STUDY TITLE: Key Dimensions of Posttraumatic Stress Disorder and Endothelial Dysfunction

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Study Protocol and Statistical Analysis Plan

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1. Study Purpose and Rationale

Cardiovascular disease (CVD) remains the leading cause of death in the United States,[1] and the mortality reductions of prior decades have stalled.[2] Stress is a long-hypothesized CVD risk factor,[3] and posttraumatic stress disorder (PTSD)—the quintessential stress-related disorder—prospectively predicts incident CVD.[4-7] Trauma and PTSD are common,[8,9] and experts have called for increased CVD surveillance after trauma and PTSD treatment trials powered to reduce CVD risk.[10] To advance surveillance and intervention efforts, we must first identify intermediary mechanisms by which PTSD influences CVD risk. Second, we must understand which dimensions of PTSD should be targeted to reduce activation of those intermediary CVD risk mechanisms. The goal of this study is to determine which PTSD dimension(s) to target to offset endothelial dysfunction, one of the earliest modifiable precursors to CVD.[11-14]

Endothelial dysfunction is implicated in the pathophysiology of atherosclerosis and CVD.[12,15] Lower flow-mediated vasodilation (FMD), a functional measure of endothelial dysfunction, is associated with increased risk of CVD events in population-based samples.[12,16-18] PTSD may increase CVD risk via endothelial cell (EC) damage. Negative emotion and stress inductions are related to lower FMD[19-21] and higher levels of EC-derived microparticles (EMPs),[20,22] a cellular measure of endothelial dysfunction. Only <u>two</u> studies in select populations of trauma-exposed individuals—one of male veterans and one of mostly male police officers—have found that elevated PTSD symptoms were associated with lower FMD.[23,24] This early work points to endothelial dysfunction as a potential intermediary mechanism of PTSD's association with CVD. The proposed study will comprehensively determine PTSD's association with functional and cellular measures of endothelial dysfunction in community-dwelling men and women exposed to a wide range of traumas.

Determining that PTSD diagnosis or total symptoms are associated with endothelial dysfunction is insufficient to guide surveillance and intervention. PTSD is heterogeneous, comprising dimensions of fear and dysphoria;[25] these factors can be decomposed into lower-order symptom dimensions (e.g., avoidance, numbing).[26] It is unclear which aspects of PTSD are most "cardiotoxic." Fear is a core component of PTSD, whereas dysphoria is considered more auxiliary.[25] Both dimensions can be assessed objectively. Fear (e.g., exaggerated fear acquisition, impaired fear inhibition and extinction) can be measured at the psychophysiological level,[27-29] and attentional bias to loss-related stimuli can provide an objective measure of dysphoria.[30,31] Elevated psychophysiological fear may promote altered autonomic nervous system activation, inflammation, and oxidative stress, all of which impact EC health. We hypothesize that this key pathological response to trauma will be most strongly associated with endothelial dysfunction. However, we will also test if dysphoria and lower-order symptom dimensions are related to endothelial dysfunction to comprehensively consider intervention targets.

This study will examine cross-sectional and longitudinal associations of PTSD and its underlying dimensions with functional (FMD) and, secondarily, cellular (EMPs) measures of endothelial dysfunction. The sample will include 160 adult men and women (80 individuals with PTSD and 80 trauma-exposed matched controls) without CVD. All participants will complete a baseline assessment, and a subset of the sample (40 with PTSD, 40 trauma-exposed controls) will complete a 2-year follow-up during which several of the initial measurements will be repeated. At both assessments, PTSD diagnosis and symptom dimensions will be interview-based.

Psychophysiological fear will be measured during fear conditioning and extinction,[29] and we will use an attention allocation task-based measure of dysphoria.[31]

Primary Aim 1: To determine if current PTSD diagnosis is associated with endothelial dysfunction.

Hypothesis: Men and women with vs. without PTSD will have lower FMD and, secondarily, greater EMP levels.

Primary Aim 2: To examine which PTSD dimensions are most associated with endothelial dysfunction.

