ACUTE STRESSOR-EVOKED INFLAMMATORY RESPONSES: PHYSIOLOGICAL ORIGINS AND RELATIONSHIP WITH CARDIOVASCULAR DISEASE RISK

by

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Submitted to the Graduate Faculty of the

Kenneth P. Dietrich School of Arts and Sciences

in partial fulfillment of the requirements for the

degree of Master of Science

University of Pittsburgh

2015

UNIVERSITY OF PITTSBURGH

DIETRICH SCHOOL OF ARTS AND

SCIENCES

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October 27th, 2015

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Individuals differ appreciably and consistently in the magnitude of their inflammatory responses to acute stressors. These individual differences in acute stressor-evoked inflammatory responses may be one biological mechanism that contributes to heightened risk for cardiovascular disease (CVD) and other inflammatory diseases. Critically, the clinical implications and physiological origins of these responses remain relatively unexplored. This thesis addresses these questions by testing whether magnitude of stressor-evoked interleukin(IL)-6 responses relate to (1) markers of cardiovascular disease risk (i.e., systemic inflammation and atherosclerotic CVD risk) or (2) stressor-evoked parasympathetic nervous system responses. Participants were 91 healthy midlife adults (30-51 years; 33% female; 68% white) who completed a laboratory stress protocol consisting of two mental stress tasks: a multisource interference task and Stroop color word task. During the protocol, cardiovascular psychophysiological measures were assessed and blood samples were drawn after a 30-min baseline and 30-min after task completion. Systemic inflammation was indicated by basal levels of C-reactive protein (CRP) and preclinical atherosclerotic CVD risk was assessed with intima-media thickness. Parasympathetic nervous system activity was measured by high frequency heart rate variability (HF-HRV). Hierarchical linear regressions controlling for age, sex, race, and BMI tested whether stressor-evoked IL-6 responses were associated with basal CRP, intima media thickness, and stressor-evoked HF-

HRV responses. Ancillary analyses assessed whether these relationships differed by sex. Primary analyses indicated that there were no significant associations between stressor-evoked IL-6 responses and CRP, intima-media thickness, or HF-HRV responses. However, ancillary analyses revealed that sex and stressor-evoked IL-6 responses interacted to predict CRP (ΔR^2 = .08, B = -1.33, β = -.39, p = .02); in males, larger stressor-evoked IL-6 responses associated with higher CRP while in females, larger stressor-evoked IL-6 responses associated with lower CRP. These findings indicate that inflammatory responses to acute stressors associate with resting levels of CRP; however, this association differs by sex. Previous literature suggests that there are sex differences in stressor-evoked IL-6 responses, but this is the first study to show sex differences in the relationship between acute inflammatory responses and systemic inflammation. The contribution of these sex differences to inflammatory disease risk warrants further investigation.

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PREFACE

This research was conducted with the help of the members of the Behavioral Neurophysiology Laboratory including Sara Snyder, Julie Johnson, Lei Sheu, and Dora Kuan.

1.0 INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the US and other developed nations**.** Inflammation plays a key role in CVD pathophysiology (Libby, Ridker, & Maseri, 2002; Ross, 1999). Higher levels of inflammatory markers in peripheral circulation, such as interleukin(IL)-6 and C-reactive protein (CRP), predict increased risk for CVD (Danesh et al., 2008; Danesh et al., 2000). Despite this relationship, the physiological mechanisms underlying systemic elevations in circulating inflammatory markers are not entirely clear. Although numerous factors contribute to systemic inflammation, there has been increased focus on the role of psychological stress. In particular, levels of circulating inflammatory markers increase after exposure to acute psychological stress (Rohleder, 2014; Steptoe, Hamer, & Chida, 2007). Moreover, the magnitude of these acute stressor-evoked increases varies between individuals (Cohen & Hamrick, 2003; Schneiderman et al., 2008). These individual differences in acute stressor-evoked inflammatory responses may be one biological mechanism that contributes to heightened systemic inflammation and greater CVD risk in general.

Critically, however, there are major gaps in our knowledge about the extent to which stressor-evoked changes in circulating inflammatory markers relate across individuals to (1) established measures of CVD risk and (2) known systemic inflammatory predictors of CVD risk measured under resting or basal conditions. Until these gaps are addressed, the relationship between acute stress-related aspects of systemic inflammation and CVD risk will remain

assumed and untested. These gaps in knowledge also extend to the physiological origins of stressor-evoked inflammatory responses. Although the physiological origins of inflammatory responses are not fully understood, indirect evidence suggests that the parasympathetic nervous system (PNS) may regulate stressor-evoked inflammatory responses; however, the relationship between PNS and inflammatory responses to stress remains unclear The proposed study thus aims to assess the relationship between acute stressor-evoked PNS and inflammatory responses, as well as the association between inflammatory responses and two known markers of CVD risk (Figure 1).

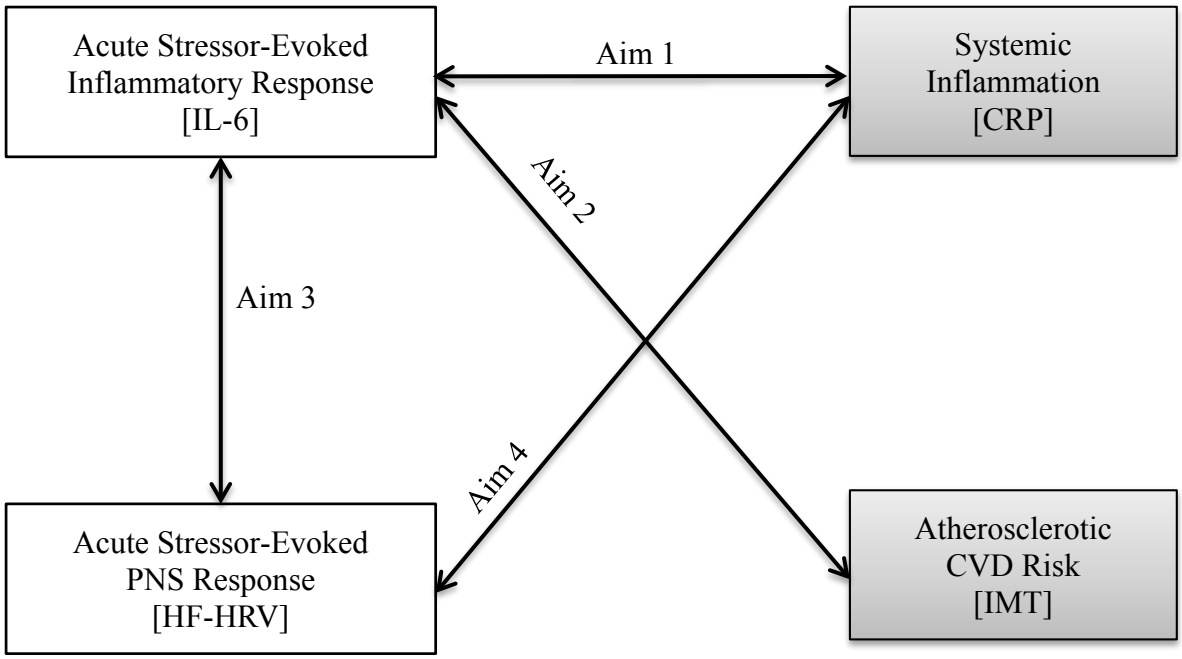

Figure 1. Specific Aims

Aim 1: Test whether acute stressor-evoked inflammatory responses, as measured by changes in IL-6, associate positively with resting levels of the circulating inflammatory marker CRP, which predicts future CVD.

Aim 2: Test whether acute stressor-evoked IL-6 responses associate positively with preclinical atherosclerosis, as measured by carotid artery intima-media thickness (IMT).

Aim 3: Test whether larger acute stressor-evoked inflammatory responses (IL-6) associate with greater stressor-evoked suppression of PNS activity, as measured by high-frequency heart rate variability (HF-HRV).

Aim 4: Test whether greater stressor-evoked suppression of PNS activity (HF-HRV) associates with higher resting levels of circulating CRP.

Ancillary Aim 1: Test for sex differences in the magnitude of stressor-evoked IL-6 responses.

Ancillary Aim 2: Test whether the relationship between stressor-evoked IL-6 response and CRP varies by sex.

Ancillary Aim 3: Test whether the relationship between stressor-evoked IL-6 response and IMT varies by sex.

Ancillary Aim 4: Test whether greater stressor-evoked blood pressure responses associate with larger stressor-evoked IL-6 responses.

1.1 CVD AND THE ROLE OF INFLAMMATION

Inflammation plays a critical role in the pathophysiology of CVD. Long before the onset of clinical symptoms, chronic inflammatory processes exacerbate damage to the lining of blood vessels and build-up of plaque within arterial walls. These changes are the chief pathophysiological basis of CVD: atherosclerosis (Libby et al., 2002; Ross, 1999). Atherosclerosis is initiated by damage to the endothelial lining of blood vessels (Ross, 1999).

This damage results in an inflammatory response that includes increased permeability of the endothelial lining, enabling the migration of lipoproteins and immune cells (e.g., monocytes) into the innermost layer of the arterial wall, the intima (Steptoe & Brydon, 2005). Upon entering the intima, monocytes mature into macrophages and ingest lipoproteins to become lipid-laden foam cells. Macrophages also secrete growth factors and cytokines, proteins that maintain the vascular inflammatory response (Ross, 1999; Steptoe & Brydon, 2005). This inflammatory state becomes chronic over time, promoting smooth muscle cell migration from the medial layer and proliferation in the intima. Over time, this results in the accumulation of cells and waste in the intima (Libby, Ridker, & Hansson, 2011; Mitchel & Schoen, 2009). These chronic processes occur over many decades and lead to preclinical arterial wall thickening and/or narrowing of the lumen, increasing the later risk of clinical CVD (e.g., angina, infarction, etc.) (Eigenbrodt et al., 2007; Libby et al., 2011). Preclinical atherosclerotic vascular changes can be assessed by measuring carotid artery intima-media thickness (IMT), taken to reflect an indirect and noninvasive predictor of CVD risk and outcomes (Lorenz, Markus, Bots, Rosvall, & Sitzer, 2007; Salonen & Salonen, 1993) The degree of preclinical atherosclerotic CVD risk varies between individuals in association with biological, behavioral, and psychosocial risk factors (Jones, Bromberger, Sutton-Tyrrell, & Matthews, 2003; Matthews, 2005; Sands et al., 2012; Thurston et al., 2014; Troxel, Matthews, Bromberger, & Sutton-Tyrrell, 2003).

The chronic inflammatory state of atherosclerosis involves continuous recruitment of immune cells and production of cytokines. Cytokines released by activated macrophages both coordinate the local inflammatory response within arterial walls and enter peripheral circulation to stimulate a systemic response. This response includes the synthesis and release of acute phase proteins, such as CRP and fibrinogen. Levels of these inflammatory markers can be reliably

detected in peripheral circulation and are widely assumed to reflect systemic levels of inflammation. However, caution should be taken in interpreting these circulating levels as a measure of immune-derived processes, as many other cell types produce inflammatory signaling proteins, including adipocytes and endothelial cells (Mohamed-Ali et al., 1997; Papanicolaou, Wilder, Manolagas, & Chrousos, 1998). For example, adipose tissue is a key source of peripheral IL-6, with adipocytes producing 10-35% of circulating levels (Mohamed-Ali et al., 1997).

Regardless of source, heightened levels of circulating inflammatory mediators confer increased CVD risk (Danesh et al., 2008; Danesh et al., 2000) and are suggested as a pathway linking stress and CVD risk. Although the majority of evidence describing these pathways focuses on circulating inflammatory markers at rest, levels of these markers also rise after exposure to acute stressors (Steptoe et al., 2007). The magnitude of acute stressor-evoked increases in inflammatory markers may correspond to an individual difference that could relate to vulnerability to inflammatory diseases like CVD. As noted, however, the extent to which acute stressor-evoked changes in inflammatory markers relate to established predictors of CVD risk is unclear. In addition, there are multiple possible physiological origins of these rises in circulating inflammatory markers. These issues must be clarified before acute stressor-evoked inflammatory responses can be used an indicator of CVD risk. Stressor-evoked changes in immune activity are mediated by the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis, which are detailed in a later section. Described next are the stressor-evoked inflammatory changes mediated by these systems.

1.2 THE ACUTE STRESOR-EVOKED INFLAMMATORY RESPONSE

Acute stressor-evoked inflammatory responses are assessed by measuring levels of circulating inflammatory markers in the blood before and after exposure to an acute stressor task in the laboratory. These tasks typically include elements of time pressure and negative feedback or social evaluation and are designed to elicit physiological stress responses (Rohleder, 2014). A meta-analytic review of such studies indicates an increase in circulating markers of systemic inflammation from pre- to post-task (Steptoe et al., 2007). Although the time course of these acute responses is not completely clear, increased circulating levels are typically observed around 30-minutes after stressor exposure and last for at least 120 minutes (Brydon, Edwards, Mohamed-Ali, & Steptoe, 2004; Carroll et al., 2011; Steptoe et al., 2007). The association between acute stressor exposure and increase in circulating markers of inflammation is particularly robust for IL-6 and IL-1β, with more studies testing IL-6 (Steptoe et al., 2007). For this reason, acute stressor-evoked changes in IL-6 will be of primary interest in the present study.

