Mental stress causes vasoconstriction in subjects with sickle cell disease and in normal controls

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Supplementary Material

S1 Methods

SCD patients were either on chronic transfusion, hydroxyurea therapy or not on any treatment. Eligible participants were 11 years and older, free of any vaso-occlusive crisis or hospitalization in the past 10 days. Subjects were excluded if they were under treatment for an anxiety disorder. Informed consent and assent was obtained prior the study. Medical history, anxiety questionnaire (STAI Y) and blood samples were obtained prior to testing. All subjects had a complete blood count, reticulocyte count, plasma hemoglobin, lactate dehydrogenase at the time of each study visit. Controls had a hemoglobin electrophoresis done at least once and SCD patients had electrophoresis done at the time of each study visit.

Experimental Setup and Study Protocol

All studies were performed in the morning in our autonomic nervous system lab with quiet and highly controlled surroundings[(1)]. Subjects were instructed to sleep well the night before to prevent fatigue during the study. Subjects were asked to avoid coffee on the study day to minimize its influence on autonomic nervous system (ANS) activity. Participants were asked to sit in a comfortable cushioned chair with the laptop in front of them. After all devices were attached, subjects were asked to sit relaxed for 5-minutes to get the baseline recording. Following the baseline recording period, a 45-minute stress induction protocol was presented in E-prime psychological software. The study protocol is diagramed in fig 1. The first and the second stress tasks were either a memory task (N-back[(2)]) or a conflict task (Stroop[(3)]). The order of presentation of the N-Back and Stroop tests was randomized. In between the first and second stress task, subjects filled out STAI Y-1 questionnaire to measure their state of anxiety immediately after completing the task. The third stress task was a pain anticipation (PA) task, which was followed by a 3-minute baseline recording.

In the N-back test the subject is presented with a temporal sequence of letters and is asked to indicate when the current letter matches the one from *n* steps earlier in the sequence. The *n* varied from least difficult at zero back (n=0) to most difficult where the subject had to identify if current letter matched one, two or three letters back (n=1, 2, 3) in the sequence. N-back difficulty sublevels were labeled as zeroback, oneback, twoback and threeback. The subject was presented with a sequence of twenty letters flashing for 500 ms on the screen one after another with 1500 ms break and responded using the keyboard when the current letter matched a letter the requested the number of steps back.

In the Stroop task, the participant was asked to identify the font color of a word presented to them but not the written name of the word. For example, when the word “orange” is printed in green font, the participant should answer “green” rather than orange. There were at least eight possible

options on the screen to select the correct answer using the mouse. Words were presented at three increasing speeds to increase the level of difficulty[4]. Stroop difficulty sublevels were labeled as onestroop, twostroop and threestroop. In addition, the position of the eight multiple options were shuffled in speed level two and level three to make the task more challenging. Subjects were also screened for color blindness before study entry.

All participants practiced N-back and Stroop tests before the study. To verify the familiarity with the tasks, they needed to achieve a minimum of 50% accuracy score in each task before proceeding for actual testing.

At the end of the entire session, the subjects were presented with a pain anticipation stimulus. The following sentence was displayed on the computer screen: "You will receive a maximum pain stimulus in one minute. When you cannot tolerate the pain any longer, say STOP and the device will cool down to normal level immediately". However, no actual pain stimulus applied.

Subjects performed a total of twelve randomized sets of N-back and Stroop with varying levels of difficulty. The exact timing of the presentation of stressors as well as the accuracy scores were controlled and recorded by the E-prime software and timing tags sent electronically to the physiology recording system. The bars in the first panel of fig 2 represent the level of difficulty for each task separately, higher the bar more difficult the task.

During the study, there was no interaction with the subject. All of the instructions were presented on computer screen. Subjects received feedback regarding their accuracy of performance at the end of each task. Accuracy is calculated by percentage of correct answers in each level. The accuracy scores shown on the screen were decreased by 10% to maximize the chances of triggering stress responses. Subjects were also told they will be financially rewarded if their accuracy scores are good.