Hypotheses: (1) Psychophysiological fear responses (high fear load after conditioning and impaired fear inhibition during extinction determined by startle and skin conductance) will be independently associated with lower FMD and, secondarily, greater EMP levels. (2) Dysphoria (determined by an attention allocation task) will be less strongly related to lower FMD and, secondarily, greater EMP levels than fear responses. Additionally, we will explore whether lower-order symptom dimensions of PTSD are associated with endothelial dysfunction.

Secondary Aim: To examine how fear, dysphoria, and lower-order symptom dimensions (and changes in these measures) predict change in endothelial dysfunction over 2 years in a subset of the sample.

Exploratory Aim: To examine whether autonomic imbalance, inflammation, and oxidative stress explain any associations of PTSD dimensions with endothelial dysfunction.

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2. Study Design

The sample for this study will comprise 160 community-dwelling adult men and women (*n*=80 with PTSD and *n*=80 trauma-exposed matched controls) without a history of CVD. Participants will be recruited from the greater Los Angeles community via a number of approaches, including websites (e.g., UC Health Clinical Trials, Clinicaltrials.gov), University of California, Los Angeles (UCLA) primary care and OB/GYN clinics, clinics at the VA Greater Los Angeles Healthcare System, community centers, and word-of-mouth referrals. All participants will complete an initial phone screening. Participants must also meet inclusion and exclusion criteria, as specified below. Approximately 220 participants will be screened and complete the Diagnostic Interview Assessment, of which 160 will be enrolled.

Inclusion Criteria

1) Aged 18+ years
2) History of exposure to a psychological trauma (e.g., natural disaster, physical assault)
3) Fluent in English
4) Willing to and capable of providing informed consent

Additional Inclusion Criteria for the PTSD Group

1) Diagnosed with current PTSD (duration of at least 1 month) using the Clinician-Administered PTSD Scale for *DSM-5* (CAPS-5)[1] at the diagnostic interview assessment
2) Score ≥33 on the PTSD Checklist for *DSM-5* (PCL-5)[2] at screening

Exclusion Criteria

1) History of CVD (i.e., diagnosis of myocardial infarction, unstable angina, heart failure, peripheral artery disease, or stroke)
2) Deemed unable to comply with the protocol (either self-selected or by indicating during screening that could not complete all requested tasks)
3) Bipolar disorder or psychotic disorder
4) Mild or more severe cognitive impairment [Mini-Mental State Exam (MMSE)[3] score ≤18]
5) Current severe substance use disorder
6) Acute, unstable, or severe medical disorder or pregnancy
7) Deemed to need immediate psychiatric intervention (e.g., active suicidality)
8) Use of antipsychotic, mood stabilizer, antidepressant, or stimulant medication in the past 4 weeks
9) Daily benzodiazepine use in the past 2 weeks

Additional Exclusion Criteria for the Trauma-Exposed Matched Control Group

1) Current diagnosis of any *DSM-5* psychiatric disorder
2) CAPS-5 total score ≥25[4]

During the initial phone screening, history of CVD will be assessed by querying if individuals have ever had a diagnosis of myocardial infarction, unstable angina, heart failure, peripheral artery disease, or stroke. Lifetime trauma exposure will be assessed with the Life Events Checklist for *DSM-5* (LEC-5).[5] This scale has good convergent validity with other trauma and trauma-related psychopathology measures.[6] For those who report trauma, the PCL-5[2] will be

used to assess current (past month) PTSD in response to the worst/most distressing trauma. A score ≥33 will indicate elevated symptoms[7,8] and inform targeted recruitment of those with probable PTSD.