Laboratory studies show that individuals differ consistently in the magnitude of their immune responses to acute stress, with some individuals showing large responses across occasions of testing and others little or no response (Black, 2003; Marsland, Bachen, Cohen, Rabin, & Manuck, 2002; Marsland, Manuck, Fazzari, Stewart, & Rabin, 1995). Larger acute stressor-evoked inflammatory responses may indicate a tendency toward greater inflammatory responses in general, or a "proinflammatory phenotype". This proinflammatory phenotype may increase vulnerability to inflammatory diseases like atherosclerosis (Miller et al., 2011). Thus, to the extent that larger acute stressor-evoked inflammatory responses are stable attributes of individuals that characterize responses to frustrations in daily life, they may relate to vulnerability to inflammatory diseases. To date, only two studies have considered this

possibility. These studies, drawn from the same cohort, reported that acute stressor-evoked inflammatory responses predict carotid artery stiffness and ambulatory blood pressure at a 3-year follow-up, suggesting that there may be a relationship between this individual difference and future CVD risk (Brydon & Steptoe, 2005; Ellins et al., 2008). The present study will conduct an initial examination of whether magnitude of IL-6 response is associated with two additional CVD risk factors: resting levels of circulating inflammatory markers and atherosclerotic CVD risk.

Heightened resting levels of circulating inflammatory markers, particularly CRP, are associated with increased risk for CVD (Kaptoge et al., 2010; Libby & Ridker, 1999; van Holten et al., 2013). However, it is unknown whether individuals who show greater stressor-evoked inflammatory responses are at increased risk for elevated resting levels of CRP and thus CVD. Accordingly, this study aims to determine whether the magnitude of an individual's acute stressor-evoked IL-6 response is associated with circulating levels of CRP (Aim 1). Based on previous reports of sex differences in stressor-evoked IL-6 responses (Edwards, Burns, Ring, & Carroll, 2006; Hackett, Hamer, Endrighi, Brydon, & Steptoe, 2012; Steptoe, Owen, Kunz-Ebrecht, & Mohamed-Ali, 2002), ancillary analyses will also test whether the magnitude of IL-6 response differs by sex (Ancillary Aim 1) and whether the association between IL-6 response and CRP varies by sex (Ancillary Aim 2).

Given the pathogenic role of inflammation in atherosclerosis, it is possible that acute stressor-evoked rises in circulating inflammatory markers are linked with atherosclerotic CVD risk. This link has a number of theoretical explanations. One possibility is the previously discussed proinflammatory phenotype model (Miller et al., 2011). An alternative explanation is that heightened levels of circulating inflammatory markers play a causal role in atherosclerosis

by exacerbating ongoing local atherosclerotic inflammatory processes. This explanation is supported by prospective findings linking elevated systemic inflammation to later CVD incidence (Ridker, Rifai, Stampfer, & Hennekens, 2000). Although the proposed study will not test how stressor-evoked inflammatory responses relate to a clinical outcome, it will provide an initial test of the possibility that individual differences in the magnitude of inflammatory reactivity associate with IMT, a preclinical marker of atherosclerotic risk (Aim 2). Ancillary analyses will assess sex differences in the association between stressor-evoked IL-6 responses and IMT (Ancillary Aim 3).

1.3 OVERVIEW OF RELATED PHYSIOLOGICAL SYSTEMS

Gaps in current knowledge of stressor-evoked IL-6 responses also extend to mechanistic control of these responses. These responses are thought to be controlled by activation of two pathways that link the central nervous and immune systems: the ANS and the HPA axis. Both of these pathways respond to acute psychological stress and may thus contribute to stress-induced inflammatory responses.

The ANS regulates physiological processes required for responding to stressors and maintaining homeostasis. Its two primary divisions are the parasympathetic nervous system (PNS) and the sympathetic nervous system (SNS). Each division innervates many different organs and regulate a wide variety of physiological functions outside of conscious control, including immune function (Kemeny, 2011). There are a number of methods of assessing both PNS and SNS activity, such as pharmacological blockade of receptors specific to each system and direct nerve recording or stimulation. Psychophysiological methods have also been used to

indirectly assess ANS activity, providing a relatively noninvasive way to examine the activity of these two systems in humans (Berntson et al., 1997; Sherwood et al., 1990; Task Force, 1996).

Heart rate variability (HRV) is one cardiovascular psychophysiological measure that indirectly assesses ANS activity. HRV is the fluctuation of time between consecutive heartbeats and is influenced by both SNS and PNS input to the heart. PNS activity is thought to be reflected by high-frequency variation in HRV (HF-HRV). HF-HRV refers to changes in the heart period that occur within 9-24 cycles per minute, a frequency band correlated with respiratory frequency. Notably, HF-HRV is closely related to a similar marker of PNS activity known as respiratory sinus arrhythmia (RSA) (Berntson et al., 1997). Although HF-HRV and RSA use different quantification methods, they are highly correlated and derived with a similar theoretical framework (Berntson et al., 1997; Task Force, 1996). Strong evidence from pharmacological blockade studies suggests that HF-HRV is almost exclusively influenced by PNS input to the heart. Specifically, administration of PNS antagonists leads to near complete elimination of HF-HRV (Berntson et al., 1997). Measures of HF-HRV are sensitive to acute psychological stress; a recent meta-analysis found that HF-HRV and two other measures of PNS input to the heart decrease during acute laboratory stressors (Brindle et al., 2014). This finding suggests that acute stress evokes withdrawal of PNS input to the heart. It should be noted that HF-HRV is useful for noninvasively assessing PNS input to the heart but it does not necessarily reflect PNS input to the rest of the body; thus, the inferences we can make with this measure are limited.

The activity of the SNS can also be measured indirectly by cardiovascular psychophysiological methods, but these measures were not assessed in the present study. Blood pressure (BP) measures are available as a gross indicator of SNS outflow to the heart and

vasculature and will be used in ancillary analyses. Specifically, these analyses will test whether stressor-evoked BP is significantly associated with stressor-evoked IL-6 (Ancillary Aim 4).

Similar to the ANS, the HPA axis response can also be triggered by stress. This response is initiated by the hypothalamus, which stimulates release of adrenocorticotropic hormone (ACTH) from the pituitary gland into peripheral circulation. ACTH travels to the adrenal cortex, where it stimulates secretion of glucocorticoids (GCs) (Cacioppo & Berntson, 2011). GCs have a number of essential functions, including regulation of immune response. Cortisol, one commonly studied GC, typically rises after acute stress, though this response may be down-regulated upon exposure to chronic stress (Miller, Cohen, & Ritchey, 2002).

1.4 MECHANISTIC BASIS OF IL-6 RESPONSES

Stressor-evoked inflammatory responses are mediated by the ANS and the HPA axis, however, the specific mechanisms that drive these changes are not entirely clear. To date, there has been an emphasis on the role of the SNS and the HPA axis in driving these responses. Although it has been suggested that acute stressor-evoked IL-6 responses and PNS activity during stress might be related, this association remains largely unexplored. Thus, the PNS will be the primary focus of the present study. The SNS and HPA axis literature will be briefly reviewed to put the emerging PNS literature into context.

Previous work suggests that the acute stressor-evoked inflammatory response parallels and may be predicted by stress-related activation of the SNS. A series of rodent studies showed that sympathetic catecholamines play an important role in stress-induced IL-6 changes (Johnson et al., 2005). Specifically, pharmacological blockade of α- and β-adrenergic receptors attenuated

IL-6 increases in rats exposed to acute stress compared with controls. In another experiment, injection of a β-adrenergic receptor agonist elevated circulating IL-6 levels after 60 minutes (Johnson et al., 2005). These findings suggest that stressor-evoked increases in catecholamines may trigger IL-6 production.

Direct human evidence for this mechanism is limited. In line with rodent findings, healthy adults show a significant association between norepinephrine (NE) and IL-6 responses to acute stress (Kop et al., 2008). Additionally, in vitro evidence supports increased activation of nuclear factor κB (NF-κB), a transcription factor that triggers IL-6 production, when peripheral blood mononuclear cells are treated with NE (Bierhaus et al., 2003). However, one study indicated that administration of a β-adrenergic antagonist does not attenuate stressor-evoked IL-6 responses in healthy adults (von Kanel et al., 2008), though this discrepancy may be due to drug timing (Rohleder, 2014). Given that NE release is triggered by SNS response to acute stress, these findings suggest that SNS activation is involved in acute stressor-evoked IL-6 responses.

In addition to the SNS, it is likely that the HPA axis plays a role in regulating the acute stressor-evoked inflammatory response. The HPA-axis secretes GCs in response to psychosocial stress, which typically inhibit the production of IL-6 and other proinflammatory cytokines. In addition, cytokines can activate the HPA-axis, creating a feedback loop that regulates stressor-evoked inflammatory responses (Darnall & Suarez, 2009; Miller et al., 2002). Indeed, there is an inverse association between cortisol and IL-6 responses to acute stress (Rohleder, 2014) and between NF-κB and cortisol responses to acute stress (Wolf, Rohleder, Bierhaus, Nawroth, & Kirschbaum, 2009). These findings suggest that the HPA axis may also play a key role in regulating acute stressor-evoked inflammatory responses.

Comparatively less is known about the role of the PNS in the regulation of the acute

stressor-evoked IL-6 response. There is, however, evidence that the PNS plays a tonic inhibitory role in regulating inflammation. This work is summarized by Tracey's (2002) characterization of the cholinergic anti-inflammatory pathway. According to this pathway, tonic stimulation of the vagus nerve promotes the release of acetylcholine (ACh), the primary neurotransmitter of the PNS. ACh has an anti-inflammatory effect, down-regulating macrophage production of proinflammatory cytokines. Through this pathway, tonic PNS activity prevents the overproduction of cytokines and protects the body from the harmful effects of excessive inflammation (Tracey, 2002).

The cholinergic anti-inflammatory pathway is supported by experimental evidence (Tracey, 2009). To summarize, in vitro evidence shows that ACh inhibits stimulated cytokine production (Borovikova et al., 2000). Rodent evidence also shows that electrical stimulation of the vagus nerve attenuates cytokine synthesis by immune cells (Borovikova et al., 2000). Additionally, vagotomy leads to exaggerated stimulated cytokine production and tissue damage (Schulte, Lichtenstern, Henrich, Weigand, & Uhle, 2014). Although it remains to be shown that the vagus nerve innervates primary immune organs in humans (Nance & Sanders, 2007), there is evidence that the PNS plays a role in down-regulating the inflammatory response. Indeed, human psychophysiological evidence shows inverse associations of circulating levels of IL-6 and CRP with resting HF-HRV (Sloan et al., 2007). Lower resting HF-HRV has also been linked with greater stimulated production of cytokines, including IL-6, in response to an in vitro inflammatory challenge (Marsland et al., 2007). These findings are consistent with a cholinergic anti-inflammatory pathway, indicating that lower levels of tonic PNS activity may relate to heightened inflammatory responses and higher resting levels of inflammatory markers.

The current psychophysiological evidence linking PNS activity and inflammatory

markers has primarily focused on resting levels of HF-HRV. As the cholinergic anti-inflammatory pathway proposes that the PNS regulates inflammatory responses in real time, it is possible that acute stressor-evoked changes in PNS activity may also regulate circulating inflammatory markers. It is unknown, however, whether there is an association between acute stressor-evoked changes in PNS activity and IL-6 responses or background levels of systemic inflammation. One study found that IL-6 responses to stress were not associated with either baseline or task levels of PNS driven HRV (Owen & Steptoe, 2003). Other than this null result, the association of stressor-evoked HF-HRV with resting and stressor-evoked inflammatory markers remains unexplored. Thus, a goal of the present study is to determine whether acute stressor-evoked decreases in HF-HRV are associated with the magnitude of acute stressor-evoked IL-6 response (Aim 3) or resting levels of CRP (Aim 4).

1.5 SCOPE OF THE PROPOSED STUDY

Although inflammatory processes play a key role in CVD pathophysiology, this relationship is primarily assessed using basal circulating levels of inflammatory markers thought to reflect sustained levels of systemic inflammation. Levels of circulating inflammatory markers increase in response to acute stress, but key information is unknown about these responses. These gaps in knowledge prevent key inferences about the relevance of stressor-evoked inflammatory responses for CVD risk.

First, the relationship between these acute stressor-evoked inflammatory responses and established CVD risk factors remains largely unstudied. The present study will address this by assessing the relationship between acute stressor-evoked IL-6 responses and two established

predictors of CVD: IMT and CRP. We hypothesize that larger acute stressor-evoked IL-6 responses will be associated with higher levels of resting CRP, a relatively stable marker of inflammation and established risk factor for CVD (Hypothesis 1). Additionally, we hypothesize that larger acute stressor-evoked IL-6 responses will be associated with greater IMT, a measure of preclinical atherosclerotic CVD risk (Hypothesis 2).

The specific physiological mechanisms that underlie acute stressor-evoked IL-6 responses are also unclear. Current research suggests that the SNS and HPA axis play a role in driving and regulating these responses, but little is known about the association between stressor-evoked PNS responses and circulating inflammatory markers. The current study addresses this in two ways: examining the association between acute stressor-evoked PNS changes and 1) stressor-evoked IL-6 changes, and 2) circulating levels of CRP. We hypothesize that greater decreases in HF-HRV during stress will be associated with larger IL-6 responses (Hypothesis 3) and higher levels of CRP (Hypothesis 4). These relationships will be examined in healthy midlife adults who participated in a laboratory study on the physiological correlates of stress and CVD.

2.0 RESEARCH DESIGN AND METHODS

2.1 PARTICIPANTS

Participants were 91 adults from the Pittsburgh Imaging Project Phase II (PIP II), a study of 176 healthy volunteers residing in Allegheny County, Pennsylvania. This subsample completed a separate protocol to assess physiological and inflammatory responses to laboratory stressors. Participants were between the ages of 30-51 and recruited through mass mail solicitations. Two participants were excluded from the analytic sample due to high IL-6 (see Analytic Plan). Descriptive statistics were assessed for the 89 remaining participants (Table 1). The sample included 53 males and 36 females; this imbalance was due to the fact that only males were recruited for PIP II study during the second half of data acquisition. The majority of participants identified as White (68.5%) and 25.8% identified as Black. For analytic purposes, the 5 participants who did not identify as White or Black were grouped with Black participants for analyses. Average annual family income was $61,119, although there was a sizable range (*SD* = $44,245). A large proportion of participants had completed college (52.3%) or graduate education (33.3%). The sample was slightly overweight, with a mean BMI of 26.10. The majority of participants had never smoked (72.2%). CRP levels were low (*M* = 0.20mg/dL) and IMT was average for this age group (*M* = 0.60mm) (Lorenz et al., 2007; O'Leary & Bots, 2010).