Physiological measurements and data processing

All the physiological monitoring sensors were attached on the subjects' left arm. Microvascular blood flow (MBF) was measured using PPG and laser Doppler flowmeter. The PPG was placed on the thumb while the laser Doppler flowmeter was placed on the dorsum of the index finger just proximal to the nailbed where there is a very high density of capillaries and arterioles. Respiration, electrocardiogram (ECG) and continuous blood pressure were recorded. In addition, Medoc Thermal Neurosensory Analyzer (TSA-II) which is capable of producing a calibrated painful stimulus was attached on their forearm for the pain anticipation task. Physiological responses were recorded continuously at 250 Hz using Biopac MP 150 AcqKnowledge data acquisition system and resampled at 30 Hz[1] for the analysis.

Recorded data from all devices were exported for processing and analysis in MATLAB. The raw waveform (figure 2, 2nd signal) and the waveform amplitude derived from the PPG signal (figure 2, 3rd signal) reflect the change in the cross-sectional area of the small arterioles, capillaries, and venules at the fingertip, which indirectly reports regional microvascular blood flow. The laser-Doppler flow (PU, figure 2, 4th signal) provides a relative measure of tissue perfusion based on red

cell velocity and density detected by light scattering. PPG amplitude was normalized to its own 95th percentile value of full study recording in order to standardize the signal to each patient's maximum MBF amplitude. Percent decrease in the amplitude of the PPG or PU waveforms from baseline mean was interpreted as vasoconstriction response [1, 5, 6] (fig 2). Average microvascular blood flow was calculated over 5-min baseline, N-back, Stroop and PA tasks.

Cardiac autonomic balance in response to mental stress was assessed by analysis of heart rate variability (HRV) [7-9]during baseline, N-back, Stroop and PA tasks. HRV analysis employs the power spectrum analysis to decompose the variability in R-R intervals (heart periods) into different frequency components. Low frequency power of HRV (LFP; 0.04-0.15 Hz) reflects a combination of cardiac sympathetic and parasympathetic activity while high frequency power (HFP; 0.15-0.4 Hz) reflects parasympathetic activity [9, 10]. LHR (LFP to HFP ratio) represents sympatho-vagal balance[10]. We calculated average R-to-R interval during baseline and tasks. Decrease in RRI from baseline indicates tachycardia during tasks.

Statistical Analysis

Results were reported as mean ± standard error (SE) for continuous variables or as proportions for categorical variables. Percent change in mean MBF and mean spectral indices were calculated during tasks compared to the baseline. Percent change in blood flow during pain anticipation was analyzed separately from cognitive tasks. Student's t-test or chi-square test was used to test for baseline group differences and task differences. Wilcoxon signed rank was used to test non-parametric differences. Regression analysis was performed with outcome being percent change in mean MBF during N-back, Stroop and PA task. Covariates such as age, gender, diagnosis, hemoglobin, hemoglobin S% and anxiety scores were assessed in univariate analysis as well as for interaction and confounders. Robust regression was only used to correlate vasoconstriction response and state anxiety during PA task due to extreme outliers. Outliers were confirmed by having large residual or high leverage. In multivariate robust regression for PA task, STAI Y1 had an interaction with diagnosis. Therefore a factorial analysis was performed separately within SCD and controls (fig 4). Repeated measures ANOVA was used to test difference in N-back and Stroop sublevel tasks and accuracy scores. All p-values are two-sided with nominal significance of $p \leq 0.05$. All statistical analyses were performed using the statistical software STATA/IC 14.1 (StataCorp LP, Texas).

S2 Figures:

Fig 1: Relationship between Baseline microvascular blood flow and baseline anxiety (STAI Y1). Highly anxious SCD responders trended to have lower baseline blood flow but not controls. SCD subjects (closed circles, —) and controls (open diamonds, - - -)

