THE IMPACT OF ACUTE AEROBIC EXERCISE ON NATURAL KILLER CELL, CATECHOLAMINE, AND CORTISOL RESPONSES IN BREAST CANCER SURVIVORS

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ABSTRACT

ELIZABETH SEREX EVANS: The Impact of Acute Aerobic Exercise on Natural Killer Cell, Catecholamine, and Cortisol Responses in Breast Cancer Survivors
(under the direction of Dr. Claudio Battaglini)

PURPOSE: The purpose of this investigation was to compare the effect of acute moderate intensity aerobic exercise on natural killer (NK) cell, catecholamine, and cortisol responses between breast cancer survivors and matched healthy controls. Additionally, relationships between post-exercise changes in NK cell responses and post-exercise changes in catecholamines and cortisol were examined. PARTICIPANTS: Data were collected from 9 women who had been treated for Stage I-III invasive breast cancer 3-6 months prior to enrollment and 9 healthy sedentary women without a history of cancer treatment. Subjects exercised for 30 minutes on a cycle ergometer at 60% of VO_{2peak}. Blood samples were obtained pre-exercise, immediately post-exercise, 2 hours post-exercise, and 24 hours post-exercise. METHODS: NK cell counts and NK cell cytotoxic activity (NKCA) were measured via flow cytometric analysis. Plasma catecholamines (epinephrine and norepinephrine) and cortisol were measured via ELISA techniques. RESULTS: Percent changes in NK cell counts were similar between groups across time ($p > 0.05$), although absolute cell counts were somewhat lower in breast cancer survivors pre-exercise and immediately post-exercise. NKCA was not significantly different across the study time points in the subset of breast cancer survivors ($p > 0.05$). Epinephrine levels were significantly elevated in the breast cancer survivor group pre-exercise, immediately post-exercise, and 24 hours post-exercise compared to controls ($p < 0.05$). Percent change in

epinephrine was somewhat lower in the breast cancer group immediately post-exercise and significantly lower in breast cancer survivors at 2 hours post-exercise ($p < 0.05$). Cortisol levels were somewhat higher in breast cancer patients immediately post-exercise, and percent change in cortisol immediately post-exercise increased in breast cancer survivors but decreased in controls ($p < 0.05$). Change in NK cell counts was only significantly correlated with change in cortisol at 2 hours post-exercise in the control group ($p < 0.05$).

CONCLUSION: These results suggest that acute moderate aerobic exercise may have similar effects on the immune system in breast cancer survivors and healthy controls, whereas it may lead to differing stress hormone responses in breast cancer survivors. Acute NK cell responses may be related to other biologic factors yet to be determined.

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CHAPTER I

INTRODUCTION

Background

Breast cancer is the most common cancer among women in the United States, other than skin cancer, and it is the second-leading cause of cancer deaths in women, after lung cancer (1). Major treatments for breast cancer include surgery, chemotherapy, and radiation therapy, and these treatments can cause significant physical and psychological distress that may persist for months or years after the completion of therapy (2). Fatigue is the most common physical distress experienced by breast cancer patients, affecting up to 70% of patients during therapy and up to 30% of patients years after the completion of therapy (3). This type of fatigue is often severe and may limit activities of daily living; therefore, in the past, patients have been advised to rest and reduce their activity levels (3). However, inactivity leads to muscular atrophy and decreased cardiorespiratory function, and prolonged rest can actually worsen fatigue, thus contributing to a cycle of further muscular atrophy, reduced cardiorespiratory functioning, and a down-regulation of daily activity levels (3-5).

Over the past 20 years, numerous studies have investigated the impact of exercise training on physical functioning in breast cancer patients and survivors (6, 7, 8). Two recent reviews by Jones et al. have examined the existing literature pertaining to the impact of exercise training on adult patients with breast cancer and other cancer types (6, 7). The primary endpoint of many of the investigations in these reviews is the effect of exercise training on cardiorespiratory endurance. Other outcomes have also been addressed,

including changes in muscular strength, body composition, functional quality of life, fatigue, anxiety, and self esteem (6). Many of these "first generation" studies have shown that exercise training appears to mitigate many treatment-related side-effects, that patients are able to make improvements in cardiorespiratory fitness, and that regular exercise seems to be safe, well-tolerated, and associated with few adverse events (6, 7). Therefore, the results of these studies thus far seem promising.

Physical exercise may also be an attractive adjunct therapy for cancer patients and survivors because of its potentially positive influence on biologic systems involved in disease protection and anti-cancer defense (9). In the field of Exercise Immunology, a "J-shaped" hypothesis has been postulated describing the inverse relationship between disease risk/cancer susceptibility (9, 10). This hypothesis states that regular, moderate exercise may lead to enhanced immune function and therefore a decreased risk of disease and cancer, whereas repeated bouts of exhaustive exercise and overtraining could lead to immunosuppression, and therefore an elevated risk of disease and cancer (9, 10).

One particular cellular component of the immune system, the natural killer (NK) cell, may be especially characterized by this J-shaped curve in response to exercise. NK cells are part of the body's innate immune response, which is activated as part of the body's immediate line of defense against pathogens. They are particularly equipped to target and kill virally-infected cells as well as cells that have undergone malignant transformation (10). In healthy individuals, both athletic and non-athletic, NK cells are extremely responsive to acute exercise. They follow a profile which typically shows marked elevations in both cell number and activity immediately post-exercise, while displaying decreases during the recovery period for up to several hours (10-14). In response to moderate levels of exercise

training, resting NK cell numbers and activity may become increased, while in response to periods of heavy exercise training, NK cell numbers and activity may become depressed (12-14). Although not all studies show entirely consistent outcomes, and the clinical implications of exercise-induced changes in NK cell responses are not entirely known, it is thought that exercise-related enhancements in NK cell function may confer a protective effect against pathogen invasion, while exercise-related decreases in NK cell function may lead to increased incidence of viral infection and potential illness (12, 13).

Research regarding immune system responses to exercise in the cancer patient population is limited. To date, 19 studies have examined the effect of exercise on some parameter of immune function (15-33). Of these, only 3 have examined the effect of exercise on NK cell function in the breast cancer patient population, and all were in response to a period of exercise training (15, 17, 23). The results of these 3 studies seem to indicate that exercise training either does not affect NK cell function, or may even improve NK cell function in breast cancer patients and survivors possibly beyond what is associated with normal recovery after cancer therapy, and could possibly be correlated with increased disease-free and overall survival (23). Studies investigating NK cell responses in patients with other types of cancer, as well as the studies investigating responses of other immune parameters to exercise have shown similar results; that exercise training either does not seem to negatively affect immune function in cancer patients and survivors, or may lead to improved immune function (16-33).

While these results seem promising, the limited scope of the existing Exercise Immunology literature in the cancer patient/survivor population makes it difficult to draw definite conclusions. For example, comparison of exercise responses between cancer

patients/survivors and healthy controls has not been performed. Also, no study has comprehensively examined immune responses to an acute bout of exercise by profiling cellular responses pre-exercise, immediately post-exercise, and at multiple time points during recovery. Furthermore, no study in the cancer patient/survivors population has examined the relationship between cellular immune parameters and other biologic factors, many of which are influenced by exercise and are also important in anti-cancer defense (9). Knowledge and understanding of how cellular immune responses behave pre- to post-exercise and then during recovery may help exercise specialists target optimal ranges of exercise intensity, frequency, and duration that would ideally strengthen the immune system. Knowledge and understanding of how biologic mediators may influence cellular immune system responses may help investigators to more fully appreciate the role of exercise in the reduction of cancer risk/recurrence/second malignancies and in the increase of survival time post-treatment (9).

Purposes of the Study

Breast cancer survivors who are enrolled in exercise rehabilitation programs often exercise multiple times per week, usually 3-5 times per week (3-5, 6, 7, 18, 22, 23, 35-39). These sessions may occur on alternate days, but occasionally they may occur on consecutive days. Previous studies describing exercise interventions for breast cancer patients have generally used moderate-vigorous intensities (50-85% of VO_{2max}, VO_{2peak}, heart rate reserve, or maximum heart rate) for durations of one hour or less per session (3-5, 6, 7, 18, 22, 23, 34-38). Current guidelines put forth by the American College of Sports Medicine (ACSM) are very similar to those for the general population; that individuals affected by cancer should be "as physically active as their conditions allow," to "avoid inactivity," and that "some physical activity is better than none" (39). With regard to aerobic exercise, the ACSM

supports the physical activity guidelines put forth by the US Department of Health and Human Services, stating that individuals should aim for 150 minutes of moderate-intensity exercise, 75 minutes of vigorous-intensity exercise, or an equivalent combination, per week (39). As one can see, these exercise prescription guidelines for cancer patients are quite general, likely because at this time, not enough research has been performed to completely understand the distinct impact of exercise on each physiological system related to every type of cancer diagnosis, every type of cancer treatment, and every cancer-related side-effect. While previous literature seems to show that aerobic exercise is safe, effective in eliciting improved physical fitness, free of significant adverse events, and could potentially be associated with improved immune function, it is not yet fully known if a breast cancer patient's immune system responds to and recovers from acute aerobic exercise in a similar manner to the immune system of a healthy individual of similar age and physical fitness who has never experienced the rigors of major cancer treatment. More specifically, it is not yet known if a breast cancer survivor's immune response to acute exercise will follow the same response profile as typically seen in healthy individuals, or if that response is more or less pronounced, compared to a healthy individual. Additionally, it is not yet understood if the recovery period after acute exercise, during which there could be immunosuppression, lasts for the same duration in breast cancer survivors and healthy individuals; a finding which could significantly impact recommendations for the amount of time a cancer patient/survivor should rest post-exercise before engaging in a subsequent bout of exercise. Furthermore, it is not yet clear whether the major factors that mediate immune responses to acute aerobic exercise in healthy individuals also influence the exercise-induced immune response in breast cancer survivors. As the research conducted in this field ultimately manifests as exercise

prescription guidelines, and in order to construct the most relevant and specific guidelines for the cancer patient/survivors populations, researchers and clinicians must understand how exercise affects all physiological systems, including the immune system, so that a cancer patient or survivor may reap the maximum health benefits of exercise without putting themselves at risk for further illness.

As NK cells are extremely responsive to acute exercise, and they provide the body with a first line of defense against pathogens and tumor cells, it is clinically relevant to understand how acute aerobic exercise may influence their cytolytic activity, as well as their entry into/exit from circulation. These parameters may give insight to whether the exercise is contributing to enhanced immune function or leading to periods of potential immunosuppression. Therefore, the purposes of this investigation were: 1) to compare the NK cell response to an acute bout of moderate-intensity aerobic exercise in breast cancer survivors and healthy controls, and 2) to compare the relationships between changes in NK cell, catecholamine, and cortisol responses to the acute bout of moderate-intensity aerobic exercise in breast cancer survivors and healthy controls.

Definition of Terms

Major cancer treatment: surgery, chemotherapy, and radiation therapy.

Adjuvant hormonal therapy: drugs used to treat women with breast cancer, particularly in tumors that are receptive to the hormone estrogen. This class of drugs may also be referred to as selective estrogen receptor modulators (SERMs).

Adjuvant trastuzumab therapy: a drug used to treat women with breast cancer, particularly in tumors that over-express the human epidermal growth factor receptor 2 protein (HER2 protein).

Breast cancer survivors: study group which includes women who have been diagnosed with Stages I-III invasive breast cancer and have completed all major cancer treatment 3-6 months prior to participation in the study.

Healthy controls: study group which includes women who are healthy, sedentary, and have never received treatment for cancer of any type.

Sedentary: not participating in regular physical activity for at least 1 year prior to enrollment in the study. Regular physical activity is considered as 30-minutes of moderate-vigorous activity, 3 days per week.

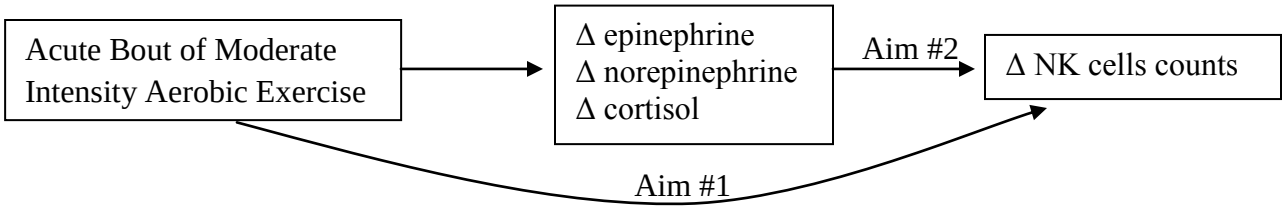
VO_{2peak}: a subject's peak aerobic capacity, measured during a VO_{2peak} test. The units of VO_{2peak} used in this study are milliliters of oxygen per kilogram of body mass per minute (mL/kg/min).

Moderate intensity aerobic exercise: aerobic exercise that is performed at an intensity of 40-60% of VO_{2peak}.

Aims and Hypotheses

The purposes of this investigation were addressed by two Research Aims. Aim #1 compared the changes in NK cell counts in response to the acute bout of moderate-intensity aerobic exercise in breast cancer survivors and healthy controls. This Aim is addressed in Chapter III. Aim #2 compared the relationships between changes in catecholamine responses, cortisol responses, and changes in NK cell counts in breast cancer survivors and healthy controls. This Aim is addressed in Chapter V. Aim #1 was considered the primary aim of this investigation and Aim #2 was considered the secondary aim of this investigation. Figure 1 below depicts the conceptual model leading to the research design, and how the relationships between the variables were addressed in each Research Aim.

Figure 1. Conceptual model leading to research aims.



Primary and Secondary Aims

Aim #1: To examine the effect of one bout of moderate-intensity aerobic exercise on NK cell counts in breast cancer survivors and healthy controls

Hypothesis 1a: There will be a significant increase in NK cell counts from pre-exercise to immediately post-exercise in both breast cancer survivors and healthy controls.

Hypothesis 1b: There will be no significant difference in NK cell counts from pre-exercise to 2 hours post-exercise in both breast cancer patients and healthy controls.

Hypothesis 1c: There will be no significant difference in NK cell counts from pre-exercise to 24 hours post-exercise in both breast cancer patients and healthy controls.

Hypothesis 1d: There will be no significant differences in NK cell counts between breast cancer survivors and healthy controls at any time point (pre-exercise, immediately post-exercise, 2 hours post-exercise, and 24 hours post-exercise).

Aim #2: To examine the relationships between changes in catecholamine responses, cortisol responses, and changes in NK cell counts in breast cancer survivors and healthy controls.

Hypothesis 2a: Changes in catecholamine levels will be positively correlated with changes in NK cell counts from pre-exercise to immediately post-exercise in both breast cancer survivors and healthy controls.

Hypothesis 2b: Changes in cortisol levels will be positively correlated with changes in NK cell counts from pre-exercise to immediately post-exercise in both breast cancer survivors and healthy controls.

Hypothesis 2c: Changes in catecholamine levels will not be correlated with changes in NK cell counts from pre-exercise to 2 hours post-exercise in both breast cancer survivors and healthy controls because changes in catecholamine levels and NK cell counts may not be significantly different from pre-exercise to 2 hours post-exercise.

Hypothesis 2d: Changes in cortisol levels will not be correlated with changes in NK cell counts from pre-exercise to 2 hours post-exercise in both breast cancer survivors and healthy controls because changes in cortisol levels and NK cell counts may not be significantly different from pre-exercise to 2 hours post-exercise.

Exploratory Analyses

In addition to performing the two Research Aims as described above, some exploratory analyses were conducted. These included investigating changes in NK cell cytotoxic activity (NKCA) to the moderate intensity aerobic exercise bout in a subset of subjects at each study time point (pre-exercise, immediately post-exercise, 2 hours post-exercise, and 24 hours post-exercise). This analysis is addressed in Chapter III along with Aim #1 as previously described. Additionally, the effect of the moderate intensity aerobic exercise bout on changes in catecholamine and cortisol levels in the breast cancer survivors and the healthy controls was examined at each study time point. This analysis is addressed in Chapter IV. As the investigation of the effect of the exercise bout on these parameters was for exploratory purposes, no specific hypotheses were tested.

CHAPTER TWO

REVIEW OF LITERATURE

Introduction

The purposes of this investigation were to compare the NK cell response to an acute bout of moderate-intensity aerobic exercise and to compare the relationships between changes in NK cell and stress hormone responses to the acute bout of moderate-intensity aerobic exercise in breast cancer survivors and healthy controls. The following literature review begins with a discussion of the role of NK cells in the destruction of pathogens, the major immune system factors that mediate the NK cell response and the general NK cell response to exercise. The literature review then focuses on three stress hormones that are major mediators of the NK cell response to aerobic exercise: the catecholamines (epinephrine and norepinephrine) and cortisol. In these sections, the general function of each hormone is described, as well as its response to acute aerobic exercise and effect on NK cells. Next, the literature review includes a discussion of the major studies which have examined the effect of acute aerobic exercise on NK cell function in healthy individuals, both sedentary and physically active. Finally, this literature review concludes with a discussion of the current literature investigating the effect of aerobic exercise interventions on immune function in the cancer patient population.

Role of Natural Killer Cells in Destroying Pathogens

Natural killer (NK) cells are a major cellular component of the innate branch of the immune system. They are derived from common lymphoid progenitor cells in the bone

marrow and are morphologically defined as large granular lymphocytes that do not express T cell receptors or surface immunoglobulins (40, 41). They represent approximately 5-20% of mononuclear cells in the blood and spleen and are not commonly found in other lymphoid organs (40, 41). NK cells are classified as lymphocyte subset, and are also referred to by their cluster of differentiation (CD) nomenclature as $CD3^{-}CD16^{+}CD56^{+}$ (42).

Functionally speaking, NK cells are able to exhibit spontaneous cytolytic activity against a wide variety of virally-infected cells and tumor cells and are able to lyse target cells without any apparent previous sensitization (10). They are able to respond rapidly to a viral challenge and can mount a proliferative and cytolytic response days before more specific adaptive immune responses can be generated (10, 43). Once a target cell has been identified, NK cells secrete a protein called perforin, which creates pores in the target cell's membrane (41). Enzymes called granzymes enter the target cell through these pores, thus triggering an endogenous pathway of apoptosis (10, 41).

Major Immune System Mediators of the NK cell Cytolytic Response

The proliferative and cytolytic activities of NK cells are mediated by soluble factors called cytokines, which are proteins that can regulate and coordinate many of the activities of the cellular components of the immune system (44). Cytokines act on other cells through transmembrane cell-surface receptors, thus activating intracellular signaling transduction pathways to induce gene transcription and synthesis of new cellular proteins (40). The major cytokines that influence proliferation and activation of NK cells are interleukins (IL)-2, 12, 15, and 18, as well as interferons (IFN)-α and β (41).

IL-15 is instrumental in mediating the differentiation and proliferation of NK cells in bone marrow (41, 45). Hematopoietic stem cells develop into NK cell precursors through

sequential acquisition of functional cell surface receptors (45). Once committed to the NK cell lineage, IL-15 stimulates receptors in these NK cell precursors, thus transforming them into mature NK cells (45). IL-2, IL-12, IL-18, IFN-α, and IFN-β, are involved in stimulating the activity of NK cells. In particular, IL-12, which is produced by other immune cells, is a powerful inducer of NK cell cytolytic activity, and IL-2 and IL-18 may act to augment the effect of IL-12 on NK cells (41). IFN-α and IFN-β also increase the cytolytic potential of NK cells, possibly through upregulation of IL-12 receptor expression, and thus NK cell responsiveness to IL-12 (41).

Once activated, NK cells also secrete a variety of cytokines. In particular, IL-12 stimulates NK cells to secrete IFN-γ which may then stimulate other immune cells called macrophages to kill phagocytosed pathogens (41, 45). NK cells may also secrete IL-1β, IL-2, IL-3, IL-4, IL-5, and IL-6; each of which plays its own role in activating inflammatory pathways and stimulating proliferation, differentiation and activation of other immune cell types (45, 46).

General NK Cell Response to Acute Aerobic Exercise

NK cells are extremely responsive to exercise, and generally produce a response that is biphasic in nature, meaning that they display one response profile immediately post-exercise, and a second response profile during recovery (10). In the first phase which manifests immediately post-exercise, NK cell counts and activity may be dramatically elevated over pre-exercise levels by anywhere from 50% to several hundred percent (10). However, this post-exercise increase is short-lived. In the second phase which occurs several hours after the completion of the exercise, NK cell counts and activity return to pre-exercise resting levels, and may even decrease below pre-exercise levels before returning to baseline

(10). The magnitude of the NK cell fluctuations relative to baseline are generally related to intensity and duration of exercise. Exercise bouts of higher intensity and/or longer duration typically produce a more pronounced biphasic response compared to exercise bouts of lower intensity and/or shorter duration (13, 47). Increases in NK cell counts and activity during exercise are primarily associated with increased cardiac output and increased catecholamine levels which cause NK cells to be released into circulation from their marginal pools in blood vessels, lungs, lymph nodes, spleen, and intestines (10, 42, 48-50).

During the recovery period, particularly after exercise of higher intensity (> 60% of VO_{2max}) and longer duration (> 1 hour), decreases in NK cell counts and activity below pre-exercise resting values have been observed, which may persist for up to 4 hours post-exercise (42). These decreases have been associated with several biological factors, including increased cortisol levels, alterations in cytokine release, and increased prostaglandin release from activated monocytes (10, 51-54). This potential suppression of NK cell function has been incorporated into the "Open Window Theory" by some researchers, which states that immunosuppression in the hours following exercise may lead to an increased susceptibility to bacterial and viral infection, thus increasing an individual's chance of acquiring subclinical or clinical infection (43, 55). Researchers have also postulated that individuals who perform repeated bouts of acute high intensity/long duration exercise within a period of time without sufficient recovery may cause further decreases in an already-suppressed immune system, thereby leading to a more chronic form of decreased immune function (43). However, much research is still needed in order to understand the impact of acute and chronic exercise, as well as the impact of other physical and psychological stressors, on long-term immune function before the open window theory can be fully accepted (55).

Catecholamines

General Function of Catecholamines

The catecholamines include the two hormones epinephrine and norepinephrine as well as dopamine. Epinephrine and norepinephrine are secreted from the adrenal medulla and released into the blood under stimulation from the sympathetic branch of the autonomic nervous system (56, 57). The ratio of adrenal secretion of epinephrine to norepineprhine is approximately 4:1; however the circulating levels of norepinephrine exceeds epinephrine by a factor of 5-10, as it is also directly released by sympathetic neurons (56-58). During times of significant physiological stress when the adrenal medulla is highly stimulated, circulating epinephrine may increase by 10-20-fold (57). Epinephrine and norepinephrine function together to elicit physiological effects by binding to α and β receptors in tissues; thereby activating second messenger systems (56, 57). The physiological responses caused by the catecholamines are often referred to collectively as "fight or flight" responses, which generally include increased activity of the cardiovascular, respiratory, muscular, metabolic, and thermoregulatory systems with decreased activity of the gastrointestinal and excretory systems (56, 57).

Acute Aerobic Exercise Response of Catecholamines

Catecholamine release during acute aerobic exercise is mediated by increases in sympathetic nervous system activity and is mostly proportional to exercise intensity and duration (56). During low intensity exercise (< 50% of VO_{2max}), increased plasma norepinephrine levels may be observed, which is likely more related to increased sympathetic nervous system activity than increased adrenal medulla secretion (57, 59). In contrast, plasma epinephrine may not significantly increase until moderate intensities are reached,

when adrenal medulla activity is also increased (57, 59). Generally speaking, plasma catecholamine levels increase significantly when exercise intensity reaches approximately 40-60% of VO_{2max} (55). For example, 20 minutes of exercise at 60% of VO_{2max} may cause plasma catecholamines to increase 2-3 times pre-exercise levels (57, 59-61).

In response to high intensity aerobic exercise, plasma catecholamine levels generally increase very dramatically. Twenty minutes of aerobic exercise at 80% of VO_{2max} may elicit a 350-500% increase in plasma catecholamines, while exercise at ≥ 100% of VO_{2max} may elicit a 1000%-1500% increase in plasma catecholamines (57, 60, 61-63). Similar increases in catecholamine levels can be observed with increasing exercise duration, where 50 minutes of moderate intensity exercise (60-70% of VO_{2max}) may elicit a 300-900% increase in epinephrine and norepinephrine, while a 90 minute exercise session at the same intensity may result in a 10-11-fold increase (57). When considering an exercise bout at a moderate intensity (50% of VO_{2max}) until exhaustion, plasma catecholamines may increase 28-fold (57, 63-65). After the completion of exercise, clearance of catecholamines from circulation occurs rather quickly with a half-life of approximately 2-3 minutes (58).