Table 1. Descriptive Statistics

	Mean or n	SD or %	N
Age (years)	40.12	6.61	89
Sex (female)	36	40.4%	89
Race			89
White	61	68.5%	
Black	23	25.8%	
Asian	4	4.5%	
Multiracial	1	1.1%	
BMI (kg/m2)	26.1	4.63	89
Smoking Status			89
Never	64	72.2%	
Former	10	11.1%	
Current	15	16.7%	
Education			89
High School	13	14.4%	
College	46	52.3%	
Graduate	30	33.3%	
Annual family income	$61,119	$44,245	87
Baseline IL-6 (pg/mL)	1.22	0.92	62
Baseline HF-HRV (ln)	5.63	1.22	84
CRP (mg/dL)	0.20	0.27	80
IMT (mean of average; mm)	0.60	0.08	89

Exclusion criteria for the study included: history of cardiovascular disease (including hypertension); prior cardiovascular or cerebrovascular surgery; chronic kidney or liver conditions; Type I or II diabetes; any pulmonary or respiratory diseases; current diagnoses of a substance abuse or mood disorder; and use of any cardiovascular, psychotropic, or lipid lowering medications. This study had a magnetic resonance imaging component and thus excluded participants with: history of neurosurgery or a neurological condition; head trauma leading to loss of consciousness; pregnancy; and claustrophobia or metallic implants. Data collection took place over multiple sessions (Figure 2) and informed consent procedures were carried out following guidelines of the University of Pittsburgh Institutional Review Board.

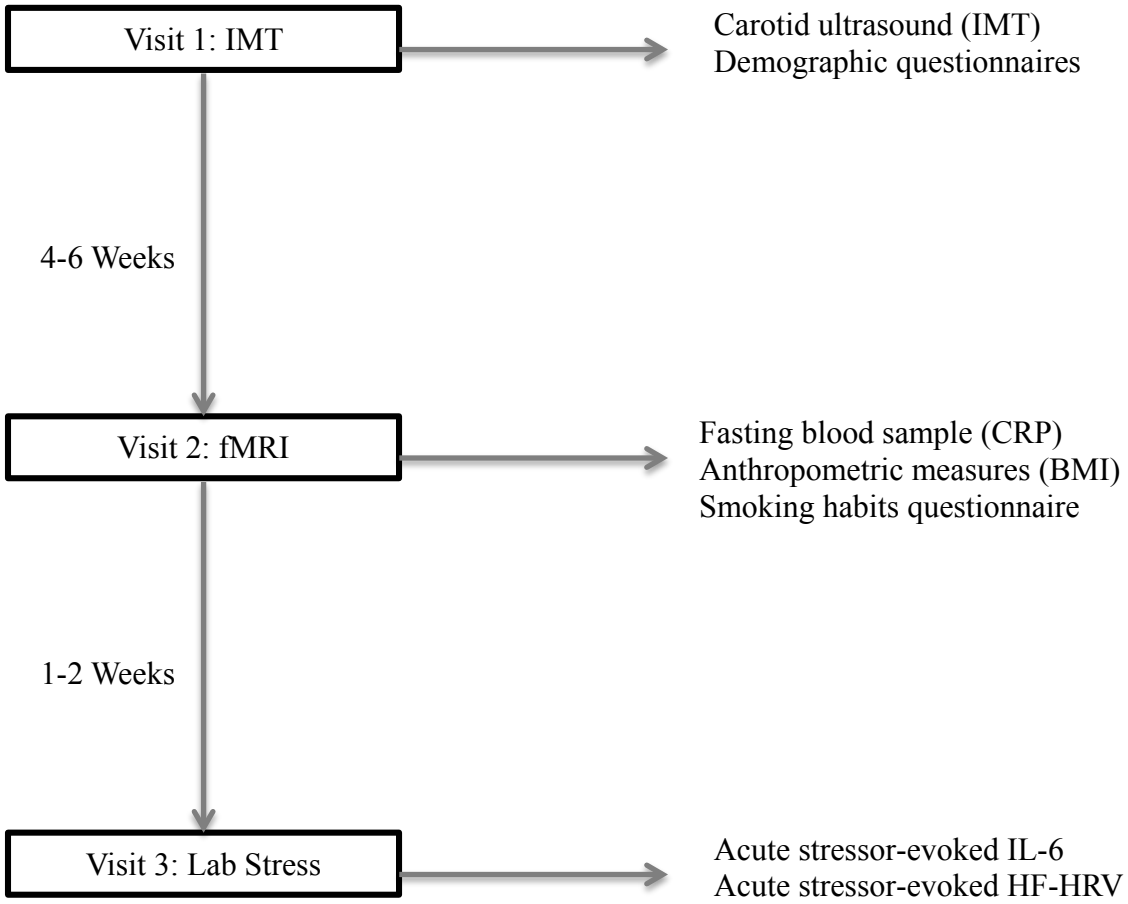

Figure 2. PIP study protocol and measures

2.2 PROCEDURES

Acute stressor-evoked inflammatory response data were collected during a laboratory session that began between 12:00 and 1:00 PM. Participants were instructed to abstain from caffeine (12 hours), strenuous physical activity (24 hours), non-prescription medications (24 hours), and alcohol (48 hours) before the session. On arrival, participants completed an acute illness-screening questionnaire; to avoid heightened inflammation due to acute illness, those with cold

or flu symptoms within 48 hours were rescheduled. Next, participants were prepared for electrocardiogram (EKG), respiration, and BP assessment. An intravenous catheter was then inserted into the antecubital vein of the left arm for the collection of blood samples. Participants then rested quietly for a 30-min baseline period, after which the first blood sample was drawn. After the baseline, participants performed two stressor tasks, separated by a 4-min rest period. Participants then rested quietly for a 30-min recovery period. The post-stressor blood sample was collected 30-min after completion of the second stressor task. EKG, respiration, and BP measurements were collected during the last 10 minutes of the baseline period, throughout both tasks, and during the first 10 minutes of the recovery period.

Participants completed two standardized mental stress tasks validated for laboratory studies of stress reactivity: a Stroop color-word task and the multi-source interference task (MSIT) (Gianaros et al., 2009; Kamarck et al., 1992). Both tasks have elements of conflict, time pressure, error feedback, and uncontrollability. Each task included alternating difficult (incongruent) and easy (congruent) conditions. In the Stroop task, participants saw one target word and four identifier words and instructed to identify the color of the target word by selecting the correct identifying word. In the MSIT task, participants saw with three numbers and instructed to select the number that is different from the other two numbers. To increase task difficulty during incongruent trials, a loud voice stated a random answer choice; the voice stated the correct answer during congruent trials. During each task, incorrect or delayed responses elicited automated negative feedback. Each task lasted approximately 9 minutes with adaptive inter-trial intervals so that accuracy was titrated to <60% for all participants (for further detail, see Sheu, Jennings, & Gianaros, 2012). These tasks have been shown to reliably elicit

cardiovascular responses in a comparable sample, with intra-class correlation coefficients of 0.75-0.85 (Sheu, Jennings, & Gianaros, 2012). Task order was randomized across participants.

2.3 MEASURES

Participants self-reported age, sex, race, education, and family income on a standard demographics questionnaire.

Baseline and 30-min post-task blood samples were used to assess circulating levels of IL-6. Immediately after each blood draw, whole blood was centrifuged at room temperature at 2500 rpm for 10 minutes and plasma was removed and stored at -80 degrees Celsius. Plasma IL-6 levels were determined using high sensitivity ELISA kits (R&D Systems, Minneapolis). Samples were run in duplicate and average intra-assay coefficients of variation for Baseline and 30-min post-task were 4% and 5%, respectively. The two samples from each participant were run on the same plate. There were 27 participants who did not provide blood samples due to refusal to undergo intravenous catheterization (N=9) or blood sampling problems (N=18). There were no statistically significant differences in age, sex, or BMI between participants who completed the blood draw and those who did not. These participants were not included any IL-6 analyses.

Resting levels of high-sensitivity CRP (mg/dL) were measured from a fasting blood sample taken during Visit 2. CRP was assayed by a CRPH reagent on a SYNCHRON LX System (Beckman Coulter, Inc.) in the Clinical Services Laboratory of the Western Psychiatric Institute and Clinic. CRP levels are stable across relatively long periods (1-5 years) (Ridker, 2007). As no participants had CRP levels indicating acute illness (>10 mg/L), all participants

with CRP data were included in analyses. There were ten participants who did not have CRP values due to blood sampling issues. These participants were not included in CRP analyses.

Preclinical atherosclerotic risk was indexed by carotid artery IMT, assessed during Visit 1 of the study. Specifically, IMT was measured as the mean IMT of the carotid vessel wall complex (Sutton-Tyrrell, Wolfson, Thompson, & Kelsey, 1992; Thompson, Sutton-Tyrrell, & Wildman, 2001). This measure has been used as both an indicator of preclinical atherosclerosis and a surrogate marker of coronary atherosclerosis (Craven et al., 1990; Probstfield et al., 1993). IMT was assessed by B-mode ultrasonography by a vascular technologist using an Acuson Antares scanner (Acuson-Simens, Malvern, Pennsylvania) and averaged across the common, bulb, and internal carotid artery segments. These values are determined using automated edge detecting scoring software (Artery Measurement System, Gothenburg, Sweden) (Wendelhag, Liang, Gustavsson, & Wikstrand, 1997). IMT assessments were carried out at the Epidemiology Ultrasound Research Laboratory at University of Pittsburgh.

HRV was derived from continuous EKG assessment during the laboratory stress protocol. A modified lead II electrode placement using three electrodes was used and EKG was sampled at 1000 Hz using MindWare software (Mindware Technologies, Gahanna, OH). Prior to calculating HRV estimates, EKG signals were inspected for irregularities and artifactual R-waves were corrected. Spectral analysis was used to determine the high frequency portion of HRV (HF-HRV) using a bandwidth of 0.15-0.40 Hz. Procedures and analyses were conducted in accordance with published guidelines (Task Force, 1996). Of the participants who completed the laboratory protocol, six participants did not have usable HF-HRV data due to equipment issues (N=5) or arrhythmia (N=1). HR and respiration rate were also collected during the laboratory stress protocol, given their known relationships with HF-HRV (Berntson et al., 1997). HR was

measured using the continuous EKG assessment described above. Respiration rate was assessed using a Sleepmate Piezo effort sensor belt and processed using Mindware analysis software. Systolic (SBP) and diastolic (DBP) BP were assessed oscillometrically by a Dinamap Automated Blood Pressure Monitor Model V100. BP readings were taken at 2-minute intervals during the rest periods and 1-minute intervals during the tasks.

Smoking status and BMI were measured and included as covariates, given their associations with systemic inflammation (O'Connor et al., 2009). Smoking status was measured via self-report of smoking habits and history. BMI was determined using participants' height and weight measurements and calculated as weight (kg)/height (m^2).

2.4 ANALYTIC PLAN

Analyses were carried out using SPSS. Resting CRP, Baseline and 30-min post-task IL-6, and HF-HRV values were non-normally distributed and were log transformed prior to analyses. A log-10 transformation was used for CRP, and natural log transformations were used for IL-6 and HF-HRV. Preliminary descriptive analyses revealed one participant with a circulating baseline IL-6 level of 11.33pg/mL and another with a raw IL-6 change of 4.95pg/mL; as these values deviated substantially (>6 *SD*) from the means for Baseline IL-6 and IL-6 change scores, these two participants were not included in analyses. No other outliers were detected for primary variables. HF-HRV, HR, respiration rate, and BP values were averaged over each period to give a single value for each of the following periods: Baseline, MSIT Incongruent, MSIT Congruent,

Stroop Incongruent, Stroop Congruent, and Recovery. These values were then averaged across the two stressor tasks, resulting in two task variables: Incongruent and Congruent[1].

Stressor task effects were assessed for IL-6, HF-HRV, HR, and respiration rate. A paired samples t-test was used to test for a significant difference in IL-6 from Baseline to 30-min post task. Repeated measures ANOVAs were used to determine main effects of the stressor tasks on psychophysiological measures. Greenhouse-Geisser corrections were applied when the sphericity assumption was violated. Planned contrasts tested whether mean values for Incongruent, Congruent, and Recovery periods differed significantly from Baseline.

Hierarchical linear regressions tested primary study hypotheses. Residualized change scores were created for IL-6 and HF-HRV. IL-6 change scores were computed by regressing 30-min post-task IL-6 levels on Baseline IL-6 levels. HF-HRV change scores were created by regressing Incongruent HF-HRV values on Baseline HF-HRV values. Age, sex, race, smoking status, and BMI were used as covariates. Dummy coding was used for sex (*0 = male, 1 = female)* and smoking status (*0 = never, 1 = former, 2 = current)*. Each aim was tested with two regression models: 1) a minimally adjusted model with age, sex, and race on Step 1 and 2) a fully adjusted model with age, sex, and race on the Step 1 and BMI and smoking status on Step 2.

To test whether acute stressor-evoked IL-6 changes associate positively with resting levels of CRP (Aim 1), regression models predicting CRP with residualized IL-6 change scores

[1] Two additional methods of combining the physiological variables during the laboratory session were also considered: 1) combining task levels across Incongruent and Congruent segments, resulting in separate MSIT and Stroop variables and 2) combining across tasks and across Incongruent and Congruent segments, resulting in a single variable. Regardless of the method, all ANOVA and regression analyses followed a similar pattern of results.

were conducted. This analysis included 57[2]; 33 participants in the analytic sample were not included due to missing IL-6 data (N=23), missing CRP data (N=6), or missing both (N=4).