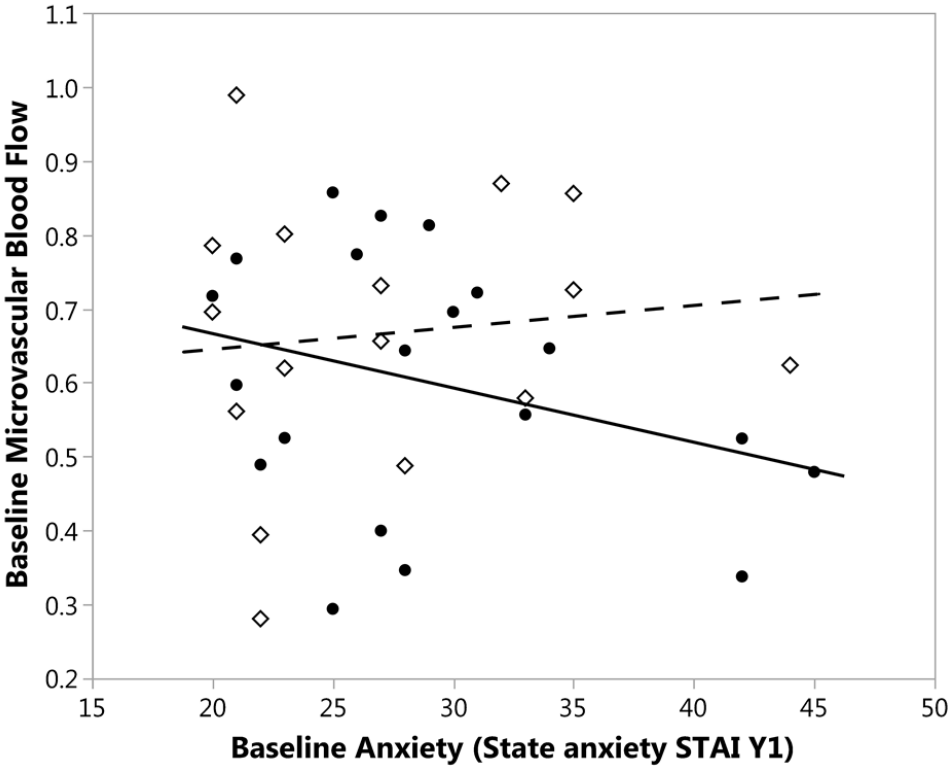


Fig 2A. Vasoconstriction response to pain anticipation (PA) task in a high anxiety SCD responder. 2nd and 3rd panel (PPG and PPG amp) shows the subject was already vasoconstricted during baseline and did not vasoconstrict further during pain anticipation task