The major physiological systems targeted by the catecholamines during acute aerobic exercise are the cardiovascular, skeletal muscle, and metabolic systems, and the major actions of these hormones on these systems are to increase cardiac output, increase blood flow to working skeletal muscles, and increase breakdown of energy substrates, and upregulate metabolic pathways in order to generate ATP from energy substrates. In the cardiovascular system, epinephrine and norepinephrine both stimulate β-adrenergic receptors in the heart, leading to increased heart rate and myocardial contractility which leads to increased cardiac output (57). Norepinephrine activates α-adrenergic receptors in the

systemic blood vessels to cause generalized vasoconstriction, while epinephrine activates β-adrenergic receptors in the arterioles of the heart and skeletal muscles to elicit vasodilation, the activities of which aim to decrease blood flow to non-essential tissues and increase blood flow to the exercising heart and muscles (57). In the skeletal muscles and other tissues associated with metabolism, epinephrine is especially important in the upregulation of metabolic processes. Epinephrine activates β-receptors in adipose tissue to cause the breakdown of triglycerides into free fatty acids to be used as energy (57, 59). Epinephrine also has major effects on glucose metabolism and blood glucose maintenance by activating β-adrenergic receptors in the liver to increase glycogenolysis and gluconeogenesis (56, 57). In the skeletal muscle, epinephrine activates β-adrenergic receptors to increase the activities of the enzyme phosphorylase which further upregulates glycogenolysis, as well as phosphofructokinase which increases the rate of glycolysis (57). Additionally, epinephrine activates α-receptors in the pancreas to decrease the release of insulin and increase the release of glucagon, further contributing to upregulation of glycogen and glucose metabolism (56, 57). Thus, increased catecholamine secretion during increasing exercise intensity and duration is essential for increasing cardiac function to deliver blood to working muscles, increasing energy usage from lipids (particularly important during prolonged exercise), increasing energy usage from carbohydrates (particularly important during high intensity exercise), and maintaining blood glucose levels (57, 59).

The Effect of Catecholamines on NK Cells

During acute aerobic exercise, the rise in catecholamine levels is associated with NK cell cytosis, and the primary mechanism of action appears to be a catecholamine-induced alteration in adhesion molecules that are expressed on NK cells, thus leading to mobilization

of NK cells into circulation (11, 13, 66, 67). The surface density of β-adrenergic receptors is high in NK cells, and the density of these receptors is further increased after exposure to increased levels of catecholamines (11, 66). When catecholamines bind to these β-adrenergic receptors, a second-messenger system involving the adenyl cyclase system and cAMP is activated (66). Changes in the configurations of one or more adhesion molecules, including CD11, CD18, CD31, CD43, CD44, CD62L, sVCAM-1, sICAM-1 and sE-selectin, causes reduced expression on the NK cell's surface, thus leading to demargination of NK cells from small venules and the spleen into circulation (66). This effect is reversed after the cessation of the exercise bout. Falling catecholamine levels are associated with a drop in NK cell counts, likely as a result of a decrease in β-adrenergic receptor density, which may return to pre-exercise levels within 1 hour post-exercise (66). A detailed review of the literature examining the association between exercise-induced catecholamine response and NK cell response is discussed in the section entitled "Acute Aerobic Exercise and NK Cell Response in Healthy Individuals."

Cortisol

General Function of Cortisol

Cortisol is a glucocorticoid hormone that is released from the adrenal cortex under control of the hypothalamic-pituitary-adrenal (HPA) axis. Physical or emotional stress or decreased blood glucose levels stimulate the release of corticotrophin-releasing factor (CRF) from the hypothalamus, which in turn stimulates the release of adrenocorticotropin (ACTH) from the anterior pituitary (56, 57). ACTH stimulates adenylate cyclase activity in the cells of the adrenal cortex, resulting in the formation of cAMP, which then stimulates the release of cortisol (56). The activity of the HPA axis is controlled through a negative feedback loop

mechanism; additionally, ACTH and cortisol levels fluctuate throughout the day due to circadian rhythms, eating, and exercise patterns, with the highest levels observed during the morning hours (56, 57, 68, 69).

Once released, the major targets of cortisol are the liver, skeletal muscle, and adipose tissue; i.e., tissues that are crucial in providing energy to the body and maintaining blood glucose levels (56, 57). Cortisol elicits its physiological effects through direct gene activation; it penetrates the target cell's membrane, and binds to a cytoplasmic receptor protein (70). The steroid-receptor complex is then activated and translocated to the target cell's nucleus, where target cell gene expression is modulated by either stimulation or inhibition of specific mRNA production, thus manifesting as the cellular response specific to the hormone's action (70). In the liver, cortisol stimulates gluconeogenesis, which results in increased blood glucose levels (57). In the liver and skeletal muscle, cortisol stimulates proteolysis (56, 57). In the adipose tissue, cortisol acts in conjunction with epinephrine to stimulate lipolysis (57). Additionally, large doses of glucocorticoids may lead to a reduced inflammatory response by inhibiting the production of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α, as well as by inhibiting the effects of these pro-inflammatory cytokines on target tissues (57, 68).

Acute Aerobic Exercise Response of Cortisol

Similar to the catecholamines, plasma cortisol levels rise proportionally with respect to exercise intensity (> 40% of VO_{2max}) and duration (70). Aerobic exercise activates the HPA axis, which causes increased ACTH release from the anterior pituitary and increased cortisol output from the adrenal cortex. Elevation in plasma cortisol levels seems to follow a threshold effect, where exercise intensities of > 40% of VO_{2max} appear to elicit increased

plasma cortisol concentrations, and intensities below this threshold are either not stressful enough, or do not cause significant decreases in blood glucose levels (57). High intensity aerobic exercise seems to elicit a dramatic increase in plasma cortisol levels. For example, a study by Hill et al. (71) observed that 30 minutes of exercise at 80% of VO_{2max} elicited an 83% increase in cortisol levels in moderately-trained men. During intense and prolonged exercise, plasma ACTH levels rise significantly, resulting in significant secretion or cortisol that may become particularly apparent after approximately 60 minutes of exercise (57, 70, 72).

During aerobic exercise activities undertaken by most individuals, the most significant effects of cortisol are to stimulate lipolysis in the adipose tissue and gluconeogenesis in the liver, thus providing the body with free fatty acids for energy and allowing the body to maintain blood glucose levels (57). During very intense and prolonged exercise, energy derived from proteins may also become significant, and cortisol-induced muscle proteolysis may provide the body with this energy source (56).

The Effect of Cortisol on NK Cells

As reported in the literature, the effect of cortisol on NK cells is mixed and may be more relevant during exercise of prolonged duration (42, 72). Increased plasma cortisol levels have been associated with decreased NK cell counts and activity during recovery, and the major mechanisms of action are not completely understood, but may be related to glucocorticoid-induced apoptosis, inhibited adhesion of effector cells to target cells, and altered cytokine responses (13, 42, 43, 73). In particular, cortisol may suppress production of the cytokines IL-2, IL-12, and IFN-γ and upregulate the actions of other cytokines including IL-4 and IL-10 which in turn may lead to a decrease in NK cell activity (13, 43, 51, 52). A

detailed review of the literature examining the association between exercise-induced cortisol response and NK cell response is discussed in the section entitled "Acute Aerobic Exercise and NK Cell Response in Healthy Individuals."

Acute Aerobic Exercise and NK Cell Response in Healthy Individuals

NK cell response to exercise is commonly quantified using two main measurements: NK cell count and NK cell activity (NKCA). Both are measured from blood samples that are usually taken at various points pre-exercise and post-exercise. To obtain NK cell count for a blood sample, two measurements are generally needed: absolute number of lymphocytes in the sample and the proportion of NK cells in the sample (measured using flow cytometry). Absolute lymphocyte number and NK cell proportion are multiplied together to determine the NK cell count, which is expressed as number of NK cells per unit blood (for example; 1 x 10^9cells/L, 1 x 10^6 cells/mL, etc.). NKCA can be obtained using several different techniques, including a whole-blood or isolated-peripheral blood mononuclear cell (PBMC) radioactive chromium-51 (^{51}Cr) release assay, fluorescence microscopy, or flow cytometry. These techniques measure the cytolytic capabilities of the NK cells in the sample by mixing NK cells (the "effector" cells) with target cells in various ratios, termed effector-to-target ratios (E:T ratios). Cells are radioactively- or fluorescently-labeled depending on the technique to be used, and NKCA is determined by measuring differences in radioactivity or fluorescence between live cells, dead cells, and control cells. NKCA is usually expressed as a percentage, commonly termed % cytotoxicity or % lysis.

An examination of the exercise immunology literature describing NK cell response to acute aerobic exercise finds that there is not one uniform method of reporting results. For example, some researchers have chosen to report NK cell counts as absolute numbers while

others have chosen to report NK cell counts as proportions of peripheral blood mononuclear cells (PBMC). In the case of NKCA, most researchers have expressed results as % cytotoxity or % lysis; however, not all researchers have used the same E:T ratios when measuring NKCA. Additionally, some studies have chosen to express NKCA in other units, such as lytic units per 10^7 mononuclear cells, lytic units per L blood, and % lysis per NK cell. Furthermore, some researchers choose to report results in table form, while others present their results in graphical form only.

This section of the literature review describes results for NK cell counts using percent changes of the absolute NK cell numbers from baseline whenever possible (i.e., [post-exercise NK cell count – pre-exercise NK cell count)/pre-exercise NK cell count]*100). NKCA will also be described using percent changes from baseline whenever possible (i.e., [post-exercise NKCA – pre-exercise NKCA)/pre-exercise NKCA]*100). The purpose for describing NK cell counts and NKCA in this fashion is to make comparisons of results between studies easier. In some cases, authors have directly reported percent changes in NK cell counts and NKCA, but in most cases, these have been calculated and estimated using data given in tables or graphs.

This dissertation project was aimed to investigate the effect of acute moderate intensity aerobic exercise on NK cell responses in women who are breast cancer survivors and women who are healthy age-matched and physical-activity matched controls. Therefore, it is important to note that relatively few studies have examined the effect of acute aerobic exercise on NK cell counts and activity in non-athletic populations, and that the majority of studies have focused on NK cell responses in elite and recreationally-active endurance athletes. However, the current literature appears to show that regardless of the study

population, acute aerobic exercise responses in healthy individuals generally follow the same trend, with increases in NK cell counts and activity immediately post-exercise compared to pre-exercise levels, and decreases in NK cell counts and activity during the recovery period. The magnitude of these changes in NK cell function appears to be largely related to the intensity and/or duration of the exercise bout, with moderate intensities and/or shorter durations eliciting smaller changes from baseline compared to higher intensities and/or longer durations.

Effect of Acute Aerobic Exercise on NK Cell Count

Moderate Intensity Exercise and NK Cell Count

Several studies have exclusively examined the effect of an acute bout of moderate intensity aerobic exercise on NK cell counts in healthy individuals, with exercise intensities and durations ranging from 60-65% of VO_{2max} and 37 minutes-4 hours, respectively. All studies measured NK cell counts from blood samples taken pre-exercise, immediately post-exercise, and at various points during the short-term recovery period (0-2 hours). One study measured NK cell counts further into the recovery period, at 24 and 72 hours post-exercise. Even at moderate intensities, NK cell counts have been shown to rise significantly immediately post-exercise to levels that can be anywhere from 76% to over 200% above pre-exercise values. During the recovery period, significant decreases in NK cell counts (usually greater than 50% below pre-exercise levels) have been described in some studies, although others have not observed significant decreases in NK cell counts. These decreases in post-exercise NK cell counts are typically short-lived, and return to near baseline levels within 2 hours post-exercise.

Huang et al. (74) investigated the effect of a short-duration, moderate intensity exercise bout on NK cell counts in 8 professional firefighters of sedentary-low fitness levels (VO_{2max} = 36.9 ± 5.8 mL/kg/min). Subjects performed a cycle ergometry ride at 60% of VO_{2max} for 37 minutes (an exercise intensity similar to what might be experienced during fire suppression activities). NK cell counts were measured 50 minutes pre-exercise, 30 minutes pre-exercise, immediately pre-exercise, immediately post-exercise, and every 15 minutes during recovery until 1 hour post-exercise. When comparing NK cell counts immediately pre-exercise and immediately post-exercise, NK cell counts increased significantly by approximately 120% ($p < 0.001$). At 1 hour post-exercise, NK cell counts were slightly below pre-exercise levels by approximately 19%; however, this difference was not significant.

Rhind et al. (49) investigated the effect of a moderate-duration, moderate intensity exercise bout on NK cell counts in 9 sedentary men both before and after participating in a 12-week exercise training program. Subjects performed a cycle ergometry ride at 60% of VO_{2max} for 60 minutes at the beginning of the intervention, and then repeated the same exercise bout at the end of the intervention. NK cell counts were measured pre-exercise, immediately post-exercise, 30 minutes post-exercise, and 2 hours post-exercise. When comparing NK cell counts pre-exercise and immediately post-exercise, NK cell counts rose 1.3-fold before the intervention ($p < 0.01$) and 1.4 fold after the intervention ($p < 0.0001$). During the recovery period, NK cell count significantly decreased by 56% below pre-exercise values which persisted throughout the recovery period, from 30 minutes to 2 hours post-exercise ($p < 0.05$). The magnitude of the post-exercise decrease in NK cells was the same both before and after the exercise intervention.

Natale et al. (50) and Scharhag et al. (75) investigated the changes in NK cell count in response to long-duration, moderate intensity exercise bouts, and although their study populations were different, the results were similar. Natale et al. (50) studied the NK cell response to exercise in 8 relatively sedentary healthy men who each performed a 2-hour cycle ergometry ride at 60-65% of VO_{2max}. NK cell counts were measured pre-exercise, immediately post-exercise, 3 hours post-exercise, 24 hours post-exercise, and 72 hours post-exercise. NK cell counts increased significantly from pre-exercise to immediately post-exercise by over 200% ($p < 0.05$). By 3 hours post-exercise, NK cell counts had returned to pre-exercise values, and no further changes in NK cell counts were observed at 24 or 72 hours post-exercise. Scharhag et al. (75) examined the NK cell response to exercise in 12 male competitive triathletes and cyclists who each performed a 4-hour cycle ergometry ride at 60% of VO_{2max}. NK cell counts were measured pre-exercise, immediately post-exercise, and during recovery at 1, 2, and 24 hours post-exercise. NK cell counts increased significantly immediately post-exercise by approximately 76% over pre-exercise levels ($p < 0.05$). During the recovery period, NK cell counts decreased significantly below pre-exercise levels by approximately 66% at 1 hour post-exercise and 38% at 2 hours post-exercise ($p < 0.05$). At 24 hours post-exercise, NK cell counts had returned to near baseline levels.

There are several potential mechanisms which may be associated with the biphasic response of NK cell counts to these aerobic exercise bouts. Increased cardiac output and increased sympatho-adrenergic system activation during exercise may be largely responsible for the marked NK cell cytosis immediately post-exercise (48-50). Particularly for exercise intensities below 60% of VO_{2max}, the increase in cardiac output may be the major factor leading to increases in NK cell counts (48). However, increased cardiac output during

exercise may act synergistically with increased catecholamine levels to affect vascular endothelial-lymphocyte adhesion, which would recruit NK cells from their marginal pools and other reservoirs (49, 75). NK cells may be especially responsive to increases in catecholamine levels during acute exercise because of their high density of β-adrenergic receptors. Additionally, changes in NK cell response after prolonged exercise may be related to changes in inflammatory cytokines, such as IL-6 and TNF-alpha (50). When comparing acute exercise responses before and after exercise training, Rhind et al. (49) explains that the increased NK cell response post-intervention may be related to exercise training-induced increases in the density of β-adrenergic receptors on NK cells, making them more responsive to the effects of catecholamines. During the recovery period, decreases in NK cell counts seem to be related to exercise-induced increases in catecholamines and cortisol; the latter of which may be mediated by changes in the inflammatory cytokine IL-6 (75).

High Intensity Exercise and NK Cell Count

The vast majority of studies examining the NK cell response to acute aerobic exercise have been performed using high intensity exercise, and of these, most have been performed using athletes and physically-active individuals. Many of these studies have exclusively examined the effect of an acute bout of high intensity aerobic exercise on NK cell response in healthy individuals, with intensities ranging from 70-100% of VO_{2max}, and durations ranging from less than 10 minutes to nearly 5 hours. All studies measured NK cell counts pre-exercise, immediately post-exercise, and at various points 0-8 hours post-exercise, although a few have measured NK cell counts 15-24 hours post-exercise.

Two studies have examined changes in NK cell counts in response to very short-term, high intensity aerobic exercise. Penkman et al. (76) examined the effect of a simulated 200-

meter rowing race in 7 female competitive rowers, and Wilson et al. (77) examined the effect of a 400-yard race-pace swim in 22 elite high school swimmers. The duration of the exercise sessions were 8.1 ± 0.3 minutes for the rowers and approximately 7 minutes for the swimmers. NK cell counts were measured pre-exercise, immediately post-exercise, and in the Penkman et al. (76) study, NK cell counts were also measured 1 hour post-exercise. Even though the exercise sessions were quite short, NK cell counts increased significantly over pre-exercise values by approximately 629% for the rowing session ($p < 0.05$) and by approximately 373% for the swimming session ($p < 0.0001$). At 1 hour post-exercise, NK cell counts were decreased below pre-exercise values by approximately 35%, but this decrease below the pre-exercise level was not significant.

Four studies have examined changes in NK cell counts to high-intensity exercise of fairly short durations (18 minutes-slightly over 30 minutes). Moyna et al. (48) examined changes in NK cell counts in response to an 18-minute cycle ergometry ride in 32 healthy men and women. Simpson et al. (79) and Campbell et al. (80) investigated changes in NK cell counts in response to treadmill runs to volitional fatigue (33.8 ± 12.3 minutes and 24 ±11 minutes, respectively) in 8 endurance-trained healthy men. Lastly, Duester et al. (81) investigated changes in NK cell counts in response to a 25-minute treadmill run in 22 healthy, physically-fit men and women. The exercise intensities for these studies ranged between 70-90% of VO_{2max} (47, 78-80). In all 4 studies, NK cell counts were measured pre-exercise, and immediately post-exercise, During the recovery period, Simpson et al. (79) and Campbell et al. (80) measured NK cell counts at 1 hour post-exercise, and Moyna et al. (48) measured NK cell counts at 2 hours post-exercise. In all 4 studies, NK cell counts were significantly elevated immediately post-exercise compared to pre-exercise levels by

approximately 53-543% ($p < 0.001$-0.05). At 1 hour post-exercise, NK cell counts were significantly decreased below pre-exercise resting levels by approximately 50-77% ($p < 0.01$) (79, 80). At 2 hours post-exercise, Moyna et al. (48) showed that NK cell counts had returned to near baseline values.

Five studies have examined changes in NK cell counts to high intensity exercise of slightly longer durations (60-75 minutes). McFarlin et al. (51, 52) performed two studies investigating changes in NK cell counts in response to a 60-minute cycle ergometry ride in samples of 13 and 8 endurance-trained men, respectively. Pedersen et al. (53) examined changes in NK cell counts in response to a 60-minute cycle ergometry ride in 6 healthy untrained men. Braun et al. (54) studied changes in NK cell counts in response to a 60-minute treadmill run in 10 male distance runners. Lastly, Ronsen et al. (82) examined changes in NK cell counts in response to a 75-minute cycle ergometry ride in 9 male endurance athletes. Exercise intensities ranged from 75-85% of VO_{2max}. In all 5 studies, NK cell counts were measured pre-exercise and immediately post-exercise. During the short-term recovery period, Ronsen et al. (82) measured NK cell counts at 1 hour post-exercise, Braun et al. (54) measured NK cell counts at 1.5 hours post-exercise, and McFarlin et al. (51, 52) and Ronsen et al. (82) measured NK cell counts at 2 hours post-exercise. McFarlin et al. (51, 52) continued to measure NK cell counts at 4 hours post-exercise, Ronsen et al. (82) also measured NK cell counts at 3 hours, 4 hours, and approximately 15 hours post-exercise, and Pedersen et al. (53) measured NK cell counts at 24 hours post-exercise. In all 5 studies, NK cell counts were significantly elevated immediately post-exercise compared to pre-exercise levels by approximately 82-460% ($p < 0.001$-0.05). During the short-term recovery period (0-2 hours), Braun et al. (54) and Pedersen et al. (53) reported that NK cell counts were

slightly below pre-exercise levels by 25-35%, but the difference was not significant. McFarlin et al. (52) also reported that NK cell counts were near pre-exercise levels at 2 hours post-exercise. However, McFarlin (51) reported a significant decrease in NK cell count at 2 hours post-exercise of approximately 53% below pre-exercise levels ($p < 0.05$). It seems though that these short-term decreases in NK cell counts appear to resolve by 4 hour post-exercise, and that NK cell counts do not seem to undergo further significant alteration between 4 and 24 hours post-exercise (50-52, 81).

Three studies investigated changes in NK cell counts in response to high intensity exercise using durations that exceeded 1.5 hours. Briviba et al. (83) investigated changes in NK cell counts in response to running a marathon or half-marathon in 22 recreational runners, with race times ranging from 1.5-2.6 hours for the half-marathon and 3.4-4.8 hours for the marathon. Kakanis et al. (84) examined changes in NK cell counts in response to a 2-hour cycle ergometry ride in 10 elite male cyclists. Lastly, Nieman et al. (85) studied changes in NK cell counts in response to a 2.5-hour treadmill in 13 experienced male and female marathon runners. The quantification of exercise intensity was slightly different among the studies. In the Briviba et al. (83) study, exercise intensity was not explicitly stated, but it is likely that subjects were running at an intense race-pace effort. In the Kakanis et al. (84) study, subjects exercised at 90% of the second ventilatory threshold. In the Nieman et al. (85) study, subjects exercised at 75-80% of VO_{2max}. Additionally, the timing of NK cell measurements was slightly different among the studies. In the Briviba et al. (83) study, NK cell counts were measured 10 days pre-race and within 20 minutes post-race. In the Kakanis et al. (84) study and the Nieman et al. (85) study, NK cell counts were measured pre-exercise and immediately post-exercise. Kakanis et al. (84) measured NK cell

counts during recovery at 2, 4, 6, 8, and 24 hours post-exercise, while Nieman et al. (85) measured NK cell counts during recovery at 1.5, 3, and 6 hours post-exercise. Results were varied among the studies. Briviba et al. (83) found that the proportion of NK cells increased pre-race to post-race in both the half-marathoners and the marathoners (17 ± 2% to 24 ± 6% and 16 ± 2% to 18 ± 2%, respectively) but the increases were not statistically significant. Kakanis et al. (84) found some rather unique results, in that NK cell counts decreased slightly immediately post-exercise compared to pre-exercise values by less than 10%, and the difference was not statistically significant. At 4-8 hours post-exercise, NK cell counts had decreased significantly below pre-exercise levels, by approximately 40% ($p < 0.05$). By 24 hours post-exercise, NK cell counts were approximately 10% below pre-exercise levels (difference not significant), and had therefore returned to near baseline levels. Nieman et al. (85) showed results that were similar to previously-mentioned studies, that NK cell counts increased significantly immediately post-exercise by approximately 83% over pre-exercise levels ($p < 0.05$). At 1.5-3 hours post-exercise, NK cell counts were decreased by approximately 50-60% below pre-exercise levels ($p < 0.05$). At 6 hours post-exercise, NK cell counts were approximately 30% below pre-exercise levels, but the difference was not statistically significant.