To test whether acute stressor-evoked IL-6 changes associate positively with IMT (Aim 2), regression models predicting IMT with residualized IL-6 change scores were conducted. This analysis included 62 participants[3]; 27 participants were excluded due to missing IL-6 data.

To test if larger stressor-evoked IL-6 changes associate with greater stressor-evoked HF-HRV suppression (Aim 3), regression models predicting residualized IL-6 change scores with residualized HF-HRV change scores were conducted. This analysis included 57 participants[4]; 32 participants were not included due to missing IL-6 (N=26), HF-HRV (N=5), or both (N=1).

To test whether greater stressor-evoked suppression of HF-HRV associates with higher resting levels of CRP (Aim 4), regression models predicting CRP with residualized HF-HRV change scores were conducted. This analysis included 76 participants[5]; 14 participants were not included due to missing HF-HRV data (N=4), CRP data (N=8), or both (N=2).

Ancillary analyses focused on sex differences tested three questions. First, are there sex differences in the magnitude of IL-6 responses (Ancillary Aim 1)? This question was tested using a repeated measures ANOVA, with sex as a between-subjects factor.

Second, does the relationship between stressor-evoked IL-6 response and CRP vary by sex (Ancillary Aim 2)? This question was tested with two regression models, one minimal model

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[2]The Aim 1 subsample was not significantly different from the rest of the analytic sample on age, sex, race, BMI, smoking status, years of schooling, or resting CRP.
[3]The Aim 2 subsample was not significantly different from the rest of the analytic sample on age, race, BMI, smoking status, years of schooling, or mean IMT. There were fewer females in the subsample compared with the overall sample ($X^2 = 5.51$, $p = .034$).
[4] The Aim 3 subsample was not significantly different from the rest of the analytic sample on age, race, BMI, smoking status, years of schooling, or baseline HF-HRV. There were fewer females in the subsample compared with the analytic sample ($X^2 = 5.34$, $p = .032$).
[5] The Aim 4 subsample was not significantly different from the rest of the analytic sample on age, sex, race, BMI, smoking status, years of schooling, resting CRP, or baseline HF-HRV.

and one fully adjusted. The minimal model included sex and residualized IL-6 change scores on Step 1 and a "sex x IL-6 change" interaction term on Step 2. The fully adjusted model included sex and residualized IL-6 change scores on Step 1, covariates (age, BMI, race, and smoking status) on Step 2, and the sex x IL-6 change interaction term on Step 3. All continuous variables were mean centered prior to regression analyses.

Third, does the relationship between stressor-evoked IL-6 response and IMT vary by sex (Ancillary Aim 3)? This question was tested using the same method as described for Ancillary Aim 2, using IMT as the outcome variable in place of CRP.

Ancillary analyses also assessed whether there were significant associations between stressor-evoked SBP and/or DBP responses and stressor-evoked IL-6 responses (Ancillary Aim 4). BP data reduction was carried out using the same process as was used for the EKG derived psychophysiological measures: mean SBP and DBP values were calculated for Baseline, Incongruent, Congruent, and Recovery. A preliminary repeated measures ANOVA tested whether there was a significant main effect of the stressor tasks on SBP and DBP. Finally, separate regression models were used to assess the association between stressor-evoked IL-6 responses and both SBP and DBP responses. Residualized BP change scores were created by regressing SBP and DBP levels during the Incongruent period on levels during the Baseline period. In the regression models, residualized BP change scores were entered as independent variables. Each association was tested using a minimally adjusted model and a fully adjusted model. As in primary analyses, the minimally adjusted models contained age, sex, and race on Step 1, with BMI and smoking status added in a second step in the full model.

3.0 RESULTS

3.1 BIVARIATE CORRELATIONS

There were a number of significant correlations between primary variables (Table 2). Older age was significantly correlated with lower Baseline HF-HRV (r = -.39, p < .001) and greater IMT (r = .45, p < .001). In addition, higher BMI was significantly correlated with higher Baseline IL-6 (r = .39, p=.001) and lower HF-HRV (r = -.22, p = .04). Higher BMI was also significantly correlated with higher CRP (r = .36, p = .001) and greater IMT (r = .30, p = .004). There was also a trend toward a positive association between Baseline IL-6 and resting CRP (r = .23, p = .08). Stressor-evoked IL-6 and HF-HRV change scores were not significantly correlated with any primary study variables.

Table 2. Bivariate Correlations

	1	2	3	4	5	6	7	8
1. Age (years)	-							
2. BMI (kg/m2)	.18	-						
3. IL-6 Baseline	.04	.39**	-					
4. IL-6 Change	.03	-.05	.01	-				
5. HF-HRV Baseline	.39***	-.22*	.03	-.04	-			
6. HF-HRV Change	-.10	-.02	-.21	-.14	.00	-		
7. CRP (mg/dL)	-.07	.36**	.23	.15	-.15	-.11	-	
8. IMT (mm)	.45***	.30**	.18	.12	-.15	-.01	-.02	-

Note: *** p < .001, ** p < .01, * p < .05

3.2 STRESSOR TASK EFFECTS

Mean values for IL-6, HF-HRV, HR, and respiration rate varied across the different task periods (Table 3; Figure 3-6). Circulating IL-6 increased significantly from Baseline to 30-min post-task (t (61) = -4.83, $p < .001$). There was a significant main effect of the stressor tasks on HF-HRV (F(2.74, 227.23) = 54.06, $p <.001$, $\eta_p = .59$). HF-HRV decreased significantly from Baseline to both Incongruent (F(1, 83) = 91.65, $p <.001$, $\eta_p = .53$) and Congruent (F(1, 83) = 55.81, $p < .001$, $\eta_p = .40$), but not from Baseline and Recovery (F(1, 83) = 1.28, $p = .26$, $\eta_p = .02$). There was also a significant main effect of the stressor tasks on HR (F(2.28, 188.93) = 24.50, $p <.001$, $\eta_p = .23$). HR increased significantly from Baseline to Incongruent (F(1, 83) = 37.51, $p < .001$, $\eta_p = .31$) and Congruent (F(1, 83) = 7.50, $p = .008$, $\eta_p = .08$), but not from Baseline to Recovery (F(1, 83) = .20, $p = .66$, $\eta_p = .002$). Finally, there was a significant main effect of the stressor tasks on respiration rate (F(2.43, 201.97) = 65.59, $p <.001$, $\eta_p = .441$). Respiration increased significantly from Baseline to Incongruent (F(1, 83) = 91.76, $p < .001$, $\eta_p = .53$) and Congruent (F(1, 83) = 51.09, $p < .001$, $\eta_p = .38$) but not from Baseline to Recovery (F(1, 83) = 3.95, $p = .05$, $\eta_p = .05$).

Table 3. Mean HR, HF-HRV, respiration rate, and IL-6 values across the testing session

	Baseline	Incongruent	Congruent	Recovery	30-min post
IL-6	1.22 (0.92)	-	-	-	1.36 (0.96)
HF-HRV	5.64 (1.21)	4.97 (1.15)	5.23 (1.27)	5.58 (1.12)	-
HR	71.9 (11.70)	74.57 (11.73)	72.83 (11.37)	72.08 (10.99)	-
Respiration	15.83 (2.75)	18.94 (3.81)	17.51 (3.60)	15.30 (3.11)	-

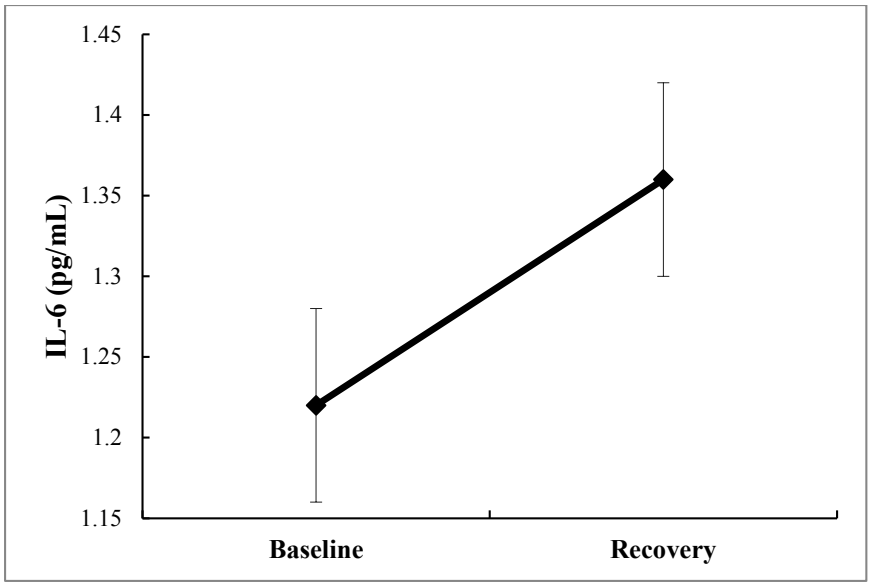

Figure 3. Effect of the acute stressor tasks on IL-6

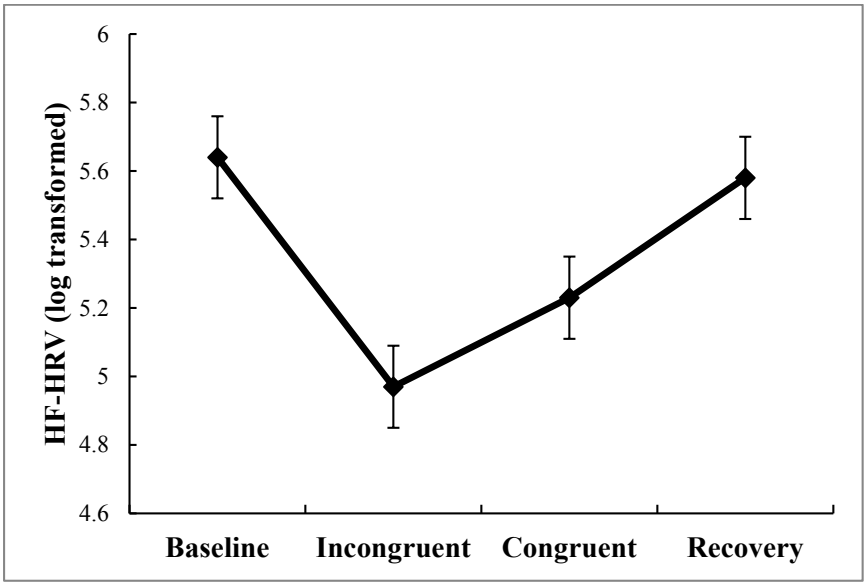

Figure 4. Effect of the acute stressor tasks on HF-HRV

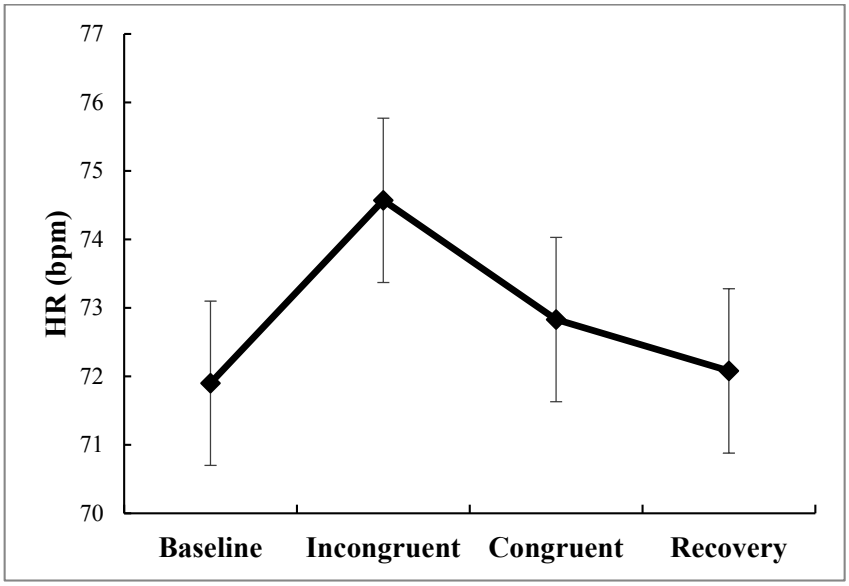

Figure 5. Effect of the acute stressor tasks on HR

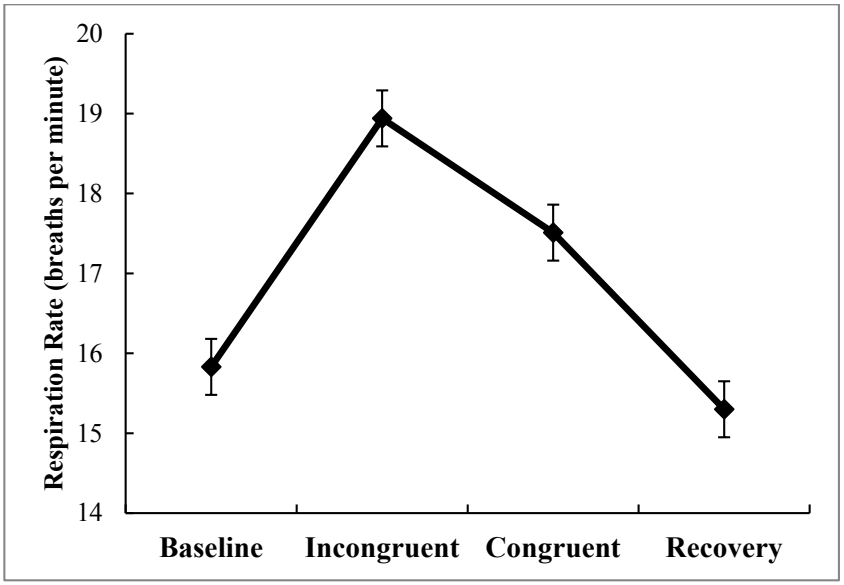

Figure 6. Effect of the acute stressor tasks on respiration rate

3.3 PRIMARY AIMS

The hypothesis that larger stressor-evoked IL-6 responses are associated with higher levels of CRP (Aim 1) was not supported. Stressor-evoked IL-6 change scores were not significantly associated with CRP in the minimally adjusted model (β = .19, p = .17) or the fully adjusted model (Table 4). In the fully adjusted model, demographic variables (Step 1) did not predict significant variance in CRP. BMI and smoking status (Step 2) accounted for 17% of the variance in CRP (ΔR^2 = .17, p = .007), largely driven by the positive association between BMI and CRP (β = .37, p = .01). Stressor-evoked IL-6 responses (Step 3) showed a trend-level association with CRP (β = .22, p = .09) (Figure 7).

