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The IMPACT Study: Inflammatory Responses, Mood and Physical Fitness after Cancer Treatment

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OVERVIEW

Inflammation, a robust and reliable predictor of all-cause mortality in older adults, is one of the key candidate mechanisms for age-related decrements in physical function and disability [1, 2]. Chronic inflammation signals a heightened risk for disability and mortality even in the absence of clinical disease [3-5]. Inflammation frequently accompanies tumor growth and can also be amplified by cancer treatments [6].

Inflammation is higher in sedentary than in active individuals [7-11]. Indeed, when cardiorespiratory fitness is assessed objectively by maximal exercise testing, poorer physical fitness (VO_2peak) is associated with higher inflammation [12-15]. In this context it is noteworthy that cancer survivors' average cardiorespiratory fitness is consistently ~30% lower than their *sedentary* age mates without a cancer history [16, 17]. The reduced physical activity and associated deconditioning that frequently occur during cancer and its treatment lead to a decrease in the capacity for physical performance [18, 19]. With deconditioning, normal activities become more fatiguing, resulting in greater fatigue and lessened functional capacity over time [18, 19]; excessive proinflammatory cytokine production is thought to promote this downward spiral [18, 19]. Consistent with this argument, breast cancer survivors with lower levels of physical activity have a higher risk of premature death [20].

An immune challenge provides a useful paradigm for studying an individual's ability to limit inflammatory responses to infection or tissue injury. We will use a typhoid vaccine as a peripheral immune stimulus to assess the magnitude and kinetics of a transient inflammatory response and associated behavioral changes that have particular importance for breast cancer survivors--depressive symptoms, fatigue, cognitive problems, and pain [21-23]. We address a novel question: does poorer cardiorespiratory fitness heighten the magnitude and duration of inflammatory responses to immune challenges, as well as magnifying maladaptive behavioral responses?

Biological and behavioral vulnerabilities intensify adverse responses to immune challenges. For example, aging and depression enhance and prolong inflammatory responses to vaccines [24-26], and thus the ability of cardiorespiratory fitness to moderate age- and depression-related proinflammatory responses will also be assessed. Our double-blind, randomized, crossover study will evaluate inflammatory and behavioral responses to typhoid and placebo inoculations as a function of cardiorespiratory fitness, age, and depression in breast cancer survivors. Our primary outcomes are inflammation, depression, and fatigue; pain and cognitive problems will be addressed as secondary outcomes.

OBJECTIVES

Specific Aim 1: To evaluate the relationships between cardiorespiratory fitness and inflammatory and behavioral responses (negative mood, fatigue, pain, and cognitive problems) to typhoid vaccine. **Hypothesis 1:** Prior to the vaccine or placebo, better cardiorespiratory fitness will be associated with lower levels of inflammation, depressive symptoms, fatigue, pain, and cognitive problems. The vaccine will produce larger inflammatory responses and greater negative behavioral changes than the placebo. Better cardiorespiratory fitness will attenuate the magnitude and duration of both proinflammatory and behavioral responses to the vaccine.

Specific Aim 2: To determine the effects of age and depressive symptoms on inflammatory and behavioral responses to typhoid vaccine and placebo. **Hypothesis 2a:** Compared to younger women, older women will have higher levels of inflammation prior to vaccine or placebo. Aging will amplify the inflammatory and behavioral responses to typhoid vaccine, with older women showing more enhanced and prolonged responses than younger women. **Hypothesis 2b:** Prior to the vaccine or placebo, women with higher levels of depressive symptoms will have higher levels of inflammation than less depressed women, as well as higher levels of fatigue. Depressive symptoms will intensify vaccine responses, such that women who are more depressed will produce larger and longer-lasting proinflammatory and behavioral responses to the vaccine than women who are less depressed. **Hypothesis 2c:** The combination of older age and high depressive symptoms will produce greater and longer-lasting increases in inflammation and negative behavioral responses to typhoid vaccine than either alone.

Specific Aim 3: To assess the ability of cardiorespiratory fitness to moderate age- and depression-related responses to typhoid vaccine. **Hypothesis 3**: Women with better cardiorespiratory fitness will show smaller age- and depression-related inflammatory and behavioral responses to typhoid vaccine.

Hyperresponsiveness of the inflammatory system reflects other risks, as documented in cardiovascular studies [27-32]. Thus, this study could significantly expand our knowledge of exercise's beneficial mechanisms. If cardiorespiratory fitness is associated with smaller proinflammatory and larger anti-inflammatory vaccine responses, then the data from this project will provide a new perspective on how cardiorespiratory fitness not only limits inflammation, but also constrains depressive symptoms, fatigue, pain, and cognitive problems in breast cancer survivors [21-23].

BACKGROUND AND RATIONALE

A.1 Importance and Relevance of Inflammatory Challenges:

Blood samples for assessment of inflammatory markers are typically drawn in the morning after individuals have fasted overnight. Although these fasting inflammatory data are significantly influenced by variables such as age, obesity, and physical (in)activity, they do not provide information about inflammatory responsiveness to immune challenges [33]. Dynamic responses to an immune challenge provide an important alternative perspective on health because they assess whether the immune system can control inflammatory responsiveness and homeostatic sensitivity; an inability to recover quickly from an immune challenge may reflect underlying pathology [33]. Furthermore, unusually quick and forceful responses can also reveal inflammatory vulnerabilities [34, 35].

In some cases, resting or baseline inflammatory markers may provide less information than the same markers assessed following a challenge [34]. Vulnerable and invulnerable individuals may look quite similar under normal or resting conditions, while a challenge unmasks problems in over-responsiveness [34].

The ability to minimize inflammatory responsiveness to an immune challenge influences the total burden that an infection or injury places on an individual. Larger, more frequent, or more persistent inflammatory responses have negative consequences for health, particularly among older adults [9, 10, 36-38]. Immune challenges occur frequently in our daily lives, e.g., high fat meals can provoke endotoxin release from the gut [39]. The vaccine paradigm tests a person's ability to limit responsiveness to inflammatory challenges as well as the associated behavioral responses. The data from the aging and cardiovascular studies described below illustrate the risks associated with an inability to limit inflammatory responses.

A.1.a. Health implications: Heightened inflammatory responses to immune challenges. Research with cardiovascular populations has demonstrated health risks associated with enhanced responsiveness to infectious challenges and/or tissue injury. For example, inflammatory responses to influenza vaccine differed in patients with histories of stable vs. unstable coronary heart disease [27]. Patients with unstable CHD who had a history of acute coronary syndromes had significantly higher serum amyloid A (SAA) concentrations 48 hours after inoculation than those with quiescent CHD.

Similarly, the SAA response was 60% higher 24 hours after an influenza vaccine inoculation among men with severe carotid artery disease (CAD) than men who did not have severe CAD [28]. Furthermore, the SAA response appeared to be independent of baseline levels, leading the authors to argue that measurement of inflammatory responses to an immune challenge might provide unique information not obtained by measuring chronic levels [28]. Together these studies suggest that a prolonged acute phase response reflects a proinflammatory precondition-–the proneness of the arterial tissue to develop vulnerable plaques, as well as the propensity for inflammatory responses to nonspecific stimuli in other tissues [27].

In accord with these vaccine studies, other researchers have shown that nonspecific inflammatory responses also have important prognostic implications. For example, coronary angioplasty or diagnostic coronary angiography increased CRP and SAA among unstable angina patients but not those with stable angina [29]. Subsequent studies showed that higher CRP responses to angioplasty were associated with a greater risk for clinically relevant restenosis [30, 31] as well as major adverse cardiac events (death, myocardial infarction, or recurrent angina) [32]. *Thus, hyperresponsiveness of the inflammatory system to immune challenges reflects other risks.*

A.1.b. Aging. Aging clearly amplifies inflammatory responses to immune challenge, illustrated by reactions to endotoxin (lipopolysaccharide or LPS) and bacterial infections [40-42]. For instance, compared to younger participants, older adults had larger and more prolonged inflammatory responses to endotoxin, and their fevers took longer to return to normal [40],

In accord with the human data, rodent data also demonstrate the adverse influence of age on endotoxin responsiveness. After endotoxin administration, older mice had exaggerated depressive-like behavioral

responses, greater deficits in learning and memory, and more substantial and prolonged decrements in social behavior compared to younger mice [43-47]. Activation of the peripheral innate immune system can induce production of proinflammatory cytokines by brain microglia, and the ensuing neurobehavioral deficits were still evident in aged mice even after younger mice had recovered [45]. Thus aging both exacerbates and prolongs maladaptive inflammatory responses. Our proposed study will provide important data about the extent to which cardiorespiratory fitness and depression alter inflammatory responsiveness in breast cancer survivors who are middle-aged and older.

A.2 Typhoid Vaccine as a Peripheral Immune Stimulus

A number of studies have used typhoid vaccine to induce transient systemic inflammation [48-60]. In studies with healthy young unmediated subjects, the maximum cytokine increases occurred 2-4 hours post-vaccination, with a 4-6 fold increase in IL-6 and up to a 30-fold increase in IL-1Ra [49, 54].

In contrast, older subjects show greater reactivity and slower recovery. For example, subjects whose average age was 58 had a 10-fold rise in IL-6 post-vaccination [60]. Moreover, among people who were around 59 years old, IL-6 was higher at 8 hours than at 4 [59]. Although subjects in their early twenties did not show significant heart rate changes, vaccination increased heart rate by 5 beats per minute in men and women whose average age was 41.5 [52]. Thus, consistent with the endotoxin studies, older participants' typhoid vaccine responses were larger in both magnitude and duration compared to younger individuals.

We anticipate that inflammatory responses to typhoid vaccine will be variable, and this variability will provide important information about psychological and biological vulnerabilities. For example, young men who had larger IL-6 responses to typhoid vaccine also had larger increases in fatigue, negative mood, systolic blood pressure, and salivary 3-methoxy-phenylglycol, a major norepinephrine metabolite, in response to a laboratory stressor [48]. Our design will allow us to assess how inflammatory responsiveness and associated symptoms differ following vaccination among breast cancer survivors who are likely to have heightened post-treatment inflammation as suggested by our data described in Preliminary Results. In this vulnerable group, the ability to minimize inflammatory responsiveness is particularly important.

A.3. Depression Primes Inflammatory Responsiveness--and Inflammation Enhances Depression and Other "Sickness Behaviors"

We have shown that mild depressive symptoms were associated with an amplified and prolonged inflammatory response following influenza vaccination in older adults [24], as described in more detail in Preliminary Results. Work from another lab replicated and extended our findings: they showed that the association between distress and heightened IL-6 following an influenza inoculation was stronger among older adults than younger adults [25]. Further studies showed that depressive symptoms predicted an exaggerated inflammatory response to influenza vaccine in pregnant women [26]. Similarly, among women who had just given birth, those who had a lifetime history of major depression showed greater increases in both serum IL-6 and the soluble IL-6 receptor after delivery than women without a history of depression [61].

Together, these studies suggest that depression sensitizes or primes inflammatory responsiveness, provoking larger responses following an immune challenge. Aging also serves as a vulnerability factor, further enhancing inflammatory responses.

Heightened inflammation can in turn alert the central nervous system to induce or intensify "sickness behaviors," including negative mood changes, fatigue, anhedonia, increased pain sensitivity, loss of appetite, and cognitive deficits [62-66]. These effects can be substantial; for example, cytokine therapies, used in the treatment of some cancers and chronic viral infections, provoke significant depressive symptoms in 30-50% of patients [67]. Importantly, depression prior to treatment is a risk factor for this pattern: higher levels of depressive symptoms prior to cytokine therapy substantially enhance the likelihood of developing significant depressive symptomatology during cytokine treatment [67-69].

Depression's association with inflammation is well-documented, but patients rarely present with only a single symptom; depression, fatigue, and pain are a common constellation [70]. These symptoms may share an underlying mechanism. For example, data from a number of studies suggest that persistent fatigue in cancer survivors is related in part to overactivation of the inflammatory network [71-73]. Depression has well-established ties to inflammation [63, 74, 75]. Furthermore, substantial evidence shows that proinflammatory cytokines heighten pain responsiveness [76, 77].

Inflammation also provokes cognitive disturbances, decreasing memory, attention, and executive function [78, 79]. Memory impairments are a well-documented adverse effect of cytokine therapy [78], but much milder inflammatory challenges also decrease performance, as illustrated in endotoxin and typhoid vaccine studies [79-81].

These interrelated symptoms build on each other, with depression heightening cognitive problems, fatigue, and pain, while fatigue and pain promote greater depression [82-85]. These symptoms all affect a patient's functioning; they not only have an adverse effect on patient outcomes, they may have a synergistic effect in the prediction of morbidity [86-88]—and this problematic symptom constellation is common among a subset of breast cancer survivors [70, 89-91].

A.4 Inflammation and Autonomic Balance: Age, Depression, and Cardiorespiratory Fitness

Age, depression, and cardiorespiratory fitness can all influence autonomic balance, one common pathway through which they may impact acute inflammatory responses. Accumulating evidence underscores the importance of autonomic balance and its relevance for inflammation. The central nervous system (CNS) can impact inflammation through both sympathetic and parasympathetic nervous system (vagal) activity [92]. Heart rate variability (HRV), the respiration-related variability in heart rate that is mediated by the vagus nerve, serves as an indicator for parasympathetic activity (also called vagal tone). Lower vagal tone (lower HRV) is related to higher levels of inflammation [93, 94]. Vagus nerve stimulation lowers serum TNF-α production, ultimately resulting in attenuation of systemic inflammation [94]; low parasympathetic activity appears to promote inflammation [93, 95-103]. Parasympathetic activity decreases during normal aging [104], and these decrements are thought to be linked to age-related inflammatory increases [92].