Finally, 4 studies have exclusively examined the effect of a graded exercise bout or VO_{2max} test on NK cell counts. Gabriel et al. (86) investigated changes in NK cell counts before and after a VO_{2max} test in 10 healthy untrained individuals. Woods et al. (87) studied changes in NK cell counts before and after a VO_{2max} test in 11 sedentary elderly men and women enrolled in a 6-month moderate-intensity aerobic exercise intervention. Van der Pompe et al. (88) investigated changes in NK cell counts in 13 healthy post-menopausal

women before and after performing a graded cycle ergometry protocol. Simpson et al. (89) investigated changes in NK cell counts in response to a VO_{2max} test in 16 healthy younger and older men. The exercise protocols used for each study were slightly different. In the Gabriel et al. (86) study, subjects performed an incremental graded cycle ergometry protocol. In the Woods et al. (87) study, subjects performed a modified Balke treadmill test. In the van der Pompe et al. (88) study, subjects performed a graded cycle ergometry protocol, with intensities progressing at 40%, 60%, 80%, and 100% of VO_{2max}. In the Simpson et al. (89) study, subjects performed a walking protocol on the treadmill. All subjects exercised until volitional fatigue. In all 4 studies, NK cell counts were measured pre-exercise and immediately post-exercise. During the recovery period, van der Pompe et al. (88) measured NK cell counts at 5, 15, and 30 minutes post-exercise, while Gabriel et al. (86) and Simpson et al. (89) measured NK cell counts at 1 hour post-exercise. All 3 studies showed significant increases in NK cell counts immediately post-exercise compared to pre-exercise levels. Gabriel et al. (86) found that NK cell counts increased by approximately 250% over pre-exercise values ($p < 0.025$). Woods et al. (87) found that the proportions of NK cells in circulation increased significantly immediately post-exercise both before and after the 6-month exercise intervention (~22 ± 3% to ~31 ± 5%, $p < 0.05$ before the intervention; and ~17 ± 2% to ~26 ± 2%, $p < 0.05$ after the intervention). No differences were observed when comparing the magnitude of the NK cell count responses pre-intervention and post-intervention. Van der Pompe et al. (88) showed that NK cell counts immediately post-exercise increased 6-fold over pre-exercise values ($p < 0.001$). Simpson et al. (89) showed that NK cell response was similar for both younger and older men, in that NK cell counts increased significantly immediately post-exercise compared to pre-exercise levels by

approximately 286% in the younger group and 297% in the older group ($p < 0.001$). At 30 minutes post-exercise, van der Pompe et al. (88) showed that NK cell counts had returned to near baseline value. At 1 hour post-exercise, Gabriel et al. (86) showed that NK cell counts decreased significantly below pre-exercise values by approximately 50% ($p < 0.01$), while Simpson et al. (89) showed that NK cell counts were significantly decreased below pre-exercise values by approximately 79% in the younger group ($p < 0.001$) and 70% in the older group ($p < 0.05$).

The potential mechanisms which may be associated with the biphasic response of NK cell counts to these high intensity aerobic exercise bouts are similar to those described for moderate intensity aerobic exercise. The marked rise in NK cell counts immediately post-exercise is likely associated with increased cardiac output and catecholamines. Epinephrine in particular may induce selective recruitment of NK cells from marginal pools by changing the adhesive interaction between endothelial cells and NK cells (48, 53). During the recovery period, cortisol may be a major factor mediating the NK cell cytopenia, possibly through alterations in cellular adhesive interactions (53, 84, 85). This may be of particular importance during exercise exceeding 60 minutes in duration, where increases in cortisol may overshadow the influence of epinephrine, thus facilitating the egress of NK cells out of circulation (85).

Two studies offered further insight into potential factors which may have lead to their observed results. Briviba et al. (83) observed non-significant increases in NK cell counts in response to half-marathon and marathon racing. The authors suggest that these results may be associated with differences in the time at which catecholamines and cortisol were released during exercise, as subjects were running the races at different paces, and therefore were

exercising for different durations (ranges of 1.5-2.6 hours for the half-marathon, and 3.4-4.8 hours for the marathon). Additionally, blood sample timing may have affected results, as pre-race NK cell counts were determined from samples taken 10 days pre-race, and post-race NK cell counts were determined from samples taken anytime between 0-20 minutes post-race. Kakanis et al. (84) did not offer any explanation of why a decrease in NK cell counts was observed immediately post-exercise. However, when the authors examined NK cells according to their subsets ($CD56^{bright}$ and $CD56^{dim}$), they observed that the $CD56^{bright}$ NK cells significantly immediately post-exercise, compared to pre-exercise levels. This increase in the $CD56^{bright}$ NK cells have implications in enhancing other innate immune responses, particularly through inducing increases in TNF-alpha, which can lead to increases in neutrophil counts.

Kakanis et al. (84) also suggests that the NK cell cytopenia observed during the recovery period from 0-8 hours post-exercise may possibly support the "open-window" theory. As NK cells are a first line of defense against pathogens, decreased NK cell numbers during recovery could provide an opportunity for pathogens to invade the body, thus potentially increasing susceptibility for infections such as upper respiratory tract infections (URTI). The observed decrease in NK cell counts 6-8 hours post-exercise may be of relevance when considering the implications of repeated bouts of exercise, as performing additional exercise bouts when NK cell numbers are suppressed may lead to chronic NK cell suppression, thus further increasing the susceptibility to infection (84).

Studies Comparing Exercise Intensities and NK Cell Count

The previously described studies have exclusively examined either the effect of moderate intensity exercise on NK cells, or the effect of high intensity exercise on NK cells.

In this section, seven studies are now discussed which have explored and compared the effects of different exercise intensities on changes in NK cell counts. These studies have examined and compared various combinations of exercise intensity, such as comparing the effects of low and high intensity, moderate and high intensity, or low, moderate, and high intensity.

Anane et al. (90) and Campbell et al. (91) compared changes in NK cell counts in response to short-duration aerobic exercise at low and high intensity in 11 and 13 healthy physically-active men, respectively. In both studies, subjects performed two 20-minute cycle ergometry rides, one at 35% of VO_{2max} and one at 85% of VO_{2max}. Anane et al. (90) measured NK cell counts pre-exercise, during the final 2 minutes of exercise, and at 15 minutes post-exercise. Campbell et al. (91) measured NK cell counts pre-exercise, immediately post-exercise, 15-minutes post-exercise, and 1 hour post-exercise. In both studies, NK cell counts increased significantly at the end of the low-intensity exercise bout levels by approximately 156% ($p< 0.05$) (89) and 158% ($p < 0.001$) compared to pre-exercise levels (91) Similarly, NK cells increased significantly immediately after high intensity exercise in both studies by approximately 829% ($p<0.05$) (89) and 935% ($p < 0.001$) (91). During the recovery period after low-intensity exercise, Anane et al. (90) observed that NK cell counts had returned to slightly above pre-exercise levels at 15 minutes post-exercise. Campbell et al. (91) also observed that NK cell counts returned to slightly above pre-exercise levels at 15 minutes post-exercise, where they remained throughout the 1-hour recovery period. During the recovery period after high intensity exercise, Anane et al. (90) and Campbell et al. (91) observed that NK cell counts were still elevated at 15 minutes post-exercise by approximately 100% ($p < 0.01$). At 1 hour post-exercise, Campbell et al.

(91) found that NK cell counts were decreased below pre-exercise values by approximately 35%; however, the difference was not statistically significant. When comparing the magnitude of the NK cell responses to the low and high intensity bouts, both studies observed marked differences, where the high intensity session produced a greater change in NK cell counts immediately post-exercise (156% vs. 829%; $p < 0.001$, and 158% vs. 935%). The studies did not directly compare the NK cell count responses between intensities during the recovery period, although it is apparent that NK cell counts returned to near baseline levels within 15 minutes post-low intensity exercise whereas they were still elevated after high intensity exercise. At 1 hour into recovery, high intensity exercise had elicited a 35% decrease in NK cells, which was not seen after low intensity exercise (91).

Nieman et al. (92) examined changes in NK cell counts in 10 male runners following two 45-minute treadmill runs, one at 50% of VO_{2max} and one at 80% of VO_{2max}. NK cell responses (reported as percentages of lymphocyte counts) were measured pre-exercise, immediately post-exercise, and during recovery at 1 hour, 2 hours, and 3.5 hours post-exercise. Both exercise bouts elicited significant increases in NK cells during exercise. In response to the moderate intensity bout, the percentage of NK cells in circulation increased from $14.0 \pm 2.4\%$ pre-exercise to $19.1 \pm 2.6\%$ immediately post-exercise ($p < 0.0125$). In response to the high intensity bout, the percentage of NK cells in circulation increased from $17.8 \pm 3.2\%$ pre-exercise to $26.7 \pm 3.2\%$ immediately post-exercise ($p < 0.0125$). During the recovery period, the percentage of NK cells decreased significantly below pre-exercise values in response to both intensities. In response to the moderate intensity exercise bout, the percentage of NK cells was $9.6 \pm 2.5\%$, at 1 hour post-exercise, while at 2 hours post-exercise, the percentage of NK cells was $8.5 \pm 1.7\%$ ($p = 0.02$ and < 0.003, respectively). At

3.5 hours post-moderate intensity exercise, the percentage of NK cells had risen to 12.7 ± 1.9%, which was still decreased below pre-exercise values, but the difference was not significantly significant. In response to the high intensity exercise bout, the percentage of NK cells was 7.8 ± 1.3% at 1 hour post-exercise, while at 2 hours post-exercise, the percentage of NK cells was 9.2 ± 1.4%, ($p < 0.003$ compared to pre-exercise values). At 3.5 hours post-high intensity exercise, the percentage of NK cells was still slightly decreased below pre-exercise levels at 12.3 ± 1.9%, but the difference was not statistically significant. The only time-point at which changes in NK cell counts statistically differed among the moderate and high intensity exercises sessions was immediately post-exercise, where the percentage of NK cells was significantly greater for the high-intensity exercise session (26.7 ± 3.2% vs. 19.1 ± 2.6%; $p < 0.0125$).

Wang et al. (93, 94) performed two studies investigating changes in NK cell counts to moderate intensity exercise and a VO_{2max} test in sedentary men. In the first study (93), 16 subjects participated in a cycle ergometry ride at 50% of VO_{2max} for 30 minutes, as well as a graded VO_{2max} test on the cycle ergometer. NK cell counts were measured pre-exercise, immediately post-exercise, and during recovery at 2 hours post-exercise. In response to the moderate intensity exercise bout, NK cell counts increased significantly immediately post-exercise by approximately 50-100% compared to pre-exercise values ($p < 0.05$). At 2 hours post-moderate intensity exercise, NK cell counts were slightly below pre-exercise values but the difference was not statistically significant. In response to the high intensity exercise bout, NK cells also increased significantly immediately post-exercise by approximately 400-500% ($p < 0.05$) compared to pre-exercise values. At 2 hours post-high intensity exercise, NK cell counts decreased significantly below pre-exercise values ($p < 0.05$). The authors do not

make any direct comparisons between the moderate and high intensity exercise bouts, but it is apparent that the magnitude of NK cell increase in response to the high intensity exercise bout is markedly greater than the response to the moderate intensity bout. Likewise, the NK cell cytopenia during recovery was greater in response to the high intensity bout compared to the moderate intensity bout.

In the second study by Wang et al. (94), 30 subjects participated in a moderate intensity cycle ergometry ride at 60% of VO_{2max} for 40 minutes. Additionally, subjects performed a graded VO_{2max} test on a cycle ergometer, as well as a combination moderate-high intensity exercise bout where subjects cycled at 60% of VO_{2max} for 20 minutes, rested for 30 minutes, and then performed a VO_{2max} test. NK cell counts were measured pre-exercise and immediately post-exercise. In response to the moderate intensity exercise bout, NK cell counts increased slightly immediately post-exercise by approximately 13% (not statistically significant). In response to the high intensity and combined moderate-high intensity exercise bouts, NK cell counts increased significantly by approximately 403% and 285%, respectively ($p < 0.05$). No direct comparisons were made between exercise bouts, but again, it is apparent that the high-intensity and combined moderate-high intensity bouts elicited a markedly greater NK cell cytosis compared to the moderate intensity bout.

Bruunsgaard et al. (95) investigated the NK cell response to two different exercise bouts in 9 recreationally-active men. Subjects performed a 30-minute cycle ergometry ride at 65% of VO_{2max}, using normal concentric pedaling, as well as a 30-minute cycle ergometry ride using eccentric pedaling at 100-150% of the workload that elicited concentric VO_{2max}. NK cell counts were measured from blood samples taken pre-exercise, during minute 20 of exercise, immediately post-exercise, and during recovery at 2 hours post-exercise. NK cell

counts increased significantly immediately post-exercise by approximately 360% compared to pre-exercise levels, in response to both types of cycle ergometry rides ($p < 0.05$). At 2 hours post-exercise, NK cell counts decreased below pre-exercise levels by approximately 23% and 34% for the concentric ride and the eccentric rides, respectively; however, neither decrease was significantly different from pre-exercise levels. NK cell counts were not significantly different between the concentric and eccentric exercise bouts pre-exercise, immediately post-exercise, or during recovery.

Lastly, Kendall et al. (96) examined changes in NK cell counts in response to low, moderate, and high intensity exercise in 22 healthy young adult men. Subjects were divided into 3 groups based on aerobic fitness. Eight subjects were classified as low-fit ($VO_{2max} < 50$ mL/kg/min), 6 subjects were classified as moderately-fit ($VO_{2max} = 50$-60 mL/kg/min), and 8 subjects were classified as highly fit ($VO_{2max} > 60$ mL/kg/min). All subjects participated in four randomly-ordered cycle ergometry rides of varying intensities and durations: a low intensity ride at 30% of VO_{2max} for 60 minutes, two moderate intensity rides at 65% of VO_{2max} for 30 minutes and 120 minutes, and a high intensity ride at 75% of VO_{2max} for 60 minutes. NK cell counts were measured 24 hours pre-exercise, immediately pre-exercise, immediately post-exercise, 30 minutes post-exercise, 2 hours post-exercise, and 24 hours post-exercise. Changes in NK cell counts were compared within exercise bouts and within groups only (i.e., pre-exercise to post-exercise responses for each exercise bout within each group), and because of multiple comparisons, the alpha level was set fairly conservatively at 0.005. When examining the NK cell response to the low-intensity exercise bout (30% of VO_{2max} for 60 minutes), only the highly-fit group experienced a significant increase immediately post-exercise compared to pre-exercise levels (~167%; $p < 0.005$). When

examining the NK response to the short-duration moderate intensity bout (65% of VO_{2max} for 30 minutes), only the low-fit group experienced a significant increase immediately post-exercise compared to pre-exercise levels (~175%, $p < 0.005$). When examining the NK cell response to the long-duration moderate intensity exercise bout (65% of VO_{2max} for 120 minutes), the low-fit and high-fit groups experienced significant increases immediately post-exercise compared to pre-exercise values (~275% and ~533% respectively; $p < 0.005$). Lastly, when examining the NK cell response to the high intensity exercise bout (75% of VO_{2max} for 60 minutes), all 3 fitness groups experienced significant increases immediately post-exercise compared to baseline values (~275-900%, $p < 0.005$). The post-exercise increases in NK cell counts were transient for all exercise bouts in all fitness-level groups, and all returned to pre-exercise levels by 30 minutes post-exercise with no further changes observed throughout the 24-hour recovery period.

In all 7 of these studies, the magnitude of NK cell response was markedly more pronounced for exercise bouts of higher intensities compared to exercise bouts of low and moderate intensities. The mechanisms leading to the observed results are similar to what has been described previously. Increases in NK cell counts during exercise may be associated with increases in cardiac output as well as catecholamines, particularly epinephrine, as NK cells possess a high number of β-adrenergic receptors, and epinephrine is a potent β-adrenergic receptor agonist (92). Furthermore, the density of the β-adrenergic receptors on NK cells may also increase in response to high intensity exercise, further contributing to the increased magnitude of the NK cell cytosis during high intensity exercise in comparison to low and moderate intensity exercise (92, 94). The NK cell cytopenia that occurs during recovery is likely associated with increased cortisol levels particularly in response to

prolonged exercise, as corticosteroids can elicit a redistribution of NK cells from the blood to other sites in the body (92, 94).

Two studies offer further explanations pertaining to their specific observed results. Campbell et al. (91) noted that during the recovery period after high intensity exercise, NK cell counts were decreased by approximately 35% below pre-exercise levels and that this decrease was not statistically significant. While the exercise intensity was quite high (85% of VO_{2max}), the duration was rather short (20 minutes). Therefore, the exercise may not have been of sufficient duration in order to elicit a significant NK cell cytopenia during recovery (91). Kendall et al. (96) observed NK cell responses of varying magnitudes to the different exercise intensities and durations in the 3 fitness-level groups. The authors speculate that the wide array of responses may be due to differences in absolute exercise workloads rather than relative workloads, as well as differences in perceived stress within fitness groups (96).

Effect of Acute Aerobic Exercise on NKCA

Moderate Intensity Exercise and NKCA

Of the four previously described studies that have exclusively examined the effect of an acute bout of moderate intensity aerobic exercise on NK cell counts in healthy individuals, one also examined the effect of acute moderate intensity exercise on NKCA. Scharhag et al. (75) examined NKCA in 12 male competitive triathletes and cyclists in response to 4 hours of cycling at 60% of VO_{2max}. Whereas NK cell counts were measured at various points pre- and post-exercise, NKCA was only measured pre-exercise and immediately post-exercise. NKCA was measured using flow cytometric analysis. Briefly, NK cells were mixed with fluorescently-labeled K562 target cells in an effector-to-target cell ratio (E:T ratio) of 50:1. NKCA (% lysis) was determined by subtracting the percentage of dead cells in the control

sample from the percentage of killed target cells in the test sample, and the number of NK cells needed to lyse one target cell were calculated as follows: needed NK cells = effector cells * % NK cells measured by flow cytometry / target cells * % specific lysis. The authors found that the number of NK cells needed to lyse one K562 target cell (i.e., single cell NKCA) did increase immediately post-exercise compared to pre-exercise values, but the increase was slight and not statistically significant. However, as described previously, the authors did observe a significant increase in NK cell counts immediately post-exercise by approximately 76% over pre-exercise levels ($p < 0.05$). These findings may indicate that significant changes often observed for NKCA in response to aerobic exercise may be more related to the numerical redistribution of NK cells rather than of single cell NKCA (75).

High Intensity Exercise and NKCA

Eleven studies have exclusively studied the effect of acute high intensity aerobic exercise on changes in NKCA in healthy individuals using both short and long duration protocols. The ^{51}Cr release assay was the most common method used to determine NKCA, either by whole-blood or isolated-PBMC techniques. However, for three of the more recent studies, flow cytometric or calcein-AM fluorescence techniques were used to determine NKCA.

Moyna et al. (48) investigated the NKCA response to an 18-minute cycle ergometry ride in 32 healthy men and women, where exercise intensity progressed until 85% of VO_{2max}. As was the case for NK cell counts, NKCA was measured pre-exercise, immediately post-exercise and during recovery at 2 hours post-exercise. NKCA was determined using a whole-blood ^{51}Cr release assay. Briefly, K562 target cells were labeled with ^{51}Cr and then mixed with heparinized whole blood in varying concentrations ($2.0x10^6$ cells/mL, $1.0x10^6$ cells/mL,

$0.5x10^6$ cells/mL, and $0.25x10^6$ cells/mL). NKCA was expressed in cytolytic units, calculated using an algorithm that includes the percentage specific lysis and the natural logarithm of the concentration of target cells. The authors found that NKCA increased significantly immediately post-exercise by approximately 100% over pre-exercise levels ($p < 0.001$). At 2 hours post-exercise, NKCA had returned to near baseline levels along with NK cell count. Percent change in NK cell count was significantly correlated with percent change in NKCA during exercise at 85% of VO_{2max} ($r = 0.60$; $p < 0.001$). Percent change in NK cell count was also significantly correlated with percent change in NKCA at 2 hours post-exercise ($r = 0.31$; $p < 0.001$).

Pedersen et al. (53), Braun et al. (54), and McFarlin et al. (51, 52, 97) examined NKCA responses to high intensity exercise of 60-75 minutes in duration. Pedersen et al. (53) examined the NKCA response to a 60-minute cycle ergometry ride at 80% of VO_{2max} in 6 healthy untrained men. As was the case for NK cell counts, NKCA was measured pre-exercise, during the last few minutes of exercise, 2 hours post-exercise, and 24 hours post-exercise. A ^{51}Cr release assay was used to determine NKCA from isolated blood mononuclear cells (BMNC). Briefly, K562 target cells were labeled with ^{51}Cr and mixed with BMNC in an E:T ratio of 100:1. NKCA was expressed as % lysis per fixed number of mononuclear cells, and was calculated by measuring differences in ^{51}Cr release from test samples and control samples. The authors found that NKCA increased significantly during exercise by more than 50% above pre-exercise values ($p < 0.05$), along with a significant increase in the percentage of $CD16^+$ NK cells ($p < 0.05$). During the recovery period, NKCA decreased approximately 40% below pre-exercise values, which was significantly decreased below the pre-exercise level ($p < 0.05$), although the percentage of $CD16^+$ NK cells had

returned to pre-exercise levels. NKCA returned to pre-exercise levels by 24 hours post-exercise as did the percentage of $CD16^+$ NK cells.

Braun et al. (54) examined the NKCA response to a 60-minute treadmill run at 85% of VO_{2max} in 10 male distance runners. As was the case with NK cell counts, NKCA was measured pre-exercise, immediately post-exercise, and 1.5 hours post-exercise. The authors investigated changes in NKCA using 3 different methods: an isolated-PBMC ^{51}Cr release assay with E:T ratios of 2.5:1, 5:1, 10:1, and 20:1; a whole-blood ^{51}Cr release assay with the same E:T ratios; and the whole-blood ^{51}Cr release assay with results expressed using an E:T ratio of 1:1 (i.e, a "per-cell" NK basis). The results for changes in NKCA varied somewhat based on the method used to determine NKCA. When using the isolated-PBMC method, NKCA (% lysis) increased significantly immediately post-exercise compared to pre-exercise levels (8.06 ± 1.39% vs. 22.24 ± 2.76%; $p < 0.05$). At 1.5 hours post-exercise, NKCA decreased below pre-exercise values but the difference was not statistically significant (8.06 ± 1.39% vs. 5.86 ± 1.06%). When using the whole-blood method, NKCA also increased significantly immediately post-exercise compared to pre-exercise levels (14.04 ± 2.07% vs. 39.49 ± 2.26%; $p < 0.05$). NKCA decreased significantly below pre-exercise levels at 1.5 hours post-exercise (14.04 ± 2.07% vs. 7.14 ± 1.00%; $p < 0.05$). When using the whole-blood method but expressing NKCA on a "per-NK cell-basis," NKCA again increased significantly immediately post-exercise compared to pre-exercise levels (16.53 ± 2.08% vs. 38.68 ± 2.89%; $p < 0.05$). At 1.5 hours post-exercise NKCA was slightly decreased below pre-exercise values, however, the difference was not statistically significant (16.53 ± 2.08% vs. 11.31 ± 1.39%).

McFarlin et al. (51, 52, 97) performed a series of 3 studies investigating the NKCA response to a 60-minute cycle ergometry ride at 70-80% of VO_{2max} in 8-13 trained subjects. All 3 studies measured NKCA pre-exercise, immediately post-exercise, and at 2 hours post-exercise. Two of the studies (51, 52) also measured NKCA at 4 hours post-exercise. NKCA was determined using two different methods. McFarlin et al. (51, 52) used a whole-blood ^{51}Cr release assay. McFarlin et al. (97) used a modified method, where whole blood was mixed with varying concentrations of K562 target cells which had been labeled with Calcein-AM fluorescent dye instead of ^{51}Cr. NKCA was expressed on a 1:1 E:T ratio (a "per-NK cell" basis) for both assays. All 3 studies showed significant increases immediately post-exercise by approximately 68-290% compared to pre-exercise levels ($p < 0.05$). At 2 hours post-exercise, NKCA decreased by approximately 40% (50), 44% (52), and 19% (97); however, only the 40% decrease was statistically significant ($p < 0.05$). At 4 hours post-exercise, NKCA had returned to near baseline levels (51, 52).