Table 4. Aim 1: Linear regression predicting CRP with stressor-evoked IL-6

	N = 57							
	B	SE	β	*p*	R^2	ΔR^2	ΔF	*Sig.* ΔF
Step 1					.04	.04	.82	.49
Age	-.01	.01	-.07	.63				
Sex	.02	.12	-.02	.87				
Race	.15	.12	.18	.21				
Step 2					.21	.17	5.41	.007
BMI	.03	.01	.37	.01				
Smoking Status	.11	.08	.19	.15				
Step 3					.26	.04	2.95	.09
IL-6 Change	.45	.27	.22	.09				

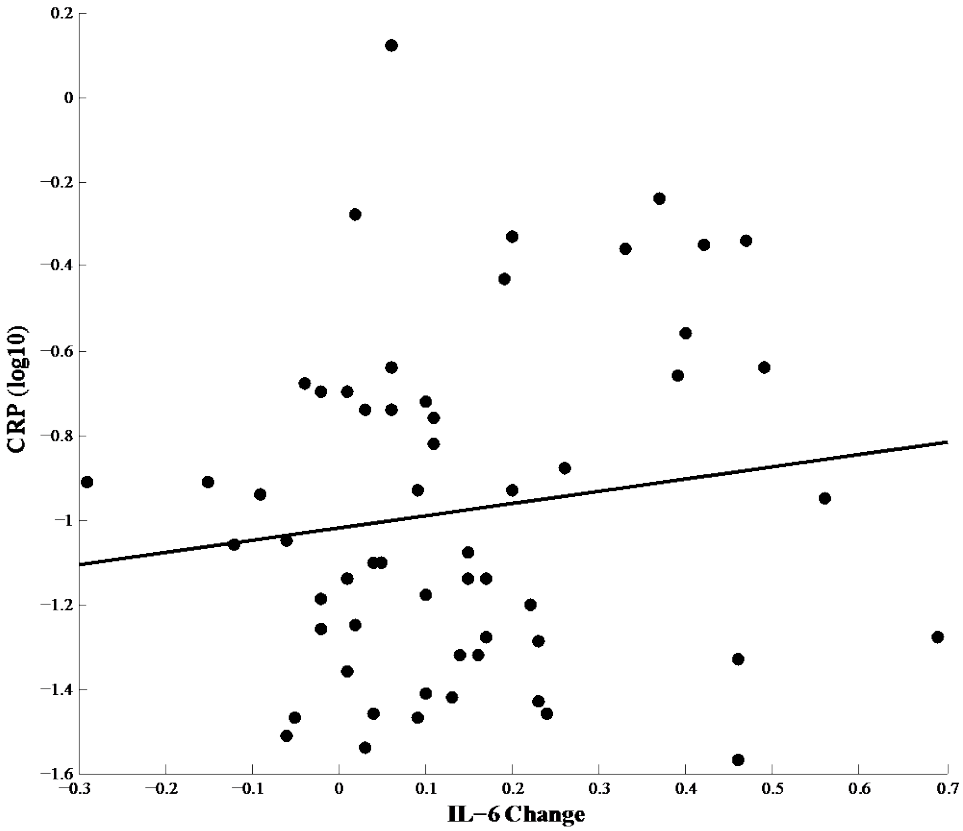

Figure 7. Association of stressor-evoked IL-6 response and CRP (Aim 1)

The hypothesis that greater stressor-evoked IL-6 responses are positively associated with IMT was not supported (Aim 2). Stressor-evoked IL-6 change scores were not significantly associated with IMT in the minimally adjusted model (β = .13, p = .30) or the fully adjusted model (Table 5). Demographic variables (Step 1) accounted for 18% of the variance in IMT (ΔR^2 = .18, p = .007), with older age significantly associated with greater IMT (β = .43, p = .001). BMI and smoking status (Step 2) accounted for an additional 19% of the variance in IMT (ΔR^2 = .19, p = .001), with BMI significantly associated with IMT (β = .43, p < .001). Stressor-evoked IL-6 change scores (Step 3) were not associated with IMT (β = .12, p = .29) (Figure 8).

Table 5. Aim 2: Linear regression predicting IMT with stressor-evoked IL-6

	N = 62							
	B	SE	β	*p*	R^2	ΔR^2	ΔF	*Sig.* ΔF
Step 1					.18	.18	4.41	.007
Age	.01	.01	.43	.001				
Sex	-.01	.02	-.05	.72				
Race	-.01	.02	-.05	.71				
Step 2					.37	.19	8.47	.001
BMI	.01	.01	.43	<.001				
Smoking Status	-.02	.01	-.20	.08				
Step 3					.38	.01	1.14	.29
IL-6 Change	.05	.04	.12	.29				

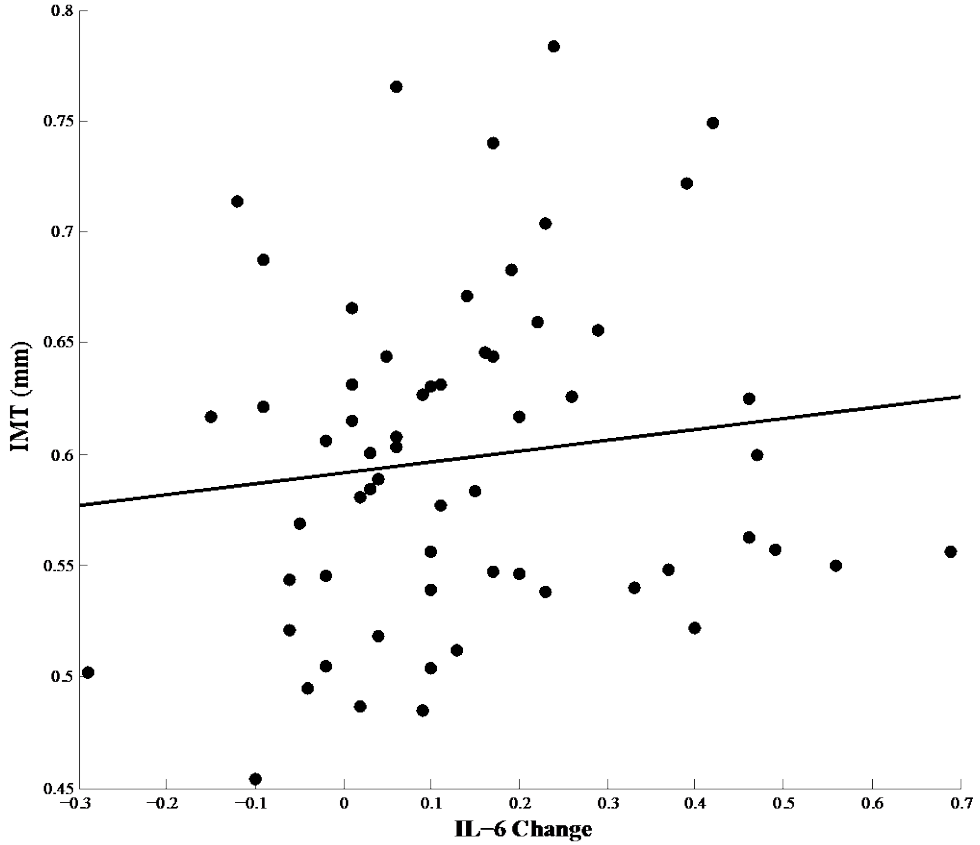

Figure 8. Association of stressor-evoked IL-6 response and IMT (Aim 2)

The hypothesis that greater stress-related HF-HRV suppression is associated with larger stressor-evoked IL-6 responses was not supported (Aim 3). Stressor-evoked HF-HRV suppression was not significantly associated with stressor-evoked IL-6 responses in the minimally adjusted model (β = -.10, p = .49) or the fully adjusted model (Table 6). None of the steps of the fully adjusted model explained significant variance in stressor-evoked IL-6 responses, although sex was significantly associated with IL-6 change scores (β = .34, p = .02), such that females showed greater IL-6 change scores. Stressor-evoked HF-HRV suppression (Step 3) was not significantly associated with stressor-evoked IL-6 (β = .09, p = .52) (Figure 9).

Table 6. Aim 3: Linear regression predicting stressor-evoked IL-6 with stressor-evoked HF-HRV

	N = 57							
	B	SE	β	*p*	R^2	ΔR^2	ΔF	*Sig.* ΔF
Step 1					.10	.10	2.05	.12
Age	-.01	.01	-.11	.42				
Sex	.13	.05	.34	.02				
Race	.01	.05	.01	.97				
Step 2					.11	.003	.08	.93
BMI	.01	.01	.05	.73				
Smoking Status	-.01	.03	-.03	.84				
Step 3					.11	.01	.41	.52
IL-6 Change	.03	.05	.09	.52				

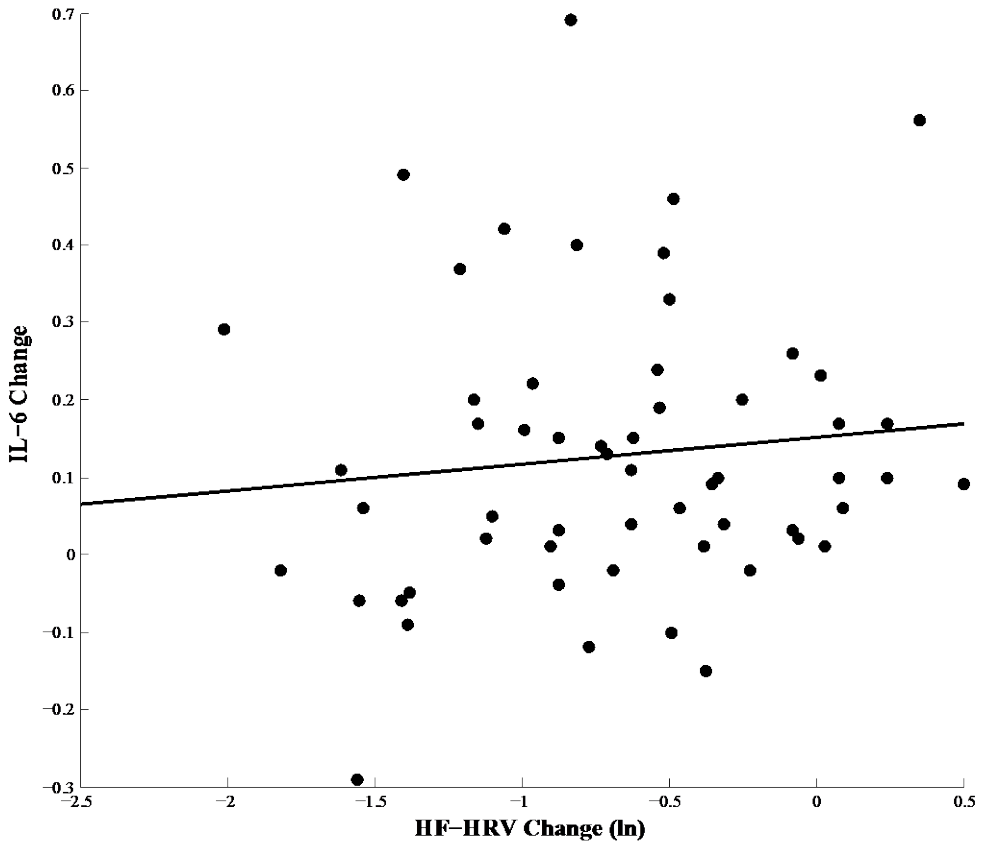

Figure 9. Association of stressor-evoked HF-HRV and stressor-evoked IL-6 response (Aim 3)

The hypothesis that greater stressor-evoked HF-HRV suppression would be associated with higher resting levels of CRP was not supported (Aim 4). Stressor-evoked HF-HRV suppression was not significantly associated with CRP in the minimally adjusted model (β = -.12, p = .31) or the fully adjusted model (Table 7). In the fully adjusted model, demographic variables (Step 1) did not account for significant variance in CRP. BMI and smoking status (Step 2) accounted for 19% of the variance in CRP (ΔR^2 = .19, p = .001), with higher BMI significantly associated with higher CRP (β = .374, p = .001). Stressor-evoked HF-HRV suppression (Step 3) was not significantly associated with CRP (β = -.14, p = .22) (Figure 10).