Healthy adults with better cardiorespiratory fitness have higher HRV than their less fit counterparts, and exercise training appears to enhance HRV [105-107]. Furthermore, regular exercise training may attenuate age-related declines in parasympathetic control [108, 109].

Greater physical fitness that enhances parasympathetic activity should serve to attenuate cytokine responses following immune challenge. Although this question has not been assessed in humans, there are several relevant rodent studies. For example, exercise training reduced the inflammatory response to endotoxin-induced sepsis in rats [110-112]. Furthermore, four weeks of treadmill running attenuated the rise in plasma TNF-α following an endotoxin injection [111]. However, whether these same relationships hold in humans is unknown.

Depression is associated with increased sympathetic activity, as well as lower parasympathetic activity [97, 113, 114]. Parasympathetic activity decreases with increasing depression severity, such that more severe depression has a greater impact on vagal tone [115]. More severe depression may also strengthen relationships between parasympathetic activity and inflammation; one large study with 682 coronary heart disease patients showed that relationships were substantially stronger among more depressed patients then among the non-depressed [97].

Inflammatory challenges acutely decrease parasympathetic activity and increase sympathetic activity [116, 117]. Importantly, stimulation of the vagus nerve attenuates the acute systemic inflammatory response to endotoxin, highlighting the importance of this cholinergic anti-inflammatory pathway [118]. The vagus nerve plays a key role in cytokine-induced sickness behavior, demonstrated by studies showing that endotoxin activates vagal afferents; vagotomy prevents endotoxin-induced hyperalgesia [65]. In data from our lab, breast cancer survivors who reported greater fatigue had lower HRV than those who were less fatigued [119].

These studies describe mechanistic pathways through which age, depression, and cardiorespiratory fitness could influence acute inflammatory responses via alterations in parasympathetic activity. We will assess HRV in this study to address how psychological and biological vulnerabilities interact to exacerbate fatigue, pain, and depression in response to typhoid vaccine in breast cancer survivors.

A.5 Innovation and Potential Impact

Attenuated reactivity to and enhanced recovery from challenge are among the mechanisms researchers have suggested to account for exercise's health benefits [120]. However, the studies addressing the benefits of cardiorespiratory fitness during acute challenge have focused on cardiovascular and cortisol reactivity and recovery from psychological laboratory stressors [120], not immune challenge and inflammatory responsiveness. This project adopts an innovative model to look at reactivity and recovery from a very different perspective: no prior research has addressed whether cardiorespiratory fitness affects people’s inflammatory responses to an

immune challenge. Furthermore, in addition to the kinetics of inflammatory reactivity and recovery, the vaccine model will provide original data on changes in mood, fatigue, cognitive problems, and pain and their relationship to cardiorespiratory fitness.

Immune challenges are a frequent occurrence, and the ability to minimize inflammatory responsiveness influences the total burden that infectious challenges or tissue injury place on an individual. Larger, more frequent, or more persistent inflammatory changes have negative consequences for health. If better cardiorespiratory fitness dampens or limits inflammatory responsiveness, then this study could demonstrate a new and novel mechanism through which regular exercise produces its substantial health benefits.

Persistent activation of innate immunity is a key feature of chronic inflammatory disorders. Use of a typhoid vaccine provides a way to safely activate the innate immune response and study subsequent downstream responses; as a consequence, the vaccine bypasses the thorny issues related to reverse causation and confounding that are so common in chronic disease studies. *Differences in acute phase responses will provide insight into the downstream effects on the initiation and progression of both acute and chronic inflammatory diseases as well as the associated behavioral symptomatology [121].*

Cardiovascular disease, diabetes, and osteoporosis, all notable common health problems among breast cancer survivors, have inflammatory links [122-127]. Our preliminary data, described in the next section, show longitudinal treatment-related inflammatory changes in survivors compared to both their own pretreatment baseline and to longitudinal data from controls. This study will extend those findings, addressing questions of biological and behavioral vulnerability related to enhanced inflammatory responsiveness. Accordingly, this study could have a very high impact.

A.6 Preliminary Studies

We have an active and productive research program addressing biobehavioral issues in breast cancer survivors [119, 128-136]. We recently completed a successful randomized controlled trial addressing the impact of yoga on inflammation, mood, and fatigue in 200 breast cancer survivors, and we retained 91% of our participants through the full trial. In another ongoing study we recruited 210 newly diagnosed breast cancer patients prior to any treatment, including surgery, and we have thus far retained 89% through the 18-month follow-up. Due to space limitations we focus below on preliminary data that are directly applicable to this proposal.

A.7.a Depression and Proinflammatory Responses to Influenza Vaccine

Our lab has published a series of biobehavioral vaccine studies [24, 26, 137-140]. Although most of the studies have addressed the impact of stress and depression on antibody and cellular responses to different vaccines, one study with direct relevance to our proposed work showed that mild depressive symptoms were associated with an amplified and prolonged inflammatory response following influenza vaccination in older adults [24]. To study the dynamics of interleukin 6 (IL-6) in response to an immunological challenge, blood samples were obtained from 119 older adults (mean age = 71.21, SD = 8.68) immediately prior to an annual influenza vaccination, and then again 2 weeks later [24]. The sample included 47 current and/or former caregivers of spouses with Alzheimer's disease, providing a range of depressive symptoms. Men and women with higher levels of depressive symptomatology had higher levels of IL-6 prior to and following vaccination than those who reported fewer symptoms; moreover, individuals reporting more depressive symptoms also showed enhancement of IL-6 two weeks later, while there was little change in IL-6 among those reporting little or no symptomatology, see Figure 1. These findings were particularly noteworthy because depressive symptoms were quite low in this sample prior to vaccination, and did not change significantly after vaccination.

In summary, the data from our influenza vaccine study suggest that even relatively modest levels of depressive symptoms may enhance and prolong alterations in the inflammatory response system in response to common infectious challenges. Sustained and amplified inflammatory responses could accelerate a range of age-related diseases. These data suggest a mechanism whereby syndromal depression and subthreshold depressive symptoms may serve as a key gateway to a broad array of health problems.

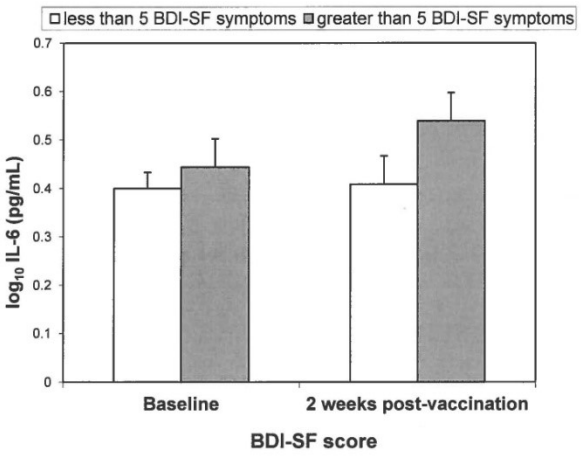

Figure 1: Participants were divided into non-depressed (open bars) and depressed groups (closed bars) based on an empirically derived cut score of 5 BDI-SF symptoms to illustrate the relationship between depressive symptoms and IL-6 production at baseline and 2 weeks after vaccination, (N =119). The time-by-depressive symptoms interaction indicated that individuals who reported more depressive symptoms demonstrated an increase in IL-6 at 2 weeks after vaccination, F(1,116) = 7.42, p = .007. Depressive symptoms were associated with elevated levels of IL-6 across baseline and post-vaccination, F(1,116) = 4.78, p = .03.

A.7.b Longitudinal Changes in Inflammation from Pre- to Post-Treatment in Cancer Survivors and Controls

We are currently completing a prospective study of inflammation, depression, and fatigue from pretreatment through survivorship in breast cancer survivors, as well as a benign comparison group (women who had a benign diagnosis following an initial abnormal mammogram). Baseline data on the stage 0-IIIA breast cancer survivors were collected before any cancer treatment including surgery, as well as 6 months and 18 months after completion of primary treatment (except tamoxifen/aromatase inhibitors). The average age in our sample is 55.39 (SD = 11.26, range = 26 to 88).

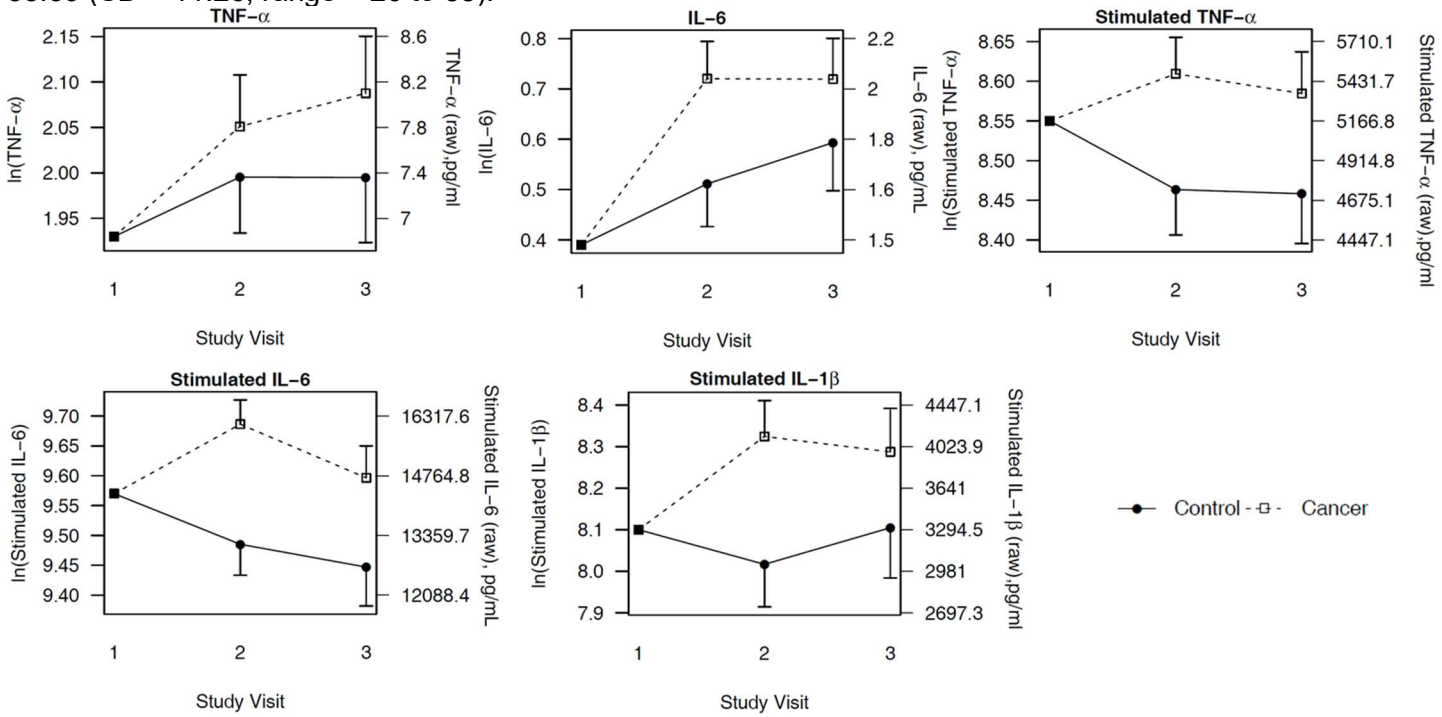

Figure 2: After adjusting for baseline levels, breast cancer survivors (N=124) had significantly higher serum IL-6 (P-value for group effect=0.009), stimulated TNF-α (P-value for group effect=0.02), stimulated IL-6 (P-value for group effect=0.004), and stimulated IL-1β (P-value for group effect=0.008) after cancer treatment compared to control subjects (N=62). Importantly, there were no differences in baseline cytokine levels for cancer subjects compared to controls after adjusting for age, BMI, depressive symptoms, and physical activity (all p-values >0.39).

These data are important because they show the inflammatory changes that followed treatment for breast cancer compared to well-matched benign controls. Insofar as we know, these are the first prospective data to demonstrate post-treatment increases in inflammation compared to a non-treated group. These data are relevant to our proposed study because they highlight the fact that survivors already have heightened baseline

levels of inflammation before an immune challenge, and thus the potential moderating influences of cardiorespiratory fitness, age, and depression are even more consequential because of their greater vulnerability.

A.7.c Inflammation, Mood, and Fatigue in Breast Cancer Survivors: A Randomized Controlled Trial

To evaluate yoga's impact on inflammation, mood, and fatigue, we conducted a randomized 3-month controlled trial with a 3-month follow-up. Our participants, 200 breast cancer survivors, were assigned to either a 12 week, twice-weekly 90 minute hatha yoga classes or a wait-list control. The main outcome measures were LPS stimulated monocyte production of interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), and IL-1β, and scores on the Multidimensional Fatigue Symptom Inventory (MFSI-SF), the vitality scale from the Medical Outcomes Study 36-item short-form, and the Center for Epidemiological Studies-Depression (CES-D) scale.