Nieman et al. (85), Briviba et al. (83), and Kakanis et al. (84) examined NKCA responses to high intensity exercise exceeding 1.5 hours in duration. Nieman et al. (85) examined the NKCA response to a 2.5 hour treadmill run at 75-80% of VO_{2max} in 13 marathon runners. As was the case for NK cell counts, NKCA was measured pre-exercise, immediately post-exercise, and during recovery at 1.5 hours, 3 hours, and 6 hours post-exercise. NKCA was determined using an isolated-PBMC ^{51}Cr release assay with E:T ratios of 40:1, 20:1, 10:1, and 5:1. When all NKCA data was combined and expressed as total lytic units per 10^7 mononuclear cells, a significant time effect was observed ($p < 001$). NKCA increased immediately post-exercise compared to pre-exercise levels, by approximately 34%. At 1.5 hours post-exercise, NKCA had decreased to approximately 49% below pre-exercise

values, and then increased to approximately 40% below pre-exercise values at 3 and 6 hours post-exercise. When NKCA was expressed on a "per-NK-cell" basis, the time effect was erased, and NKCA did not differ significantly from pre-exercise values at any time point post-exercise.

Briviba et al. (83) examined the NKCA to running a marathon or half-marathon in 22 recreational runners. As was the case for NK cell counts, NKCA was measured 10 days pre-race and within 20 minutes post-race. NKCA was measured using flow cytometric methods. Briefly, PBMC cells were isolated and mixed with fluorescently-labeled K562 target cells in E:T ratios of 50:1, 25:1, and 12.5:1. NKCA (% lysis) was determined by examining differences in fluorescence for damaged and undamaged target cells. For individuals competing in the half-marathon, NKCA increased significantly pre-race to post-race (61 ± 6% lysis vs. 69 ± 7% lysis; $p = 0.009$). For individuals competing in the full marathon, NKCA increased slightly pre-race to post-race, but the difference was not significant (61 ± 7% lysis vs. 62 ± 5% lysis).

Kakanis et al. (84) examined changes in NK cell counts in response to a 2-hour cycle ergometry ride in 10 elite male cyclists exercising at 90% of their second ventilatory threshold. As was the case with NK cell counts, NKCA was measured pre-exercise, immediately post-exercise, and during recovery at 2, 4, 6, 8, and 24 hours post-exercise. NKCA was determined using flow cytometric methods with isolated PBMC and an E:T ratio of 25:1. NKCA (% lysis) remained largely unchanged from pre-exercise values for all time points post-exercise.

Finally, Woods et al. (87) and van der Pompe et al. (88) examined NKCA responses to graded exercise up to VO_{2max} in 11 elderly individuals before and after a 6-month

intervention and 13 post-menopausal women, respectively. Both studies measured NKCA pre-exercise and immediately post-exercise, and van der Pompe et al. (88) also measured NKCA at 5, 15, and 30 minutes post-exercise. Both studies used the ^{51}Cr release assay to determine NKCA. Woods et al. (87) used an isolated PBMC assay with E:T ratios of 50:1, 25:1, and 12.5:1, while van der Pompe et al. (88) used a whole-blood assay. Woods et al. (87) found that for the VO_{2max} test performed before the 6-month intervention, NKCA (25:1 E:T ratio) increased significantly immediately post-exercise compared to pre-exercise levels by approximately 70% ($p < 0.05$). In contrast, NKCA increased by only ~10% in response to the VO_{2max} test performed after the 6 month intervention (result not statistically significant). Similarly, van der Pompe et al. (88) found that NKCA only increased slightly during exercise, and NKCA did not change significantly throughout the 30-minute recovery period.

The mechanisms mediating the changes in NKCA in response to acute exercise are generally similar to those previously described for changes in NK cell counts. Increases in catecholamines during exercise may also be associated with increases in NKCA immediately post-exercise, while increases in cortisol levels may be associated with decreases in NKCA during recovery (53). Additionally, other biological factors may influence the biphasic response of NKCA, which may also be associated with the exercise-induced changes in stress hormone levels (51, 52). During exercise, activated T lymphocytes may release inflammatory cytokines such as IL-2 and IFN-γ, which may act to increase NKCA (51). During recovery, the number of cytotoxic T lymphocytes producing IL-2 and IFN-γ may decrease, thus allowing the actions of other cytokines including IL-4 and IL-10 to prevail, which in turn may lead to a decrease in NKCA (51, 52). Furthermore, prostaglandin release from monocytes may also cause a decrease in NKCA during recovery (53, 54).

Some authors have suggested that differences in techniques used to determine NKCA may influence results. Moyna et al. (48), Braun et al. (54), and McFarlin et al. (52, 53) suggest that whole-blood techniques may be preferable over isolated-PBMC techniques when evaluating NKCA. When isolated-PBMC assays are performed, cells must be washed and are therefore not incubated with other immune and endocrine factors that were present when the blood sample was initially obtained, and it is possible that NKCA determined using these methods may not completely represent what would actually be occurring in vivo (54). In contrast, whole-blood assays do not require washing of cells, so therefore, the "blood borne milieu" is retained, and therefore NKCA determined using these methods may more fully reflect the in vivo environment (48, 54). As previously described, Braun et al. (54) compared NKCA results using both isolated-PBMC and whole-blood assays, and found that both methods resulted in significant elevations in NKCA immediately post-exercise; however, only the whole-blood technique resulted in a significant decrease in NKCA during recovery. The authors do caution that it can be difficult to compare whole-blood techniques with isolated-PBMC techniques because of the inability to establish equivalent E:T ratios (54).

In addition to differences in assay techniques, the expression of NKCA as total % lysis or % lysis on a "per-NK cell" basis may affect interpretation of results. Nieman et al. (85) suggests that hormone-induced effects on NK cell numbers may affect NKCA by either increasing or decreasing the number of NK cells in each assay well, and that is may be preferable to express NKCA on a per-cell basis. As described previously, several studies have shown that when NKCA is expressed on a 1:1 E:T ratio or per-NK cell basis, significant time effects are removed (54, 85). This may indicate that changes in NKCA, particularly

during recovery, may be more related to a redistribution of NK cell counts rather than an impairment in NK cell functionality (54, 75).

Several authors give further insight into their specific NKCA outcomes. Moyna et al. (48) observed that the exercise-induced increases in NK cell counts and NKCA were markedly different in magnitude; NK cell counts increased by over 500% while NKCA increased by only ~100%. The authors hypothesize that the NK cells that were recruited into circulation during exercise may have had decreased cytotoxic function compared to the NK cells circulating pre-exercise. In addition to the variable changes observed in NK cell count responses, Briviba et al. (83) also observed variable NKCA responses to the half-marathon and marathon runs; likely related to the wide range of race times and therefore time-dependent effects of catecholamines and cortisol on recruitment and cytotoxicity of NK cells. Finally, van der Pompe et al. (88) observed that while NK cell counts increased significantly during exercise, NKCA did not. The exact mechanisms driving this result are not known; however, the authors speculate that a component of the signaling pathways triggered by agonist stimulation of the β-adrenergic receptors on NK cells may be impaired in post-menopausal women (88).

Studies Comparing Exercise Intensities and NKCA

Nieman et al. (92) examined and compared NKCA responses to moderate and high intensity treadmill running in 10 male runners. Subjects performed two 45-minute treadmill runs, one at 50% of VO_{2max} and one at 80% of VO_{2max}. As was the case with NK cell counts, NKCA was measured pre-exercise, immediately post-exercise, and during recovery at 1 hour, 2 hours, and 3.5 hours post-exercise. NKCA was determined using an isolated-PBMC ^{51}Cr release assay with E:T ratios of 40:1, 20:1, 10:1, and 5:1. The authors reported NKCA

results using 3 different quantifications: 1) number of lytic units contained in 10^7 mononuclear cells, where lytic units was defined as the number of effector cells required to lyse 20% of 10,000 target cells, 2) lytic units per NK cell x 10^{-5}, (i.e., NKCA on a "per-NK cell" basis), and 3) lytic units per L blood x 1000. As reported in other studies, observed changes in NKCA in response to exercise seemed to vary depending on the quantification method used to describe NKCA. When NKCA was expressed as lytic units per 10^7 mononuclear cells (i.e., unadjusted for changes in the proportion of NK cells), NKCA increased immediately post-exercise above pre-exercise values, and then decreased below pre-exercise resting values in response to both exercise intensities. For the moderate intensity exercise bout, NKCA increased by approximately 40% immediately post-exercise (difference from baseline not significant), while decreasing by approximately 30% at 1 and 2 hours post-exercise (difference from baseline not significant). At 3.5 hours post-exercise, NKCA had risen to near baseline levels. For the high intensity exercise bout, NKCA increased significantly by approximately 80% ($p < 0.0125$), while decreasing by approximately 45% at 1 hour post-exercise (difference from baseline not significant). NKCA returned to near baseline levels by 2-3.5 hours post-exercise. Additionally, the pattern of change in NKCA over time was not significantly different between exercise intensities. When NKCA was expressed on a "per-NK cell-basis," very different results were observed. For the moderate intensity exercise bout, NKCA did not significantly differ from pre-exercise values for any time point, and the magnitude of change in NKCA over time was slight. For the high intensity exercise bout, NKCA did not change significantly from pre-exercise levels until 2 hours post-exercise, when NKCA increased by approximately 60% ($p < 0.0125$). Again, the pattern of change in NKCA over time was not significantly different between exercise

intensities. Lastly, when NKCA was expressed as lytic units per L blood, changes in NKCA appeared more similar to the changes in NKCA expressed as unadjusted for the changes in proportion of NK cells. For the moderate intensity exercise bout, NKCA increased significantly immediately post-exercise by approximately 47% compared to pre-exercise values ($p < 0.0125$), while at 1-2 hours post-exercise, NKCA decreased by approximately 46-60% compared to pre-exercise values ($p < 0.0125$). At 3.5 hours post-exercise, NKCA had returned to near baseline values. For the high intensity exercise bout, NKCA also increased significantly immediately post-exercise by over 200% compared to pre-exercise values ($p < 0.0125$), while at 1-2 hours post-exercise, NKCA had decreased by approximately 46-60% below pre-exercise values ($p < 0.0125$). At 3.5 hours post-exercise, NKCA had once again returned to near baseline values. The pattern of change in NKCA over time was significantly different between exercise intensities only immediately post-exercise ($p = 0.009$).

The varied results obtained in the Nieman et al. (92) study further support the idea that the relationship between NKCA and acute exercise may depend on the type of assay used, and how the results are quantified. This particular study employed an isolated-PBMC ^{51}Cr assay to measure NKCA, as have other previously-described studies (53, 54, 85, 87). In this type of assay, at certain E:T ratios, even though the total number of mononuclear cells (i.e., effector cells) in the pre- and post-exercise assay wells remain constant, the proportions of mononuclear cell subsets (i.e., monocytes, lymphocytes, and lymphocyte subsets) may vary considerably (92). Therefore, increases and decreases in NKCA may depend on the actual number of NK cells that are present in each assay well (92). This may be why significant increases during exercise and significant decreases during recovery were observed for unadjusted NKCA and NKCA per L blood, as they tend to reflect the increased

recruitment of NK cell numbers into circulation during exercise, as well as the egress of NK cell numbers out of circulation during recovery. When the results were normalized to a "per-NK cell basis," an increase in NKCA was observed during recovery from high intensity exercise, which is somewhat of a novel finding, the mechanisms for which are not entirely known (92).

Bruunsgaard et al. (95) examined and compared NKCA responses to two bouts of cycle ergometry exercise in 9 recreationally active men; the first being a 30-minute cycle ergometry ride at 65% of VO_{2max} using normal concentric pedaling, and the second being a 30-minute cycle ergometry ride using eccentric pedaling at 100-150% of the workload that elicited concentric VO_{2max}. As was the case with NK cell counts, NKCA was measured pre-exercise, during minute 20 of exercise, immediately post-exercise, and during recovery at 2 hours post-exercise. NKCA was determined using an isolated-PBMC ^{51}Cr release assay with E:T ratios of 100:1, 50:1, and 25:1. In response to the concentric exercise bout, NKCA increased significantly immediately post-exercise by approximately 100% compared to pre-exercise levels ($p < 0.05$). At 2 hours post-exercise, NKCA had returned to near baseline levels. Similarly, in response to the eccentric exercise bout, NKCA increased significantly immediately post-exercise by approximately 67% ($p < 0.05$), and at 2 hours post-exercise, NKCA had returned to near baseline values. The pattern of change in NKCA over time was not significantly different between exercise bouts at any time. The authors speculate that the return of NKCA to near baseline levels during the recovery period may be consistent with the fact that the exercise bouts were of fairly short duration and moderate intensity (94).

Correlation between Changes in NK Cell Response with Changes in Catecholamines, and Cortisol

Four of the studies described in the above sections have examined correlations between changes in hormonal parameters and changes in NK cell response to acute aerobic exercise. Kendall et al. (96), Moyna et al. (48), and Scharhag et al. (75) have explored correlations between catecholamines and NK cell responses. Nieman et al. (85) explored correlations between catecholamines, cortisol, and NK cell response. Sharp increases in both epinephrine and norepinephrine levels were observed immediately post-exercise in all studies (48, 75, 85, 96). Moyna et al. (48) and Scharhag et al. (75) showed that increases in epinephrine levels were significantly correlated with increases in NK cell count ($r = 0.30$; $p < 0.002$ and $r = 0.74$; $p = 0.005$, respectively), however, Moyna et al. (48) also found that epinephrine was not significantly correlated with NKCA. Kendall et al. (96) and Scharhag et al. (75) observed that increases in norepinephrine levels were significantly correlated with increases in NK cell count ($r = 0.40$; $p < 0.0002$ and $r = 0.65$; $p = 0.02$, respectively). In contrast, Nieman et al. (85) did not observe significant correlations between epinephrine and NK cell count or NKCA ($r = 0.18$; $p = 0.34$ and $r = 0.09$; $p = 0.64$, respectively); however, increases in norepinephrine were significantly correlated with changes in NK cell counts ($r = 0.37$, $p = 0.044$). Nieman et al. (85) also observed that cortisol levels increased sharply immediately post-exercise and were elevated at 1.5 hours post-exercise. Post-exercise cortisol levels (average of immediate and 1.5 hr post-exercise levels) were negatively correlated with NK cell counts at 3 hrs post-exercise ($r = -0.38$, $p = 0.04$). However, no significant correlations between cortisol and NKCA were found.

The results of these studies largely support the idea that catecholamines and cortisol are related to NK cell responses to acute aerobic exercise. Increases in catecholamines during exercise may be associated with increases in NK cell counts by altering the adhesive interactions between NK cells and endothelial cells, thus recruiting them from their marginal zones into circulation (48). Increased cortisol may be associated with decreased NK cell counts, possibly through alterations in cellular adhesive interactions (53, 84, 85).

It may be important to note that while many of the correlations reported in these studies were of statistical significance, their respective strengths were rather weak. Kendall et al. (96) suggests that the relative weakness of these correlations may be related to the timing of blood samples taken immediately post-exercise. Catecholamine levels decrease rapidly upon cessation of exercise, and even delaying blood sampling by 2-3 minutes may affect results (96). The non-significant correlations reported by Nieman et al. (85) may be due to the fact that the authors tested subjects in two conditions; a placebo condition and a carbohydrate-supplemented condition, and correlational analyses were performed for all subjects together instead of separately by group. Additionally, Nieman et al. (85) propose that epinephrine may not have been associated with changes in NK cell counts because the exercise duration used in their study was longer than 60 minutes, and that for exercise bouts of longer durations, the effect of epinephrine may be overtaken by the effect of cortisol on diminishing NK cell numbers in circulation.

Nieman et al. (85) comment that NKCA may not have been significantly correlated with cortisol levels because the time of exposure to elevated levels of cortisol may not have been long enough. The authors do suggest that NKCA could have been indirectly affected by increased post-exercise cortisol levels, as post-exercise increases in cortisol did influence a

decrease in post-exercise NK cell counts, and observed decreases in NKCA were also observed (85).

Aerobic Exercise and Immune Function in Cancer Patients and Survivors

NK Cell Responses

The existing literature describing the effects of aerobic exercise on immune system function in cancer patients is limited. Currently, there are 19 original investigations examining the impact of aerobic exercise on some component of immune function in patients and survivors of various cancers including breast, lung, gastric, prostate, and leukemia (15-33), and of these only 3 have performed these investigations using breast cancer patients and survivors (15, 17, 23). Furthermore, only one of these 6 studies investigated the NK cell response to an acute bout of aerobic exercise (19). In the other 5 studies, resting NK cell counts and/or NKCA were measured before and after an exercise training intervention in an effort to determine whether exercise training could potentially result in enhanced immune function in this population of individuals.

Shore et al. (19) examined the NK cell response to acute aerobic exercise at anaerobic threshold in 6 pediatric cancer patients, mostly treated for acute lymphoblastic leukemia. Four of the six patients were currently undergoing chemotherapy. All 6 subjects participated in a cycle ergometry ride at anaerobic threshold for 30 minutes. NK cell counts and NKCA were measured from blood samples were drawn pre-exercise, immediately post-exercise, and at 30 minutes post-exercise. Three of the patients participated in a 12-week exercise training intervention that consisted of moderate-vigorous aerobic activities (70-85% of maximum heart rate) for 30 minutes per session for 3 sessions per week. The other 3 patients served as a control group. At the end of the 12-week period, the exercise bout at anaerobic threshold

was repeated. The authors observed that both NK cell counts and NKCA were suppressed in the 3 patients who were receiving chemotherapy, compared to the two patients who were not receiving chemotherapy as well as to responses seen in healthy children. However, the authors do not describe a detailed NK cell response to the aerobic exercise bouts; i.e, the magnitude the change in NK cell counts and activity relative to pre-exercise levels.

Peters et al. (15), Nieman et al. (17), and Fairey et al. (23) all examined the effect of aerobic exercise training on NK cell counts and/or NKCA in breast cancer patients and survivors. Peters et al. (15) examined the effect of a 7-month moderate aerobic exercise intervention on NK cell counts and NKCA in 24 breast cancer patients who were at least 6 months post-surgery, Subjects performed moderate-intensity cycling (60-86% of heart rate reserve) for 30-40 minutes per session for 2-5 sessions per week. Resting NK cell counts and NKCA were measured before the intervention, 5 weeks into the intervention, and at completion of the intervention. Resting NK cells increased by approximately 15% from pre-intervention to post-intervention; however, the increase was not statistically significant. Resting NKCA, measured using an isolated-cell ^{51}Cr release assay, did significantly increase by approximately 83% from pre-intervention to post-intervention ($p < 0.05$).

Nieman et al. (17) investigated the effect of an 8-week moderate aerobic exercise intervention on NK cell counts NKCA in 12 breast cancer survivors who were an average of 3 years post-diagnosis. Six of the subjects participated in moderate-intensity walking (75% of maximum heart rate) for 60 minutes per session for 3 sessions per week. The other six subjects served as a sedentary control group. Resting NK cell counts and NKCA were measured before and after the intervention. Resting NK cell counts and NKCA did not change significantly over the course of the intervention period for either the exercise group or

the control group, although subjects in the exercise group were observed to have slightly higher NKCA % lysis compared to the control group ($p = 0.06$). The authors concluded that the exercise intervention may not have been of long enough duration or high enough intensity to elicit a change in resting NK cell function, although the safety of having cancer patients participate in high-volume, high-intensity exercise training is not yet known.

Fairey et al. (23) studied the effect of a 15-week moderate to vigorous exercise intervention on multiple immune system parameters including NK cells, in 53 post-menopausal breast cancer survivors who were an average of 14 months post-treatment. Twenty-five subjects participated in moderate to high intensity cycling (50-75% of VO_{2peak}) for 15-35 minutes per session for 3 sessions per week. The other 28 subjects served as a sedentary control group. Resting NKCA was measured before and after the intervention using a ^{51}Cr release assay with 5 E:T ratios ranging from 3.125:1 to 50:1. Significant differences between the exercise group and the control group were observed for changes in NKCA pre-intervention to post-intervention at all E:T ratios, where subjects in the exercise group experienced increased NKCA and subjects in the control group largely experienced decreases in NKCA ($p < 0.001$-0.41). The authors found that exercise training improved NKCA at all 5 E:T ratios by 6.45%. Lytic units, the number of NK cells required to lyse 30% of the target cells, decreased by 2.72 from pre-intervention to post-intervention, indicating the NKCA increased on a per-cell basis.

Na et al. (20) and Lee et al. (33) both investigated the effect of exercise training on NK cell response in gastric cancer patients. Na et al. (20) examined the effect of a 2-week moderate exercise intervention on NKCA in 35 stomach cancer survivors who had recently undergone curative surgery. Seventeen subjects participated in moderate intensity arm and

cycle ergometry exercise (60% of maximum heart rate) for 30 minutes twice per day for 5 days per week. The other 13 subjects served as a sedentary control group. Resting NKCA was measured on Days 1, 7, and 14 of the intervention using a ^{51}Cr release assay with an E:T ratio of 50:1. The authors observed that NKCA did not change from Day 1 to Day 7 for either the control group or the exercise group. From Day 7 to Day 14, NKCA increased in the exercise group by approximately 91%, whereas NKCA decreased in the control group by approximately 26% ($p < 0.05$ comparing the changes between groups). The authors suggested that NKCA may have shown significant increases after the exercise intervention because NKCA levels were already low at baseline (% lysis = 16.2%). The low NKCA observed for subjects in the study was likely attributed to the recent stress of gastric surgery; therefore, sensitivity to exercise-induced immunological change could have been increased.

Lee et al. (33) examined the effect of a 24-week light to moderate exercise intervention on multiple immune parameters including NK cell counts, in 21 gastric cancer survivors who were at least 2 years post-surgery. Subjects participated in weekly tai chi exercise sessions of 50-60 minutes in duration. Resting NK cell counts were measured before and after the intervention. The percentage of NK cells in circulation did not change over the course of the intervention ($p = 0.786$).

The NK cell response to chronic aerobic exercise in the cancer patient population has shown varied results. Peters et al. (15), Nieman et al. (17), and Lee et al. (33) did not observe statistically significant changes in resting NK cell counts from pre- to post-intervention. Similarly, Nieman et al. (17) did not observe significant changes in resting NKCA from pre-to post-intervention. However, Peters et al. (15), Fairey et al. (23), and Na et al. (20) did observe marked increases in NKCA over the course of their respective

intervention periods. Reasons for these differences in magnitude and direction of the NK cell response to exercise training may result from a variety of factors, including differences in the timing of the exercise interventions relative to the amount of time that had elapsed since patients had completed treatment, differences in the exercise session intensity, frequency, and duration of the interventions, differences in total intervention time, and differences in experimental design and patient population (23). NKCA may also be influenced by other biological factors including nutritional state, anti-cancer therapy, circadian rhythm, fatigue, and depression, which could have also differed among these studies (20). For those studies which have observed changes in resting NKCA due to exercise training, several mediating mechanisms have been proposed, including exercise-induced immune modulation of cytokines, activation and changes in signal transduction of NK cells, macrophages, neutrophils, and tumor infiltrating lymphocytes, changes in the expression of cell adhesion molecules, and alteration in prostaglandins (20). While the clinical implications of altered NK cell responses to exercise are still not completely known, it seems that exercise training may improve NKCA beyond what is associated with normal recovery after cancer therapy, and this enhanced NKCA may be of great importance because the role of NK cells in the destruction of tumor cells and potential correlation with disease-free and overall survival (15, 23).

Other Immune System Responses

Additional immune system outcomes that have been investigated in the cancer patient population include exercise-induced responses of the other cellular components of innate immunity (i.e., neutrophils and monocytes), the cellular components of adaptive immunity (i.e., T and B lymphocytes), and soluble factors such as inflammatory cytokines and C-

reactive protein (16-19, 21-33). As was the case for the studies involving NK cells, these other studies have largely examined changes in immune function in response to exercise training interventions that were generally of moderate to vigorous intensity for 15-60 minutes per session, 3-5 sessions per week for 3 weeks-7 months. The results of these studies have been mixed. One study has shown that exercise training could lead to decreases in some immune parameters (19). Some studies have shown that exercise training does not seem to significantly alter resting immune function in cancer patients or survivors (16, 21, 23, 24, 26, 28, 29, 32, 33). Other studies have shown that exercise training may lead to improvements in resting immune function, including decreased resting C-reactive protein and inflammatory cytokine levels, increased total leukocyte and lymphocyte counts, increased lymphocyte activation, increased monocyte phagocytosis, and decreased duration of neutropenia (16, 18, 22, 24, 30, 31, 33). The overall conclusions of these studies seem to be that exercise training generally does not lead to decreased immune function in cancer patients and survivors, and that in some cases, exercise training may lead to improvements in cell counts and activity as well as decreases in markers of systemic inflammation. However, especially when considering cancer patients who may have depressed immune system parameters at the beginning of an intervention, exercise prescription should proceed with caution (19).