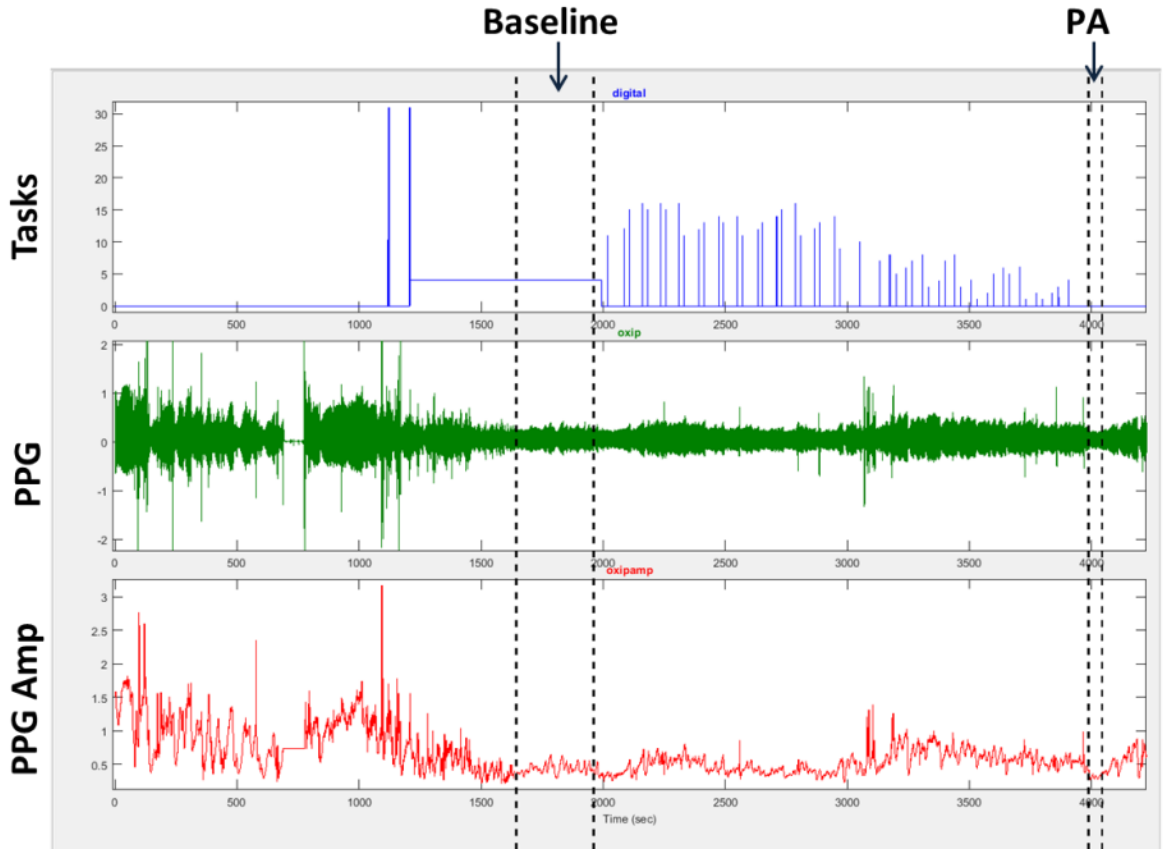
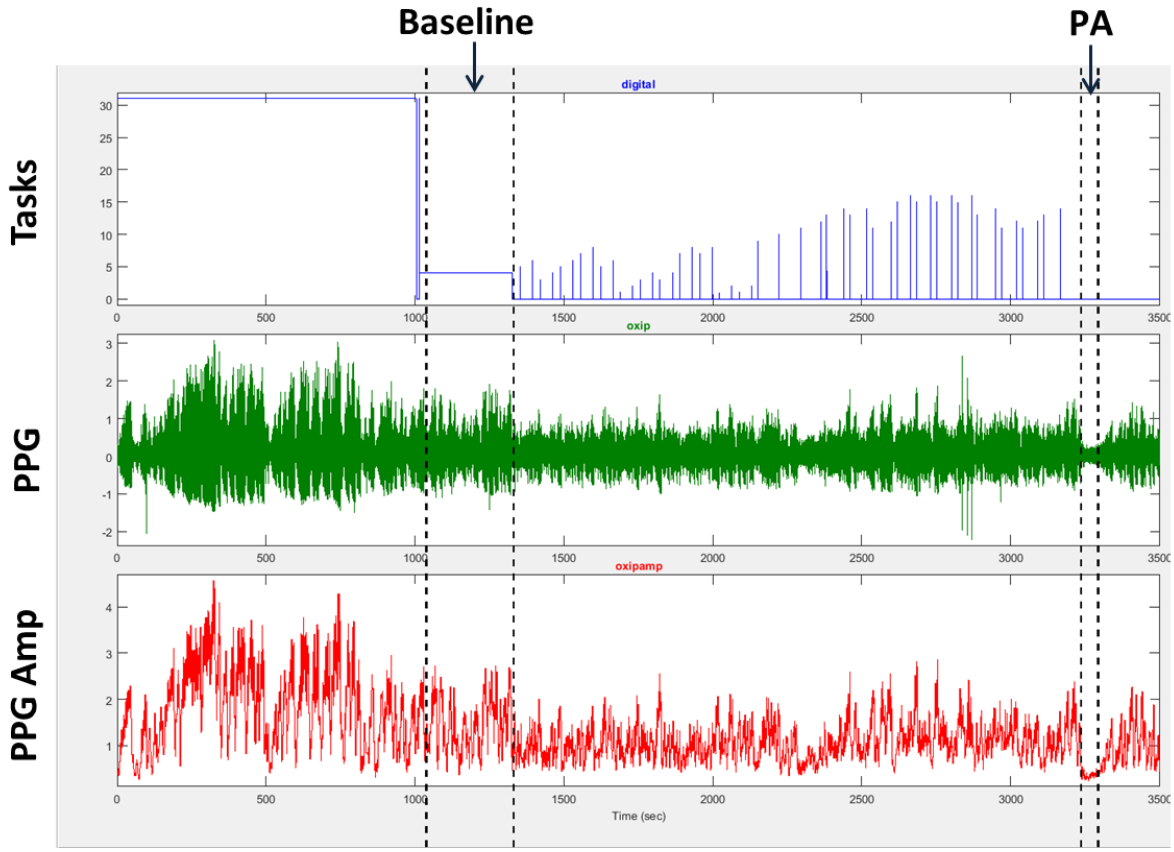


Fig 2B. Vasoconstriction response to pain anticipation (PA) task in a low anxiety SCD responder. 2nd and 3rd panel (PPG and PPG amp) shows the subject did not have vasoconstriction during baseline and showed significant vasoconstriction during pain anticipation task



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