After the intervention the average MFSI-SF fatigue score was 49% lower and vitality was 12% higher in the yoga group compared to the control group (both P=0.007), and the geometric means for IL-6 (P=0.02), TNF-α (P=0.03), and IL-1β (P=0.07), were 11%, 10%, and 15% lower, respectively, in yoga participants compared to the wait-list group. The change in depression scores was not significantly different between groups (P=0.18). Planned secondary analyses showed that yoga practice frequency had stronger associations with MFSI-SF fatigue (P=0.0009), vitality (P=0.004), and depression scores (P=0.03) than simple group assignment, with more frequent yoga practice producing larger changes. Increasing yoga practice also led to decreased IL-6 (P=0.03) and IL-1β (P=0.04) production, with a marginal effect on TNF-α (P=0.07).

Monocyte cytokine production decreased significantly in yoga participants compared to the wait-list group. Blood monocytes provide a direct source for whole body inflammation, and their function may provide a proxy for macrophages' inflammatory responses in adipose tissue[141]. Monocyte cytokine production increases with age [2]. and persistent subclinical inflammation appears to enhance risk for chronic disease and disability among older adults [2]. Thus the reduced inflammatory response in the yoga group could have broad clinical implications.

Chronic inflammation has been suggested as one key biological mechanism that may fuel declines in physical function leading to frailty, disability, and, ultimately, death. This RCT demonstrated that yoga could significantly modulate inflammation and concurrently reduce fatigue and depressive symptoms.

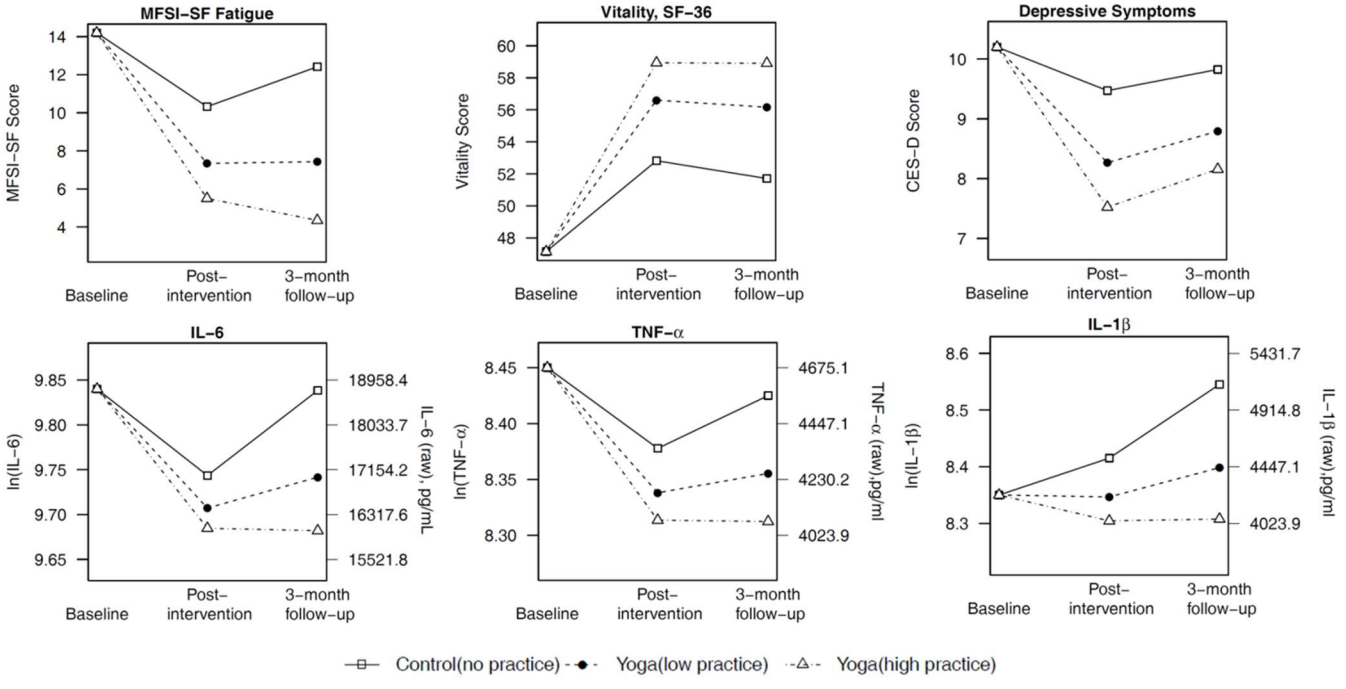

Figure 3: Changes in MFSI-SF fatigue scores, vitality scores, depressive symptoms, and LPS-stimulated cytokine production (IL-6, TNF-α, and IL-1β) across the post-intervention and 3-month follow-up as a function of yoga practice frequency, 25th percentile (low practice) and 75th percentile (high practice) vs. the wait-list control.

In summary, our lab has pioneered biobehavioral vaccine studies [24, 26, 137-140]. We can recruit and retain the breast cancer survivors needed for this study. Inflammation has been our primary immunological focus during the last decade [24, 119, 142-151], and we have extensive experience with inflammatory markers across multiple studies. We are well-prepared to conduct this important trial.

METHODS

B. Participant selection

Participants: A total of 240 women ages 45-65 who have been diagnosed with stage I-IIIA breast cancer will be recruited 1-8 years after the completion of all primary cancer treatment except for longer-term hormonal therapies (tamoxifen, aromatase inhibitors). We will recruit women who have received one of the two most common stage I-IIIA chemotherapy regimens, either docetaxel/cyclophosphamide or doxorubicin/cyclophosphamide followed by paclitaxel to provide uniformity of prior treatment. All women will be postmenopausal, defined as having FSH and estradiol within the institutional postmenopausal range at the time of study entry and no menstrual cycle in the last 12 months.

Cardiorespiratory fitness is a key variable, and it is thus important to select women who represent the full range of fitness. We anticipate that recruiting women at the lower end of fitness will not be a problem, but may represent a greater challenge in recruiting the most fit women. We will screen women prior to any in-person appointments using the CHAMPS [152-155] to maximize selection of more active women, particularly at higher BMIs. Although central adiposity will be inversely associated with VO_{2peak}, we will make sure to carefully select women who have lower central adiposity but are less fit, as well as women with higher central adiposity who have higher levels of activity and are more fit [156-159].

Most women who had estrogen receptor positive breast cancer will be on aromatase inhibitors or tamoxifen. We will collect information about these medications. Some evidence suggests that aromatase inhibitors and tamoxifen may affect cardiovascular risk [160, 161], while other studies did not observe a difference in risk [162] suggesting that this link requires further research at this time [163]. To our knowledge, the effect of these medications on acute cardiovascular changes has not been tested. Accordingly, we will include aromatase inhibitor and tamoxifen use as covariates in secondary analyses to assess whether these medications impact cardiovascular and inflammatory outcomes.

Exclusion criteria: Exclusions will include a prior history of any other malignancy except basal or squamous cell skin cancers, strokes, diabetes, current heart disease or uncontrolled hypertension, peripheral vascular disease, liver disease, autoimmune and/or inflammatory diseases including rheumatoid arthritis and ulcerative colitis, and other medical conditions that would limit participation in the assessments (e.g., pulmonary disease, orthopedic problems, major psychiatric illness, major cognitive dysfunction, or an acute medical problem). Other exclusions will include anemia (defined as having a hemoglobin level less than 11.7 g/dL for white women, following the OSU hospital's criteria, and 11.5 for African American women, based on data from Beutler and Waalen [164]), alcohol or drug abuse, smoking, individuals who routinely take fish oil, krill oil, or flaxseed (oil, pills, or powder) or consume more than two portions of oily fish per week [165, 166]. Consistent with the American Red Cross deferral criteria, we will exclude women with blood pressures above 180/100 or below 80/50. Medication exclusions will include steroids as well as statins and other medications with anti-inflammatory actions [167, 168].

We will not exclude antidepressant users who have been medicated for at least three months. Recent CDC data suggest that more than 1 in 10 Americans take antidepressants, and some breast cancer survivors are given antidepressants for menopausal symptoms. We found very small effect sizes for antidepressants on inflammatory markers in our previous studies, with the effect on IL-6 no larger than d=0.17 and the effect on TNF-α no larger than d=0.29 in several large studies (N ranging from ~150 to 300). Despite this small anticipated effect, to ensure results are not confounded by antidepressant use we will include it as a covariate if imbalance is present. We will carefully evaluate all other medications and health problems [169] to assure that participants are low risk and can fully participate.

Women who have received typhoid vaccine within three years or any other vaccine within three months will be excluded. Although we do not anticipate relationships between recency of typhoid vaccination and responses based on evidence that prior exposure is not reliably associated [170], we will assess this possibility

We cannot list all possible exclusions; however, we will carefully evaluate all medications and health problems as we have in past studies to assure that participants are low risk and will provide solid data.

Recruitment: We will initially identify eligible women in our current studies who have agreed to be contacted for participation in future studies. We foresee this recruitment avenue yielding only a small number of eligible participants, so the primary recruitment sources will be the breast cancer clinics of the Stefanie Spielman Comprehensive Breast Center of the James Cancer Hospital. The primary recruitment sources will be the breast cancer clinics of the Stefanie Spielman Comprehensive Breast Center of the James Cancer Hospital. Dr. Lustberg, co-I on this project, provides expertise in breast cancer treatment and survivorship as well as close contacts with her breast oncology colleagues to facilitate recruitment. They will provide us access to their clinic schedules in IHIS so that we can screen and identify women, scheduled for follow up oncology appointments, who meet eligibility criteria.

Additionally we will submit requests to the Information Warehouse and Cancer Registry to identify eligible breast cancer survivors in the OSUMC system who may not be active on clinic schedules. The request to contact eligible patients identified through the Information Warehouse will be made to the appropriate physician, either the physician who has recently seen the patient, or the physician who is scheduled to see the patient as follow-up. With physician approval, potential women will then either be approached by one of our researchers at their next appointment or will receive one of our IRB approved recruitment letters. For patients who express interest, the research staff member will then fully explain the study and invite their participation.

If we need to identify additional participants, we will open up recruitment to the greater Columbus area. We will place ads in and around medical centers and appropriate businesses. We will utilize email and online media recruitment ads and advertise to breast cancer survivors through events geared for this population such as the Race for the Cure, Relays for Life, Army of Women, etc. We will also recruit participants through the ResearchMatch website.

Diagnostic, treatment, and staging data will be obtained from medical records. These data will include (but are not limited to): types and dates of diagnostic procedures, histology, cytology, tumor grade, available genetic tests, comorbidities, major medications, and hormonal treatment status. Where possible we will use the James Cancer Registry and Outcomes Management Department and/or the OSU Information Warehouse to provide us with information regarding our participants' treatment.

Online/phone screening: To confirm eligibility, we will collect data on cancer stage, time since treatment, and treatment regimen. We will also assess vaccination history, medication use, comorbidities, and physical activity.

Screening session: When subjects come for their in-person screening appointment at the Clinical Research Center (CRC), a hospital research unit, nurses will draw blood for assessment of anemia and hemoglobin A1C (to confirm < 6.5). The nurses will also assess any potential phlebotomy problems, and measure blood pressure, height, and weight. Women will be interviewed using the mood and anxiety disorder modules from the Structured Clinical Interview for DSM-V [171, 172], nonpatient version.

Cardiorespiratory fitness: Peak oxygen consumption (VO_2peak)will be evaluated at a screening visit using a graded cycle ergometry exercise test, starting at 25 watts and increasing by 25 watts every two minutes, with continuous monitoring via 12-lead EKG (MedGraphics Cardio2, Cardio Perfect). VO_2peak [173] will be calculated from 10-second averages of breath-by-breath expired air (MedGraphics Cardio2, Breeze Suite). Declines in cardiorespiratory fitness may result directly from chemotherapy's toxic effects on pulmonary, cardiac, hematological, vascular, and skeletal muscle function, as well as deconditioning following treatment [174]. Cardiorespiratory endurance is the gold standard measure of physical fitness; low values predict mortality [175-177]. To avoid influencing vaccine/placebo responses, this session will not occur on the day before a full-day CRC visit.

C. Study Design and Procedures

Study Design: This study will have a balanced, randomized, double-blind, crossover design. The two ~9.5-hour sessions will be scheduled 10-30 days apart.

Randomization and masking: Following completion of the screening appointments, women will be randomly assigned to either the vaccine/placebo or the placebo/vaccine sequence using a treatment sequence prepared and maintained by the Data Manager. The research assistants will not have access to the condition assignments. The CRC nurse who administers the vaccine or placebo will not be the same nurse who will be measuring the vital signs throughout the day and drawing blood. The Data Manager will not be involved in any

other aspects of the research, including data collection or blood sample analyses. Blinding will be assessed for subjects, nurses, and research assistants.

Standardized activity prior to study days: Participants will be asked to consume their last meal no later than 7:30 pm the night before each of the day-long CRC admissions. Participants will avoid alcohol use and any strenuous physical activity two days prior to their appointments [168]. Aspirin, nonsteroidal anti-inflammatory drugs, and vitamins will be discontinued at least one week prior to each evaluation.

Anyone with a recent illness will be rescheduled. A temperature greater than 99.7°F on admission will result in cancellation of the current session.