There are two studies which have examined the effect of acute aerobic exercise on immune parameters other than NK cells, both in pediatric cancer patients. Along with examining the effect of exercise on NK cells, Shore et al. (19) investigated the effect of the exercise bout at anaerobic threshold along with the 12-week exercise intervention on lymphocyte subsets. The authors observed that resting immune function, including total leukocyte count, total lymphocyte count, helper T lymphocytes, cytotoxic T lymphocytes,

and B lymphocytes, were depressed in the 4 patients receiving chemotherapy compared to normative data. In contrast, the resting immune function of the two patients who had completed chemotherapy fell within normal ranges. The authors also observed that the acute exercise bout produced similar lymphocyte responses compared to that seen in healthy children, with overall leukocyte and lymphocyte counts increasing post-exercise. However, cell counts were significantly lower for the patients undergoing chemotherapy, and there were no increases seen in total T lymphocytes, helper T lymphocytes, or cytotoxic T lymphocytes. Lymphocyte activation was also suppressed post-exercise in the patients undergoing chemotherapy, whereas it was within normal ranges for the patients who had completed chemotherapy. When examining the effect of the 12-week exercise training intervention on resting immune function, the 3 patients undergoing chemotherapy experienced even further decreases in resting total T lymphocytes, helper T lymphocytes, and cytotoxic T lymphocytes, although cell counts were still above the threshold needed for a major increase in susceptibility to infection or spontaneous neoplasia. Lymphocyte cytolytic activity did appear to increase over the course of the intervention period. The authors suggest that the small number of patients in the study may not be sufficient to draw definitive conclusions; however, caution should be applied when prescribing exercise in this patient population, especially for individuals whose immune systems may already be weakened by chemotherapy.

Ladha et al. (25) studied the effect of acute moderate-to-vigorous aerobic exercise on neutrophil response in 4 pediatric patients with acute lymphoblastic leukemia who were receiving maintenance therapy and 6 matched healthy controls. Subjects participated in a 30-minute treadmill exercise bout which alternated 10 minutes of running at 85% of VO_{2peak}, 10

minutes of walking at 70% of VO_{2peak}, and 10 minutes of running, again at 85% of VO_{2peak}. Neutrophil counts and activity were measured from blood samples taken pre-exercise, immediately post-exercise, and during recovery at 1 hour and 2 hours post-exercise. The authors observed that absolute neutrophil counts increased significantly from pre-exercise to immediately post-exercise by 26% in the patient group and 47% in the control group ($p = 0.011$). At 1 hour post-exercise, neutrophil counts decreased slightly below pre-exercise values by 3% in the patient group, whereas neutrophil counts remained elevated above pre-exercise levels by 33% in the control group. At 2 hours post-exercise, neutrophil counts were slightly above baseline values by 6% in the patient group, while remaining elevated by 49% in the control group. There were no significant differences in neutrophil counts between the two study groups at any time point. Neutrophil activity was markedly higher in the control group across all time points compared to the patient group by 54-70%, and neutrophil activity did not appear to vary considerably across time in either study group. The authors suggest that the decreased neutrophil function observed in the patient group may be related to immunosuppressive effects of chemotherapy. When examining the ratio of active neutrophils to resting neutrophils across time, the patient group displayed a considerably greater increase in neutrophil oxidative burst compared to the control group ($p = 0.048$-0.074). These results suggest that the patients with acute lymphoblastic leukemia experienced a greater per-cell activity compared to the control group upon neutrophil stimulation. The physiological significance of this finding is not clear, although it could be in part due to the low neutrophil oxidative burst activity observed in the patient group at rest. The authors conclude that the pediatric leukemia patients seem to respond in a generally similar fashion to the acute exercise bout compared with healthy matched controls, which

could be clinically important for a pediatric patient who wishes to engage in sports and exercise activities similar to their peers. As does Shore et al. (19), Ladha et al. (25) caution that further research is needed to understand the effects and optimal prescription of acute exercise on immune function in the cancer patient population.

After examining the current exercise immunology literature in the cancer patient population, it is apparent that many questions have yet to be answered. In particular, the effect of acute aerobic exercise on cellular immune system responses must be further elucidated, as only two studies to date have attempted to examine this particular research question in cancer patients. Additionally, no study has attempted to examine potential mediators of the exercise-induced immune response in cancer patients, including catecholamines and cortisol. In order to determine whether exercise in cancer patients and survivors may reduce the risk of illness and cancer recurrence/secondary malignancies and increase overall survival, researchers and clinicians must understand how exercise affects all physiological systems, including the immune system, so that the most relevant and specific exercise prescription guidelines can be developed that will hopefully allow cancer patients and survivors to reap the maximum health benefits of exercise without putting themselves at risk for further illness.

Conclusion

In summary, NK cells are a major component of the innate immune system necessary for protecting the body against the invasion of foreign pathogens and for killing tumor cells. These immune cells are also extremely responsive to acute aerobic exercise. They display marked increases immediately post-exercise and then returning to pre-exercise levels or decreasing below pre-exercise levels during the recovery period. Many studies have been

performed examining the NK cell response to aerobic exercise in healthy individuals, both athletic and non-athletic, and it seems that higher intensity and/or longer duration exercise produces a more pronounced response compared to exercise of moderate intensity/shorter duration. Additionally, these NK cell responses are associated with exercise-induced changes in the stress hormones epinephrine, norepinephrine, and cortisol. However, the literature examining exercise-induced NK cell responses in cancer patients and survivors is limited, and no study has examined biological factors that might mediate the immune response in this clinical population. As a result, many questions remain to be answered; such as, will the immune system of cancer patients/survivors respond similarly to that of healthy individuals when undergoing acute aerobic exercise? Will similar biological factors be at work to mediate the exercise-induced immune response in cancer patients/survivors and healthy individuals? These questions, among others, are addressed in the three manuscripts included in this dissertation.

CHAPTER III

THE IMPACT OF ACUTE AEROBIC EXERCISE ON NATURAL KILLER CELL RESPONSES IN BREAST CANCER SURVIVORS

Summary

Current research examining the effect of aerobic exercise on immune responses in cancer patients and survivors is limited. The purpose of this study was to examine the effect of one acute bout of aerobic exercise on natural killer (NK) cell numbers in breast cancer survivors and healthy controls. For a subset of subjects, NK cell activity (NKCA) was also explored. Participants included 9 women who had completed major treatments for Stage I-III invasive breast cancer within 3-6 months of enrollment and 9 sedentary women without a history of cancer. Subjects completed a 30-minute bout of exercise on the cycle ergometer at 60% of VO_{2peak}. Whole blood samples were taken pre-exercise, immediately post-exercise, 2 hours post-exercise, and 24 hours post-exercise. Complete blood counts were obtained at each time point. Proportions of lymphocytes that carry the NK cell phenotype were obtained via flow cytometric analysis. NK cell activity (NKCA) was assessed for 6 breast cancer survivors and 1 control using a whole-blood flow cytometric assay. In both study groups, NK cell counts showed significant increases immediately post-exercise ($p < 0.05$). At 2 hours and 24 hours post-exercise NK cell counts had returned to near pre-exercise levels in both groups ($p = 0.161$-0.381). Absolute NK cell counts were somewhat lower in breast cancer survivors pre-exercise and immediately post-exercise ($p = 0.069$-0.090), although the magnitude of change in NK cell counts was similar between groups across time. NKCA was

not significantly different across the study time points in the subset of breast cancer survivors. Although some differences in absolute NK cell counts may exist between the groups, recent breast cancer survivors seem to exhibit a normal NK cell response to acute moderate intensity aerobic exercise as compared with similar women without a history of cancer.

Introduction

Breast cancer is the most common cancer among women in the United States, other than skin cancer, and it is the second-leading cause of cancer deaths in women, after lung cancer (1). Major treatments for breast cancer, that include surgery, chemotherapy, and radiation therapy, can cause significant physical distress that may persist for months or years after the completion of therapy (2). To this end, numerous studies over the past 20 years have attempted various strategies to alleviate these complications, with many focusing on exercise. Many of these "first generation" studies have shown that exercise training can mitigate many treatment-related side-effects, improve physical functioning, and that regular exercise seems to be safe, well-tolerated, and is associated with few adverse events (6-8).

Exercise may be an attractive adjunct therapy for cancer patients and survivors because of its potentially positive influence on the immune system (9). One particular component of the immune system, the natural killer (NK) cell, is an especially relevant because of its ability to target and kill virally-infected cells as well as cells that have undergone malignant transformation (10). In healthy individuals, both athletic and non-athletic, NK cells are extremely responsive to acute aerobic exercise. They follow a profile which typically shows marked elevations in both cell number and activity immediately post-exercise, while displaying decreases during the recovery period for up to several hours (10-

14). Although not all studies show entirely consistent outcomes, and the clinical implications of exercise-induced changes in NK cell responses are not entirely known, it is thought that exercise-related enhancements in NK cell function may confer a protective effect against pathogen invasion, while exercise-related decreases in NK cell function may lead to increased incidence of viral infection and potential illness (12, 13).

NK cells are extremely responsive to acute exercise, and they provide the body with a first line of defense against pathogens and tumor cells, thus, understanding how acute aerobic exercise may influence their cytolytic activity, as well as their entry into/exit from circulation is clinically relevant. Examining and understanding NK cell responses to acute aerobic exercise in breast cancer survivors is particularly important when considering how to prescribe exercise to this population. Firstly, aerobic exercise prescriptions for cancer patients and survivors are largely modeled after those for the general population (39). While much research has been conducted in healthy individuals, immune responses to exercise have not been systematically measured in breast cancer survivors. Since cancer treatments can lead to immune system deficiencies which may continue for extended periods of time post-treatment (23, 24, 98), one could hypothesize that a cancer patient's weakened immune system may not respond to exercise in the same way as that of a healthy individual. Secondly, aerobic exercise prescriptions are constructed of an accumulation of multiple acute exercise sessions per week. Therefore, exercise specialists and clinicians need to understand how long it takes for a breast cancer survivor's immune system to recover from one bout of aerobic exercise before prescribing the next bout of aerobic exercise. Current studies examining NK cell responses to exercise in breast cancer patients and survivors have shown promising results (15, 17, 23). However, these studies only examined resting NK cell

function at the beginning and end of an exercise training period, none examined the acute exercise response, and none compared these responses to individuals who had never experienced the physiological toll of cancer treatments. Studying these parameters may provide insight to whether the exercise is contributing to enhanced immune function or leading to periods of potential immunosuppression. Therefore, the purpose of this investigation was to compare the effect of one acute bout of aerobic exercise on NK cell numbers in breast cancer survivors and healthy controls. For a subset of subjects, the effect of the acute bout of aerobic exercise on NK cell activity (NKCA) was also explored.

Methods

Subjects

Subjects were recruited into two study groups: a breast cancer survivor group and a control group. Subjects in the breast cancer survivor group included women who had been diagnosed with Stage I, II, or II invasive breast cancer, had received chemotherapy, and had completed all planned surgery, chemotherapy, and radiation therapy 3-6 months prior to enrollment. Subjects in the control included women who did not have a history of cancer diagnosis or treatment, were sedentary (i.e., had not participated in regular organized physical activity within the past year), and were free from cardiovascular and musculoskeletal disease that would render aerobic exercise participation unsafe. All subjects, regardless of study group, were between the ages of 40-70 years, were not regular users of anti-inflammatory medications, and were either post-menopausal or had not experienced a menstrual period for approximately 1 year prior to enrollment. Subjects in the breast cancer survivor group were recruited from the Medical Oncology clinic at the North Carolina Cancer Hospital, the Radiation Oncology clinic at the North Carolina Cancer

Hospital, and from the waitlist for the Get REAL and HEEL Breast Cancer Program in the Department of Exercise and Sport Science at UNC-Chapel Hill. Subjects in the control group were recruited from the faculty, staff, and student populations at UNC-Chapel Hill, as well as from across the Triangle region of North Carolina (Raleigh, Durham, Chapel Hill, and surrounding areas). Approval from the Protocol Review Committee in the Lineberger Comprehensive Cancer Center and the Institutional Review Boards in the Department of Exercise and Sport Science and School of Medicine at UNC-Chapel Hill were obtained before proceeding with subject recruitment.

Overview of Procedures

Each subject visited the laboratory on three separate occasions. Prior to each laboratory visit, all subjects were asked to refrain from eating for at least 2 hours prior to testing, exercise and caffeine for at least 12 hours prior to testing, alcohol use for at least 48 hours prior to testing, and to maintain adequate hydration. Visit 1 included an orientation to the study, medical and physical screening, and the assessment of peak aerobic capacity on the cycle ergometer. During visit 2, the subject performed a moderate bout of aerobic exercise at 60% of VO_{2peak} for 30 min on the cycle ergometer. Blood samples were collected during this laboratory visit; immediately prior to exercise, immediately post-exercise, and 2 hours post-exercise. Visit 3 was a resting session, during which one blood sample was collected from the subject 24 hours post-exercise. All laboratory visits occurred in the Integrative Exercise Oncology Research Laboratory (IEORL) and/or in the Applied Physiology Laboratory (APL) in the Department of Exercise and Sport Science at UNC-Chapel Hill.

Visit 1: Medical/Physical Screening and VO_{2peak} test

During the first visit to the laboratory, subjects received an explanation of the study protocol, information regarding the potential risks of participation in the study, and were asked to sign the informed consent documents. All subjects completed a comprehensive medical history questionnaire, underwent a 12-lead resting electrocardiogram (ECG) and a physical exam by either a physician or other certified professional. Subjects were screened for exclusion based on the criteria put forth by the American College of Sports Medicine (ACSM) as contraindications to exercise testing (99).

Age, race, height (portable stadiometer, Perspective Enterprises, Portage, MI), and body mass (calibrated balance-beam scale, Detecto, Webb City, MO), were recorded for all subjects upon enrollment onto the study. Height and body mass were used to calculate body mass index (BMI). Percent body fat was assessed using a Discovery Dual Energy X-ray Absorption (DEXA) scanner (Hologic, Inc., Bedford, MA). For subjects in the breast cancer survivor group, cancer treatment type was also recorded.

The final component of visit 1 was an exercise test to measure VO_{2peak}. The purpose of measuring VO_{2peak} was to assess peak aerobic capacity and for estimating the submaximal workload that subjects would perform during laboratory visit 2. The VO_{2peak} test was performed on an electronically-braked cycle ergometer (Lode, Gronigen, The Netherlands) using the Astrand Cycle Ergometer Maximal Test Protocol (100). Expired gases were collected and analyzed using a TrueMax 2400 Metabolic System (Parvo Medics, Salt Lake City, UT). Subjects began the test sitting quietly on the cycle ergometer for 3 minutes while resting metabolic data was collected. Subjects then began the first stage of the test by cycling at 50 Watts for 3 minutes. At the end of the 3-min stage, the workload was increased by 25

Watts every 3 minutes until volitional fatigue. Heart rate, rating of perceived exertion or RPE (101), expired gases, and 12-lead ECG monitoring were performed throughout the test. Heart rate and RPE were recorded at the end of every minute. The highest VO_2 measured by the metabolic system during the last stage of the test was recorded at the subject's VO_{2peak}, and the corresponding workload on the cycle ergometer was recorded as the subject's peak workload. Once the VO_{2peak} test was complete, the subject pedaled at a very low workload (<20 Watts), with ECG and blood pressure monitoring continued until they had returned to near-baseline levels (102).

VO_{2peak} was calculated by the metabolic system, while the peak workload (W) was the workload at which the subject was exercising when VO_{2peak} was achieved. From these results, the workload (W) at 60% of VO_{2peak} was estimated following the Karvonan procedures, by regressing VO_2 values for each stage against the corresponding workload for that stage. This intensity was chosen because it is widely used in exercise prescriptions for this population (3, 4, 6, 17, 39, 103-108). .

Visit 2: Acute Aerobic Exercise Session

During the second visit to the laboratory, subjects performed an acute bout of intermittent, moderate exercise on the cycle ergometer at a workload corresponding to 60% of the subject's VO_{2peak} for 30 minutes. To ensure that all subjects would be able to complete the exercise session, a discontinuous protocol was employed such that subjects alternated ten, 3-minute intervals of exercise with 1.5 minutes of rest, for a total of 30 minutes of exercise in a 43.5-minute period. All subjects started the exercise session between 7:00-10:00 am in order to control for daily variations in the study variables.

At the beginning of visit 2, subjects rested in the supine position for approximately 20 minutes while a catheter was inserted into an antecubital vein in the arm for blood sampling. For blood sampling a three-stage technique was used. The first draw was a small amount to discard the contents of the catheter. The second draw contained the sample. The third injected a small amount of sterile saline to keep the catheter patent between sampling. A pre-exercise blood sample was drawn into K_3EDTA Vacutainer® tubes which were used for the measurement of NK cell counts. For a subset of subjects, blood was also drawn into a sodium heparin Vacutainer® tube and was used for the measurement of NK cell activity (NKCA).

After the pre-exercise blood sample was obtained, subjects sat quietly on the cycle ergometer for 3 minutes while resting metabolic data was collected. Subjects then performed a warm-up period, consisting of 4-5 minutes of light pedaling at about 30 Watts on the cycle ergometer. Subjects were also given the opportunity to stretch the lower body in a manner that was most comfortable for them. The subjects then began the 30-minute moderate-intensity exercise bout at the prescribed workload. Heart rate and RPE were recorded at the end of every 3-minute period of exercise. Expired gases were monitored during the first, third, seventh, and tenth exercise interval. If necessary, adjustments in workload were made to ensure that the subject was exercising as close to 60% of VO_{2peak} as possible. Immediately upon completion of the exercise bout, subjects dismounted the cycle ergometer and returned to the supine position. Blood sampling immediately post-exercise was performed in the same manner as described above.

Subjects were then allowed to rest comfortably in the laboratory. They were allowed to drink water ad libitum; however, no food or other beverage could be ingested. A final

blood sample was obtained at 2 hours post-exercise in the same manner as the other two samples. The catheter was then removed and any necessary bandaging was performed.

Visit 3: 24-hour Follow-up Session

The purpose of the third visit was to obtain a blood sample 24 hours after the completion of the aerobic exercise bout. The blood sample was drawn from an antecubital vein using a standard venipuncture technique.

Determination of Immunological Parameters

Complete blood counts (CBC) were obtained from all blood samples using a COULTER® Ac•T diff ™ Hematology Analyzer (Beckman Coulter, Inc., Brea, CA). Whole blood samples were stained using fluorescently-labeled monoclonal antibodies for the cell surface markers CD3, CD16, and CD56. The specific fluorescent labels were, fluorescein isothyocyanate (FITC) for the CD3 marker, phycoerythrin (PE) for the CD16 marker, and allophycocyanin (APC) for the CD56 marker. Fluorescent staining was done in order to identify the proportion of lymphocytes that express the $CD3^{-}CD16^{+}CD56^{+}$ phenotype which is characteristic of NK cells. Stained samples were further prepared for flow cytometric analysis according to procedures adapted from protocols made available by Becton Dickenson (109). Samples were fixed in a 1% paraformaldehyde solution and were analyzed within 24 hours of preparation using a CyAn 3 laser/9 color flow cytometer (Beckman Coulter, Inc., Brea, CA). Flow cytometry data was viewed and analyzed using Summit 4.3 software (Dako North America, Inc., Carpinteria, CA). Absolute NK cell counts were calculated by multiplying total lymphocyte counts by the NK cell proportions.

NK cell activity (NKCA) was determined for a subset of subjects (1 control and 6 breast cancer survivors) using a whole-blood flow cytometric assay adapted from Fondell et

al. (110). K562 cells were used as the target cells and were adjusted to a concentration of 200,000 cells/mL in a complete culture medium (RPMI + penicillin-streptokinase + 10% fetal bovine serum). The following reactions were prepared for each subject: 1) 500 μL whole blood + 500 μL whole blood (one tube for each study time point); 2) 500 μL K562 cells + 500 μL complete culture medium (two tubes); and 3) 500 μL whole blood + 500 μL K562 cells (two tubes for each study time point). The tubes were centrifuged at 200 x g for 1 minute and then placed in an air incubator at 37°C in 5% CO_2 for 2 hours. Samples were then stained for 15 minutes with FITC-labeled anti-CD71 (a fluorescently-labeled monoclonal antibody that only stains the K562 target cells). Two mL of 10X BD FACS Lysing Solution diluted to a 1X concentration were added to each tube in order to lyse the red blood cells, and the samples were incubated for 20 minutes. The tubes were then centrifuged at 500 g for 5 minutes, and the supernatant was removed. The remaining cell pellets were re-suspended in 500 μL of phosphate-buffered saline (PBS). Samples were analyzed within 2 hours of preparation using a CyAn 3 laser/9 color flow cytometer, and data was viewed and analyzed using Summit 4.3 software. NKCA, measured as % NK cytolysis, was calculated using the following equation:

%NK cytotox = 100*[target cells – (mixed cells – effector cells)] / target cells.

The "target cells" refers to the number of K562 cells measured in the samples with target cells only and "mixed cells" refers to the number of remaining K562 cells in the samples with a mixture of whole blood and K652 cells. "Effector cells" refers to cells in the samples with whole blood only which may be present in the region for $CD71^+$ K562 cells.

Calculation of Plasma Volume Shifts

Plasma volume shifts (changes in plasma volume due to exercise) were calculated according to the equation by Dill and Costill (111). The hematocrit and hemoglobin values that were used in these calculations were obtained from the CBC data pre-exercise immediately post-exercise, 2 hours post-exercise, and 24 hours post-exercise. Plasma volume shifts were reported in order to estimate the effect that exercise and posture had on fluid shifts, which may affect concentrations of leukocytes.

Statistical Analysis

All statistical analyses were performed using SPSS Statistics version 18.0. Comparison of physical characteristics (age, height, weight, BMI, and percent body fat) between the two study groups was performed using independent samples t-tests. Comparison of VO_{2peak}, peak workload, submaximal VO_2, and submaximal workload between the two study groups was also performed using independent samples t-tests. Paired-samples t-tests were used to compare the estimated submaximal VO_2 and workload to the actual submaximal VO_2 and workload that each study group performed during the acute aerobic exercise bout. Plasma volume shifts were compared between groups at the three post-exercise time points using independent samples t-tests. Immune cell counts (total leukocyte, granulocyte, monocyte, lymphocyte, and NK cell counts) were compared across the four study time points and between the two study groups using 2x4 mixed-model ANOVAs. NKCA was compared across time for the subset of breast cancer survivor subjects using a repeated-measures ANOVA. Pairwise comparisons were made between groups using independent samples t-tests and within groups across time using repeated-measures ANOVAs. Percent changes were also calculated for NK cell counts and NKCA for both study groups in order to examine

how these parameters were changing from pre-exercise to each of the three post-exercise time points. Percent changes were compared between groups using independent samples t-tests.

Results

Subjects

This study included a total of 18 subjects, with 9 subjects in the breast cancer survivor group and 9 subjects in the control group. Physical characteristics for the 18 subjects are presented in Table 3.1. Descriptive statistics are presented as mean ± standard deviation (SD). Age was the only physical characteristic for which there was a statistically significant difference between groups, where the average age of the control group was approximately 9 years older than age of the breast cancer survivor group ($p = 0.002$). Although the BMIs do not suggest obesity, the percentage of body fat (~42%) for both groups was very high. In addition, the VO_{2peak} results suggest very low aerobic fitness for both groups.

All subjects in the breast cancer survivor group received surgery, chemotherapy, and radiation therapy and had completed these treatments within 3-6 months prior to enrollment. Four subjects underwent mastectomy and five underwent lumpectomy. Six subjects received the chemotherapy combination of Adriamycin, Cytoxan, and Taxol, and one of these subjects also received Carboplatin. One subject received the chemotherapy combination of Cytoxan and Taxotere. Two subjects received the chemotherapy combination of Carboplatin and Taxotere. Six subjects were receiving adjuvant hormonal therapy, with 5 receiving Tamoxifen and one receiving Femara. Two subjects were receiving adjuvant trastuzumab in the form of Herceptin. Lastly, two subjects received additional medications related to their

cancer treatments, with one receiving Lapatinib (a protein kinase inhibitor) and one receiving Bevacizumab (an angiogenesis inhibitor).