Table 7. Aim 4: Linear regression predicting CRP with stressor-evoked HF-HRV

	N = 76							
	B	SE	β	*p*	R^2	ΔR^2	ΔF	*Sig.* ΔF
Step 1					.04	.04	.86	.47
Age	-.01	.01	-.02	.90				
Sex	.06	.10	.07	.54				
Race	.17	.11	.18	.13				
Step 2					.22	.17	8.34	.001
BMI	.04	.01	.37	.001				
Smoking Status	.11	.06	.20	.07				
Step 3					.24	.02	1.57	.22
IL-6 Change	-.10	.08	-.14	.22				

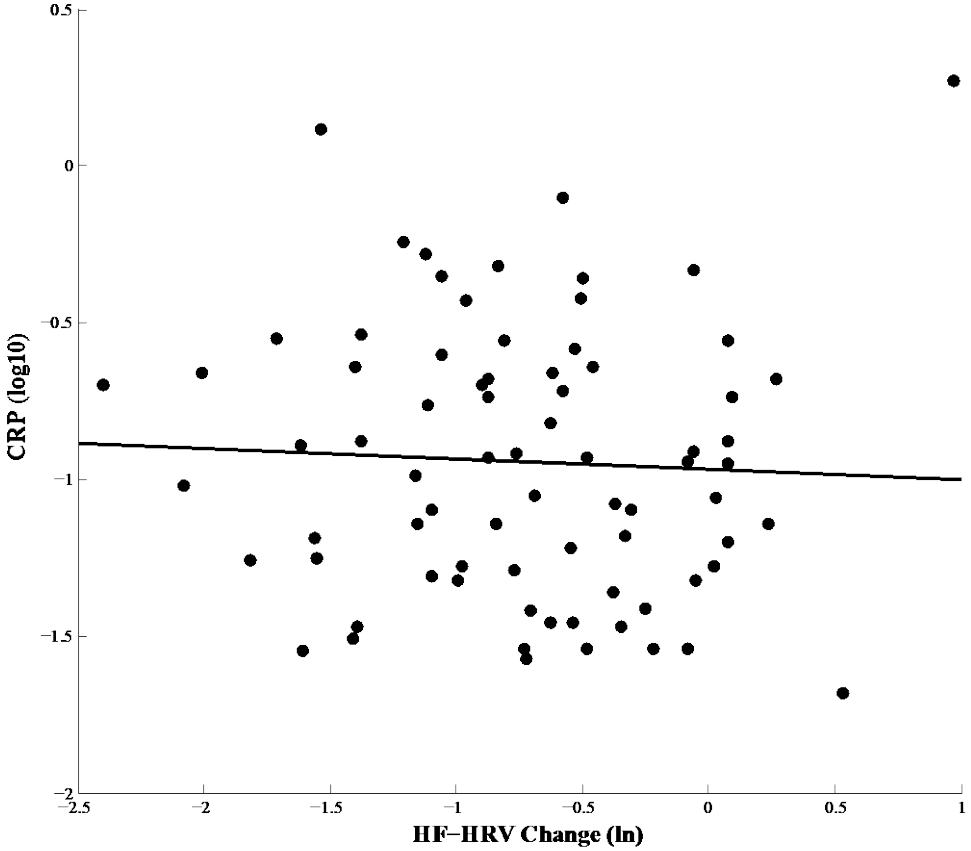

Figure 10. Association of stressor-evoked HF-HRV and CRP (Aim 4)

3.3.1 Respiration Adjustment of HF-HRV

As respiration is known to have a significant influence on HF-HRV, controlling for respiratory parameters is recommended when using HF-HRV as a marker of PNS activity (Grossman, Karemaker, & Wieling, 1991). Since the repeated measures ANOVAs reported above indicate a significant main effect of the stressor-tasks on respiration rate, additional analyses were carried out to account for the possible confounding of respiration and HF-HRV. Respiration adjusted HF-HRV values were calculated by regressing HF-HRV values for each period onto respiration rate for the same period. A repeated measures ANOVA was carried out using the respiration adjusted HF-HRV values for Baseline, Incongruent, Congruent, and Recovery. There was no significant main effect of the stressor tasks on HF-HRV independent of respiration rate ($F(3, 252) = .00$, $p = 1.00$, $\eta_p = .00$). In other words, respiration rate accounts for all of the variation in HF-HRV from Baseline to the Incongruent, Congruent, and Recovery periods. For this reason, primary regression analyses including HF-HRV (Aims 3 and 4) should be interpreted with caution. These analyses were re-run with the inclusion of residualized respiration rate change score as a covariate; the pattern of results remained the same.

3.4 ANCILLARY ANALYSES

There were significant sex differences in the magnitude of stressor-evoked IL-6 responses ($F(1, 60) = 4.85$, $p = .03$, $\eta_p = .08$) (Table 8). Specifically, females show a greater increase in IL-6 from Baseline to 30-min post-task compared with males (Figure 11).

Table 8. Mean IL-6 stratified by sex

	Baseline	30-min post task	Raw Change	N
Males	1.21 (1.00)	1.31 (1.07)	0.10	42
Females	1.23 (.75)	1.44 (.70)	0.21	20
Overall	1.22 (.92)	1.36 (.96)	0.14	62

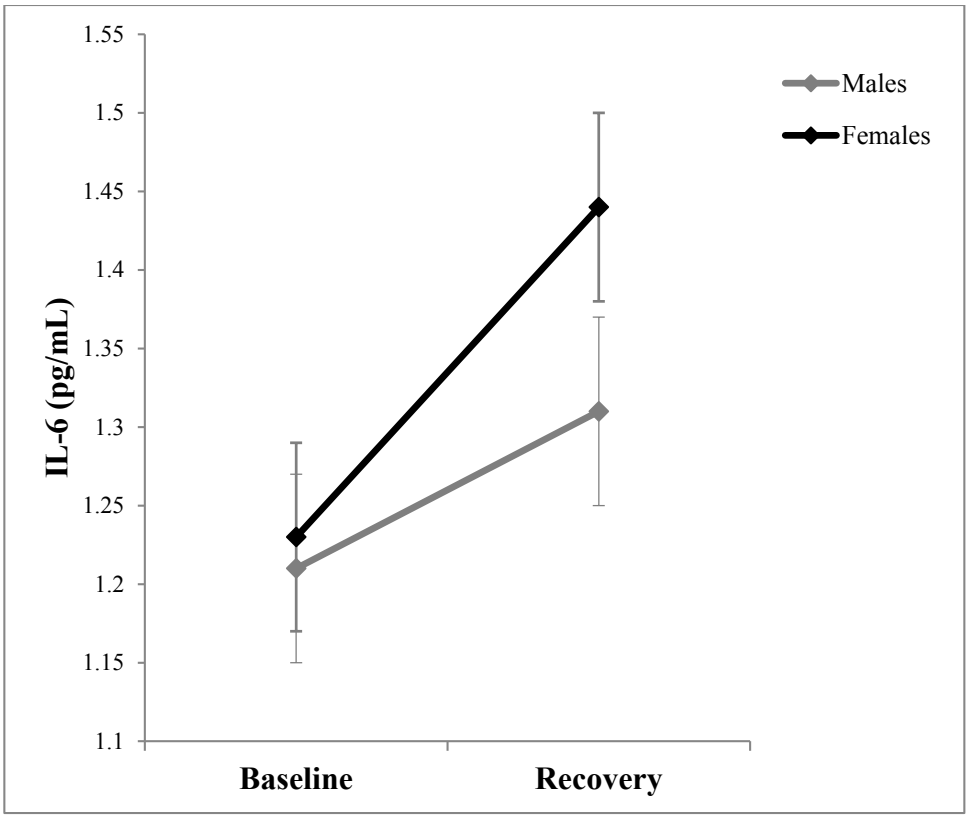

Figure 11. Sex differences in stressor-evoked IL-6 responses (Ancillary Aim 1).

The relationship between stressor-evoked IL-6 response and resting CRP varies by sex. There was a significant interaction between sex and stressor-evoked IL-6 responses in predicting CRP in the minimal model (β = -.36, p = .04) and the fully adjusted model (Table 9). In the fully adjusted model, covariates (Step 2) accounted for 21% of the variance in CRP (ΔR^2 = .21, p = .01), with higher BMI significantly associated with higher CRP (β = .37, p = .006). The sex x

IL-6 change interaction term (Step 3), accounted for 8% of the overall variance in CRP (ΔR^2 = .08, B = -1.33, β = -.39, p = .02). This indicates that the slope of the relationship between IL-6 change and CRP differs by sex (Figure 12). In males, the slope of the association between stressor-evoked IL-6 change and CRP was 0.94; for every unit increase in IL-6 change, there is a 0.94 *increase* in resting CRP. In females, the slope is -0.39; for every unit increase in IL-6 change, there is a 0.39 unit *decrease* in resting CRP.[6] Thus, the slope coefficient in males indicates the relationship predicted in Aim 1 of the primary analyses (greater stressor-evoked IL-6 responses are associated with higher levels of CRP). The slope coefficient in females indicates a negative association between stressor-evoked IL-6 response and resting CRP, such that greater stressor-evoked IL-6 responses are associated with lower resting CRP.

[6] Note that both stress-evoked IL-6 and resting CRP values used in regression analyses do not reflect the raw values of these variables and interpretation of slopes does not reflect raw units of these variables; IL-6 change scores are residualized scores accounting for baseline levels of IL-6, while the CRP values are log-10 transformed.

Table 9. Ancillary Aim 2: Linear regressions predicting CRP with stressor-evoked IL-6 x sex interaction

	N = 57							
	B	SE	β	*p*	R^2	ΔR^2	Δ*F*	*Sig.* Δ*F*
Minimal Model								
Step 1					.04	.04	1.16	.32
Sex	-0.12	.12	-.14	.31				
IL-6 Change	0.39	.29	.19	.18				
Step 2					.12	.08	4.47	.04
Sex x IL-6 Change	-1.23	.58	-.36	.04				
Fully Adjusted Model								
Step 1					.04	.04	1.16	.32
Sex	-0.12	.12	-.14	.31				
IL-6 Change	0.39	.29	.19	.18				
Step 2					.26	.21	3.60	.01
Age	-0.01	.01	-.15	.26				
Race	0.05	.11	.06	.64				
BMI	0.03	.01	.37	.006				
Smoking Status	0.12	.07	.21	.11				
Step 3					.34	.08	6.11	.02
Sex x IL-6 Change	-1.33	.54	-.39	.02				

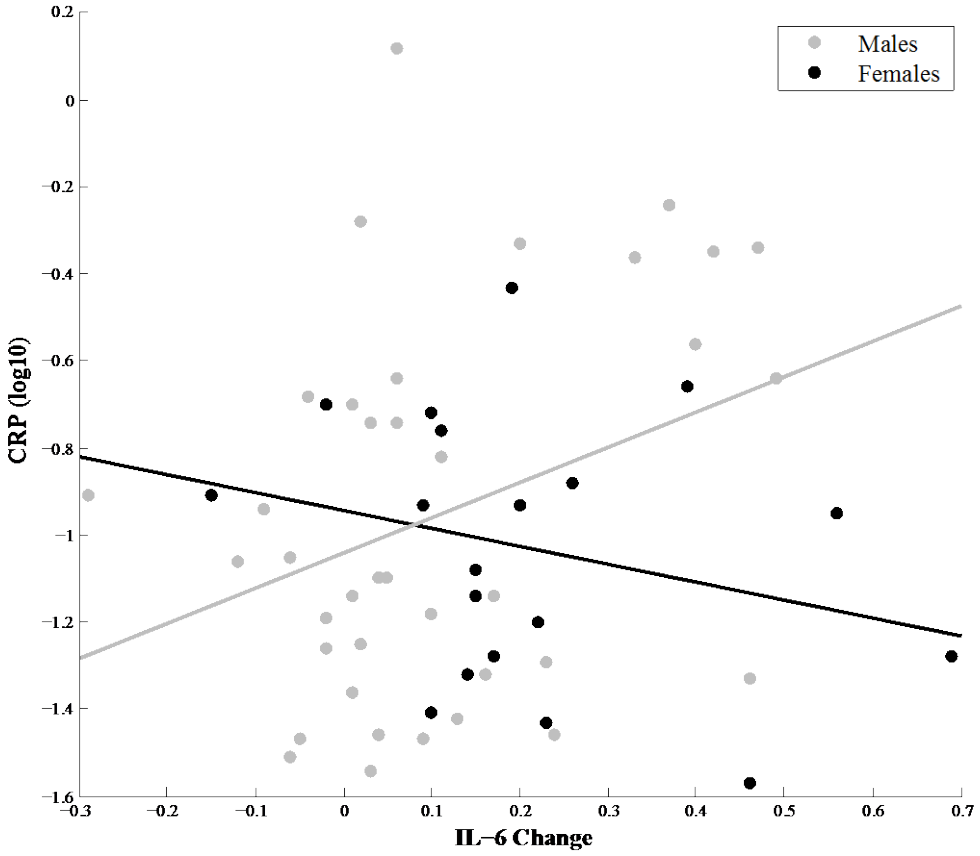

Figure 12. Sex specific associations of stressor-evoked IL-6 and CRP (Ancillary Aim 2).

There was no evidence for an interaction between sex and stressor-evoked IL-6 response in predicting IMT. The sex x IL-6 change interaction term was not significantly associated with IMT in the minimal model (β = -.08, p = .63) or the fully adjusted model (Table 10; Figure 13). In the fully adjusted model, covariates (Step 2) accounted for 38% of the variance in IMT (ΔR^2 = .38, p < .001), with greater IMT significantly associated with age (β = .35, p = .004) and BMI (β = .43, p <.001). The sex x IL-6 change interaction term (Step 3) was not significantly associated with IMT (β = -.04, p = .80).