Individuals who are interested in participating in this study and who meet the study eligibility criteria detailed above will complete a diagnostic interview assessment at the UCLA Psychology Department. Due to the COVID-19 pandemic, we will offer an online interview via Zoom in an effort to reduce the number of in-person research visits at baseline. Written informed consent will first be obtained from each participant after a thorough explanation of procedures by the clinical assessor conducting the diagnostic interview assessment. Individuals will be informed of the nature of the investigation and the types of assessments and procedures involved, and they will have the opportunity to ask and receive answers to questions. During the diagnostic interview assessment, a highly trained clinician will assess current PTSD (and lower-order symptom dimensions) and other mental conditions using the: 1) CAPS-5,[1] the gold standard in PTSD assessment. This 30-item structured interview has 0-4 Likert items for PTSD symptoms based on symptom frequency and intensity. The CAPS has good reliability and validity;[9] and 2) Structured Clinical Interview for *DSM-5* (SCID-5),[10] the gold-standard semi-structured interview for making the major *DSM-5* psychiatric disorders. If there are concerns regarding any cognitive impairment, the MMSE[3] will be administered as well. Interviews will be audio-recorded for reliability purposes. However, participants can decline to have their interview recorded. Socio-demographics (e.g., age, sex, race, ethnicity, marital status, education), medical history (e.g., diabetes, hypertension, pregnancy), medications (e.g., medications for high cholesterol, hypertension, and diabetes, antidepressants, vitamins), and health behaviors (e.g., cigarette smoking, physical activity, alcohol use, COVID-19 information) will also be documented. Food allergies will be queried as well to inform snack selection if the participant is eligible for the study and participates in the laboratory session. The interview will be recorded for the purposes of determining inter-rater reliability, although participants have the opportunity to decline having their interview be recorded. In addition, participants will complete self-report questionnaires assessing childhood adversity (Childhood Trauma Questionnaire,[11] Childhood Experiences of Care and Abuse inventory,[12] U.S. Department of Agriculture Food Security Scale,[13] and Conflict Tactics Scale[14]), depressive symptoms (Patient Health Questionnaire-8[15]), PTSD symptoms (PCL-5[2]), sleep (Pittsburgh Sleep Quality Index[16] with Addendum for PTSD,[17] Insomnia Severity Index,[18] and RU-SATED[19]), race-related discrimination (Everyday Discrimination Scale[20]), and dispositional optimism (Revised Life Orientation Test[21]). These questionnaires will be offered online in response to the COVID-19 pandemic. Individuals will be paid $50 for the diagnostic interview assessment.

After completing the diagnostic interview assessment, participants will schedule a baseline laboratory session within 2-3 weeks of the diagnostic interview assessment. Participants will be mailed a urine collection kit for a first morning urine sample that they will collect at home and bring to the lab on the day of the laboratory session. Participants will present to the UCLA Department of Psychology for the baseline laboratory session at 8:00am. They will be asked to avoid exercise, food/drink, THC and CBD use, and smoking for 8 hours prior to the session, and this will be confirmed by the clinical coordinator. Coordinators will also confirm that they have not used any medications or vitamins (PRN or prescribed) 48 hours before the lab session, with the exception of diabetic medications, blood thinners, statins, and birth control. These procedures are consistent with previous studies of brachial artery FMD.[22,23]

Anthropometrics (i.e., hip and waist circumferences, height, weight, nondominant arm circumference) will be measured, followed by resting clinic blood pressure. Blood samples (2-3 tablespoons) will then be collected by a phlebotomist. A blood sample will be used to process circulating EMPs (measured using flow cytometry), specifically EMPs expressing

CD62E. Prior to conducting lab visits with study participants, we will demonstrate reliability of assaying EMPs. For approximately 10 volunteers, we will collect blood samples from the same individual, 2 days in a row, and quantify EMPs twice on both days to assess within-session and between-session reliability. Blood-based measures of pro-inflammatory biomarkers (C-reactive protein, interleukin-6, tumor necrosis factor-α receptor-II), along with other cardiovascular risk factors such as lipids, glucose, and HbA1C, will also be assessed as part of the study visit. A measure of oxidative stress—F2-isoprostanes—will be measured using an ELISA assay from the urine sample. (Note: we are not validating any assays, we are not trying to determine predisposition to disease, and no whole genome/exome sequencing will be conducted.) Measures of inflammation and oxidative stress will be examined as potential mechanisms underlying associations of PTSD dimensions with endothelial dysfunction in exploratory analyses.