Table 3.1. Subject physical characteristics (mean ± SD)

Characteristic	Breast Cancer Survivor Group (n = 9)	Control Group (n = 9)
Age (years)	50 ± 6*	59 ± 5*
Race (# of women)	Caucasian (8) African American (1)	Caucasian (9)
Height (cm)	164.7 ± 5.8	163.8 ± 5.9
Weight (kg)	76.9 ± 12.6	77.7 ± 13.3
Body Mass Index (kg/m^2)	28.4 ± 5.0	28.9 ± 4.6
Percent Body Fat (%)	41.6 ± 4.5	42.1 ± 4.0
VO_{2peak} (mL/kg/min)	18.1 ± 2.7	18.5 ± 5.1
Peak Workload (W)	107 ± 19	106 ± 17

*$p < 0.05$ for comparing age between groups

Metabolic Responses

The submaximal VO_2 and workloads that were used during the aerobic exercise session are presented in Table 3.2. Estimated submaximal VO_2 and workload were calculated from the results of the VO_{2peak} test. Actual submaximal VO_2 and workload were the subjects actually performed during the exercise session. Estimated and actual submaximal VO_2 were similar between groups ($p > 0.05$) as were estimated and actual submaximal workload ($p > 0.05$). Within the breast cancer survivor group, actual submaximal VO_2 was significantly higher than estimated submaximal VO_2 ($p = 0.016$). Within the control group, there was no significant difference between estimated and actual submaximal VO_2 ($p = 0.376$).

Table 3.2. Metabolic responses during the submaximal aerobic exercise session (mean ± SD)

Parameter	Breast Cancer Survivor Group	Control Group
Estimated VO_2 (mL/kg/min)	$10.9 \pm 1.6^*$	11.1 ± 3.1
Actual VO_2 (mL/kg/min)	$12.1 \pm 1.3^*$	11.1 ± 1.8
Estimated Workload (W)	63 ± 12	66 ± 11
Actual Workload (W)	59 ± 9	62 ± 11

$^*p < 0.05$ for estimated vs. actual VO_2

Leukocyte Subset Cell Counts and Plasma Volume Shifts

Cell counts for total leukocytes, granulocytes, monocytes, and lymphocytes, as well as plasma volume shifts at each study time point are presented in Table 3.3. As mentioned previously, total leukocyte, granulocyte, monocyte, and lymphocyte counts were adjusted to reflect the exercise-induced shifts in plasma volume. In the breast cancer survivor group, blood samples were not able to be obtained from one subject immediately post-exercise (0h post-exercise) and from one subject at 2 hours post-exercise. In the control group, NK cell count at 24 hours post-exercise was not able to be determined for one subject because of difficulty obtaining enough blood for sample preparation and analysis. Therefore, mean substitution was performed to account for these missing data points. Exercise and recovery patterns of plasma volume shifts were similar between breast cancer survivors and controls ($p > 0.05$). Total leukocyte and leukocyte subset cell counts were significantly elevated immediately post-exercise compared with pre-exercise values ($p < 0.05$). At 2 hours post-exercise, total leukocyte, granulocyte, and lymphocyte counts remained significantly elevated relative to pre-exercise values in the breast cancer survivor group, while total leukocytes and lymphocytes counts remained significantly elevated in the control group ($p < 0.05$). When comparing groups, monocyte counts were somewhat lower in breast cancer survivors pre-

exercise ($p = 0.059$), while lymphocyte counts were consistently lower in the breast cancer group across time ($p = 0.013$-0.090).

Table 3.3. Immune cell counts and plasma volume shifts across time (mean ± SD)

Parameter	Breast Cancer Survivor Group	Control Group
Total Leukocytes (cells/μL)		
Pre-exercise	3666.7 ± 1659.1	4377.8 ± 1072.1
0h post-exercise	4890.9 ± 1833.4*	5459.5 ± 1092.6*
2h post-exercise	5239.8 ± 1524.0†	5505.6 ± 1173.1†
24h post-exercise	3611.9 ± 1372.7	4251.2 ± 1094.3
Total Granulocytes (cells/μL)		
Pre-exercise	2633.3 ± 1320.0	2844.4 ± 610.6
0h post-exercise	3554.0 ± 1342.9*	3517.6 ± 478.2*
2h post-exercise	3967.5 ± 1192.6†	3579.0 ± 677.4
24h post-exercise	2538.4 ± 1068.0	2683.8 ± 736.5
Total Monocytes (cells/μL)		
Pre-exercise	255.6 ± 133.3	400.0 ±165.8
0h post-exercise	364.6 ± 117.4*	442.5 ± 168.3*
2h post-exercise	307.1 ± 96.8	395.8 ± 178.5
24h post-exercise	306.4 ± 133.0	361.7 ± 138.0
Total Lymphocytes (cells/μL)		
Pre-exercise	788.9 ± 355.1	1111.1 ± 401.4
0h post-exercise	973.0 ± 462.7^{*a}	1508.6 ± 512.5^{*a}
2h post-exercise	941.3 ± 430.8$^{\dagger b}$	1530.6 ± 649.2$^{\dagger b}$
24h post-exercise	755.4 ± 309.8^{c}	1195.0 ± 355.4^{c}
NK Cells (cells/μL)		
Pre-exercise	70.3 ± 37.9	108.9 ± 51.7
0h post-exercise	170.5 ± 110.4*	282.5 ± 132.3*
2h post-exercise	91.8 ± 76.2	130.1 ± 80.2
24h post-exercise	90.1 ± 57.8	122.2 ± 57.8
Plasma Volume Shifts (%)		
0h post-exercise	-12.0 ± 3.6	-11.5 ± 4.6
2h post-exercise	-3.4 ± 6.2	-0.1 ± 9.2
24h post-exercise	-7.3 ± 6.7	-6.6 ± 7.1

$^{*}p < 0.05$ for pre-exercise vs. 0h post-exercise
$^{\dagger}p < 0.05$ for pre-exercise vs. 2h post-exercise
$^{a}p < 0.05$ for comparing 0h post-exercise between groups
$^{b}p < 0.05$ for comparing 2h post-exercise between groups
$^{c}p < 0.05$ for comparing 24h post-exercise between groups

NK Cell Counts

Cell counts for NK cells are also presented in Table 3. There was no significant group by time interaction effect ($p = 0.106$). In both study groups, NK cell counts were significantly elevated immediately post-exercise compared to pre-exercise levels ($p = 0.006$ and 0.001 for the breast cancer survivor group and the control group, respectively). At 2 hours and 24 hours post-exercise, NK cell counts were similar to pre-exercise levels in both groups ($p = 0.161$-0.381). When comparing groups, NK cell counts were somewhat lower in the breast cancer survivor group pre-exercise ($p = 0.09$) and immediately post-exercise ($p = 0.069$).

Percent changes for NK cell counts between study groups and across the three post-exercise time points are displayed in Table 3.4. These are presented in order to more clearly appreciate the magnitude of the NK cell response that took place post-exercise, relative to pre-exercise values. Percent change in NK cell counts post-exercise were similar between groups ($p = 0.651$-0.894).

Table 3.4. Percent change in NK cell counts (mean ± SD)

Percent change (%)	Breast Cancer Survivor Group	Control Group
0h post-exercise	165.6 ± 147.6	179.9 ± 89.3
2h post-exercise	26.8 ± 48.0	23.3 ± 61.0
24h post-exercise	31.4 ± 66.3	19.3 ± 42.2

NKCA % Cytolysis and Percent Changes

NKCA for a subset of 7 subjects (1 control and 6 breast cancer survivors) is presented in Table 3.5. There were no significant differences in NKCA (expressed as % cytolysis in 500 μL blood) across the study time points for the group of 6 breast cancer survivors ($p = 0.607$).

Table 3.5. NK cell activity across time for six breast cancer survivors and one control subject (mean ± SD)

Parameter	Breast Cancer Survivors (n = 6)	Control (n = 1)
NKCA (% target cell cytolysis in 500 μL blood)		
Pre-exercise	73.6 ± 30.3	95.2
0h post-exercise	74.4 ± 21.0	95.5
2h post-exercise	67.6 ± 12.5	91.4
24h post-exercise	60.2 ± 37.1	60.8
NKCA Percent Change (%)		
0h post-exercise	17.2 ± 47.8	0.4
2h post-exercise	22.7 ± 95.9	-4.0
24h post-exercise	16.9 ± 128.7	-36.1

Discussion

To the authors' knowledge, this investigation is the first to examine the effect of acute aerobic exercise on any cellular immune parameter in breast cancer survivors. Absolute NK cell counts were consistently lower in the breast cancer survivor group at each study time point compared to the control group, and this difference was most notable for the pre-exercise and immediately post-exercise time points. This study found that the magnitude of change for NK cell counts appears similar across time (0-24 hours post-exercise) when comparing the breast cancer survivor subjects and the control subjects, as evidenced by similar NK cell count percent changes for both study groups. Additionally, NKCA did not seem to change significantly from pre-exercise to any post-exercise time point in the group of 6 breast cancer survivors.

Changes in NK Cell Counts

As expected, NK cell counts increased significantly immediately post-exercise compared with pre-exercise, and then decreased towards pre-exercise levels at 2 hours and 24 hours post-exercise. A key finding was that the magnitudes of change in NK cell counts

(evidenced by the percent changes) were similar in the breast cancer survivor group and the control group. Immediately post-exercise, NK cell counts increased by an average of 166% in the breast cancer survivor group and 180% in the control group. At 2 hours and 24 hours post-exercise, NK cell counts were still approximately 19-31% above pre-exercise levels in the two groups. These findings are encouraging, in that they suggest that the breast cancer survivors seemed to experience similar NK cell number recruitment compared to the controls during and after 30 minutes of moderate intensity aerobic exercise. Additionally, neither the breast cancer survivors nor the controls appeared to experience below-baseline decreases in NK cell counts that are typically seen during the recovery period from exercise (10-14). These results are similar to those of previous studies, which have examined changes in NK cell counts in response to moderate intensity and moderate duration aerobic exercise in healthy men and women of various fitness levels (74, 94-96, 112, 113).

The current study did not directly explore the effects of potential biologic mediators of the NK cell response to the moderate-intensity acute aerobic exercise bout. However, it is still worth noting that there are several biological mechanisms which may be associated with the rise and fall of NK cell counts during and after moderate-intensity acute aerobic exercise. Increased cardiac output during exercise may act synergistically with increased catecholamine levels to affect vascular endothelial-lymphocyte adhesion, which would recruit NK cells from their marginal pools and other reservoirs (48-50, 75). During the recovery period, decreases in NK cell counts could be related to exercise-induced changes in catecholamines and cortisol; the latter of which may be mediated by changes in the inflammatory cytokine IL-6 (75). In the current investigation it was interesting to note that even though absolute NK cell counts were lower in the breast cancer survivor group, the

magnitude of change in NK cell counts was similar between groups. This suggests that even though the cancer treatments may have affected their resting immune counts (9), the exercise bout resulted in a response that paralleled the controls who had never experienced cancer treatment.

Changes in NKCA

NKCA as observed for the subset of 6 breast cancer survivors did not change significantly across the four study time points. This exploratory finding is interesting, since it was expected that NKCA would respond in a similar manner as NK cell counts. From a biological standpoint, the exact mechanisms driving these NKCA results are not clear. A similar result was observed by van der Pompe et al. (88) during and after graded exercise up to VO_{2max} in post-menopausal women, in which the authors speculated that a component of the signaling pathways triggered by agonist stimulation of the β-adrenergic receptors on NK cells may be impaired in this population of subjects (88). Other authors who have observed relatively small changes in NKCA post-exercise despite large changes in NK cell counts hypothesized that the NK cells that were recruited into circulation during exercise may have had decreased cytotoxic function compared to the NK cells circulating pre-exercise (48). In the current study, the sharp increases in NK cell counts post-exercise accompanied by the relatively constant NKCA across time may likely be more related increased cardiac output on NK cell cytosis rather than β-adrenergic stimulation, especially considering that the exercise bout was discontinuous and of moderate intensity and duration. It is interesting to note that NKCA for the group of breast cancer survivors appears quite different from the NKCA reported for the one control subject in the subset. However, no definite conclusions can be drawn from this comparison without data from more controls. Even so, it appears that the

aerobic exercise bout performed in this study did not compromise NKCA in the breast cancer survivors.

The type of assay used in this current investigation to measure NKCA was a whole blood flow cytometric assay. Some authors have suggested that whole-blood techniques may be preferable over isolated-PBMC techniques when evaluating NKCA (48, 52-54). When isolated-PBMC assays are performed, cells must be washed and are therefore not incubated with other immune and endocrine factors known to have autocrine and paracrine effects on immunity, thereby suggesting that NKCA determined using these methods may not completely represent what would actually be occurring in vivo (54). In contrast, whole-blood assays do not require washing of cells, so therefore, the "blood borne milieu" is retained, and therefore NKCA determined using these methods may more fully reflect the in vivo environment (48, 54). Additionally, there may be difficulty in comparing results using whole-blood techniques versus isolated-PBMC techniques because of the inability to establish equivalent effector to target cell ratios (54). The assay used in the current study could be considered novel, as whole-blood flow cytometric assays for NKCA do not appear to be common in the literature. Further investigation of NKCA in breast cancer survivors using other assay techniques may be warranted in order to allow for a more comprehensive picture of NK cell function in this population.

Strengths and Limitations

As with any study, there are inherent strengths and limitations. One strength is the homogeneity of the study sample and the inclusion of a comparative control group of women. With the exception of age, the two study groups were matched very closely with regard to the other physical characteristics and aerobic fitness. This study is one of the few

to describe immune responses to acute aerobic exercise in the oncology patient population and is the only study to investigate the effect of acute aerobic exercise on NK cells in breast cancer survivors. Additionally, this study is one of the few to also include NK cell responses to acute aerobic exercise in healthy post-menopausal women, as well as in a study sample with a low VO_{2peak} (< 20 mL/kg/min). One limitation to this study included small sample sizes in each group. Despite this, there was sufficient statistical power at the 0.80 power level to detect statistical in the primary outcome variable, which was the difference between NK cell count fold change and the null value of 1.0. Additionally, NK cell count data were not available for two cancer survivor subjects (one immediately post-exercise and one 2 hours post-exercise) and one control subject at 24 hours post-exercise because of difficulties obtaining blood samples from those subjects at those times. Furthermore, NKCA results were only available for 6 breast cancer survivors and one control subject, due to the difficult nature of measuring this parameter. As a result, group comparisons for NKCA could not be made. While the control subject for whom NKCA was measured would not have been considered an outlier for any other parameter, one cannot necessarily apply her NKCA results to the entire control group. Finally, potential biologic mediators of the NK cell response were not explored in this study. Therefore, biologic mechanisms driving these results are largely speculative, especially when concerning results pertaining to the breast cancer survivor group.

Conclusions

In summary, 30 minutes of intermittent, moderate intensity aerobic exercise seems to elicit similar changes in NK cell counts in recent breast cancer survivors compared to physically similar women without a history of cancer diagnosis or treatment. Some differences in absolute NK cell counts appeared to exist between the groups pre-exercise and immediately post-exercise. NKCA did not appear to change significantly in response to the acute exercise bout. These findings suggest that moderate intensity and duration aerobic exercise affected the study groups similarly, and that this type of aerobic exercise is safe to include in exercise prescriptions for recent breast cancer survivors.

CHAPTER IV

THE IMPACT OF ACUTE AEROBIC EXERCISE ON CATECHOLAMINE AND CORTISOL RESPONSES IN BREAST CANCER SURVIVORS

Summary

The hormones of the adrenal gland are especially sensitive to the effects of acute aerobic exercise, and are responsible for eliciting many cardiovascular and metabolic responses associated with exercise. Current literature examining the effect of acute aerobic exercise on adrenal gland hormones in the cancer patient population is limited. Therefore, the purpose of this study was to compare the effect of one bout of aerobic exercise on catecholamine (epinephrine and norepinephrine) and cortisol responses in breast cancer survivors and healthy controls. Nine women who had completed major treatments for Stage I-III invasive breast cancer within 3-6 months of enrollment and 9 sedentary women without a history of cancer diagnosis or treatment completed a 30-minute bout of aerobic exercise on the cycle ergometer at 60% of VO_{2peak}. Whole blood samples were taken pre-exercise, immediately post-exercise, 2 hours post-exercise, and 24 hours post-exercise. Plasma concentrations of catecholamines and cortisol were measured at each time point using ELISA techniques. Epinephrine increased significantly immediately post-exercise in the control group ($p < 0.05$), but did not change significantly in the breast cancer survivor group. Norepinephrine responses were similar between groups ($p > 0.05$). Cortisol decreased significantly immediately post-exercise and 2 hours post-exercise in the control group and at 24 hours post-exercise in the breast cancer survivor group ($p < 0.05$). These findings imply that catecholamine output from the adrenal medulla and the sympathetic nervous system as

well as hypothalamic-pituitary-adrenal (HPA) axis stimulation of cortisol release from the adrenal cortex may have been different for breast cancer survivors compared with healthy controls.

Introduction

The American Cancer Society estimated that there are over 2.9 million breast cancer survivors alive in the United States (1). However, major cancer treatment can affect virtually all of the body's physiological systems, causing significant physical and psychological distress that may persist for months or years after the completion of therapy (2). Over the past two decades, numerous studies have investigated the impact of exercise training on physical functioning in breast cancer patients and survivors (6, 7, 8). The primary endpoint of many of these investigations was the effect of exercise training on cardiorespiratory capacity. Other outcomes have also been addressed, including changes in muscular strength, body composition, functional quality of life, fatigue, anxiety, and self esteem (6).

In recent years, studies examining the impact of aerobic exercise on endocrine parameters in cancer patients have begun to emerge as well (27, 28, 30, 34, 114-120). These studies do suggest that positive changes in hormone levels after aerobic exercise training may be associated with favorable outcomes including decreased stress and decreased risk for developing cardiovascular or metabolic disease (117, 120). These findings are pertinent, as cancer survivors may be at a higher risk for developing comorbid conditions including cardiovascular disease as a result of their cancer treatments (27, 120). Additionally, examining the impact of aerobic exercise on the endocrine system in cancer patients and survivors may be important because of the role of certain hormones in mediating immune responses that are associated with anti-cancer defense (9).

Regarding response to acute aerobic exercise, one set of hormones that may be especially important to study are hormones of the adrenal gland, namely the catecholamines (epinephrine and norepinephrine) and cortisol. In healthy individuals, these hormone levels rise proportionally with respect to exercise intensity and duration, targeting multiple organs including the heart, blood vessels, skeletal muscle, adipose tissue, liver, and pancreas (56, 57). Thus, these hormones elicit a wide array of responses involving the cardiovascular and metabolic systems. For breast cancer survivors, certain cancer treatments may lead to deregulation in adrenal gland hormone release and hypothalamic-pituitary-adrenal (HPA) axis function (121-124). Therefore, it is plausible that their release during acute aerobic exercise could be affected as well, although studies examining hormonal responses to acute aerobic exercise in breast cancer survivors are scarce.

Understanding how these hormones respond to and recover from acute aerobic exercise will aid researchers and clinicians in constructing the most relevant and specific exercise guidelines for cancer patients and survivors; thus allowing a cancer patient or survivor to reap the maximum health benefits of exercise. Furthermore, since current exercise guidelines put forth by the American College of Sports Medicine for cancer patients and survivors are very similar to those for the general population, it is important to investigate and compare physiological responses to exercise between cancer patients and survivors and healthy individuals without a history of cancer. Therefore, the purpose of this study was to compare the catecholamine and cortisol responses to an acute bout of moderate-intensity aerobic exercise in breast cancer survivors and healthy controls.

Methods

Subjects

Participants in this study included women breast cancer survivors and healthy women who had never been diagnosed with or treated for cancer. All subjects were between the ages of 40-70 years, were not regular users of anti-inflammatory medications, and had not experienced a menstrual period for approximately 1 year prior to enrollment. Inclusion in the breast cancer survivor group further required that subjects had been diagnosed with Stage I, II, or II invasive breast cancer, had received chemotherapy, and had completed all planned surgery, chemotherapy, and radiation therapy 3-6 months prior to enrollment. Inclusion in the control group further required that subjects did not have a history of cancer diagnosis or treatment, were sedentary, and were free from cardiovascular and musculoskeletal disease that would render aerobic exercise participation unsafe. Subjects were recruited from the University of North Carolina at Chapel Hill (UNC-Chapel Hill) and the surrounding areas.

General Procedures

Study details have been previously discussed in Chapter III. Briefly, each subject completed three laboratory visits during the course of the study. During visit 1, subjects were introduced to the study, received a comprehensive medical and physical screening, and completed a VO_{2peak} test on the cycle ergometer to assess maximal aerobic power. During visit 2, subjects completed a 30-minute aerobic exercise bout on the cycle ergometer at a workload corresponding to 60% of VO_{2peak}. Blood samples were obtained immediately pre-exercise, immediately post-exercise, and 2 hours post-exercise. During visit 3, one resting blood sample was obtained at 24 hours post-exercise. Before each visit, all subjects were asked to follow a set of pre-assessment guidelines which included the following: no eating

for at least 2 hours prior to testing, no exercise for at least 12 hours prior to testing, maintaining adequate hydration, no caffeine for at least 12 hours prior to testing, and no alcohol use for at least 48 hours prior to testing. All subject testing took place in the laboratory facilities housed within the Department of Exercise and Sport Science at UNC-Chapel Hill.

Visit 1: Medical/Physical Screening and VO_{2peak} test

Subjects were informed of the study objectives, the potential risks of participation, and were asked to sign the informed consent documents previously approved by the Institutional Review Board. Subjects then completed a comprehensive medical history questionnaire, underwent a 12-lead resting electrocardiogram (ECG) and a physical exam. Subjects were screened for exclusion based on the criteria put forth by the American College of Sports Medicine (ACSM) as contraindications to exercise testing (99).

After subjects were cleared for participation, physical characteristics (i.e., age, race, height, body mass, and percent body fat) were recorded. For subjects in the breast cancer survivor group, details of their cancer treatments were recorded to include surgery type, chemotherapy medications received, whether or not radiation therapy was received, and the use of other medications relevant to the cancer treatment. Height and body mass were measured using a portable stadiometer (Perspective Enterprises, Portage, MI) and a calibrated balance-beam scale (Detecto, Webb City, MO). Body mass index (BMI) was calculated from height and body mass. Percent body fat was assessed using a Discovery Dual Energy X-ray Absorption (DEXA) scanner (Hologic, Inc., Bedford, MA).

Subjects then completed a cardiopulmonary exercise test to measure VO_{2peak} with the purposes of assessing peak aerobic capacity and calculating the submaximal workload that

subjects would perform during visit 2. The Astrand Cycle Ergometer Maximal Test protocol was performed on an electronically-braked cycle ergometer to assess VO_{2peak} (Lode, Gronigen, The Netherlands) (100). This is a multi-stage protocol, where subjects began by pedaling at a workload of 50 Watts for 3 minutes, and then progressed by increments of 25 Watts every 3 minutes until volitional fatigue. Resting metabolic data were collected for 3 minutes immediately before beginning the first stage. Heart rate and Rating of Perceived Exertion (RPE) (101) were recorded at the end of every minute of the test. Expired gases were collected and analyzed using a TrueMax 2400 Metabolic System (Parvo Medics, Salt Lake City, UT). Minute-by-minute heart rates were obtained from 12-lead ECG monitoring (GE CASE Cardiosoft V. 6.6 ECG diagnostic system, General Electric, Palatine, IL). VO_{2peak} was the highest VO_2 measured by the metabolic system during the last stage of the test. Peak workload was the workload on the cycle ergometer that corresponded to the subject's VO_{2peak}. Once the test was complete, subjects cooled down by pedaling at a very light workload (< 20 Watts). Monitoring continued for at least 5 minutes post-exercise and until heart rate and blood pressure had returned to near-baseline levels (102).

Visit 2: Acute Aerobic Exercise Session

During visit 2, subjects completed a 30-minute bout of moderate intensity aerobic exercise at a workload corresponding to 60% of VO_{2peak}. The 30-minute exercise bout was comprised of ten 3-minute exercise intervals alternating with 1.5 minutes of rest. This type of discontinuous protocol was used in order to ensure that all subjects would be able to complete the entire 30 minutes of exercise. All exercise sessions began between 7:00-10:00 am in order to control for daily fluctuations in the study variables.