Clinical Research Center (CRC) visits, overview: Women will have a small intravenous catheter inserted on admission to the CRC, and baseline samples will be drawn following a 20-30 minute adaptation period. Following the baseline blood draw, and prior to breakfast, women will complete baseline mood questionnaires and brief cognitive tests. Following completion of baseline measures a nurse will inject **saline (the placebo)** or **Typhoid capsular polysaccharide vaccine (Typhim-Vi, Aventis Pasteur)** into the non-dominant deltoid muscle. Women will receive a standardized breakfast (and, later, lunch).
Blood pressure, heart rate, and temperature will be assessed before the injection and then every half hour. Nurses will be asked to avoid providing any feedback to participants or research assistants on vital signs, aside from baseline (pre-inoculation) data. Blood draws will occur at baseline prior to vaccine or placebo administration, and then every 90 minutes for the next 7.5 hours. At the end of each research day a final teaspoon of blood will be drawn from the arm which does not have the catheter inserted; this is to confirm that immune changes are not just reflecting local changes at the catheter site. This end-of-day draw will not occur for women who have an arm restriction. Women will rate the intensity of physical symptoms (muscle aches, headache, feverishness, pain, and fatigue on 0-7 scales) and mood (using the PANAS) at each blood draw.

Participants will remain in the CRC for ~7.5 hours after the inoculation. Cognitive assessment tasks will be completed at 3 and 4.5 hours post-injection, and the cold pressor test will be scheduled following the first of these post-injection assessments. During intervals when participants do not have experimental tasks, we will show them segments of the “Planet Earth” series, providing a uniform activity to reduce variance.

HRV will be measured beginning before vaccine/placebo administration and through the end of each visit. Five-minute samples will be taken hourly when subjects are in a supine position. **Resting HRV** will be measured non-invasively with the Polar s810 wristwatch and Wearlink 31 belt band using previously described methods for processing and analysis [119]. The 1000 Hz sampling rate provides valid and reliable ECG data [178, 179].

Visit	CRC Screen #1	VO2max Screen #2		Visit 1	Visit 2
Length	2 hours	2 hours		9.5 hours	9.5 hours
Activity	Consenting Anemia/Diabetes blood draw Questionnaires Body measure-ments	VO2 max test (exercise bike) Questionnaires	Eligibility Determined	Blood draws Questionnaires Metabolic Measure-ments Computer tasks Heart Rate/blood pressures/temp ***Condition:*** Typhoid vaccine or Placebo/saline	Blood draws Questionnaires Metabolic Measure-ments Computer tasks Heart Rate/blood pressures/temp ***Condition:*** Typhoid vaccine or Placebo/saline

D. Data Collection and Management Process

Self- Report Data – Primary Measures: ***Unless otherwise indicated, all measures will be administered during both CRC visits.***

The **Structured Clinical Interview for DSM-V (SCID), nonpatient version** [171, 172] mood and anxiety disorder modules, administered during screening, will provide data on lifetime prevalence and current status

[180]. Our laboratory has been administering SCIDs for 25+ years. We have well-established procedures for training interviewers, and we use regular consensus meetings to obtain diagnoses and maintain consistent, reliable, and valid interview data. All interviews are recorded and used as part of the consensus process.

The **Center for Epidemiological Studies Depression Scale (CES-D)** [181, 182] will provide data on baseline depressive symptoms at both CRC visits.

The **Positive and Negative Affect Schedule** (**PANAS**) includes two mood scales [183]. The PANAS will be administered each time blood is drawn across the two CRC admissions to assess changes in both positive and negative affect related to the vaccine/placebo. We will add 5 additional negative mood words to the PANAS taken from the Profile of Mood States depression subscale [184], following work by Eisenberger et al. [185]. Participants will rate the extent to which they feel: unhappy, blue, lonely, gloomy, and worthless on a scale from 0 (not at all) to 4 (extremely), and experimenters will also rate the participant's mood using two of the adjectives, unhappy and gloomy [185]. We will also ask participants to rate their mood each time blood is drawn for cytokine assays, using pictographs for mood, and anxiety; in rating mood the figure's face is continuously modulated from an extreme frown to a broad smile, while for anxiety or arousal the figure varies from inactivity to rapid movement [183, 186, 187]. Participants mark circles underneath the graphic figure that most closely represents their experience at that moment, for example providing valence ratings on a scale of 1 (pleasant, "happy") to 8 (unpleasant, "sad") .

We are interested in both customary or usual ("trait") fatigue as well as changes in fatigue in response to the vaccine/placebo. We will administered the full **SF-36 (RAND Health Survey** [188]; **its energy/fatigue (vitality) scale** measures fatigue over the last month. As the most commonly used scale in studies of cancer fatigue and inflammation [71, 72, 189-194], it has good convergent and discriminant validity with other energy/fatigue scales, and it discriminates among patient groups that differ in the severity of fatigue-related medical and psychiatric diagnoses [195]. This is the only fatigue scale that has a well-defined cut score to separate fatigued versus nonfatigued [72, 189-193, 196-199]. In addition, to measure changes in fatigue across the day, women will be asked to rate their current level of fatigue on a 0-7 Likert-type scale each time blood is drawn.

The 30-item **Multidimensional Fatigue Symptom Inventory-Short form (MFSI-SF**) [200, 201] assesses behavioral, cognitive, physical, and affective expressions of fatigue [200, 201]. It provides a very good measure of the multidimensional aspects of fatigue, allows for comparisons between fatigued and non-fatigued individuals, and has subscales measuring general, physical, emotional, mental, and vigor aspects of fatigue, as well as a total score. The brief NIH PROMIS fatigue measure may be an alternative to the MFSI.

The **Fatigue Catastrophizing Scale** (FCS) will be used to assess whether people who catastrophize fatigue report greater fatigue [202]. This 10-item scale asks respondents to rate on a 5-point scale (1 = never true to 5 = all of the time true) how often each item is true for them when they have experienced fatigue (e.g. "I find myself expecting the worst when I'm fatigued"). We will derive a total score by computing the mean of the 10 ratings. This scale has high internal reliability (alpha = .85).

The **Brief Pain Inventory Short-Form** has two subscales measuring pain severity and pain interference that will provide baseline pain data at both CRC visits. Reliability and validity are excellent [203, 204]. In addition, women will rate overall pain as well as muscle aches and headache at each blood draw as described earlier.

At baseline at each visit, participants will rate the severity of memory and concentration problems they experienced in the last week, using a two-item **Cognitive Function Self-Report Measure** [205] that was developed for use with cancer patients, as well as the brief PROMIS cognitive function measure [206].

The **Functional Assessment of Cancer Therapy – Cognitive Function (FACT-Cog)** [207] questionnaire will be used to measure self-reported cognitive function. Participants rate how often they have been bothered by a list of common treatment-related cognitive problems, which were identified by oncology treatment providers and patients. The scale includes items on mental acuity, concentration, memory, verbal fluency, functional interference, change from previous function, and comments from others.

We will use two exercise questionnaires, one during screening and another during the 9.5 hour visits. The **Community Healthy Activities Model Program for Seniors (CHAMPS) Questionnaire and Seven-Day Physical Activity Recall** assess the weekly frequency and duration of various physical activities [152-155, 208, 209]. The seven-day physical activity recall is one of the most widely used physical activity assessments in epidemiological research as well as exercise science; the instruments have an excellent history of validation and testing. In addition, the brief (6-item) **Godin Leisure-Time Exercise Questionnaire** measures light,

moderate, and vigorous activity over the past week and has good reliability and validity [210, 211]; it has been used extensively in research with cancer survivors, and provide somewhat different activity estimates.

Self-Report Data – Secondary/covariate measures:

Information regarding medical comorbidities, treatment-related functional limitations, and relevant health behaviors will be collected in order to assess the unique contributions of each variable to inflammation and cardiovascular health. The **Charlson Index** [212 , 213, 214] will provide data on comorbidities. This widely used measure has good concurrent and predictive validity, test-retest reliability, and inter-rater reliability [215]. The **Pittsburgh Sleep Quality Index** [216] assesses sleep quality and disturbances over a one-month interval; it has good diagnostic sensitivity and specificity in distinguishing good and poor sleepers. We will also collect information about sleep the night before each CRC assessment. The **revised Breast Cancer Prevention Trial (BCPT) symptoms checklist** will provide information about treatment-related symptoms [217, 218]. The **Diet History Questionnaire (DHQ)**, a food frequency questionnaire (FFQ) developed by NCI staff [219, 220], will be completed by participants at the first CRC visit. The standard FFQ format asks about intake in the last year, and includes questions about portion size.

People who lack social companionship and feel lonely experience more pain, depression, fatigue, and cognitive problems than people who are more socially connected [132, 221, 222]. Accordingly, we will measure loneliness and social connection in a number of ways. The **Couples Satisfaction Index** is a self-report instrument that will provide information about social connection within a romantic relationship. The scale was developed by combining items from eight well-validated relationship satisfaction scales using item response theory analysis, and has both a full 32-item form as well as 16 and 4-item short forms. The scale has strong correlation with traditional measures of relationship satisfaction, but has greater measurement precision [223]. The **Revised UCLA Loneliness Scale** [224, 225] is a 20-item scale which has been used with a number of populations including both college students and older adults [225]. The UCLA scale will assess baseline levels of loneliness. The **Social Connection** scale has 10-items and is designed to assess whether a person currently feels socially connected to other people. The scale is unpublished, but unpublished data suggest that the reliability of the scale ranges from .86-.93. The social connection measure is included to account for changes in social connection throughout the day.

Lower perceived social support is associated with poorer immune function and depression risk [226, 227]. The relationship between social support and depression is especially true for women, and thus very relevant for our study [228]. Accordingly, we will assess several different aspects of social support. The **Interpersonal Support Evaluation List (ISEL)** will be used to assess four types of social support: self-esteem, appraisal, tangible, and belonging [229]. This brief six-item scale assesses perceptions of support, as rated on a four-point scale. While social support is generally protective against poor health and depression, problematic relationships or support can lead to increases in depressive symptoms and inflammation [227, 230, 231]. To provide a measure of negative or upsetting support, we will use the **Test of Negative Social Exchange (TENSE)** [232]. The four subscales have good test-retest reliability, internal consistency, and convergent and discriminant validity. Separately, network size is related to overall health and mortality as well as response to immune challenge [233]. The **Social Network Index Interview (SNII)** will be used to assess the number of each participant's roles and her number of relationships across roles [234]. The SNII has been widely used in research on social networks.

Chronic concerns about rejection are linked to dysregulated immune function and depression [235, 236]. Accordingly, we will assess these concerns in order to account for their relationships with our primary outcomes. Attachment insecurity will be assessed using a modified version of the **Experiences in Close relationships (i.e. ECR-M16)**. The ECR-M16 was designed to assess attachment insecurity in patients of diverse ages across a variety of medical settings. The 16-item self-report attachment measure assesses general attachment insecurity in close relationships; it contains two 8-item subscales, one assessing attachment anxiety and the other assessing attachment avoidance. The anxiety subscale includes items such as the following: "I worry about being abandoned" and "I need a lot of reassurance that I am loved by people with whom I feel close to." The following items are representative of the avoidance scale: "I get uncomfortable when other people want to be very close to me," and "I don't feel comfortable opening up to other people." Both scales have been shown to have both high internal and test-retest reliability. Cronbach's alpha has been shown to be .84 for attachment anxiety and .83 for attachment avoidance. Pearson correlations assessed within a 4-6 month time were $r = .82$, $p < .01$ for attachment anxiety, and $r = .73$, $p < .01$ for attachment avoidance. The **Brief Fear of Negative Evaluation (BFNE) Scale** measures the extent to which a person is concerned about negative social evaluation

from others [237]. The scale has 12 items. Internal consistency is acceptable at .80 [236]. The scale also has good convergent validity and is correlated with measures that assess loneliness and depression.

The **Rosenberg Self-Esteem (RSE)** questionnaire is a 10-item measure that assesses a person's global feelings about himself/herself [238]. The measure is one of the most widely used self-esteem questionnaires in the social sciences. Test-retest correlations are range from .82 to .88. The scale also has adequate reliability with alphas' ranging from .77 to .88. People with lower self-esteem are more likely to be depressed than people with higher self-esteem [239]. Including the RSE will allow us to account for self-esteem as a potential confounding factor.

Chronic stress is associated with maladaptive salivary cortisol and inflammatory responses [240, 241] as well as depressive symptoms [242]. To assess the presence of recent chronic stressors, we will use the **Trier Inventory of Chronic Stressors (TICS)**, a 57-item questionnaire that includes 9 scales assessing various aspects of chronic stress, including work overload, social overload, performance pressure, work discontent, overextended at work, lack of social recognition, social tension, social isolation, chronic worrying, and a chronic stress screening scale.

Lower socioeconomic status (SES), typically measured by education, income, and occupation, is associated with higher rates of clinical depressive disorders as well as depressive symptoms [243], as well as most major causes of morbidity and mortality across populations [244] . We routinely collect data on objective SES dimensions described above. In addition, recent evidence suggests that subjective socioeconomic status is also related to a variety of mental and physical health indices including response to immune challenge [245]. We will use the well-established **MacArthur Scale of Subjective Social Status** [246], which asks participants to place themselves on a rung of a ladder based on where they think they stand in society.

Higher self-regulation or attentional control, or self-regulatory capacity is protective against depression [247]. Accordingly, those who are better able to regulate their attention and mood may experience smaller increases in depression. We will use the **Attentional Control Scale** [248] to measure attention focusing and shifting; participants rate each item on a 4-point scale in terms of how well it describes them. The scale has demonstrated adequate internal validity and test-retest reliability [248].