Next, FMD will be measured, using a high-resolution, semi-automatic ultrasonography system (UNEXEF38G; UNEX Co., Nagoya, Japan).[24-26] Participants will be tested between 8am and 10am to account for circadian effects on FMD. The participant will lie supine, and an appropriate-sized blood pressure cuff will be placed around the right forearm. After a 15-min rest, the brachial artery will be scanned longitudinally. The UNEXEF38G was designed with features to ensure image consistency and optimization. The baseline longitudinal image of the artery will be acquired for 30 s, and then the blood pressure cuff will be inflated to 50 mm Hg above systolic pressure for 5 min. The longitudinal image of the artery will be recorded continuously until 5 min after cuff deflation. Pulsed Doppler velocity signals will be obtained for 20 s at baseline and after cuff deflation. Changes in brachial artery diameter are calculated automatically by the analysis software as the maximum percentage change relative to baseline vessel diameter before cuff inflation.

Participants will be given a snack after FMD assessment. Participants will then undergo a heart rate response to deep breathing (HRDB) protocol to measure heart rate variability (HRV) a prior to completing fear and dysphoria tasks. HRV will be measured using the Biopac MP160 ECG module; respiration will be measured with a respiration belt. The ECG data will be collected using electrodes above the right collarbone and on a left lower rib. ECG and respiration data will be collected during a 1-min resting baseline and a 10-sec respiratory cycle (5-sec inhale, 5-sec exhale) repeated 6 times.[27] High frequency HRV will serve as an indicator of autonomic imbalance and will be examined as a potential mechanism underlying associations of PTSD dimensions with endothelial dysfunction in exploratory analyses.

After the assessment of HRV, participants will complete task-based measures of fear and dysphoria. Specifically, a well-established fear conditioning protocol that has been shown to successfully generate a robust psychophysiological fear response will be employed.[28-30] This paradigm consists of two phases: Fear Acquisition and Fear Extinction. During Fear Acquisition, participants first complete a habituation phase in which two conditioned stimuli (CSs; colored shapes in this task paradigm) and a 108-dB, 40-ms noise probe alone (NA) are presented to familiarize participants to the stimuli. This phase contains 4 trials each of the two CSs and NA. Next, participants undergo a conditioning phase that has 36 trials, with 3 blocks of 4 trials each of the following: the reinforced CS (CS+), the nonreinforced CS (CS-), and NA. Both CSs are presented on a computer monitor for 6 s. The startle probe, a 108-dB, 40-ms burst of broadband noise delivered binaurally via headphones, is delivered on every trial after 6 s. During conditioning, the CS+ is reinforced with a US on every trial, whereas the CS- is not. The US is a 250-ms airblast of 140-psi intensity directed at the larynx, which has been shown to consistently produce robust FPS.[28,29] On the CS+ trials, the 250-ms airblast (US) coterminates with the CS+, and the startle probe precedes the airblast by 0.5 s. The CS- trials terminate immediately after the presentation of the startle probe. Fear Extinction occurs 10 minutes after Fear Acquisition.

Fear Extinction consists of 72 trials, with 6 blocks of 4 trials of each type (CS+, CS-, NA). During this phase, the CS+ is presented without the US. In all phases, the inter-trial intervals are randomized to be 9-22 s. Psychophysiological fear responses will be collected using Biopac MP160 System (Biopac Inc., Goleta, CA) wireless modules for electromyography (EMG) and SC. EMG of the corrugator muscle will provide an index of negative facial expressions, and corrugator and orbicularis EMG will be used to measure FPS. Startle magnitude will be assessed as the peak amplitude of the EMG contraction 20 to 200 ms following the acoustic startle probe. SC data will be collected using SC electrodes on the hypothenar surface of the non-dominant hand to measure arousal and sympathetic nervous system activity. Consistent with previous research,[31] acquired data will be filtered, rectified, and smoothed using the MindWare software suite (MindWare Technologies, Gahanna, Ohio) and exported for statistical analysis.