Subjects rested in the supine position for approximately 20 minutes so that an angiocatheter could be placed in an antecubital vein in the arm for blood sampling. A pre-exercise blood sample was drawn into two 3-mL K_3EDTA Vacutainer® tubes. Subjects then sat quietly on the cycle ergometer for 3 minutes while resting metabolic data was collected. Subjects were then allowed to warm up for 4-5 minutes by pedaling at approximately 30 Watts for 4-5 minutes on the cycle ergometer, after which they were encouraged to lightly stretch the muscles of the lower body in a manner of their choice. After this was completed, subjects performed the 30-minute aerobic exercise bout at the workload which corresponded to 60% of VO_{2peak}. Expired gases were monitored during the first, third, seventh, and tenth exercise interval. Workload was adjusted as needed in order to ensure that subjects were exercising as close to 60% of VO_{2peak} as possible. Heart rate and RPE were recorded every 3 minutes. When the exercise bout was complete, subjects immediately dismounted the cycler ergometer and returned to the supine position. An immediate post-exercise blood sample was drawn into two 3-mL Vacutainer® tubes.

For the remainder of the visit, subjects rested in the laboratory. Subjects were not allowed to ingest any food or beverage with the exception of water. At 2 hours post-exercise, another blood sample was obtained in the same manner as previously described. The angiocatheter was removed and any necessary bandaging was performed.

Visit 3: 24-hour Follow-up Session

Subjects returned to the laboratory in order to obtain a resting blood sample at 24 hours post-exercise. A standard venipuncture technique was used to draw the blood was into two 3-mL K_3EDTA Vacutainer® tubes. Blood was sampled from an antecubital vein in the arm.

Perceived Stress Scale

At the beginning of visits 2 and 3, subjects were asked to complete the Perceived Stress Scale (PSS). The PSS is a 10-item questionnaire, where scores may range from 0-40, with higher scores denoting higher levels of stress. The PSS is a widely-used psychological instrument used tor measuring the perception of stress (125, 126). The purpose for administering the PSS was to assess subjects' perceptions of the stressors in their daily life, as these stressors could potentially impact resting values of the stress hormone variables in this study (epinephrine, norepinephrine, and cortisol).

Determination of Plasma Levels of Catecholamines and Cortisol

At the conclusion of laboratory visits 2 and 3, whole blood samples were centrifuged at 4°C at 3000 rpm for 10 minutes using an IEC Centra-8R refrigerated centrifuge (International Equipment Company, Needham Heights, MA). The plasma portion was isolated and stored in vials at -80°C until the time of analysis. Catecholamines were measured using a competitive binding, single antibody, solid phase epinephrine/norepinephrine ELISA kit (Abnova, Tapei City, Taiwan) with analytical sensitivities of 10 pg/mL and 50 pg/mL for epinephrine and norepinephrine, respectively. The reported mean intra-assay coefficients of variation for epinephrine ranged from 6.9-15.8%, and the reported mean inter-assay coefficients of variation ranged from 13.2-18.2%. The reported mean intra-assay precisions coefficients of variation for norepinephrine ranged from 9.8-16.1%, and the reported mean inter-assay coefficients of variation ranged from 8.5-15.0%. Samples were run in duplicate, with the exception of two control samples which were run in singlet due to limited plasma volume. Cortisol was measured using a competitive binding, double antibody, combination solid and mobile phase ELISA kit

(Abnova, Taipei City, Taiwan) with a sensitivity of 0.44 ng/mL. The reported mean intra-assay coefficients of variation for cortisol ranged from 6.2-9.4%, and the reported mean inter-assay coefficients of variation ranged from 8.6-15.0%. Samples were run in one batch and in duplicate.

Calculation of Plasma Volume Shifts

Plasma volume shifts (changes in plasma volume due to exercise) were calculated according to the equation by Dill and Costill (111). Complete blood counts were performed at each study time point using a COULTER® Ac•T diff ™ Hematology Analyzer (Beckman Coulter, Inc., Brea, CA), which yielded the hematocrit and hemoglobin values that were used in these calculations. Plasma volume shifts were reported in order to indicate the effect that exercise may have on fluid shifts, which may affect concentrations of catecholamines and cortisol.

Statistical Analysis

Age, height, body mass, BMI, percent body fat, VO_{2peak}, peak workload, submaximal VO_2, and submaximal workload were compared between the two study groups using independent samples t-tests. Submaximal VO_2 parameters and submaximal workload parameters were compared within the groups using paired-samples t-tests. Scores from the PSS were compared between the two study groups using independent samples t-tests and score comparisons within the groups were made using paired-samples t-tests. Plasma volume shifts immediately post-exercise, 2 hours post-exercise, and 24 hours post-exercise were analyzed between groups using independent-samples t-tests. Plasma concentrations of catecholamines (epinephrine and norepinephrine) and cortisol were analyzed using a 2x4 mixed model ANOVA. Pairwise comparisons were made within and between the groups

using repeated-measures ANOVAs and independent samples t-tests, respectively. Percent changes were also calculated for catecholamines and cortisol in order to examine how these parameters were changing from pre-exercise to each of the three post-exercise time points. Percent changes in hormone concentrations were compared between groups using independent samples t-tests. SPSS Statistics version 18.0 was used to perform all statistical analyses.

Results

Subjects

The 18 subjects were included in this study were equally divided into the breast cancer survivor group and the control group. Although the physical characteristics have been previously presented in Chapter III, Table 4.1 presents these for the reader's edification. Similarly, treatment details for the breast cancer survivor group are presented in Table 4.2 simply for edification. The control group was significantly older than the breast cancer survivor group by an average of 9 years ($p = 0.002$). There were no other statistically significant physical differences between the groups.

Table 4.1. Subject physical characteristics (mean ± SD)

Characteristic	Breast Cancer Survivor Group (n = 9)	Control Group (n = 9)	p-value
Age (years)	50 ± 6	59 ± 5	0.002
Race (# of women)	Caucasian (8) African American (1)	Caucasian (9)	--
Height (cm)	164.7 ± 5.8	163.8 ± 5.9	0.754
Weight (kg)	76.9 ± 12.6	77.7 ± 13.3	0.906
Body Mass Index (kg/m^2)	28.4 ± 5.0	28.9 ± 4.6	0.817
Percent Body Fat (%)	41.6 ± 4.5	42.1 ± 4.0	0.823
VO_{2peak} (mL/kg/min)	18.1 ± 2.7	18.5 ± 5.1	0.847
Peak Workload (W)	107 ± 19	106 ± 17	0.897

Table 4.2. Details of cancer treatments received by breast cancer survivor group (n = 9)

Treatment	Number of Subjects Receiving Treatment
Surgery	
Mastectomy	4
Lumpectomy	5
Radiation Therapy	9
Chemotherapy	
Adriamycin	6
Cytoxan	7
Taxol/Taxotere	9
Carboplatin	3
Hormonal Therapy	
Tamoxifen	5
Femara	1
Other	
Herceptin	2
Lapatinib	1
Bevacizumab	1

Metabolic Responses and PSS Scores

Submaximal VO_2 and workload were compared to determine if there were any differences in exercise intensity between the groups during the aerobic exercise session. Estimated submaximal VO_2 (i.e., what was calculated as 60% of VO_{2peak}) was similar between breast cancer survivors and controls (10.9 ± 1.6 mL/kg/min vs. 11.1 ± 3.1 mL/kg/min, p = 0.865), as was actual submaximal VO_2 (i.e., what was actually performed, 12.1 ± 1.3 mL/kg/min vs. 11.1 ± 1.8 mL/kg/min, p = 0.261). Actual submaximal VO_2 was significantly higher than estimated submaximal VO_2 in the breast cancer survivor group (p = 0.016). No significant differences existed between the groups for estimated or actual submaximal workload, within either group for estimated vs. actual submaximal workload, or within the control group for estimated vs. actual submaximal VO_2 (p = 0.084-0.626). Additionally, there were no statistically significant differences in PSS scores between the breast cancer survivor group and the control group for either laboratory visit 2 (15 ± 4 vs. 13

± 6, p = 0.511) or laboratory visit 3 (15 ± 5 vs. 12 ± 7, p = 0.305). Similarly, no significant differences existed when comparing PSS scores within the groups for laboratory visit 2 or 3 (p = 0.261-0.829).

Plasma Levels of Epinephrine, Norepinephrine, and Cortisol

Plasma concentrations of epinephrine, norepinephrine, and cortisol are shown in Table 4.3. As mentioned previously, hormone concentrations were adjusted to reflect shifts in plasma volume. Catecholamine and cortisol concentrations were not able to be determined for one breast cancer survivor subject immediately post-exercise and one breast cancer survivor subject 2 hours post-exercise because blood samples were not able to be obtained. Cortisol concentration was not able to be determined for one control subject 24 hours post-exercise because of limited plasma volume. Therefore, mean substitution was performed to account for these missing data. There was a significant Group by Time interaction effect for epinephrine (p = 0.040). Epinephrine levels were significantly elevated immediately post-exercise in the control group only (p = 0.022). At 2 hours post-exercise, epinephrine levels had somewhat decreased in the breast cancer survivor group (p = 0.053). When comparing groups, epinephrine levels were significantly elevated in the breast cancer survivor group pre-exercise, immediately post-exercise, and 24 hours post-exercise compared to the control group (p < 0.0005, p = 0.032, and p < 0.0005 respectively).

In the breast cancer group, plasma norepinephrine levels were somewhat elevated immediately post-exercise (p = 0.068) and were significantly elevated at 2 hours and 24 hours post-exercise compared to pre-exercise levels (p = 0.030 and 0.035, respectively). Post-exercise norepinephrine levels were not significantly different from the pre-exercise

levels in the control group (p = 0.120-0.363). Norepinephrine levels were similar between groups at each time point (p = 0.203-0.410).

For plasma cortisol, there was a significant group-by-time interaction effect (p = 0.018). In the breast cancer survivor group, cortisol levels at 24 hours post-exercise were significantly lower than pre-exercise levels (p = 0.004). In the control group however, cortisol levels were significantly decreased below pre-exercise levels immediately post-exercise and 2 hours post-exercise (p = 0.003 and 0.013, respectively). When comparing groups, cortisol levels were somewhat higher in the breast cancer survivor group immediately post-exercise (p = 0.064).

Table 4.3. Plasma hormone levels across time (mean ± SD)

Parameter	Breast Cancer Survivor Group	Control Group
Epinephrine (pg/mL)		
Pre-exercise	49.7 ± 9.9[a]	27.1 ± 4.9[a]
0h post-exercise	54.5 ± 12.7[b]	39.2 ± 14.9[*b]
2h post-exercise	42.8 ± 3.0	36.5 ± 15.6
24h post-exercise	47.9 ± 6.2[c]	28.6 ± 6.7[c]
Norepinephrine (pg/mL)		
Pre-exercise	258.0 ± 109.6	518.0 ± 555.3
0h post-exercise	507.2 ± 395.5	741.0 ± 728.1
2h post-exercise	524.9 ± 350.7[†]	709.0 ± 485.0
24h post-exercise	350.7 ± 105.3[‡]	282.2 ± 147.3
Cortisol (ng/mL)		
Pre-exercise	137.7 ± 73.2	147.7 ± 49.2
0h post-exercise	131.1 ± 77.5	75.3 ± 16.3[*]
2h post-exercise	93.3 ± 64.3	85.2 ± 31.2[†]
24h post-exercise	84.9 ± 49.8[‡]	116.4 ± 61.8

[*]$p < 0.05$ for pre-exercise vs. 0h post-exercise
[†]$p < 0.05$ for pre-exercise vs. 2h post-exercise
[‡]$p < 0.05$ for pre-exercise vs. 24h post-exercise
[a]$p < 0.05$ for comparing pre-exercise values between groups
[b]$p < 0.05$ for comparing 0h post-exercise values between groups
[c]$p < 0.05$ for comparing 24h post-exercise values between groups

Percent Changes in Plasma Hormones

Percent changes in plasma epinephrine, norepinephrine, and cortisol for the three post-exercise time points are shown in Table 4.4. These were calculated in order to more clearly understand how these parameters changed compared to pre-exercise levels for each group. When comparing groups, percent change in hormone levels after the acute aerobic exercise bout were generally similar, with a few exceptions. Percent change in epinephrine was somewhat lower in the breast cancer survivor group immediately post-exercise ($p = 0.081$) and significantly lower at 2 hours post-exercise ($p = 0.040$). Percent change in cortisol was somewhat increased in the breast cancer survivor group immediately post-exercise and in the opposite direction from the control group ($p = 0.054$).

Table 4.4. Percent change in plasma hormone levels post-exercise (mean ± SD)

Parameter	Breast Cancer Survivor Group	Control Group
Percent change in epinephrine (%)		
0h post-exercise	12.2 ± 30.9	44.8 ± 42.5
2h post-exercise	-11.5 ± 14.7[a]	35.9 ± 57.4[a]
24h post-exercise	-1.4 ± 18.7	6.0 ± 18.2
Percent change in norepinephrine (%)		
0h post-exercise	101.1 ± 110.5	122.4 ± 172.1
2h post-exercise	111.4 ± 107.6	131.5 ± 136.6
24h post-exercise	56.4 ± 75.5	12.5 ± 103.8
Percent change in cortisol (%)		
0h post-exercise	19.4 ± 76.4	-40.8 ± 34.0
2h post-exercise	-13.2 ± 66.7	-35.3 ± 30.9
24h post-exercise	-36.6 ± 24.8	-16.8 ± 45.0

[a] $p < 0.05$ for comparing 2h post-exercise values between groups

Discussion

To the authors' knowledge, this is the first study to examine the effect of acute aerobic exercise on any hormonal parameter in breast cancer survivors. Breast cancer survivors exhibited significantly higher plasma epinephrine levels compared to controls pre-exercise, immediately post-exercise and 24 hours post-exercise, as well as somewhat higher

cortisol levels immediately post-exercise. Compared to pre-exercise levels, plasma epinephrine increased significantly immediately post-exercise in the control group, whereas plasma norepinephrine significantly increased 2 hours and 24 hours post-exercise in the breast cancer survivor group. Plasma cortisol became significantly decreased in the breast cancer survivor group at 24 hours post-exercise, but was significantly decreased immediately post-exercise and 2 hours post-exercise in the control group.

Changes in Plasma Catecholamines

There were several novel findings regarding the catecholamine responses to the acute aerobic exercise session. Firstly, absolute plasma epinephrine levels were consistently higher in the breast cancer survivor group compared to the control group across time, and this difference was significant at the pre-exercise, immediate post-exercise, and 24 hours post-exercise. Since the majority of the epinephrine originates in the adrenal gland, this finding implies that the breast cancer survivors were experiencing increased adrenal gland activity compared to the controls. It is possible that these elevated epinephrine levels could be associated with elevations in pro-inflammatory cytokines (i.e., interleukin 1, 6, and tumor necrosis factor alpha); a scenario that is not uncommon in breast cancer survivors who are experiencing fatigue, cachexia, depression, and/or pain (127-129). The current study did not ask the breast cancer survivors to report treatment-related side effects, and inflammatory cytokines were not measured, but such associations certainly warrant further investigation.

With regard to metabolism and substrate utilization, Tosti et al. (130) observed that breast cancer survivors exhibited elevated blood glucose responses compared to healthy controls before and after short duration aerobic exercise sessions at 40-70% of VO_{2max}. Since epinephrine upregulates the process of glycogenolysis in order to release glucose into the

bloodstream, the findings of this current study and those of Tosti et al. (130) may be related. Blood glucose and lactate measurements were not taken during this study; however, such parameters should be investigated further.

The plasma norepinephrine significantly increased in absolute concentrations from pre-exercise to post-exercise time points in the breast cancer survivor group but not in the control group. While the intended intensity of the aerobic exercise session was estimated at 60% of VO_{2peak}, the breast cancer survivor group's actual submaximal VO_2 was slightly higher than 60% of VO_{2peak} (~67% vs. ~61%, $p = 0.016$), whereas the control group's estimated and actual submaximal VO_2 did not differ. Therefore, it is possible that by working at a higher relative intensity, the breast cancer survivors experienced increased sympathetic nervous system activity, and hence significant fluctuations in plasma norepinephrine levels.

Interestingly, the magnitude of catecholamine release during the 30-minute aerobic exercise bout was notably lower than what may be typically observed. For example, it is not uncommon for 20 minutes of exercise at 60% of VO_{2max} to cause plasma catecholamines to increase 2-3 times pre-exercise levels (57, 59-61). In the current study, average magnitudes of catecholamine increase immediately post-exercise ranged from 12-122% above pre-exercise levels. This somewhat "attenuated" response may be most directly related to the discontinuous nature of the aerobic exercise bout which alternated 3 minute intervals of exercise with 1.5 minute intervals of rest. Therefore, even though subjects exercised for a total of 30 minutes at approximately 60% of VO_{2peak}, the cumulative effect of the exercise session may not have elicited the same amount of physiological stress as would 30 minutes of continuous exercise at the same intensity, especially for healthy control subjects.

Additionally, the submaximal intensity performed during the aerobic exercise session was based off the subjects' VO_{2peak} test results, which may not have been equal to the subjects' true VO_{2max}.

Taken together, these results indicate that there were some significant differences in absolute plasma catecholamine concentration between the breast cancer survivors and healthy controls, (i.e., higher epinephrine levels in the breast cancer survivors) but that percent changes in catecholamines post-exercise were either notably lower in the breast cancer survivors (i.e., epinephrine immediately post-exercise and 2 hours post-exercise) or were similar between groups. After observing some of these group differences, one question that arises is whether or not there were any differences in perceived stress of the exercise between the groups. To examine this, an exploratory comparison of heart rate and RPE responses between groups was performed. No significant differences were observed between the breast cancer survivors and the controls for either % exercise intensity based on heart rate reserve (53.1 ± 14.6% vs. 56.1 ± 15.3%, $p = 0.679$) or RPE response (12 ± 1 vs. 12 ± 1, $p = 0.536$). Additionally, PSS scores reported by both groups during laboratory visit 2 were similar. This suggests that the higher absolute epinephrine concentrations seen in the breast cancer survivor group could be related to some pre-existing condition (i.e., a treatment-related side effect), but that the relative stress of the exercise on the sympathetic nervous system and the adrenal medulla was not necessarily greater.

Changes in Plasma Cortisol

In the control group, plasma cortisol significantly decreased immediately post-exercise and 2 hours post-exercise, but the reverse occurred in the breast cancer survivor group. Additionally, percent change in cortisol immediately post-exercise was somewhat

different between groups. These divergent responses were somewhat unexpected, and the exact mechanisms driving these results are not entirely clear. Elevation in plasma cortisol levels seems to follow a threshold effect, where exercise intensities of > 50% of VO_{2max} appear to elicit increased plasma cortisol concentrations, and intensities below this threshold are either not stressful enough, or do not cause significant decreases in blood glucose levels (57). The fact that the breast cancer survivors exercised at a slightly higher relative intensity during the exercise trial may have led to increased activation of the hypothalamic-pituitary-adrenal (HPA) axis, thus resulting in higher plasma cortisol release during exercise. Although HR and RPE response and relative output of catecholamines were largely similar between groups, the exercise seemed to place a greater amount of stress on the HPA axis of the breast cancer survivors, thus yielding greater plasma cortisol concentrations. Additionally, the discontinuous nature of the aerobic exercise session may not have elicited the same amount of physiological stress as would a continuous aerobic exercise session of the same intensity and duration. For the control group, this could mean that the exercise may not have reached the threshold that is necessary for eliciting an increase in plasma cortisol concentration, and that the decreases in plasma cortisol may have occurred because the rate of removal exceeded the rate of secretion (57).

Strengths and Limitations

As with all studies, there were strengths and limitations inherent to this study. One strength was that breast cancer survivors and controls were very closely matched regarding aerobic fitness (VO_{2peak}), and body composition. This study is one of the few to describe endocrine responses in a study sample of women who are of very low aerobic fitness (< 20 mL/kg/min), and it is the first to describe endocrine responses to acute aerobic exercise in

breast cancer survivors at multiple time points post-exercise. One limitation of this study may be the average age difference between the study groups. While all attempts were made to match subjects closely on all physical characteristics, the control group was older than the breast cancer survivor group by an average of 9 years. Previous studies have also observed post-exercise decreases in cortisol in healthy older females (131-132). Additionally, this study employed a relatively small sample size, and there may not have been adequate statistical power to detect all within- or between-group differences in all hormone parameters. Furthermore, some data points were not available for all subjects because of limited availability of blood samples, or the inability to obtain blood samples. As a result, mean substitution was performed to account for these missing data points. Lastly, while all subjects had fasted for at least 2 hours prior to all Laboratory Sessions, it is likely that some subjects had fasted for a longer period of time than others; a factor which could affect epinephrine and cortisol release.

Conclusions

In summary, 30 minutes of moderate intensity aerobic exercise seems to elicit generally similar changes in plasma epinephrine concentrations in recent breast cancer survivors compared to physically similar control women without a history of cancer diagnosis or treatment. Some differences in absolute plasma epinephrine levels were measured between groups, which could be related to underlying physiological differences specifically associated with cancer and its treatments. Plasma cortisol responses during the exercise session differed between breast cancer survivors and controls, possibly due to differences in age and exercise intensity. These findings suggest that acute aerobic exercise may have a more pronounced or "stressful" effect on the adrenal glands in recent breast cancer survivors compared to healthy women without a history of cancer, even though other measures of exercise intensity (i.e., HR and RPE) are similar.

CHAPTER V

THE RELATIONSHIPS BETWEEN CHANGES IN NATURAL KILLER CELL NUMBERS, CATECHOLAMINES, AND CORTISOL AFTER ACUTE AEROBIC EXERCISE IN BREAST CANCER SURVIVORS

Summary

Current research examining the endocrine factors that mediate immune responses in cancer patients and survivors is limited. The purpose of this study was to investigate the relationships between changes in NK cell, catecholamine, and cortisol responses to the acute bout of moderate-intensity aerobic exercise in nine breast cancer survivors (3-6 months post-treatment for Stage I-III invasive breast cancer) and nine healthy controls. Subjects performed 30 minutes of discontinuous aerobic exercise on the cycle ergometer at 60% of VO_{2peak}. NK cell counts and plasma concentrations of epinephrine, norepinephrine, and cortisol were determined from blood samples taken pre-exercise, immediately post-exercise, and 2 hours post-exercise. Changes in NK cell counts were correlated with changes in epinephrine, norepinephrine, and cortisol concentrations using Spearman Rank Correlations. Changes in NK cell counts were not significantly correlated with changes in catecholamines or cortisol in the breast cancer survivor group ($r = -0.417$-0.433, $p = 0.244$-0.831). Change in NK cell count was significantly correlated with change in cortisol concentration in the control group at 2 hours post-exercise ($r = 0.767$, $p = 0.016$). These results suggest that exercise-related changes in NK cell counts may not be related to the hormones of the adrenal gland in breast cancer survivors, but rather to other biologic factors yet to be examined.

Introduction

The American Cancer Society estimates that at this time, there are more than 2.9 million breast cancer survivors in the United States (1). The increase in breast cancer survival rates over recent years is encouraging; however, many of these survivors are left to deal with treatment-related side effects which may persist for months or years (2). In addition to their effects on the cardiopulmonary and musculoskeletal systems, cancer treatments can compromise the immune system which may continue for extended periods of time beyond treatment completion (23, 24, 98). Since immune function and disease-free overall survival are positively related, exploring complementary therapies that may boost or suppress a breast cancer survivor's immune system is of particular importance (9, 23).

Aerobic exercise is an attractive adjunct therapy for cancer patients and survivors because of its potentially positive influence on biologic systems involved in disease protection and anti-cancer defense (9). In particular, natural killer (NK) cells are an especially relevant parameter to study because of their ability to destroy virally-infected cells and tumor cells, as well as their extreme responsiveness to acute aerobic exercise (10-14). NK cells are thought to be associated with multiple biologic factors including hormones of the adrenal gland, and in particular, the catecholamines (epinephrine and norepinephrine) and cortisol. Catecholamines affect NK cell numbers through upregulating the surface density of β-adrenergic receptors on the NK cells themselves, thus causing changes in the configurations of adhesion molecules, which lead to demargination of NK cells from small venules and the spleen into circulation (11, 66). Cortisol affects NK cell numbers, possibly through glucocorticoid-induced apoptosis, inhibited adhesion of effect or cells to target cells, and altered cytokine responses (13, 42, 43, 73). During acute aerobic exercise, plasma

concentrations of both catecholamines and cortisol increase; therefore, increased catecholamines are often associated with increased NK cell numbers during exercise, whereas increased cortisol is often associated with decreased NK cell numbers during recovery (11, 13, 42, 43, 66, 67, 73).