The **Beck Anxiety Inventory** assesses both cognitive and physiological symptoms [249]. Developed to discriminate anxiety from depression while displaying convergent validity [249], the measure appears to be better at discriminating anxiety from depression than other self-report anxiety scales. The scale has good internal consistency (coefficient alpha = .92), as well as good test-retest reliability. Evidence suggests that high levels of anxiety are related to poor cognitive function through its adverse effects on executive functions involving attentional control [250].The brief NIH PROMIS anxiety scale may be used as another anxiety measure, along with the PROMIS fear and anger scales [251]. Similarly, the 16-item **Penn State Worry Questionnaire** addresses an individual's proneness to undue, disproportionate, and uncontrollable worry; high scorers report higher negative affect. The respondent rates items such as ‘‘I worry all the time’’ on five-point scales with 1 = not at all typical of me and 5 = very typical of me [252]. The scale is designed to provide a trait assessment of pathological worry [253].

The 15-item **Impact of Events Scale (IES)** taps two distinct factors: avoidant and intrusive thoughts following a stressful experience[254]. The avoidance items (Cronbach's alpha= .82) assess attempts to suppress thoughts about the experience of their recent life events, while the intrusion items (Cronbach's alpha= .79) assess the unintended thoughts. We will administer the IES asking people how they feel about their breast cancer screening test and any subsequent treatment [255, 256]. Cancer researchers have found that higher scores, particularly intrusive thoughts, are related to depressive symptoms, while non-cancer studies have reported links among intrusive thoughts, elevated stress hormones, and poorer immune function [257-259].

Dietary Assessment.

In order to assess typical dietary behavior of subjects for this study, a series of **24-hour multi-pass recall(s)** will be administered twice between the two admissions [260, 261]. This method estimates food intake within less than 10% error for actual intake for total energy and fat for obese and non-obese women [262]. The 24-hr multi-pass recall is respondent-driven meaning that the initial listing of foods is self-defined. There is repetition with minimal burden on the subjects; yet, frequently forgotten foods are usually recalled through the multi-pass method. At the initial interview at 6 months in the parent study, subjects are given a printed guide developed by the United States Department of Agriculture [263] for portion sizes of foods commonly consumed. The study coordinator will briefly review how the 24-hr recall will be administered and portions of commonly consumed foods will be reviewed using the written guide as well as 3-dimensional food models. The Study Coordinator will

then administer the two subsequent recalls that precede each of the two CRC sessions by telephone using the 5-step system; averaging data across these interviews will provide reliable data about normal dietary intake for prospective analyses. In brief, the interviewer asks the subject to list all foods eaten the previous day by asking: "What was the first food or beverage consumed and what event(s) or location was it associated?" The quick list is then reviewed and the interviewer probes for foods that may have been forgotten. The third step is to identify the time and occasion of food and beverages consumed. In the fourth step, the interviewer asks the subject to add detail for each food listed (one food described at a time). Step 5 is the final probe where interviewer asks for anything else consumed. The manually recorded data are entered into the NDSR nutrient data base system (Minneapolis, MN) that is available at the CRC that will analyze the diet records for macro- and micronutrients as well as numerous bioactive food components and fatty acid composition.

Central adiposity: Body composition will be assessed at the first CRC visit using **dual x-ray absorptiometry (DXA)** (model DPX-NT/software version 5.60, GE Lunar, Madison, WI).

Experimental Pain Sensitivity: Experimental pain sensitivity predicts clinical pain [264] and thus a well-established model of experimental pain will be used in this study, the **cold pressor test**. We will follow the procedures described by Mechlin et al.[264]. Briefly, participants immersed their foot in 4°C ice water and are asked to say when it first becomes painful (pain threshold) and when they are no longer able to tolerate the pain (pain tolerance). The maximum time for immersion is 5 minutes. The cold pressor test will be administered around the expected peak time for IL-6 on each of the 2 visits.

Objective Cognitive Assessments: We will measure aspects of cognitive functioning that have been responsive to endotoxin administration and chemotherapy exposure as suggested by other studies and our neuropsychology consultant, Robert Bornstein PhD, including verbal memory, reaction time, attention, and executive function [79, 265-270]. At each visit, we will include both baseline and post-injection assessments. Unless otherwise noted, assessment tasks will be completed at baseline, 3 hours, and 4.5 hours post-injection, to coincide with expected peak inflammatory responses and prior studies [79, 267, 271]. The order in which tasks are administered will remain the same across subjects and conditions. Alternate versions of the tests will be administered to avoid practice effects, and test versions will be counter-balanced. The **Hopkins Verbal Learning Task** will assess verbal learning and short- and long-term verbal memory [266, 272]. Subjects are asked to immediately recall a presented word list, then to recall it after a 20-minute delay. The **Conners' Continuous Performance Test** is widely used to measure attention, concentration, and reaction time [273]. On this computerized task, subjects are instructed to press the space bar when a target stimulus appears, but to not press it when any other stimulus appears. To reduce participant burden, we will only administer this task at baseline and 3hr post-injection. The **Stroop Task** will be used to measure executive function. The Stroop requires participants to name a color of a word while inhibiting the natural inclination to read the actual word itself [274]. Significant practice effects exist with the Stroop Task [275], so participants will only be tested at 3h post-injection at both visits, consistent with other studies [265]. For the emotional Stroop, participants name the color in which negative or threatening words are printed, rather than reading the word itself [276]. Following work by Mogg et al. [277] we will use words from their lists from each of the following categories: depression words (desolate, discouraged, dismal, forsaken, glum, grief, mournful, suicide, tearful, tormented, blame, despair, desperate, drained, forlorn, helpless, hopeless, listless, pessimistic, sorrow), neutral (uncategorized) words (adjacent, amount, assembled, bearings, bent, bilateral, boulder, casement, celery, circuit, alignment, angle, border, boundary, bowl, branched, brochures, capacity, contents, density), and negative words (anxiety, assassin, assault, betrayal, brutal, chaotic, crisis, dangerous, death, destruction, ambulance, atrocious, brutal, devil, hazardous, horrible, hostile, hurricane, maniac, menacing). The word types were matched for length and frequency based on published norms [278]. The words will be presented in random order. The depression and negative word interference scores for each subject and assessment time point will be calculated by subtracting the mean latency for the neutral words from those for depression-related words, and the same procedure will be used for negative words [277]. Thus, larger values indicate slower responses to emotion-relevant words than neutral household words [277].

To measure working memory, we will administer the **N-back Task** [279]. In this task, participants view a continuous stream of stimuli (e.g., letters); each stimulus is directly followed by the next on a computer screen. The participant indicates whether the current stimulus matches the stimulus presented a specified number ('n') of trials before it, which requires her to monitor, retain, and update information in memory. **Cognitive Function Task:** In both visits, participants will engage in a brief established lexical decision task that assesses their cognitive functioning after the inoculation [280]. Participants are presented with a series of strings of letters via

computer monitor (e.g., "dog, mipt, break, prant..."); for each string, they are asked to decide whether or not it is a word in English, responding by presssing one key on the computer keyboard for "word," another key for "nonword."

Social withdrawal: Heightened inflammation can impact social behavior [62]. We will use two computer tasks to assess changes in social approach and withdrawal preferences in response to the vaccine. These tasks will provide data on both automatic motor responses as well as subjective interpretations of social stimuli. An **implicit joystick task** will be used to measure automatic motor responses to standardized photographs of happy, sad, angry, and fearful facial expressions [281, 282]. Prior research suggests that participants are faster to push the lever forward for aversive stimuli (indicating withdrawal tendency), and pull the lever backward more quickly in response to appetitive stimuli (indicating approach tendency) [283, 284]. On a different **explicit rating task**, participants will view the same standardized facial expression stimuli, and will explicitly rate their tendency to approach or avoid the person on a scale of -4 to 4 [281, 282]. The tasks both have adequate reliability, and have been used in prior research on social motivation [281, 282].

Daily stressors: The **Daily Inventory of Stressful Events (DISE)** provides an interview-based approach to measuring daily stressors [285]. In a national sample of adults in the United States, participants reported at least one daily stressor on 40 percent of the days they were studied, and multiple stressors on 11 percent of days, with interpersonal tension the most common stressor [285]. Importantly, interpersonal tension was one of the unique predictors of self-reported health symptoms as well as negative mood in that sample [285], consistent with the immune and endocrine data we have collected from married couples following disagreements. The DISE methodology utilizes investigator-rated measures of stressor severity and threat, which provides a way to reduce the bias inherent in self-ratings of stressor severity and appraisal [285]. The threat dimensions used for the interview were developed from the contextual threat dimensions utilized by Brown and Harris [286]. Daily stressors are rated by investigators for type of threat (loss, danger, disappointment, frustration, and opportunity) and severity of threat ("none" to "extreme") that the event would pose to an average individual [285]; as with the Brown and Harris methodology, an extensive electronic “dictionary” is used by investigators to anchor ratings.

This interview's excellent sampling of interpersonal stressors has key advantages for the population and questions being addressed, in that interpersonal events are highly salient. Moreover, it provides a nice parallel with the use of the stressful life events interview. By following the model employed by Almeida [285], we will use regression analyses to examine the unique prediction of various aspects of the stress experience; for example, to examine mood, on the first two steps the objective measures of daily stressors were entered, while severity, threat, and appraisal measures were entered on the next three steps. Using this strategy, higher levels of daily physical symptoms and more intense negative moods were associated with more frequent interpersonal, network, respondent-focused stressors as well as more severe stressors that pose greater risk to physical health and safety.

Respiratory Quotient (RQ) and Resting Metabolic Rate (RMR). RQ and RMR will be obtained via indirect calorimetry with the Deltatrac Metabolic Cart prior to the meal (SensorMedics, Yorba Linda, CA). Inspired and expired airflow of oxygen and carbon dioxide (VO_2 and VCO_2) will be measured with a facemask following a short calibration of the equipment. The subject will lie supine on a bed at a 20 degree angle and the facemask will be fastened around the back of the head. The subject will then relax and breathe normally for the duration of the measurement.

Total baseline testing time for RMR, resting and measurement, will last 40-50 minutes. RMR will be determined during the steady state, defined during the measurement as the consecutive 5 minute interval in which EE is the lowest while oxygen consumption (VO2) and carbon dioxide production (VCO2) change by <10% [287, 288]. Subsequent measurements to assess the thermic effect of food will be taken for ~10 minutes of every 30 minutes for 5-6 hours. Deltatrac is a well-respected instrument with accuracy of 3.0% for gas exchange and 0.2% for RQ [289].

Cardiovascular Data. Blood pressure and heart rate will be assessed, providing data on autonomic activity. During each of the CRC assessments, participants will wear a Polar™ S810i HR monitor which will provide a continuous recording of heart rate data when downloaded after the session, with sampling at 15 sec intervals, and will also provide data on heart rate variability (HRV).

Participants will spend the five minutes of the baseline period breathing slowly (2 seconds for inhalation and 2 seconds for exhalation) in time with a pre-recorded tape. This 5-minute period will serve as the formal

measure of tonic RSA (indexing vagal tone). Paced breathing during a baseline assessment provides a way to standardize respiratory parameters and thus yields more accurate assessments of between-person differences in tonic RSA [290].

In addition, during the CRC admissions we will assess blood pressure using an automated system, the Dinamap/Critikon 1846SX/P. Blood pressure will be assessed at baseline and roughly every hour after breakfast.

Data Storage and Protection: Our data are stored on a password-protected server behind the OSUMC firewall. All data are coded by subject number, and questionnaires do not include names or other identifying information. Identifiers are stored on an Access database that is separated from the remainder of data, and individuals in the lab only have access to those parts of the data that are necessary to their particular job.

Quality Control Procedures*:* All data will be stored in EXCEL and/or ACCESS databases. Monitoring will be conducted by the data manager; external audits and a random chart review may be conducted periodically by the data manager to assure that tabulated data and source document entries match.

E. Specimen Collection

Assays and Timing of Blood and Saliva Samples: Fasting levels of glucose and insulin will be assessed prior to breakfast. HOMA-IR [291] will be used as a covariate in our analyses as an estimate of insulin resistance. The assays will be conducted by the hospital labs using standard methods. Insulin resistance will be assessed, based on data that suggests that higher proinflammatory cytokine responses to stress occurred among individuals with greater insulin resistance [292].

Typhoid vaccine provokes a transient acute phase response, with increases in proinflammatory cytokines followed by rises in anti-inflammatory cytokines [48-60]. To assess the magnitude and duration of these changes, blood will be drawn for **IL-6, TNF-α, IL-10, IL-1Ra, sTNF-RII, and SAA** in serum samples measured using an electrochemilluminescence method with Meso Scale Discovery kits at the fasting baseline and then every 90 minutes post-inoculation for 7.5 hours. The choice of assays was based on changes observed following typhoid inoculation and related endotoxin studies, and was designed to provide data on kinetics of both pro- and anti-inflammatory markers [48-60]. Our laboratory has had many years of experience in measuring proinflammatory cytokines [142, 143, 146, 147, 165]. The stored serum samples for each subject will be assayed for all the cytokine markers in one run, thus using the same controls for all time points for each person. High-sensitivity **CRP assays** will be performed by the hospital lab using standard procedures. Across both full-day admissions we will have a total of 12 **CBCs with differential**, coinciding the timing with the cytokine assays; typhoid vaccination studies show sharp increases in the white cell count [49, 51, 52, 57, 60].

