (Sigma-Aldrich). PBMCs were plated for three hours in fibronectin-coated dishes with endothelial basal medium (EBM-2 Cambrex), supplemented with EBM-2MV SingleQuot and 20% FBS to remove adherent circulating cells and shed endothelial cells. At the end of the three hours, the initially non-adherent cells were harvested and frozen in liquid nitrogen at a concentration of 1 x $10^7$ cells/mL in EBM-2 with EBM-2MV SingleQuot, 20% FBS and 10% DMSO. Prior to functional assays, PBMCs were thawed and cultured on fibronectin-coated dishes as previously described (6-8), for 7 days in EBM-2 media with EBM-2MV SingleQuot and 20% FBS, refreshing the media every two days. Notably, we did not add hydrocortisone to the media, as per the SingleQuot, to prevent confounding of cortisol experiments. Prior to the migration assay CACs were isolated by washing off non-adherent cells. VEGF was placed in the lower portion of the Boyden chamber to induce a chemotactic gradient, whereas cortisol was placed in both chambers (non-gradient). RU486 (Sigma) was used for the glucocorticoid receptor antagonist experiment. Previous studies by our lab (7, 9) have characterized the surface phenotype of these 7-day CAC cultures as follows: *Ulex europeus* agglutinin lectin binding and acetylated LDL uptake (>95% double-positive), CD14 (68-70%), CD11b (54-55%), CD45 (85-93%), KDR (24-49%), CXCR4 (57-59%), CD31 (49-63%), CD3 ((≤1%), CD34 (≤1%), and CD133 (≤1%).

*CAC Migration*

These assays have been validated in a previous study contrasting older patients with coronary artery disease with older healthy and younger healthy men and women (8). 600 µL of EBM-2 media was added to the bottom of a 24-well transwell chamber (Corning). 2 x $10^5$ cells (post 7-day culture) were resuspended in 100 uL EBM-2 supplemented with 0.5% BSA, added to each Boyden chamber insert (8 µm pores, Corning) and placed in the corresponding 24-well tissue culture plate. For the primary migration assay conducted among all 32 participants, three conditions were tested in triplicate: 1) no VEGF control, 2) 50 ng/mL VEGF (Sigma) placed at the bottom of the chamber, establishing a chemotactic gradient, 3) VEGF (bottom chamber only) + 1 uM cortisol (Sigma), applied with an even distribution (top and bottom chamber). Cells migrated during a 6-hour incubation period at 37°C. Cells attached to the underside of the insert membrane were fixed in 4% formaldehyde and cells remaining on the topside of the membrane were removed with a cotton swab. The membrane was mounted on a glass slide and stained using Hoechst 33342 (Invitrogen). The number of migrated cells in 5 standardized fields per membrane was counted using fluorescence microscopy. The final migration count was averaged across the triplicates, using the median of the 5 fields.

*VEGF protein in CAC-Conditioned Media*

Prior to the primary experiment, samples of EBM + 5% FBS were tested, and were found not to yield detectable levels of VEGF protein, thus verifying that the culture medium did not contribute

significant background noise. To investigate the impact of culture time and cortisol dose, we first cultured PBMCs from two participants in standard EBM media with 20% FBS and Single-Quot for 12 and 14 days (7), and non-adherent cells were discarded. In the last 48 hours, the medium was completely replaced with EBM with no additional growth factors and 5% FBS, with or without cortisol present (at 300 or 1000 nM). CAC-conditioned media was collected and frozen for later VEGF ELISA (Human VEGF Quantikine ELISA kit, R&D Systems, Inc.). The intra- and interassay coefficients of variation respectively range from 3.5-6.5 and 5.0-8.5, and the limit of detection is less than 5.0 pg/mL. Both the 300 nM and 1000 nM doses of cortisol inhibited VEGF secretion, but there was no significant difference in the magnitude of the inhibition. To replicate the initial finding that 300nM cortisol reduced VEGF secretion in CAC-CM after 12 days of culture, we then conducted a second experiment with PBMCs from another three participants (results expressed as pg/day x $10^6$ cells).

*Data Analyses*

Stress reactivity in the larger sample (N=106) was assessed using repeated measures to establish whether anxiety and attention changed significantly in response to the task. To assess the relationship between stress reactivity, the CAR, and CAC function, residualized change variables were created inputting attention and anxiety pre-stress task as predictors of attention and anxiety post-stress task, and saving the standardized residuals. As in previous publications (10), the CAR was calculated as the difference score of cortisol measured at 30 minutes post-awakening minus immediately post-awakening. Twenty-five participants who reported valid adherence data (i.e., sampling times and potential confounds) were included in the analysis. Associations between the CAR and CAC function were assessed using regression analyses, which controlled for day-of factors (previous night's sleep quality and adherence to protocol timing) (4, 11). Age and gender were not significantly associated with either the CAR or CAC-GS, but were included as covariates in final regression analyses. Group differences in cell culture experiments are expressed as the mean ± standard error of the mean, and were tested using repeated measures ANOVA with a critical alpha of .05. CAC glucocorticoid sensitivity (CAC-GS) was quantified by subtracting the number of cells that migrated to VEGF with cortisol (1000 nM) from the number migrating to VEGF alone, and multiplying by -1 so that higher scores represent greater glucocorticoid sensitivity. For correlational analyses, CAC migration to VEGF, CAC-GS and threat appraisals were natural-log transformed to improve the normality of the distribution.

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