Participants will also complete a dysphoria-relevant attention allocation task. Attention allocation will be measured using an established eye-tracking task[32,33] adapted for dysphoria,[34] using a remote high-speed eye-tracker (EyeLink 1000 Plus; SR Research, Ontario, Canada). Eye-tracking provides the most direct measure of attention.[35,36] The task consists of free-viewing of 4x4 matrices of happy and sad faces from the NimStim set.[37] Each trial begins with a fixation-cross, shown until a 1000-ms fixation is recorded, verifying that trials begin when gaze is fixated at the matrix's center. Matrices are presented for 6000 ms, followed by an inter-trial interval of 2000 ms. Participants freely view 60 different matrices, presented in two blocks of 30 matrices each, with a break of 60 s between blocks. Eye-tracking data will be used to define fixations as at least 100 ms of stable fixation within 1 degree visual angle. Dwell time for two Areas of Interest (AOIs), one for the 8 sad faces and one for the 8 happy faces, will be calculated for each matrix. Total dwell time for each AOI will average dwell time on each AOI across the 60 matrices. This task has been found to be a valid measure of dysphoria-related attention: more fixations on the sad AOI and fewer fixations on the happy AOI.[34]

The order of the task-based measures of fear and dysphoria will be counterbalanced across participants. Participants will be paid $175 for completing the laboratory visit and reimbursed for travel. At the baseline laboratory session, participants will indicate if they are willing to be contacted for a 2-year follow-up. We will remain in contact with interested participants over the study (e.g., birthday cards) and invite them to return for a follow-up visit 2 years later. We will aim to conduct follow-up visits with as many participants as possible, with a minimum sample size of 80 (40 with PTSD, 40 trauma-exposed controls) with follow-up data. At the follow-up, participants will complete a laboratory session to repeat assessments of endothelial dysfunction (FMD), anthropometrics, and heart rate variability (HRDB). Participants will then complete a diagnostic interview for current PTSD and additional questionnaires to assess current medical history, medications, and health behaviors. These assessments are identical to those described above. Participants will be paid $175 for completing the follow-up visit and reimbursed for travel.

All devices referenced in this protocol are routinely used in clinical practice, will be used in accordance with labeling, and are cleared/approved for marketing.

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3. Statistical Procedures

Statistical Analysis for Primary Aim 1: To determine if current PTSD diagnosis is associated with endothelial dysfunction. Hypothesis: Men and women with vs. without PTSD will have lower FMD and, secondarily, greater EMP levels.

The primary exposure will be a dichotomous PTSD diagnosis variable, although we will also consider continuous PTSD severity measures as secondary predictors. The primary outcome will be FMD, calculated as the maximum change in diameter after cuff release normalized to the baseline diameter (% of baseline diameter). Analyses are powered for the primary outcome. The concentration of EMPs expressing CD62E (#/uL in blood) will be a secondary outcome. Two sample t-tests will compare those with and without PTSD on 1) FMD and 2) concentration of EMPs expressing CD62E. Alternatively, we will apply the Wilcoxon Rank-Sum test if the outcome variables are not normally distributed. We will then conduct linear regression models.

Unadjusted models will be examined first, followed by a series of adjusted models (see section on covariates below).

Power. The estimated effect size used in power calculations was based on the two prior studies of PTSD and FMD. One study found a Cohen's d of 0.75 for the difference in percent change in FMD in those with severe PTSD symptoms vs. the lowest symptom levels,[1] and another study found a Cohen's d of 0.48 for the difference in percent change in FMD in patients with vs. without probable PTSD.[2] These estimates correspond to medium to large effect sizes. We conservatively used the smaller effect size estimate in our calculations. Power analysis for an independent two sample t-test was conducted in G*Power 3.1 to determine a sufficient sample size using an alpha of .05, power of .80, a medium effect size (Cohen's d=0.48), and two tails. Participants will be equally allocated in the PTSD and no PTSD groups. Based on these assumptions, the desired sample size was 70 participants per group. We conservatively selected 80 participants per group.

Statistical Analysis for Primary Aim 2. To examine which PTSD dimensions are most associated with endothelial dysfunction. Hypotheses: (1) Psychophysiological fear responses (high fear load after conditioning and impaired fear inhibition during extinction determined by FPS and SC responses) will be independently associated with lower FMD and, secondarily, greater EMP levels. (2) Dysphoria (determined by an attention allocation task) will be less strongly related to lower FMD and, secondarily, greater EMP levels than fear responses. Additionally, we will explore whether lower-order symptom dimensions of PTSD are associated with endothelial dysfunction.