Cortisol increases over 3 to 4 hours after an inflammatory challenge as illustrated in multiple endotoxin and cytokine therapy studies, and the magnitude of the change may signal vulnerability [35, 79]. For example, HPA hyperreactivity to the first interferon alpha (IFN-α) injection in cancer patients was a strong predictor of the development of major depression later in IFN-α therapy [35]. Patients who responded to the initial administration of IFN-α with larger ACTH and cortisol responses were more likely to develop major depression. Furthermore, changes in cortisol were correlated with depressive symptoms even after controlling for cytokine changes following a much milder inflammatory stimulus, low-dose endotoxin [79]. We will collect **salivary cortisol** samples across each of the two CRC visits using previously described procedures for collection and assay [293]. Samples will be collected in conjunction with each blood draw, as well as immediately before the cold pressor test, and 15 and 30 minutes after completion.

Additional assays that will be considered if funding is available include the following, designed to collect preliminary data for future related studies:

Measurement of Cytokines Produced by PBMCs stimulated with Con A and LPS. Histopaque 1077 gradients will be used to separate PBMCs from whole blood samples. The PBMC cultures, $1x10^6$ cells/ml, will be incubated for 24 hours in two 2 mls RPMI 1640 median containing 5% autologous plasma either with or without Con A, 5μg/ml. In order to measure the expression of TH-1/TH-2 cytokines, IFN-g, IL-2, IL-4 and IL-10 will be measured 24 hours after treatment.

The same number of cells will be treated with 1.0 μg/ml LPS for 24 hours in order to measure the proinflammatory markers [294]; IL-6, IL-10, TNF-α, IL-1β, TGF-β, sIL-6r, and IL-1ra. A control cell culture will be

incubated in media alone. After 24 hours, the cells will be pelleted by centrifugation (1000 rpm for 10 minutes) then frozen and thawed three times by freezing on dry ice and thawing in a 37°C water bath.

Previous studies have shown that the concentration of LPS used to stimulate the PBMCs and how the cells/supernatants are harvested after the 24 hour incubation are important for obtaining reliable results. It has been shown that using low concentrations of LPS, approximately 1 μg/ml, to stimulate PBMCs is important to show an effect of diet supplementation with EPA and DHA. In addition, pooling cell lysates after freezing and thawing with cell supernatants significantly increases the yield [294-297].

The stored serum and cell lysate samples will be assayed for all the cytokine markers as one run at the same time using the same controls for all time points for each person. This will result in well controlled conditions across all time points for each subject. Our primary battery for stimulated cytokine production will be levels of IL-6 and TNF-α, with additional cytokines measured if we have sufficient funds.

Measurement of endogenous endotoxins. Dietary researchers showed that a high-fat high-cholesterol meal provoked oxidative stress and inflammation, in contrast to a high-fiber and fruit meal which did not elicit these changes [298]. Extending these studies, they found that a high saturated fat drink increased lipopolysaccharide (LPS) concentrations and expression of Toll-like receptors (TLR)-4, the LPS receptor; when LPS binds to TLR-4 it activates a signaling pathway that leads to the production of proinflammatory cytokines [299]. The authors argued that saturated fats play an important role in postprandial inflammation because they increase permeability of the intestinal epithelium and thus promote the breakdown of the intestinal barrier [299]. Based on these and related studies reviewed by Laugerette [300], we propose to measure changes in endogenous endotoxins before and after the meal because of their potential contribution to postprandial inflammation. We will follow standard endogenous endotoxin assay protocols for human studies [39, 301]. We will also measure the endotoxin receptor sCD14 and expression of TLR-2 and TLR-4 following published protocols[298, 299]. TLR4-dependent cell activation by endotoxin requires lipopolysaccharide-binding protein (LBP) and CD14-dependent delivery of endotoxin to cells containing MD-2 and TLR4, and thus LBP will also be assessed.

Glucocorticoid insensitivity. Glucocorticoid hormones regulate the expression of proinflammatory cytokines, in part, by regulating NF-κB activity. This transcription factor is a well characterize molecule that regulates target genes that have inflammatory, anti-apoptotic, cell cycle and proangiogenic properties and thus may contribute to tumorigenesis [302]. A recent study has suggested that NF-κB is a “molecular bridge” between inflammation and cancer [303].

Glucocorticoid (GC) insensitivity is a state of unresponsiveness to the regulatory activity of GC hormones. There are a number of mechanisms that can make immune cells insensitive to GC. They range from repeated glucocorticoid therapy for diseases such as asthma [304], to behavioral responses to chronic stressors [305]. GC insensitivity of cells of the immune system can be measured *ex vivo*, by using peripheral blood mononuclear cells (PBM). PBM are incubated with bacterial lipopolysaccharide (LPS) together with varying concentrations of the natural hormone cortisol, or a synthetic version of the hormone named dexamethasone. GC insensitive monocytes produce abnormally high levels of proinflammatory cytokines when their Toll-like receptors (TLR) are ligated by mitogen [306]. TLR stimulation of GC insensitive cells leads to enhanced NF-κB activity and over expression of proinflammatory genes. Using this *ex vivo* assay, studies have found that circulating immune cells are GC insensitive in spousal caregivers of dementia patients [307], in parents of children undergoing cancer treatment [308], and in chronic stress-related syndromes such as vital exhaustion and depression [305, 309, 310].

The extant literature strongly supports an association between chronic inflammation and carcinogenesis. Chronic inflammatory responses deplete anti-oxidant resources resulting in the generation of reactive oxygen and nitrogen radicals with a strong potential for cell damage [302]. Furthermore, proinflammatory cytokines such as TNF-α and IL-6 are pleiotropic molecules that contribute to host defense mechanisms, but their expression also has been associated with tumors, e.g., TNF-α has been detected in tumors of the breast, prostate, colorectum, bladder, and other tumors [311], while IL-6 has been shown to be elevated in patients with colon cancer and positively correlated with tumor burden [312]. Proinflammatory cytokines, perhaps through the activation of intracellular kinases and transcription factors, have been shown to contribute to inflammation-associated carcinogenesis [313, 314].

Thus, following a fast-food-type meal, on inflammatory gene expression in circulating monocytes, it would be expected that individuals with higher levels of depression would have elevated numbers of GC insensitive monocytes compared to control individuals. A corollary hypothesis suggests that similar findings of GC

insensitivity will be found in obese vs. lean individuals.

Antibody titers to latent EBV, HSV-1, CMV, and VZV. Measurement of IgG antibody titers to latent EBV, HSV-1, CMV, and VZV provides an indirect measure of the ability of the virus-specific memory immune response to control the steady state expression of these latent viruses. Our laboratory was one of the first to establish that psychological stress can reactivate latent EBV [315, 316]. We and others have shown that psychological stress can also reactivate latent HSV-1 and VZV [316]. Importantly, we have shown that different herpesviruses react differently to different types of stressors [316-320]. The mechanisms underlying these differences are not known. We will study different strains of herpes viruses to explore the possibility that there might be an interaction between stress-induced immune dysregulation and herpes virus latency. For this study, we will utilize aliquots of serum samples to perform the indirect immunofluorescence (IF) test using virus-infected cells as the antigen(s).

Real-time RT-PCR analyses. Total RNA from untreated and LPS-treated PBMCs will be extracted using TRIzol reagent (Invitrogen Life Technologies) and cDNAs will be synthesized using Superscript III RNase H-reverse transcriptase (Invitrogen Life Technologies). TaqMan Gene Expression Assays (Applied Biosystems) will be used for the gene expression analyses of IL-6, IL-1β, TNF-α, and a housekeeping gene. In order to pick the appropriate housekeeping gene to use as an internal positive control, we will test at least 3 housekeeping genes (e.g., 18S rRNA, Glyceraldehyde-3-phosphate dehydrogenase, and ß-actin) for stability of expression after treatment with LPS. The housekeeping gene whose expression remains stable regardless of the treatment will be selected and used as a positive control for all the samples. TaqMan Gene Expression Assays do not detect genomic DNA sequences; therefore, they are very specific to mRNA. Levels of mRNA will be compared between samples using quantitative real-time RT-PCR with Taqman fluorogenic probes, TaqMan PCR Reagent Kit and the 7300 Real-time PCR System (Applied Biosystems). All PCR reactions will be normalized with the housekeeping mRNA level and the relative expression of mRNA species will be calculated using the comparative C_T method [321].

Telomere/Telomerase. Recent studies suggest that chronic stress and adverse lifestyles, including nutrition and obesity may be associated with premature aging of immune cells. Human telomeres are 10-15 kb long tandem hexanucleotide (TTAGGG) repeats and their binding proteins are located at the ends of chromosomes. Due to incomplete replication of chromosomal termini, telomeres shorten with each cell division. As telomeres are essential for maintaining chromosomal integrity, cells with critically shortened telomeres cease division (senescence) and are prone to apoptosis. Thus, the length of telomeres serves as a limit for the number of divisions that a cell can undergo. Telomeres are synthesized by telomerase that compensates the loss of telomere length from cell divisions. Telomere attrition with age and with cell division has been observed in many types of cells including lymphocytes. Telomerase activity and telomere length, two cellular markers associated with aging, were measured in peripheral blood mononuclear cells (PBMCs) obtained from mothers caregiving for a chronically ill child, as well as mothers of healthy children [322]. Caregivers reported greater stress than controls, but higher reports of perceived stress were associated with lower telomerase activity and shorter telomere length regardless of whether the mother's child was ill or healthy. High reports of stress were also associated with higher oxidative stress activity as measured by levels of F_2-isoprostanes (another independent measure associated with aging) [322]. Moreover, telomerase activity has been associated with inflammation and immune senescence of cells.

To extend these studies, PBMCs will be isolated from subjects and cryopreserved. The composition of T and B lymphocytes, natural killer cells, and monocytes in each PBMC will be analyzed by flow cytometry (BD Calibor and Cellquest software). Measurement of telomere length of PBMC will be carried out following standard procedures [323]. The levels of induced telomerase activity of T cells will be measured by the increase of telomerase activity before and after stimulation (anti-CD3 plus IL-2, 20 U/ml for 2 days) based on a modified protocol [324].

Measurement of F_2-isoprostanes (F2-IsoPs)[325]. It has been suggested that leukocyte telomere changes reflect the joint burden of oxidative stress and inflammation[326]. F2-IsoPs provided the most reliable index of in vivo oxidant stress when compared against other well-known biomarkers[325]. An NIH-funded multi-investigator study, the Biomarkers of Oxidative Stress Study (BOSS) concluded that quantification of plasma or urinary isoprostanes was the most accurate method for assessment of in vivo oxidant stress status[325]. Plasma samples will provide data on the analysis of isoprostane levels as a measure of oxidative stress following the published protocol [325] used at Vanderbilt.

Cytokine/CRP Genotyping In these studies, SNPs known to be associated with expression levels of IL-6,

TNF-a (one each), and CRP (two each) will be determined. The IL-6 and TNF-α SNPS will be determined using the ABI SNaP genotyping kit described above. The CRP SNPs will be determined through classic restriction fragment length polymorphism (RFLPs) assays. Both assays are routinely performed for genotyping polymorphisms in the type one complement receptor [327-329]. The specifics of these genotyping assays are as follows:

TNF-α polymorphism. A SNP occurs at -308G□A in the promoter region of TNF-α, and the 308A allele is associated with higher TNF-α production [330]. This SNP will be identified by first amplifying 50 ng genomic DNA using a sense primer from -462 to -444 (5' ACCCCGTTTTCTCTCCCTC) and an antisense primer from -187 to -207 (5' GGGGACACACAAGCATCAAGG). The resulting 276 bp fragment will be treated with *Exo*1 and alkaline phosphatase, and then cycle-extended one ddNTP using a 27 base sense SNaP primer (5' GGAGGGAATAGGTTTTGAGGGGCATGG) that abuts the 308G□A polymorphism, and incorporation of a ddGTP or ddATP will be determined.

CRP polymorphisms. Two SNPs occur in the CRP gene (641G□C and 1846G□A) that are independently associated with circulating CRP levels. Both SNPs will be characterized through RFLP assays as recently published [331]. Briefly, for the 641G□C SNP, a 279 bp amplicon will be amplified from 50 ng genomic DNA using the sense primer (5' TCAGGGATCGTGGAGTTC) and antisense primer (5' GCCTTGCACTTCATACTTC). This fragment contains a restriction site for *Tsp*451 which involves the 641G□C polymorphic residue such that restriction digestion with this enzyme yields restriction fragments of 90 bp and 189 bp for the wildtype 641G allele while leaving the 641C variant allele uncut. For the 1846G□A SNP, a 227 bp amplicon will be amplified from 50 ng genomic DNA using the sense primer (5' GGAGTGAGACATCTTCTTG) and the antisense primer (5' CTTATAGACCTGGGCAGT). The 227 bp fragment contains a *Hpy*CH4III restriction site involving the 1846G□A polymorphic residue such that the wildtype 1846G is cut to yield 97 bp and 113 bp fragments, while the 1846A variant is uncut. Both RFLP reactions will analysed in 3% agarose gels, and the bands will be visualized by UV light following ethidium bromide staining.

IL-6 polymorphism. A SNP occurs at -174G□C in the promoter region of the IL-6 gene. The presence of the G allele induces higher constitutive IL-6 expression levels, and higher levels after LPS or IL-1 mediated induction, relative to the C allele [332]. This SNP will be identified by first amplifying 50 ng genomic DNA using a sense primer from -332 to -312 (5' CTTCGTGCATGACTTCAGCTT) and an antisense primer from -80 to -101 (5' GATAAATCTTTGTTGGAGGGTG). The resulting 253 bp fragment will be treated with *Exo*1 and alkaline phosphatase, and then cycle-extended one ddNTP using a 23 base sense SNaP primer (5' TTTTCCCCCTAGTTGTGTCTTGC) that abuts the -174G□C polymorphism, and incorporation of a ddGTP or ddCTP will be determined by analysis on an ABI Prism 3730 DNA analyzer. The G allele showed roughly four times greater expression of IL-6 than the C allele in lymphocytes stimulated by liposaccharide or IL-1 [332]. In a subset of 102 subjects, GG homozygotes had twice the level of fasting plasma IL-6 than CC homozygotes. ($p<.02$) [332].