Table 10. Ancillary Aim 2: Linear regressions predicting IMT with stressor-evoked IL-6 x sex interaction

	N = 62							
	B	SE	β	*p*	R^2	ΔR^2	Δ*F*	*Sig.* Δ*F*
Minimal Model								
Step 1					.02	.02	.71	.49
Sex	0.02	.02	.09	.49				
IL-6 Change	0.04	.05	.10	.46				
Step 2					.03	.01	.23	.63
Sex x IL-6 Change	-0.05	.11	-.08	.63				
Fully Adjusted Model								
Step 1					.02	.02	.71	.49
Sex	0.02	.02	.09	.49				
IL-6 Change	0.04	.05	.10	.46				
Step 2					.38	.36	8.05	<.001
Age	0.01	.001	.35	.004				
Race	-0.01	.02	-.02	.85				
BMI	0.01	.002	.43	<.001				
Smoking Status	-0.02	.01	-.19	.09				
Step 3					.39	.00	.06	.80
Sex x IL-6 Change	-0.02	.09	-.04	.80				

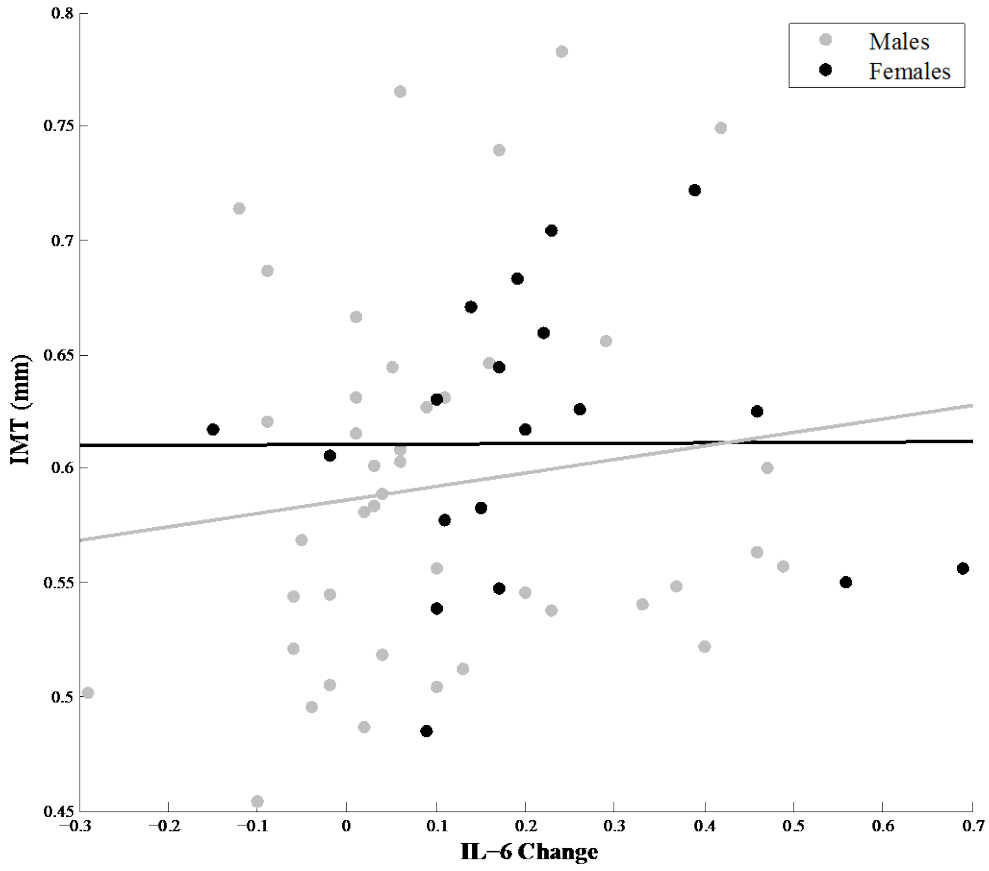

Figure 13. Sex specific associations of stressor-evoked IL-6 and IMT (Ancillary Aim 3).

There was a significant main effect of the stressor tasks on both SBP (F(2.08, 181.21) = 41.79, p <.001, η_p = .32) and DBP (F(2.05, 178.03) = 16.32, p <.001, η_p = .16) (Table 11). SBP increased significantly from Baseline to Incongruent (F(1, 87) = 81.49, p <.001, η_p = .48) and Congruent (F(1, 87) = 72.55, p <.001, η_p = .46); SBP levels remained significantly elevated during Recovery (F(1, 87) = 21.99, p <.001, , η_p =.20). DBP also increased significantly from Baseline to Incongruent (F(1, 87) = 31.51, p <.001, η_p = .27) and Congruent (F(1, 87) = 21.74, p <.001, η_p = .20); these elevations remained marginally elevated from Baseline to Recovery (F(1, 87) = 3.70, p = .06, η_p = .04).

There was no evidence for a significant association between IL-6 responses and either SBP or DBP (Table 12). In the minimally adjusted models, stressor-evoked SBP was not significantly associated with IL-6 (β = -.06, p = .63) nor was DBP (β = .18, p = .15). None of the steps of the fully adjusted model explained significant variance in stressor-evoked IL-6 responses. Stressor-evoked SBP and DBP (Step 3) were not significantly associated with stressor-evoked IL-6 responses (SBP: β = -.07, p = .59; DBP: β = .18, p = .17)

Table 11. Mean SBP and DBP across the testing session

	Baseline	Incongruent	Congruent	Recovery
SBP	111.08	116.26	115.82	113.71
DBP	66.21	68.41	68.05	66.83

Table 12. Ancillary Aim 4: Linear regressions predicting stressor-evoked IL-6 with SBP and DPB

	N = 62							
	B	SE	β	*p*	R^2	ΔR^2	ΔF	*Sig.* ΔF
Step 1					.09	.09	1.98	.13
Age	-0.01	.01	-.09	.49				
Sex	0.11	.06	.28	.05				
Race	-0.05	.05	-.12	.37				
Step 2					.10	.002	.07	.94
BMI	0.00	.01	.01	.93				
Smoking Status	-0.01	.04	-.05	.72				
Step 3 (separate models)								
SBP	-0.01	.01	-.07	.59	.10	.005	.29	.60
DBP	0.01	.01	.18	.17	.13	.03	1.92	.17

4.0 DISCUSSION

This study examined interrelationships between acute stressor-evoked inflammatory responses, CVD risk factors, and stressor-evoked psychophysiological responses. The stressor tasks elicited the expected responses: decreases in HF-HRV and increases in IL-6, HR, respiration, and BP. The interrelationships between these responses and CVD risk factors were explored with four primary aims. Aim 1 assessed whether the magnitude of acute stressor-evoked inflammatory responses positively associates with resting levels of systemic inflammation. Across all participants, results revealed no significant association of stressor-evoked IL-6 responses with circulating levels of CRP. However, ancillary analyses showed that sex moderated the magnitude of the association. Specifically, males showed a positive association between stressor-evoked IL-6 responses and CRP while females showed a negative association. Aim 2 examined whether stressor-evoked IL-6 responses associate with preclinical atherosclerotic CVD risk, as measured by IMT. Here, results showed no significant association and that sex does not moderate the relationship. Aim 3 examined whether stressor-evoked decreases in HF-HRV, a marker of parasympathetic activity, associate with magnitude of IL-6 response following the task. Results did not show a significant association between task-related decreases in HF-HRV and increases in IL-6. Finally, in Aim 4, we examined whether stressor-evoked decreases in HF-HRV relate to circulating levels of systemic inflammation. Again, we observed no significant association between stressor-evoked HF-HRV and CRP. Although our study hypotheses were largely

unsupported, below we consider a number of interpretive caveats and limitations that may clarify these results.

4.1 AIM 1: STRESSOR-EVOKED IL-6 RESPONSES AND CRP

Stressor-evoked IL-6 responses were not significantly associated with resting CRP when males and females were analyzed together. However, ancillary analyses revealed a significant interaction between sex and stressor-evoked IL-6 responses, such that males showed a positive association between magnitude of IL-6 responses and resting CRP while females showed a negative association. In addition, females showed greater stressor-evoked IL-6 responses compared with males, replicating previously reported sex differences in stressor-evoked IL-6 responses (Hackett et al., 2012; Steptoe et al., 2002)

Prior work suggests that there are sex differences in IL-6. Previous reports on resting levels of IL-6 have been mixed, with some studies finding higher levels in females (Chapman et al., 2009), others finding higher levels in males (Gruenewald, Seeman, Ryff, Karlamangla, & Singer, 2006; Thorand et al., 2006), and still others reporting no difference (Sadeghi, Daniel, Naujokat, Weimer, & Opelz, 2005). These reports suggest that sex differences in resting levels of IL-6 are inconsistent across samples. Prior work suggests that there may be more consistent sex differences in magnitude of stressor-evoked IL-6 responses. In the present study, females showed significantly larger stressor-evoked IL-6 responses than males. While two other laboratory stress studies found similar differences (Hackett et al., 2012; Steptoe et al., 2002), another found that males had higher levels of IL-6 than females at 30-min post-stress followed by a rapid decrease, while females showed higher levels at 60-min post-stress (Edwards et al., 2006). As both the

present study and the study by Steptoe and colleagues (2002) measured IL-6 only at 45-min post-stressor, it is possible that this single post-stressor blood sample did not capture an earlier circulating IL-6 peak in males. However, this hypothesis is inconsistent with an additional report that females had higher levels of IL-6 immediately following the stressor task (Hackett et al., 2012). In sum, although the exact pattern varies between studies, our findings support other reports that females show greater stressor-evoked IL-6 responses.

The mechanisms underlying sex differences in stressor-evoked circulating IL-6 responses are not entirely clear. However, our mechanistic understanding may be informed by studies of sex differences in stimulated monocyte production of IL-6 in vitro. Although the in vitro work does show some parallels with our results, it should be noted that it is unclear whether responses to an immune stimulant are relevant for understanding variability in circulating levels of IL-6 in response to a psychological stressor. Considering that caveat, it does appear that females show larger stimulated IL-6 responses. One in vitro study found that lipopolysaccharide (LPS) stimulated IL-6 production is greater in females than in males (O'Connor, Motivala, Valladares, Olmstead, & Irwin, 2007). Another study assessed sex differences in stimulated IL-6 production 30 minutes after an acute laboratory stress paradigm, finding that females showed greater IL-6 production; however, these sex differences were only detected between males and post-menopausal females (Prather et al., 2009). Thus, it is possible that females mount larger inflammatory responses to immune stimulants than males.

A number of possible mechanisms may account for differential IL-6 response magnitude by sex. First, sex differences in HPA axis responses to stress may play a role. After stress exposure, GCs secreted by the HPA-axis inhibit IL-6 production (Argarwal et al., 1998; Miller, Cohen, & Ritchey, 2002). A review of the literature on stressor-evoked cortisol responses

indicates that males show greater responses compared to females (Kudielka & Kirschbaum, 2005). Thus, it is possible that enhanced cortisol responses in males may more effectively downregulate stressor-evoked IL-6 responses. However, in vitro studies show there are individual differences in the sensitivity of immune cells to GC signaling, leading to differences the ability of GCs to regulate inflammatory responses (Miller, Cohen, & Ritchey, 2002). Reductions in GC sensitivity are related to both chronic and acute stress exposure (Dickerson, Gable, Irwin, Aziz, & Kemeny, 2009; Miller et al., 2002). Notably, there is evidence for acute stressor-evoked sex differences in GC sensitivity. Specifically, one study found that males show acute stressor-evoked increases in GC sensitivity of IL-6 production while females showed no stressor-evoked changes (Rohleder, Schommer, Hellhammer, Engel, & Kirschbaum, 2001). This finding suggests that, after acute stress, GCs in females may be less effective in shutting down stimulated IL-6 responses. Thus, to the extent that stimulated IL-6 responses are related to stressor-evoked increases in circulating IL-6 levels, stressor-evoked differences in GC sensitivity may be one mechanism contributing to larger stressor-evoked IL-6 responses in females.

Second, sex differences in IL-6 response magnitude may be explained by differences in autonomic activity. Support for this mechanistic explanation is mixed. A recent meta-analysis reported no sex differences in stressor-evoked PNS responses, but significantly greater stressor-evoked SNS responses in males (Brindle et al., 2014). As greater SNS activation is thought to predict heightened inflammatory responses to stress, these meta-analytic results suggest that males should have larger IL-6 responses than females. Our data shows the opposite pattern, suggesting that sex differences in IL-6 response might not be driven by differential stressor-evoked SNS activation. In addition to stressor-evoked autonomic responses, it has also been suggested that basal autonomic activity may account for sex differences in IL-6 responses. In a

one study, females showed both greater stimulated IL-6 production and a positive association between resting HF-HRV and stimulated IL-6 production, while males showed no association (O'Connor et al., 2007). The significant association in females runs contrary to the predictions of the cholinergic anti-inflammatory pathway (Tracey, 2002; 2009), which suggests that vagal activation should downregulate IL-6 production. Follow up analyses in the present study tested whether there were sex differences in the association between basal HF-HRV and stressor-evoked IL-6 response. Analyses showed a marginally significant interaction, such that females show a negative association between resting HF-HRV and stressor-evoked IL-6 response while males show no association (ΔR^2 = .05, β = -.25, p = .09). Taken together, these mixed findings are inconclusive and warrant continued exploration of sex differences in autonomic activity as a mechanism for sex differences in stressor-evoked IL-6 response.

Third, it is possible that IL-6 expression is influenced by different levels of reproductive hormones, such as estrogens. In general, estrogens are thought to have an anti-inflammatory effect (Gubbels Bupp, 2015; Straub, 2007), inhibiting IL-6 production and gene expression (Liu, Liu, & Bodenner, 2005). Estrogen levels are generally lower in males compared with females, and lower in post-menopausal compared with pre-menopausal females (Darnall & Suarez, 2009; Gubbels Bupp, 2015). Thus, one would anticipate greater IL-6 production in post-menopausal females compared with males (Prather et al., 2009). Moreover, pre-menopausal females (i.e., those with the highest plasma concentrations of estrogens) should have the lower levels of IL-6 production compared with males; however, this was not the case in the study by O'Connor et al. (2007). Additionally, females in the present sample were predominantly premenopausal (85.7%), and showed larger stressor-evoked IL-6 responses compared to males. To assess whether this small number of females influenced results, analyses were repeated excluding the post-

menopausal females; without these participants all significant results remained significant. Thus, if estrogens do influence stressor-evoked IL-6 responses, their effect may not be simply anti-inflammatory. In summary, the mechanisms that underlie sex differences in the magnitude of IL-6 responses are not entirely clear, but interactions with the HPA axis, the ANS, and estrogens are likely involved.