For Hypothesis 1, separate linear regression models will be conducted with FMD as the primary outcome and each of the following FPS scores as the independent variables: 1) FPS to the CS+ during early extinction (fear load), and 2) FPS to the CS+ during late extinction. The first measure reflects expression of conditioned fear; greater startle during early extinction (first 2 blocks of extinction phase) indicates a greater fear load to extinguish. The second measure reflects fear inhibition; lower startle to the CS+ during late extinction indicates greater inhibition. Exploratory analyses will also examine FPS to the CS- during late fear acquisition; elevated FPS to the CS- indicates poor discrimination and inhibition. FPS will be calculated using a difference score by subtracting startle magnitude to the NA from startle magnitude to the CS in each block to account for individual differences in startle magnitude and habituation. Comparable scores for SC response will be examined as secondary predictors. SC response will be calculated by subtracting the SC prior to stimulus onset from the maximum SC during CS presentation. The concentration of EMPs expressing CD62E will be a secondary outcome. Unadjusted and then adjusted linear regression models will be examined (see section on covariates below). We will also include dysphoria measures to see if fear measures independently predict endothelial dysfunction.

For Hypothesis 2, we will use the same approach as for Hypothesis 1 but our independent variables will be total dwell time for the sad and happy AOIs. We will also explore how the 5 lower-order symptom dimensions of the dysphoric arousal model[3] relate to our endothelial dysfunction measures and if these findings with interview-based symptom dimensions parallel those with the objective indices. As above, a series of adjusted analyses will be conducted. Final models for the lower-order symptom dimension analyses will include the other symptom dimensions as covariates.

Power. Given our hypothesis that psychophysiological fear is the key PTSD dimension that will be related to endothelial dysfunction, we considered power for this analysis. We assumed a SD of 70 for FPS scores[4] and a SD of 3.5% for FMD.[2] With *N*=160 and a two-sided significance level of 0.05, we have 80% power to detect a change of 0.011% in FMD for each unit of FPS

score increase.[5] This is a small effect size,[2] so our sample is large enough to allow for modeling psychophysiological fear responses and FMD in adjusted models.

Statistical Analysis for Secondary Aim: To examine how fear, dysphoria, and lower-order symptom dimensions (and changes in these measures) predict change in endothelial dysfunction over 2 years in a subset of the sample.

We will conduct two sets of analyses. First, we will examine how FPS measures, total dwell times, and lower-order symptom dimensions at baseline predict change in FMD, our primary outcome. Second, we will examine how changes in PTSD symptoms over follow-up predict change in FMD. Unadjusted and then adjusted linear regression models will be examined (see section on covariates below). Although we are not powering our analysis to this Secondary Aim, with a sample size of 80 and assuming a SD of 70 for FPS scores4 and a SD of 1.0% for change in FMD over 2 years,6 we have 80% power to detect a difference of 0.0043% in the change of FMD for each unit of startle score increase at a two-sided significance level of 0.05. Prior research on FMD change over 2 years observed a mean change of 1.6%;6 thus, we will be powered to detect change even smaller than that.

Statistical Analysis for Exploratory Aim: To examine if autonomic imbalance, inflammation, and oxidative stress explain any associations of PTSD dimensions with endothelial dysfunction, we will estimate total and direct effects of the fear, dysphoria, and lower-order symptom dimensions on endothelial dysfunction and investigate if there are significant indirect effects of PTSD dimensions on endothelial dysfunction via these pathways using bootstrapping methods.[7]

Covariates: For all analyses, we will examine a series of sequentially adjusted models. The base adjusted model will include socio-demographics (age, sex, race, ethnicity); subsequent models will sequentially control for: 1) other socio-demographics (e.g., education, marital status); 2) CVD medical risk factors (e.g., diabetes, hypertension); 3) medications (e.g., medication for hypertension, high cholesterol); and 4) anthropometrics and health behaviors (e.g., body mass index, smoking, physical activity). To avoid overfitting, we will fit lasso regression, which performs both variable selection and regularization of regression coefficients, to identify important predictors of endothelial dysfunction and improve interpretability of the regression model.[8]

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