Flow Cytometry. Using separated PBMCs, 0.5 – 1x 10^6 cells will be aliquoted into tubes. The cells will be incubated with each mAb and then washed and resuspended with 2% Formalin solution and stored at 4C until analyzed with a BD FACSCalibur flow cytometer. We will measure the percentage and absolute number of leukocyte activation markers CD11A, CD11B, CD62L, CD14, sCD14, and CD66B [333].

Adhesion Molecules. We will assess sICAM-1, sVCAM-1 and E-selectin, as well as serum amyloid A (SAA), a family of apolipoproteins associated with HDL in plasma.

Gene Expression Studies Using Focused Microarrays. These experiments are designed to detect and quantify gene expression for inflammatory cytokines, stress and appetite hormones, chemokines, growth factors and accessory molecules that are produced in the subjects. Peripheral blood leukocytes (PBLs) will be obtained at each time point and used for molecular studies. Purified RNA prepared from the PBLs will be used to do gene expression using focused microarrays.

The following oligo arrays will be used to characterize gene expression in peripheral blood mononuclear cells (PBMC)s: *Array 1*) Inflammatory Cytokines & Receptors. This array profiles the expression of 113 key genes involved in the inflammatory response. The chemokines, interleukins, and TNF ligands involved in the inflammatory response are represented as well as their receptors. *Array 2)* T-cell and B-cell Activation. This array profiles 113 genes representing T-cell and B-cell activation, which are both key parts of adaptive immunity. This array includes most of the genes known to influence the maturation of the B and T cell response and many of these 'activating' genes are produced by the macrophage or dendritic cells. *Array 3)* NFkB Signaling Pathway. This array profiles the expression of 113 genes related to NFkB-mediated signal transduction. The

array includes genes that encode members of the Rel, NFkB, and IkB families, NFkB-responsive genes, extracellular ligands and receptors that activate the pathway, and kinases and transcription factors that propagate the signal. We may also explore use of focused microarrays for expression of stress and appetite hormones, as well as obesity-related genes, because they may play a role in the strength of the immune system's proinflammatory cytokine responses to stress, as well as dietary challenges [334]. New nutrigenomics research involves a study of gene expression in response to changing conditions such as nutritional intervention studies to better understand the effects of diet on gene expression [335]. For example, one study showed a decrease in oxidative stress and inflammation genes in response to a low-calorie diet [335]. Approximately $1x10^6$ PBMCs (1-2ml heparinized whole blood) will be used for each array.

Note that we are not proposing to provide feedback to participants about the results of genetic testing. We carefully considered the issue of whether we should provide feedback on the results of their cytokine genotype profile, and decided that we would not, based on several factors. First, the health relevance of the cytokine/CRP polymorphisms is still a matter of considerable debate in the literature; for instance, though most studies of the IL-6 G174C polymorphism indicate that the 174G allele is associated with increased IL-6 levels and is a risk factor for various diseases, two studies oppositely state that it is the 174C allele that is associated with high levels, and three studies indicate that the C allele is the disease risk factor; indeed, our own data from older adults also show contrary results . Furthermore, there might be both an age and sex effect on these associations, although there is even some contradiction as to the specifics in these studies. As for TNF-α, there are also many pathologies involving TNF-α that do not associate with the 308 polymorphism (eg., rheumatoid arthritis), and at least one study that states that this polymorphism doesn't affect levels [336]. Thus, it would be premature to provide feedback to the study participants when the actual impact of the polymorphisms is still unclear. Similarly, the same considerations apply for the microarray studies; these are very preliminary studies in new areas that involve small samples, and any results would be extremely preliminary and in need of replication. Accordingly, our consent form states that we will not provide feedback.

Glucose: The CRC uses Immobilized Enzyme Technology using the YSI 2300 Stat Plus Glucose and Lactate Analyzer from YSI International, Yellow Springs, Oh. Assay coefficient variation is 2% with calibration occurring every 15 minutes. The sensitivity of this instrument is 2.5 mg/dl.

Insulin: Chemilluminescence methodology using the Immulite 1000 (Siemens Medical Solutions Diagnostics) Sensitivity for this assay is 2μIU/ml. Intra-assay coefficient of variation is 5.7%and Inter-assay coefficient variation is 6.7%. Insulin resistance (IR) will be determined from fasting serum insulin and glucose measures as indexed by the Homeostasis Model Assessment (HOMA) [337, 338]. Saturated fat worsens insulin sensitivity and adversely effects glucose metabolism [339].

Fatty acid composition of red blood cells. Omega-3 enhances triglyceride clearance following high-fat meals [340], and better triglyceride clearance is related to lower postprandial inflammation, but relationships between dietary omega-3 and postprandial inflammation have not been studied. The fatty acid composition of red blood cells drawn as part of the fasting blood sample will provide data on habitual fatty acid intake [341], assayed as described previously [342].

Endocrine Data. Stressors can substantially elevate epinephrine and norepinephrine, as well as other stress hormones [343-346]. The ability to "unwind" after stressful encounters, i.e., quicker return to one's neuroendocrine baseline, influences the total burden that stressors place on an individual [347]. Catecholamine samples will be collected at set intervals during the protocol if we have sufficient resources. Serial blood samples will be obtained at the OSU CRC. Plasma catecholamines will be assayed using HPLC with ElectroChemical Detection using standards and chemicals (Alumina extraction) purchased from ChromSystems (Munich, Germany, U.S .affiliate Thermo-Alko). C-18 Columns (Waters), an HPLC pump and detector (ESA), and a 717-plus Autosampler (Waters Corporation) are the configuration that will be utilized for these determinations. The intra-assay coefficient variation for norepinephrine is 3% and the inter-assay coefficient variation is 6%. The intra-assay coefficient variation for epinephrine is 6% and inter-assay coefficient variation is 13%. Sensitivity for norepinephrine is 15 pg/ml and 6 pg/ml for epinephrine.

Related to our immunological focus on changes in inflammatory cytokines, we also hope to assay leptin, ghrelin, and adiponectin, depending on available funds. Higher concentrations of plasma adiponectin are associated with lower waist-hip ratios, lower insulin resistance, lower diastolic pressure, lower triglyceride concentrations, and lower TNF-α receptor concentrations [348].

Glucocorticoids and insulin work together in the regulation of serum leptin; increases in leptin from the lowest to highest point daily are related to insulin changes induced by meals [349]. Sufficient endogenous

cortisol secretion is important for insulin's effects on leptin production [349]. However, leptin-resistant obesity may eventually be caused by leptin stimulation related to glucocorticoids [349].

Leptin, ghrelin, and ghrelin (total) will be assayed by the CRC using the respective RIA kits (Millipore Corporation, St. Charles, MO 63304). For leptin, the intra-assay coefficient of variation is 4.2% and inter-assay coefficient of variation is 4.5%; sensitivity is 0.5 ng/ml. For adiponectin, the intra-assay coefficient of variation is 3.8% and inter-assay coefficient of variation is 8.5%; sensitivity is 1 ng/ml. For ghrelin, the intra-assay coefficient of variation is 6.4% and inter-assay coefficient of variation is 16.3%; sensitivity is .09 ng/ml.

Saliva will be collected for cortisol assay 15-18 times during each visit using a salivette (Sarstedt, Newton, North Carolina), an untreated sterile cotton roll placed in the subject's mouth for ~2 minutes to ensure saturation. Each subject's saliva samples will be frozen after collection and analyzed by the CRC within the same assay using the Cortisol Coat-A-Count RIA (Siemens Medical Solutions Diagnostics, Los Angeles); the intra-assay coefficient of variation is 4.3%, the inter-assay coefficient of variation is 5.2%, and sensitivity is .025 ug/dl.

Salivary α-amylase will be assayed from saliva to provide a marker for the activity of the sympathetic nervous system[350]. Amylase has also been directly associated with ratings of satiety and fullness, and inversely associated with hunger and desire to eat. It will be measured by the CRC lab using an enzyme kinetic assay.

Specimen Storage and Protection: Specimens will be stored in a -80^{o} freezer that is located at the Institute for Behavioral Medicine Research. All laboratory samples have only the study's identifying number and date and sample number, and individuals in the lab only have access to those parts of the data that are necessary to their particular job.

F. Human Subject Information

Potential Risks: The study has only modest risks associated with the procedures. Personal questions contained in questionnaires and interviews and cognitive tests may make participants uncomfortable or could produce stress. The blood draws and the catheter put subjects at small risk for pain, infection, a bruise at the draw site, or fainting. There is a minor risk of an allergic response to the tape used to hold the catheter in place that may include redness or a rash, swelling, small blisters, itching, and discomfort on the arm where the skin was covered by the tape. A single DXA scan produces a very small amount of radiation, approximately 1/10th of that experienced during a cross-country air flight or 1/120th of what participants would experience during a dental scan. For the cold pressor test, the primary risk is transient pain/discomfort related to the water temperature, no other known risks are associated with this procedure. For the cardiac stress testing, risks may include feeling fatigued after the testing and/or shortness of breath, as well as transient muscle soreness. The discomforts and/or risks associated with a maximal exercise test are comparable to those encountered during any strenuous athletic event and include occasional disorders of heartbeat, abnormal blood pressure, and a very remote chance of heart attack. For the typhoid vaccine: The Vi polysaccharide typhoid fever vaccine is commonly administered when people travel to areas where typhoid fever is endemic; the vaccine is well-tolerated in adults [351, 352]. The safety profile for the typhoid vaccine is well-established, and is supported by over 20 years of immunogenicity trials, efficacy trials, and post-marketing surveillance data [352]. For example, out of more than 22 million doses of the Vi vaccine that were distributed between 1986 and 1996, only 140 nonserious adverse events and 20 serious adverse events were reported [351]. Side effects are typically mild and infrequent [351, 353], occurring in less than 10% of all adults [352]. The most common side effects are local pain, redness, induration, or swelling at the injection site [170]. A small subset of people who receive the vaccine report feeling feverish; in two studies comparing Vi vaccine to placebo, feverish feelings, malaise, and headache were reported about 10% more often in those who received the typhoid vaccine [352]. However, objectively confirmed fever is uncommon, occurring in less than 1% of subjects after a 25-μg dose [352]. Furthermore, in a study of 104 Vi vaccinated participants, none required time off of work or any form of medical treatment as a result of the vaccine [351]. Other systemic reactions such as skin disorders (including rash), muscle or joint pain, or gastrointestinal complaints are rare, and occur in less than 2% of individuals who receive the vaccine [170, 352].

Protection Against Risks: The following steps will be taken to protect against risks.

Recruitment and Informed Consent. Written informed consent will be obtained at the beginning of the screening session. The consent form will be posted on the web along with the study description at the recruitment web site, so the participants have the opportunity to read the form carefully prior to applying for participation. In addition, once a participant has been scheduled for screening, they will receive a copy of the

informed consent form, the HIPAA form, and the Notice of Privacy Practices along with the letter confirming their appointment date/time. On arrival in the CRC for their screening appointment, the key points of the study will be reviewed and they will be asked to sign the informed consent prior to any other activities.

Confidentiality. The risk of breaching confidentiality is low. All data are coded by subject number, and questionnaires do not include names or other identifying information. All laboratory samples have only the study's identifying number and date and sample number. Identifiers are stored on an Access database that is separated from the remainder of the data, and individuals in the lab only have access to those parts of the data that are necessary for their particular job. Data will only be reported as averages, with no identifying individual data.

For questionnaires and interviews. As an additional precaution, as part of the packet given to individuals at their first session, along with a copy of the informed consent, we will give all subjects a list of mental health agencies when they are finished with their initial appointment on the project. Note that the consent form also informs participants that we will contact appropriate authorities if the participant appears to be at imminent risk for harm to herself or others. Suicide risk is assessed in the SCID interview, and if the subject endorses suicidality, we have a protocol in place that will be activated. The protocol includes notifying CRC staff, assessment by clinically trained personnel, and/or notifying authorities as appropriate.

For blood draws and the catheter. Blood draws and catheter insertions will be conducted by experienced nurses in the Clinical Research Center to minimize the risks associated with these procedures.

Discovery of a previously unknown condition. Any potentially abnormal results from the hospital lab data will be first discussed with Dr. Malarkey, the physician on the study, to confirm the nature and seriousness of the problem; if there appears to be reason for concern, participants would be informed of the abnormal value and advised to seek treatment from their personal physician.

DXA scan. A single DXA scan produces a very small amount of radiation, approximately $1/10^{th}$ of that experienced during a cross-country air flight or $1/120^{th}$ of what participants would experience during a dental scan.

For the typhoid vaccine. Consistent with any vaccine procedure, we will exclude individuals who have a severe allergy to any component of the vaccine. In addition, our study will exclude subjects who are currently ill or immunocompromised. Vaccinations will be conducted by experienced nurses in the Clinical Research Center to minimize the risks associated with these procedures. Side effects will be carefully monitored in the CRC for 7.5 hours after vaccine administration.