These same mechanisms may also be relevant to sex differences in the association between IL-6 responses and CRP. Exploratory analyses revealed that the relationship between stressor-evoked IL-6 responses and resting CRP differs by sex. It is surprising that, even though females showed larger stressor-evoked IL-6 responses, only males showed the hypothesized positive association between IL-6 responses and CRP. In males, our results support the assumption that larger acute stressor-evoked inflammatory responses are linked with greater resting levels of systemic inflammation. These results are consistent with previous work showing that larger stressor-evoked IL-6 responses are associated with other CVD risk factors (Brydon & Steptoe, 2005; Ellins et al., 2008). Importantly, these results are cross-sectional in nature and prevent us from making any claims about the direction of the relationship between stressor-evoked IL-6 responses and CRP. As IL-6 is a precursor of CRP, it is plausible that, over time larger stressor-evoked IL-6 responses cause heightened systemic levels of CRP. Conversely, it is possible that heightened levels of systemic CRP may somehow prime immune cells to produce larger amounts of IL-6 in response to acute stress. Although it is impossible to answer such mechanistic questions with these data, future longitudinal work could help elucidate the temporal relationship between stressor-evoked IL-6 responses and resting systemic inflammation. Such work should consider that longitudinal associations may also differ by sex.

Unlike males, females showed a negative association between stressor-evoked IL-6 responses and CRP. The reason for these sex differences is uncertain; demographics and biobehavioral characteristics (i.e., age, race, BMI, and smoking status) do not seem to explain the differential relationship. This pattern of sex differences is of particular interest, as CVD incidence rates in females show an approximate 10-year lag behind incidence in males (Gubbels Bupp, 2015; Lerner & Kannel, 1986; Roger, Go, & Lloyd-Jones, 2012). This lag is often attributed to hormonal changes that occur during and after menopause; this may be particularly important here, as the present sample includes predominantly premenopausal females. Premenopausal females may be protected against inflammatory atherosclerotic processes by higher estrogen levels, as higher concentrations of estrogens tend to have anti-inflammatory effects (Gubbels Bupp, 2015). Thus, it may be that even though females show greater acute stressor-evoked IL-6 responses, this may not result in greater resting levels of systemic inflammation (i.e., CRP) due to higher levels of anti-inflammatory estrogens.

In interpreting these sex differences, it is important to emphasize the limitations of the present sample. Specifically, the Aim 1 subsample included 57 participants, only 19 of whom were females. This small group of females may not be representative of the broader population. However, we did not find any systematic differences between these females and the rest of our analytic sample. Regardless, the small sample size is a major limitation of the present work; future studies should test these questions in a larger, more generalizable sample.

4.2 AIM 2: STRESSOR-EVOKED IL-6 RESPONSES AND IMT

Our results showed that there is no significant association between stressor-evoked IL-6 responses and IMT; in contrast to Aim 1, there were no sex differences obscuring a potential relationship. These results are contrary to previous evidence that stressor-evoked IL-6 responses are associated with preclinical CVD risk factors (Brydon & Steptoe, 2005; Ellins et al., 2008). The explanation for the null relationship between stressor-evoked IL-6 responses and IMT is less clear. The lack of association may be due to the cross-sectional nature of this study; it is possible that stressor-evoked IL-6 responses are indeed related to IMT, but not until a future time point. Cross-sectional analyses assume that ongoing inflammatory processes have occurred over a long enough period for stressor-evoked IL-6 responses to influence atherosclerotic processes. It is possible that participants in the study are not old enough for stressor-evoked IL-6 responses to have had an effect on atherosclerotic processes or for endothelial injury to have occurred. However, IMT was specifically selected as a marker of preclinical atherosclerotic risk because the degree of thickness has been show to be predictive of CVD risk, even in younger, healthier adults; this reduces the likelihood that the age of participants plays a major role. These questions could be addressed with longitudinal assessment of atherosclerosis and other markers of CVD risk. It is also possible that atherosclerotic processes are not influenced by these small, stressor-evoked increases in IL-6. The duration of these elevations in circulating IL-6 have yet to be conclusively determined; it is possible that circulating IL-6 levels return to baseline after only a few hours and do not have any lasting impact. Finally, the lack of significant association may be due to the small sample. A post-hoc power analysis showed that, with a subsample of 62, we did not achieve adequate power to detect the small effect size ($f^2 = .02$). A sample size of 240 would be required to detect such a small effect size.

4.3 AIMS 3 & 4: STRESSOR-EVOKED HF-HRV AND INFLAMMATION

Aims 3 and 4 focused on possible parasympathetic regulation of systemic inflammation. The lack of association of HF-HRV responses with both baseline CRP and stressor-evoked IL-6 responses is surprising and does not align with the regulatory parasympathetic mechanism proposed by the cholinergic anti-inflammatory pathway (Tracey, 2002; 2009). Also contrary to the cholinergic anti-inflammatory pathway and previously reported associations (Sloan et al., 2007), we failed to find any significant correlations between Baseline HF-HRV and resting levels of inflammatory markers (i.e., Baseline IL-6, resting CRP). There are three primary implications of these null findings. First, it is possible that the PNS does not play a meaningful role in regulating either baseline or stressor-evoked levels of circulating inflammatory markers. If this is indeed the case, it may be that stressor-evoked inflammatory responses are primarily driven by the SNS and HPA-axis. This would align with previous work arguing that there is insufficient evidence for parasympathetic innervation of any primary immune organs (Nance and Sanders, 2007). Second, our in vivo methods of assessing inflammatory markers are markedly different from the in vitro evidence and animal models that support the cholinergic anti-inflammatory pathway (Tracey, 2002; 2009). It may be that the PNS does regulate inflammatory cytokine production at the cellular level, but we are unable to detect such relationships using gross measurements of circulating inflammatory markers. Third, it may be that PNS activation is not accurately measured by HF-HRV. As previously noted, HF-HRV is a marker of PNS input to the heart and does not reflect system-wide PNS activation. Thus, it is possible that more direct and invasive measurements of stressor-evoked PNS activation would yield different results.

The validity of HF-HRV as a marker of PNS activation during acute stress is also brought into question by our analyses of respiratory control of HF-HRV. Specifically, there was no

significant change in HF-HRV across the task periods after controlling for respiration rate. Respiratory control in HRV measurement has been strongly suggested by the psychophysiological literature (Berntson et al., 1997; Grossman, Karemaker, & Wieling, 1991). This suggestion was initially based on observational evidence that HF-HRV and RSA are highly correlated with respiratory parameters. The observational evidence is further supported by experimental evidence that RSA is generated by two different respiratory mechanisms: 1) central respiratory drive modulates the activity of cardiac vagal neurons and 2) lung inflation inhibits vagal input to the heart (Hayano & Yasuma, 2003; Horner, Brooks, Kozar, Gan, & Phillipson, 1995). Based on this experimental evidence, it has been posited that RSA is an essential physiological mechanism of the cardiopulmonary system in resting animals that optimizes pulmonary gas exchange efficiency. Further, this optimization process may not be reflected by measures of RSA during stress or physical strain, as these conditions often evoke significant variations in respiratory parameters (Hayano & Yasuma, 2003; Yasuma & Hayano, 2004). Experimental evidence supports this assertion: when there is significant variation in stressor-evoked respiration, RSA only reflects vagal input to the heart when respiratory parameters are experimentally or statistically controlled (Berntson et al., 1997; Grossman et al., 1991). For this reason, respiratory control of stressor-evoked HRV measures is thought to be particularly important (Berntson et al., 1997). Notably, it has also been argued that controlling for respiratory parameters is an overly conservative approach (Berntson et al., 1997; Horner et al., 1995).

If controlling for respiratory parameters is indeed essential, our results indicate that there were no significant stressor-evoked changes in PNS input to the heart. This finding is somewhat at odds with work showing consistent stressor-evoked decreases in HF-HRV in a recent meta-analysis (Brindle et al., 2014). However, this meta-analysis did not report whether the reviewed

studies corrected for respiratory parameters. This is not surprising, as many studies that utilize HF-HRV as a marker of PNS control of the heart do not measure or control for respiratory parameters. Our findings reiterate that stressor-evoked changes in HF-HRV are highly influenced by respiratory parameters. In addition, these findings raise questions about the validity of using HF-HRV change scores as a marker of stressor-evoked PNS change. Given these methodological issues, the null association of HF-HRV change scores with CRP and stressor-evoked IL-6 responses reported here does not necessarily rule out PNS regulation of systemic inflammation.

4.4 ANCILLARY ANALYSES: BP AND STRESSOR-EVOKED IL-6

The ancillary BP analyses in this study also failed to elucidate whether the SNS drives stressor-evoked IL-6 responses. Specifically, our results showed no significant association between stressor-evoked IL-6 responses and either SBP or DBP responses. These findings are contrary to mechanisms suggested by rodent and in vitro evidence (Bierhaus et al., 2003; Johnson et al., 2005) and suggest that the SNS may not be the primary driver of the stressor-evoked IL-6 response. However, there are several other possible explanations for this null result. First, BP was included as a crude indicator of stressor-evoked SNS activity, even though it also reflects some degree of PNS input. Future work exploring the underlying mechanisms of stressor-evoked IL-6 responses should employ a more specific psychophysiological indicator of SNS activity (i.e., pre-ejection period) (Cacioppo, Uchino, & Berntson, 1994). A second explanation for this null result is that there were significant methodological issues with the BP collection. Because the intravenous catheter was inserted in participants' left arm for the duration of the laboratory protocol, BP measurements had to be taken from the right arm (i.e., the same arm used to

respond to the computerized stress tasks). For many participants, this did not significantly interfere with BP collection. For some participants, however, the small amount of movement needed to press buttons on the keyboard interfered with BP readings. This interference and previous evidence that motor activity can lead to increases in BP (Shapiro et al., 1996), suggests that our BP readings may not be entirely accurate. For these reasons, it is impossible to rule out SNS stimulation of stressor-evoked IL-6 responses.

4.5 LIMITATIONS AND FUTURE DIRECTIONS

There are a number of limitations to the current study design. Data collection for this study was largely cross-sectional, preventing any causal inferences about the relationships between study variables. Future work should explore these research questions using longitudinal assessments of CVD risk variables to better understand whether stressor-evoked IL-6 responses predict future CVD risk. In particular, longitudinal assessment of CRP would be very informative for determining whether the significant interaction between sex and stressor-evoked IL-6 responses can predict systemic inflammation over time. Such longitudinal work is imperative, as systemic inflammation predicts future CVD risk (Kaptoge et al., 2010; Libby & Ridker, 1999; van Holten et al., 2013). In addition, this study did not include a non-stress control group. Although a control group could strengthen these data, previous work has shown that IL-6 levels do not increase significantly over time in non-stress control participants (Steptoe et al., 2001). This study also did not assess stressor-evoked IL-6 responses on multiple occasions. Previous research indicates that stressor-evoked IL-6 responses do not habituate over repeated testing sessions (von Kanel et al., 2006) but may sensitize on exposure to repeated stressors (McInnis et al., 2014). Repeated

measurements of IL-6 responses on multiple occasions could clarify these issues and increase the reliability of individual differences in these responses.

Mechanistic interpretations of these data are also limited by our method of measuring IL-6. Levels of IL-6 were determined by assessing levels in the peripheral circulation. This is a highly nonspecific method that gives no indication of which cells in the body produced the IL-6. While our interpretations assume that stressor-evoked IL-6 is produced by immune cells, IL-6 is also produced by endothelial cells and adipocytes (Mohamed-Ali et al., 1997; Papanicolaou et al., 1998). Indeed, previous in vitro work indicates that human adipocytes treated with adrenergic agonists secrete IL-6 (Mohamed- Ali et al., 2001), suggesting that adipocytes may be an important source of stressor-evoked IL-6 increases. As it is not possible to test this question with the present data, our understanding of the source of stressor-evoked IL-6 responses is limited.

The limitations of this sample should also be noted. First, this is a relatively healthy middle-aged sample, composed primarily of White and Black participants. Thus, these results may not generalize to disease populations or non-Black ethnic minorities. In addition, there were a number of participants with missing data in this sample; this was primarily due to the 30% of participants who were unable to provide both pre- and post-task blood samples for IL-6. These participants were not included in any of the IL-6 analyses but did not differ significantly from the rest of the sample on age, sex, or BMI. Finally, the relatively small sample may not have provided sufficient power to detect small effect sizes between study variables. Testing these questions with a larger sample could elucidate whether the nonsignificant findings reported here were due the small sample size or an actual null relationship between study variables.

While many questions remain, this study can inform future work on the underlying mechanisms and CVD risk correlates of individual differences in stressor-evoked inflammatory

responses. Although it is still unclear whether stressor-evoked inflammatory responses predict future CVD risk, our results emphasize the importance of examining sex differences in these relationships. In particular, it seems that stressor-evoked IL-6 responses are larger in females, but may not show the expected positive associations with systemic inflammation. Finally, our findings did not support the hypothesis that PNS activity during stress predicts stressor-evoked inflammatory responses or systemic inflammation; however, our results emphasize the importance of respiratory control of HF-HRV. In conclusion, stressor-evoked inflammatory responses may have important implications for systemic inflammation, but additional work is needed to understand the underlying mechanisms of these responses and whether they can predict future CVD risk.

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