Exercise testing. The risks of serious injury during exercise testing in healthy subjects are negligible. Even in populations with chest pain or established coronary artery disease, fatal arrhythmias or myocardial infarction occur in less than 1 of 10,000 exercise tests. There is no alternative to the exercise stress testing; stress testing is the gold standard for evaluating cardiopulmonary endurance. All participants will be carefully screened and those with serious medical conditions will not be eligible for participation. In addition, we will conduct all exercise testing studies in the CRC under the supervision of an exercise physiologist and a physician, and emergency coverage is readily available.

For blood draws and the catheter. Blood draws and catheter insertion will be conducted in the General Clinical Research Center to minimize the risks associated with these procedures.

For the cold pressor test. Participant will be informed that they may remove their foot from the cold water when the pain or discomfort becomes too great. The procedure lasts 5 minutes, not long enough for any lasting effects due to the water temperature.

Benefits: Participants will receive a report based on their answers to the food recall interviews. The report will tell them about their eating patterns, such as how closely their number of servings in each food group consumed daily matches USDA recommendations. In addition, this report will show your average daily intake of different nutrients and recommendations for improving your diet. Participants will also receive the results of their exercise test. The results obtained from the exercise test will provide them with information about their physical fitness, cardiorespiratory endurance, and ability to sustain prolonged exercise related to others in their age group.

Compensation for time: The total protocol will take 23-24 hours, and participants will receive up to $600 for participation in the full study.

Reporting of Adverse Events: Reporting of adverse events will occur as detailed in the Data Safety Monitoring Plan listed below.

Premature Removal of Participant: Study participants who conclude their participation either prematurely or at full completion, and who have an unresolved adverse event will be followed by telephone by a member of the study team until the event(s) is (are) resolved.

Study Termination Procedures: It is not anticipated that the study will (need to) be terminated prematurely, either due to acceptance of the null hypothesis and/or adverse events, or other, and that subjects' safety will not be compromised.

Annual data checks will assess significance and effect sizes for key areas; if we unexpectedly found that none of these was significant or could be significant given the planned sample size, then we would stop the trial.

G. Statistical Methods

Power and Sample Size

NOTE: our projected sample for the two CRC admissions is 180, reflected in the sample size calculations below which are based on typhoid vaccine administration. The protocol describes a sample of 240 because any screening participants who are ineligible and/or who elect not to continue are counted in the total by the IRB.

Power calculations were based on previously published data estimating the effect of typhoid vaccine on inflammation (IL-6) and mood. Wright [56] evaluated both proinflammatory cytokine responses and mood following injection of typhoid vaccine or placebo in 30 healthy male volunteers. The effect of vaccine on IL-6 was large; at 3h the mean IL-6 in the vaccine group was 1.59 (SD=0.81) compared to 0.53 (SD=0.30) in the placebo group, yielding an effect size of Cohen's d = 1.5. The effect of vaccine on negative mood was also large, though smaller than the effect on inflammation. At 3h post-injection the mean negative mood was 12.8 (SD=7.7) in the vaccine group compared to 7.4 (SD=5.8) in the placebo group, yielding an effect size of d = 0.76. Based on these results, we expect the effect of vaccine on mood to be smaller than the effect on IL-6, thus power calculations were designed to ensure adequate sample size to detect effects of vaccine on mood, which will ensure adequate power for IL-6 as well.

The three specific aims for the proposed study are to evaluate (1) the effect of cardiorespiratory fitness on vaccine response, (2) the effect of age and depressive symptoms on vaccine response, and (3) the moderating effect of fitness on the age- and depression-related effects on vaccine response. Since each woman will serve as her own control by receiving both the vaccine and the placebo injection, the corresponding hypotheses all involve complex interactions: (1) the two-way interaction between fitness and injection type (vaccine/placebo), (2) the two-way interaction between age/depression and injection type, and (3) the three-way interaction of fitness by age/depression by injection type. Sample size requirements are considerably higher for testing higher-order interaction terms, thus we based required sample size on the tests of the third specific aim: the three-way interaction between fitness, age/depression, and injection type, while ensuring adequate power for the first two aims as well. Throughout we assumed 2-tailed tests with an 0.05 level of significance and used SAS PROC GLMPOWER for calculations.

Data from Wright [56] gives us an estimate of the overall (averaged across fitness and age/depression) effect of vaccine and placebo on negative mood: at 3h post-injection the mean in their study was 12.8 (SD=7.7) for vaccine and 7.4 (SD=5.8). We anticipate larger effects in our sample as we are recruiting older subjects who are breast cancer survivors, thus we assume that average negative mood responses to both the vaccine and placebo will be 20% higher in our sample of older breast cancer survivors. In order to calculate required sample size for the three-way interaction, we hypothesized the mean negative mood post-vaccine and also post-placebo for each of four groups: (a) high fitness/low depression, (b) high fitness/high depression, (c) low fitness/low depression, (d) low fitness/high depression. We hypothesized that the highest negative mood post-injection (either type) would be in group (d) low fitness/high depression, and the lowest negative mood would be in group (a) high fitness/low depression. Additionally we hypothesized that the average effect of depression on mood would be larger than the effect of fitness on mood and (as in Hypothesis 3) the effect of depression among women with high fitness would be smaller than the effect of depression among women with low fitness. The hypothesized means are shown in the **Table** below. We note that even the largest hypothesized mean is only approximately 1.25 SD above the estimated overall mean and the smallest is only ¾ SD below this mean, thus these estimates seem reasonable.

Table: Hypothesized group means for negative mood for power calculations

	VACCINE RESPONSE (SD=7.7)			PLACEBO RESPONSE (SD=5.8)		
	Low Depression	High Depression	Average	Low Depression	High Depression	Average
High Fitness	10	15.2	12.6	8.5	9.1	8.8
Low Fitness	11.2	25.2	18.2	8.7	9.3	9
Average	10.6	20.2	15.4	8.6	9.2	8.9

These means yield effect sizes for the vaccine response of d = 0.73 for the average effect of fitness and d = 1.25 for the average effect of depression. Importantly, in line with Hypothesis 3, they yield an effect size for depression of d = 1.8 for women with low fitness and a smaller effect of d = 0.68 for women with high fitness. Additionally, we will have baseline (pre-injection) measures of outcomes which will be used as covariates in models. In an ongoing study of breast cancer survivors, baseline negative mood was highly correlated with subsequent measures throughout the day, with r = 0.79-0.85. Inflammatory markers were similarly highly correlated, with correlations of r > 0.9. To be conservative in the power calculations we assume a single covariate with a correlation of r = 0.75. ***Based on these hypothesized means and one covariate, a sample of size n = 180 will give us 81% power to detect the three-way interaction effect of fitness by depression by injection type on mood. We will also have 94% power for the two-way interaction of depression by injection type and >99% power for the two-way interaction of fitness by injection type.*** As previously noted, effects on IL-6 are expected to be larger and group differences should be as big or larger, thus this sample size should provide us the ability to detect the effects on IL-6 as well.

These calculations are conservative for several reasons. As previously noted, our sample is older than the sample on which we base our estimates, thus we will likely see even larger effects and more dramatic effects of age, depression, and fitness and should have higher power. Additionally, power calculations were made assuming fitness and depression were dichotomized; we will obtain continuous measurements of these covariates and thus expect higher power. We will also collect serial measurements of outcomes over time and thus expect to have higher power than if we only obtained a single post-injection measurement.

Data Analysis Plan Each subject will have two visits (vaccine, placebo), and within each visit the primary outcomes (inflammation, behavioral responses) will be measured before injection and then serially (every 90 minutes) over the subsequent ~7.5 hours. Primary data analyses will use area under the curve (AUC) to summarize within-visit inflammatory and behavioral responses for each outcome. Hierarchical linear regression models (HLMs) will then be used to capture within-subject correlation across the two visits. If significant effects are found, secondary analyses will use more complex multi-level HLMs to model the individual time points within visit in order to identify at what time in the post-injection period effects arise.

For Hypothesis 1, baseline relationships between cardiorespiratory fitness and inflammation and behavioral outcomes will be evaluated using HLMs; subjects will have two baseline measurements, one before each injection, so an HLM is needed to control for the within-subject correlation. Outcomes in these models will be baseline (pre-injection) inflammation, depressive symptoms, cognitive problems, pain, and fatigue, and predictors will include cardiorespiratory fitness and visit (first or second). Tests of the main effect of cardiorespiratory fitness will investigate baseline relationships between these variables. The effect of vaccine on these outcomes over time will also be investigated using HLMs. AUC for each outcome at each visit will be the dependent variable, and predictors will include injection type (vaccine/placebo), cardiorespiratory fitness, and their interaction, as well as the baseline (pre-injection) outcome level. Separate models will be fit for each of the primary outcome AUCs (inflammation, behavioral responses) and the effect of interest will be the two-way interaction of cardiorespiratory fitness by injection type (which is a between-subject*within-subject interaction). This term will allow us to investigate differences in response for the vaccine compared to placebo (injection type effect) and how these differences differ by fitness level (cardiorespiratory fitness by injection type interaction).

For Hypothesis 2a, baseline relationships between inflammation and age as well as the effect of age on vaccine responses will be investigated using HLMs similar to those described for Hypothesis 1 with age

replacing cardiorespiratory fitness. For Hypothesis 2b similar HLMs will again be used, with depressive symptoms replacing cardiorespiratory fitness. For Hypothesis 2c, the HLMs with inflammation and behavioral AUC responses as outcomes will be modified by including both age and depression and their interaction as predictors. The test of the age by depression interaction will test whether the combination of older age and high depressive symptoms produces greater responses than the effect of either alone.

HLMs with AUC for inflammatory markers as well as behavioral responses as outcomes will also be used to test Hypothesis 3. Predictors will include injection type (vaccine/placebo), cardiorespiratory fitness, and age or depression, as well as all interactions and the baseline outcome level. Separate models will be used to test moderation of age and depression effects. To test whether cardiorespiratory fitness moderates the effect of age/depression on outcomes we will test the three-way interaction of injection type by age/depression by cardiorespiratory fitness. This is a three-way interaction, and we note that the primary interest is actually in the contrast testing the age/depression by cardiorespiratory fitness interaction for the vaccine injection. The post-placebo information serves to reduce error variance by allowing each woman to serve as her own control.

In all models we will control for BMI and insulin resistance to avoid possible confounding. Additionally, secondary analyses will control for use of aromatase inhibitors or tamoxifen; by including these variables as covariates, we will assess whether these medications impact cardiovascular (i.e., blood pressure, HRV) and inflammatory responses to the vaccine. Separately, we will assess whether tamoxifen or aromatase inhibitor use is related to cardiovascular variables and inflammation at baseline.

In secondary analyses, we will also assess antidepressant use as a possible confounder and control for this in analyses. We note again that the crossover design allows each woman to serve as her own control, thus mitigating the effect of possible confounders.

The analyses described above involve a number of tests of hypotheses and primarily focus on complex interactions. We recognize the importance of ensuring such tests have adequate statistical power for detection of effects as well as controlling, to the extent possible, the frequency of Type I errors. To control Type I error rates and minimize their impact, when appropriate, multivariate analysis of variance (MANOVA) will be used and/or alpha rates will be adjusted within subsets of hypotheses so as to control Type I error rates across sets of hypotheses tests. In addition, emphasis will be given to estimates of effect size so that findings that are statistically significant but of little practical relevance will not receive great attention.

H. Data and Safety Monitoring Plan

Possible adverse events (AE) are described above under “Risks” in the Human Subject section, and procedures to protect against risks are also outlined above. The PI will maintain close contact with all study personnel to monitor participant safety across all phases of the study.

Any adverse events (AE) occurring during any part of the protocol will be reported immediately to the PI. A written report of all adverse events will be completed within 48 hours and submitted to the OSU IRB.

Any potentially abnormal results from the blood, cardiorespiratory, or exercise session data will be first discussed with Dr. Malarkey to confirm the nature and seriousness of the problem, and then the subject will be informed of the abnormal value and advised to seek treatment from his/her physician. Any time a participant is informed of an abnormal value, a short memo to the file will document the incident.

Annually, IRB reports will include the occurrence of (un-) anticipated adverse events, and the timely progress of the trial, e.g. listing of all serious adverse events that occurred with clinical summaries, enrollment, withdraws, completions, etc.

Adverse event grading will be done using this common grading scale:

No adverse event or within normal limits or not clinically significant
Mild AE, did not require treatment
Moderate AE, resolved with treatment
Severe AE, resulted in inability to carry on normal activities and required professional medical attention
Life threatening or disabling AE
Fatal AE

The PI will determine the relationship of the AEs to the test intervention/agent(s)/device(s) as unrelated, possibly related, probably related, or definitely related, using standard criteria for clinical trials.

Each submitted AE Report will not contain any personal identifiers of the study participant(s), but will possess confidential patient identifiers (e.g., participant study identification number) that can be used by the investigator and study personnel to identify the patient(s).

Plan for assuring data accuracy and protocol compliance: The clinical research coordinator and/or investigator will be responsible for collecting and recording all relevant data for the protocol. As these results are collected, all toxicities and adverse events will be identified and reported to the principal investigator. Adverse events will be reported as described above. The principal investigator will determine the relationship of the event(s) to the procedure(s), and/or agent(s) of the protocol and decide the appropriate course of action for the study participant(s).

If an unanticipated serious adverse event occurs that may be related to the study (intervention and/or procedures), further enrollment will cease until the attribution is more fully determined and a resolution is proposed to either continue the study with or without modification or to stop the study. Such an event will be reported to the OSU IRB, medical monitor (William Malarkey, MD) and CRC RSA (Carson Reider, PhD) and to the NIH Project Officer.

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