To date, no study has examined associations between exercise-induced immune responses and endocrine mediators in the breast cancer patient population. Cancer and its treatments may be associated with chronic inflammation, altered hypothalamic-pituitary-adrenal axis and sympathetic nervous system function, and immune system deficiencies (23, 24, 98, 121-124, 127-129). Understanding how exercise affects the relationships between these parameters may help investigators devise exercise prescriptions that increase functioning in both the endocrine and immune systems, with the ultimate goals of reducing cancer risk/recurrence/second malignancies and increasing survival time post-treatment. Therefore, the purpose of this investigation was to compare the relationships between changes in NK cell, catecholamine, and cortisol responses to the acute bout of moderate-intensity aerobic exercise in breast cancer survivors and apparently healthy controls.

Methods

Subjects

Participants included 9 breast cancer survivors and 9 apparently healthy controls. All were women 40-70 years of age, were not regular users of anti-inflammatory medications, and had experienced amenorrhea for approximately 1 year prior to enrollment. The breast cancer survivor group had been diagnosed with Stage I-III invasive breast cancer, had received chemotherapy, and had completed all planned surgery, chemotherapy, and/or radiation therapy within 3-6 months prior to enrollment. The control group had never

experienced cancer treatment or diagnosis, was sedentary, and was free from any disease that would pose as a contraindication to aerobic exercise participation.

Laboratory Visits

Study procedures for the laboratory visits were previously described in Chapter III. Briefly, subjects reported to the laboratory facilities in the Department of Exercise and Sport Science at UNC-Chapel Hill on two separate occasions. Prior to each laboratory visit, subjects were asked to refrain from 1) eating for at least 2 hours prior to testing, 2) exercise and caffeine for at least 12 hours, and 3) alcohol for at least 48 hours. Visit 1 included an introduction to the study, assessment of baseline physical characteristics (age, race, height, weight, and body composition), a comprehensive medical and physical screening, and a VO_{2peak} test using the Astrand Cycle Ergometer Maximal Test Protocol to assess VO_{2peak} (100) and extrapolate to a workload equal to 60% of VO_{2peak}. During the VO_{2peak} test, oxygen uptake was determined using a Parvo Medics TrueMax 2400 Metabolic System (Parvo Medics, Salt Lake City, UT). Heart rate and Rating of Perceived Exertion or RPE (101) were recorded at the end of every minute of the test. ECG monitoring was performed continuously using a GE CASE Cardiosoft V. 6.6 ECG diagnostic system (General Electric, Palatine, IL).

Visit 2 began between 7:00-10:00 am in order to control for daily variations in the study variables. Upon arrival, subjects completed the Perceived Stress Scale (PSS) in order to assess how subjects perceived stress in daily life, as such stress could impact the resting values of epinephrine, norepinephrine, and cortisol. Subjects rested for 3 minutes on the cycle ergometer while resting metabolic data was collected. Subjects then performed a warm-up on the cycle ergometer, followed by light stretching of the muscles of the lower

body. Subjects then performed the 30 minute aerobic exercise bout at a workload which corresponded to 60% of VO_{2peak}. Since all subjects were sedentary, the exercise was divided into ten 3-minute intervals with1.5 minutes of rest. Metabolic data was collected during intervals 1, 3, 7, and 10, and workload was adjusted as needed in order to maintain the exercise intensity as close to 60% of VO_{2peak} as possible. Heart rate and RPE were recorded at the end of each 3-minute exercise interval.

Blood Sampling

Blood samples were taken through a catheter placed in an antecubital vein. Blood samples were obtained using the three-syringe technique (waste, sample, saline) pre-exercise before the warm-up, immediately post-exercise, and 2 hours post-exercises. Subjects were not allowed to ingest any food or beverage with the exception of water, as these could potentially affect study variables. At the conclusion of the laboratory session, whole blood samples were centrifuged at 4°C for 10 minutes at 3000 rpm using an IEC Centra-8R refrigerated centrifuge (International Equipment Company, Needham Heights, MA). The plasma portion was aliquoted and stored at -80°C.

Determination of NK Cell Counts

Routine complete blood counts (CBC) were performed for the blood samples taken at each time point using a COULTER® Ac•T diff ™ Hematology Analyzer (Beckman Coulter, Inc., Brea, CA). From these, the numbers of total lymphocytes were obtained. To determine NK cell counts, blood samples were stained to identify the subset of lymphocytes which express the $CD3^{-}CD16^{+}CD56^{+}$ phenotype. Whole blood samples were fluorescently stained using fluorescein isothyocyanate (FITC) for the CD3 marker, phycoerythrin (PE) for the CD16 marker, and allophycocyanin (APC) for the CD56 marker. Samples were further

prepared using procedures made available by Becton Dickenson (109). Within 24 hours of sample preparation, flow cytometric analysis was performed using a CyAn 3 laser/9 color flow cytometer (Beckman Coulter, Inc., Brea, CA). Summit 4.3 software (Dako North America, Inc., Carpinteria, CA) was used to view and analyze flow cytometry data.

Determination of Plasma Catecholamine and Cortisol Concentrations

Epinephrine and norepinephrine were assayed using a commercially available ELISA kit (Abnova, Tapei City, Taiwan) which has sensitivities of 10 pg/mL and 50 pg/mL for epinephrine and norepinephrine, respectively. The reported mean intra-assay coefficients of variation for the epinephrine ELISA was 6.9-15.8%, and the reported mean inter-assay coefficients of variation was 13.2-18.2%. The reported mean intra-assay coefficients of variation for the norepinephrine ELISA was 9.8-16.1%, and the reported mean inter-assay coefficients of variation was 8.5-15.0%. All samples were run in duplicate except for two control samples which were run in singlet due to limited plasma volumes.

Cortisol was also assessed using a commercially available ELISA kit (Abnova, Taipei City, Taiwan) that has a sensitivity of 0.44 ng/mL. The reported mean intra-assay coefficients of variation for the cortisol ELISA was 6.2-9.4%, and the reported mean inter-assay coefficients of variation ranged from 8.6-15.0%. All samples were run in duplicate.

Statistical Analysis

All analyses were performed using SPSS Version 18.0. Subject characteristics, VO_2 (peak and submaximal), workload (peak and submaximal), and PSS scores were compared between groups using independent samples t-tests. Submaximal VO_2 and workload data were also compared within groups using paired-samples t-tests. Absolute lymphocyte counts (and therefore NK cell counts), epinephrine, norepinephrine, and cortisol concentrations were

adjusted for plasma volume changes due to exercise using the method by Dill and Costill (111). Fold changes for NK cell counts, catecholamine, and cortisol concentrations at each post-exercise time point (immediately post-exercise and 2 hours post-exercise) were calculated by dividing the post-exercise value by the pre-exercise value and were compared to a null value of 1.0 using one-sample t-tests. Between-group comparisons of fold changes for each parameter were made using Wilcoxon Rank Sum tests. Relationships between fold changes in NK cell counts and fold changes in epinephrine, norepinephrine, and cortisol were evaluated at each post-exercise time point using Spearman Rank correlations.

Results

Subject Characteristics, VO_2, workload, and PSS Scores

Between-group comparisons of subject characteristics, VO_2, workload, and PSS scores (mean ± standard deviation [SD]) are shown in Table 5.1. The control group was older than the breast cancer survivor group by an average of 9 years ($p = 0.002$). All other comparisons regarding physical characteristics, VO_2, and workload were similar ($p = 0.261$-0.906). Additionally, PSS scores between groups were similar ($p = 0.511$).

Subjects in the breast cancer survivor group had been treated for Stage I, II or III invasive breast cancer and had completed all planned surgery, chemotherapy, and radiation therapy 3-6 months prior to enrollment. Surgery type included mastectomy (n = 4) and lumpectomy (n = 5). Chemotherapy agents included Adriamycin (n = 6), Cytoxan (n = 7), Taxol/Taxotere (n =9), and Carboplatin (n = 3). Hormonal therapies included Tamoxifen (n = 5) and Femara (n = 1). Other cancer treatment-related medications included Herceptin (n = 2), Lapatinib (n = 1), and Bevacizumab (n = 1). All subjects received radiation therapy.

Table 5.1. Comparison of subject characteristics, VO_2, workload, and PSS scores (mean ± SD)

Characteristic	Breast Cancer Survivor Group (n = 9)	Control Group (n = 9)
Age (years)	50 ± 6*	59 ± 5*
Race (# of women)	Caucasian (8) African American (1)	Caucasian (9)
Height (cm)	164.7 ± 5.8	163.8 ± 5.9
Weight (kg)	76.9 ± 12.6	77.7 ± 13.3
Body Mass Index (kg/m^2)	28.4 ± 5.0	28.9 ± 4.6
Percent Body Fat (%)	41.6 ± 4.5	42.1 ± 4.0
VO_{2peak} (mL/kg/min)	18.1 ± 2.7	18.5 ± 5.1
Peak Workload (W)	107 ± 19	106 ± 17
Estimated Submaximal VO_2 (60% of VO_{2peak}, mL/kg/min)	10.9 ± 1.6†	11.1 ± 3.1
Actual Submaximal VO_2 (mL/kg/min)	12.1 ± 1.3†	11.1 ± 1.8
Estimated Submaximal Workload at 60% of VO_{2peak} (W)	63 ± 12	66 ± 11
Actual Submaximal Workload (W)	59 ± 9	62 ± 11
PSS Score	15 ± 4	13 ± 6

*$p < 0.05$ for comparing age between groups
†$p < 0.05$ for comparing calculated and actual submaximal VO_2 in the breast cancer survivor group

Changes in NK Cell Counts, Plasma Catecholamines, and Cortisol

Pre-exercise values and fold changes for NK cells, plasma epinephrine, norepinephrine, and cortisol are shown in Table 5.2. In the breast cancer survivor group, blood samples were not able to be obtained from one subject immediately post-exercise and from one subject 2 hours post-exercise; therefore, mean substitution was performed to account for these missing data points. Plasma volume shifts did not differ significantly between groups and were as follows: -12.0 ± 3.6% vs. –11.5 ± 4.6% immediately post-exercise and -3.4 ± 6.3% vs. -0.1 ± 9.2% 2 hours post-exercise for the breast cancer survivor group and control group, respectively. Although plasma volume shifts were similar between

groups, cell counts and hormone concentrations were adjusted to account for shifts in plasma volume due to exercise. In the breast cancer survivor group, pre-exercise NK cell counts were somewhat lower, whereas pre-exercise epinephrine concentration was significantly higher compared to the control group ($p = 0.090$ and < 0.0005, respectively).

Within the breast cancer group, NK cell counts and norepineprhine displayed significant increases (i.e., fold changes significantly greater than 1.0) immediately post-exercise relative to pre-exercise values ($p = 0.010$ and 0.025 respectively). At 2 hours post-exercise, epinephrine was significantly decreased ($p = 0.047$), while norepinephrine remained significantly elevated relative to pre-exercise values ($p = 0.018$). Within the control group, NK cell counts and epinephrine were significantly elevated immediately post-exercise ($p < 0.0005$ and $p = 0.013$, respectively, while norepinephrine was somewhat elevated ($p = 0.065$). At 2 hours post-exercise, epinephrine was somewhat elevated ($p = 0.098$), while norepinephrine remained significantly elevated relative to pre-exercise levels ($p = 0.023$. Cortisol was significantly decreased (i.e., fold change significantly less than 1.0) relative to pre-exercise levels immediately post-exercise and 2 hours post-exercise ($p = 0.007$ and 0.009 respectively). All other fold changes were not significantly different from the null value of 1.0.

When comparing groups, the control group showed a significantly larger magnitude of increase in epinephrine 2 hours post-exercise ($p = 0.005$). All other comparisons were similar between groups.

Table 5.2. NK cell counts, plasma catecholamine, and cortisol concentrations at baseline (pre-exercise) and fold changes across time (baseline to immediately post-exercise [0h post-exercise] and baseline to 2 hours post-exercise [2h post-exercise])

Parameter	Breast Cancer Survivor Group (mean ± SD)	Control Group (mean ± SD)
NK Cells		
Pre-exercise (cells/μL)	70.3 ± 37.9	108.9 ± 51.7
Fold change		
0h post-exercise	2.7 ± 1.5†	2.8 ± 0.9†
2h post-exercise	1.3 ± 0.5	1.2 ± 0.6
Epinephrine		
Pre-exercise (pg/mL)	49.7 ± 9.9*	27.1 ± 4.9*
Fold change		
0h post-exercise	1.1 ± 0.3	1.4 ± 0.4†
2h post-exercise	0.9 ± 0.1†‡	1.4 ± 0.6‡
Norepinephrine		
Pre-exercise (pg/mL)	258.0 ± 109.6	518.0 ± 555.3
Fold change		
0h post-exercise	2.0 ± 1.1†	2.2 ± 1.7
2h post-exercise	2.1 ± 1.1†	2.3 ± 1.4†
Cortisol		
Pre-exercise (ng/mL)	137.7 ± 73.2	147.7 ± 49.2
Fold change		
0h post-exercise	1.2 ± 0.8	0.6 ± 0.3†
2h post-exercise	0.9 ± 0.7	0.8 ± 0.5†

*$p < 0.05$ for comparing pre-exercise values between groups
†$p < 0.05$ when compared to the null value of 1.0
‡$p < 0.05$ when comparing the fold change at 2 hours post-exercise between groups

Correlations between Changes in NK Cell Count, Catecholamine, and Cortisol

The correlations between changes in NK cell counts and changes in catecholamines and cortisol immediately post-exercise and 2 hours post-exercise are shown in Table 5.3. In the breast cancer survivor group, changes in NK cell counts were not significantly correlated with changes in epinephrine, norepinephrine, or cortisol concentrations. In the control group, changes in NK cell counts were not correlated with fold changes in epinephrine or norepinephrine concentrations. Changes in NK cell counts were not correlated with fold changes in cortisol levels immediately post-exercise. However, fold changes in NK cells

were significantly correlated with fold changes in cortisol at 2 hours post-exercise ($r = 0.767$, $p = 0.016$).

Table 5.3. Spearman correlations (p-values) between fold changes in NK cell counts, epinephrine, norepinephrine, and cortisol immediately post-exercise and 2 hours post-exercise

	Epinephrine	Norepinephrine	Cortisol
NK cell counts immediately post-exercise			
Breast Cancer Survivor Group	0.083 (0.831)	0.233 (0.546)	0.433 (0.244)
Control Group	-0.033 (0.932)	-0.367 (0.332)	0.483 (0.187)
NK cell counts 2 hours post-exercise			
Breast Cancer Survivor Group	0.350 (0.356)	-0.417 (0.265)	0.183 (0.637)
Control Group	0.167 (0.662)	0.150 (0.700)	0.767 (0.016)

Discussion

The key findings of this study were that no significant relationships were observed between post-exercise changes in catecholamines and NK cell counts, and that a significant relationship was observed between post-exercise changes in cortisol and NK cell counts in the control group only. It was initially hypothesized that for both groups immediately post-exercise, there would be significant positive correlations between changes in catecholamines and cortisol with changes in NK cell counts. Additionally, it was hypothesized that for both groups at 2 hours post-exercise, there would be no significant correlations between changes in catecholamines and cortisol with changes in NK cell counts because hormone concentrations and NK cell counts would have returned to near pre-exercise levels. Therefore, these findings were somewhat unexpected.

Other investigators have examined the relationship between exercise-induced changes in NK cell counts and catecholamines, showing results that are reflective of what was

hypothesized for this study. Moyna et al. (48) and Scharhag et al. (75) showed that increases in NK cell counts were significantly correlated with increases in epinephrine levels ($r = 0.27$-0.74, $p \leq 0.005$). Scharhag et al. (75) and Kendall et al. (96) have also shown that increases in NK cell counts were significantly correlated with increases in norepinephrine levels ($r = 0.40$-0.65, $p \leq 0.02$). However, it should be noted these studies were performed in healthy men and women with substantially higher VO_{2peak} than the subjects in the current investigation. Additionally, these studies included exercise protocols that were continuous rather than discontinuous (as the latter was done in the current study), and the exercise intensities and durations tended to be notably greater than that of the current study. Therefore, the differences in subject population and type of exercise performed between these studies and the current investigation may certainly lead to differing results.

Increases in plasma catecholamines during acute aerobic exercise generally occur when the intensity reaches approximately 40-60% of VO_{2max} (55). In relation to NK cells, the rise in catecholamine levels has been associated with increases in circulating NK cells, the primary mechanism of action appearing to be a catecholamine-induced alteration in adhesion molecules that are expressed on NK cells thus recruiting them from their marginal zones into circulation (11, 13, 48, 66, 67). Weak or non-significant correlations between NK cell counts and catecholamines may be related to the timing of the blood samples taken immediately post-exercise. Catecholamine levels decrease rapidly upon cessation of exercise, and even delaying blood sampling by 2-3 minutes may affect results (96). Additionally, plasma concentrations of catecholamines do not always indicate what is occurring at the tissue level (96). In the current study, increases in NK cell counts may have been more closely related to increases in cardiac output rather than increases in

catecholamines, as the discontinuous nature of the aerobic exercise session might have elicited an attenuated catecholamine response compared to a continuous exercise bout.

The current study found that changes in NK cell counts were correlated with changes in cortisol concentrations in the control group only. Similar to the catecholamines, plasma cortisol levels rise proportionally with respect to exercise intensity and duration (70). Increased plasma cortisol levels are typically thought to be associated with decreased NK cell counts and activity during recovery from acute aerobic exercise, particularly after longer duration or more intense exercise. In the current study, cortisol levels decreased from pre-exercise to immediately post-exercise and remained below baseline 2 hours post-exercise. In contrast, NK cell counts increased from pre-exercise to immediately post-exercise and remained 2 hours post-exercise.

Strengths and Limitations

As is the case with all studies, this investigation possessed some inherent strengths and limitations. With the exception of age, the study groups were extremely similar with respect to physical characteristics and aerobic fitness level. The small sample size (n = 9 per group) may not have provided enough statistical power to adequately analyze all relationships between study variables. Additionally, mean substitution had to be performed for two subjects (once immediately post-exercise and once at 2 hours post-exercise) as blood samples were unable to be obtained from them at these times. Furthermore, the current study employed a discontinuous exercise protocol at only one intensity, whereas comparing the NK cell response and stress hormone response at multiple intensities may be needed in order to provide a more conclusive view of the relationships between these parameters.

Conclusions

In summary, the exercise-induced changes in plasma catecholamines do not seem to be associated with changes in NK cell counts in breast cancer survivors, similar to normal healthy women. In contrast, changes in NK cell counts were not associated with plasma cortisol in the breast cancer survivor group, but were in the control group. These findings suggest that other biologic mechanisms might be playing a more significant role in directing exercise-induced NK cell responses, particular in breast cancer survivors.

CHAPTER VI

RESEARCH SYNTHESIS

Major Findings

These investigations have revealed several key findings regarding the impact of acute aerobic exercise on NK cell, catecholamine, and cortisol responses in breast cancer survivors. NK cell counts were somewhat lower in breast cancer survivors pre-exercise (70.3 ± 37.9 cells/μL vs. 108.9 ± 51.7 cells/μL, $p = 0.09$) and immediately post-exercise compared with healthy controls (170.5 ± 110.4 cells/μL vs. 282.5 ± 132.3 cells/μL, $p = 0.069$). Breast cancer survivors and healthy controls displayed similar magnitudes of change in NK cell counts immediately post-exercise (165.6 ± 147.6% vs. 179.9 ± 89.3%, $p = 0.807$), 2 hours post-exercise (26.8 ± 48.0% vs. 23.3 ± 61.0%, $p = 0.894$), and 24 hours post-exercise (31.4 ± 66.3% vs. 19.3 ± 42.2%, $p = 0.651$). NKCA was similar across time in the subset of 6 breast cancer survivors (pre-exercise: 73.6 ± 30.3%, immediately post-exercise: 74.4 ± 21.0, 2 hours post-exercise: 67.6 ± 12.5, 24 hours post-exercise: 60.2 ± 37.1, $p = 0.607$). These results suggest that despite some slight differences in absolute cell concentrations, the moderate intensity aerobic exercise elicits similar changes in NK cell counts in recent breast cancer survivors compared to physically similar women without a history of cancer diagnosis or treatment.

Second, the aerobic exercise bout led to some interesting differences in catecholamine and cortisol responses between the two groups. Absolute plasma epinephrine levels were significantly higher in the breast cancer survivor group compared to the control group pre-

exercise (49.7 ± 9.9 pg/mL vs. 27.1 ± 4.9 pg/mL, $p < 0.0005$), immediately post-exercise (54.5 ± 12.7 pg/mL vs. 39.2 ± 14.9 pg/mL, $p = 0.032$), and 24 hours post-exercise (47.9 ± 6.2 pg/mL vs. 28.6 ± 6.7 pg/mL, $p < 0.0005$). Percent changes in epinephrine were somewhat lower in the breast cancer survivor group compared to the control group immediately post-exercise (12.2 ± 30.9% vs. 44.8 ± 42.5%, $p = 0.081$) and significantly lower at 2 hours post-exercise (-11.5 ± 14.7% vs. 35.9 ± 57.4%, $p = 0.040$). Absolute plasma norepinephrine concentrations and post-exercise percent changes were similar between groups ($p = 0.203$-0.759). Plasma cortisol levels were somewhat higher in the breast cancer survivor group compared to the control group immediately post-exercise (131.1 ± 77.5 ng/mL vs. 75.3 ± 16.3 ng/mL, $p = 0.064$). Lastly, the percent change in cortisol was positive in the breast cancer group and negative in the control group (19.4 ± 76.4% vs. -40.8 ± 34.0%, $p = 0.054$). These results demonstrate that the exercise session could have led to a greater stress hormone response in the cancer survivors, even though HR and RPE responses were similar between groups. It should be noted however, that although absolute concentrations of epinephrine and cortisol were significantly elevated in the breast cancer survivors at various time points relative to the controls, the magnitude of the percent change in epinephrine and cortisol was somewhat blunted in breast cancer survivors.

Third, no significant correlations were observed for changes in NK cell count with changes in epinephrine or norepinephrine in either study group post-exercise ($r = -0.017$-0.400, $p = 0.286$-0.966) . Similarly, no significant correlations were observed for changes in NK cell count with changes in cortisol in the breast cancer survivor group post-exercise ($r = -0.417$-0.350, $p = 0.265$-0.932). Change in NK cell count was not correlated with change in cortisol in either group immediately post-exercise or in the breast cancer survivor group at 2

hours post-exercise ($r = 0.183$-0.483, $p = 0.187$-0.637) but was significantly correlated at 2 hours post-exercise in the control group ($r = 0.767$, $p = 0.016$). These results suggest that exercise-induced changes in catecholamines and cortisol may not be principal biologic factors driving exercise-induced changes in NK cell counts, particularly in the breast cancer survivors.

Significance of the Study and Implication of Results

The American Cancer Society estimates that there are currently more than 2.9 million breast cancer survivors living in the United States (1). Many of these survivors may experience immune system dysfunction and adrenal gland hormone dysfunction which may be related to the stress of cancer and its treatments (23, 24, 98, 121-124, 127-129). Exercise has already been shown to improve physiological and psychological outcomes including cardiorespiratory endurance, body composition, muscular strength, functional quality of life, fatigue, anxiety, and self esteem (6). While a few previous studies have examined NK cell responses to aerobic exercise training in the breast cancer patient population (15, 17, 23), The current study adds valuable cross-sectional data to the literature, as it is the only study to date that has examined acute aerobic exercise responses of any immune or hormonal parameter in these individuals. Additionally, it compares these exercise responses of the breast cancer survivors to those of healthy women who have never experienced the physical toll of cancer and its treatments. The latter point is particularly noteworthy because exercise prescriptions for the cancer patient population are largely modeled after those for the general population (39). Therefore, if exercise specialists are to continue to apply guidelines that exist for healthy individuals to cancer survivors, then it must be determined whether or not exercise affects these population groups similarly. This investigation focused on the effect of acute

moderate intensity aerobic exercise on NK cell, catecholamine and cortisol responses, both as separate parameters and in correlation with each other.

One of the major findings of this investigation was that acute moderate aerobic exercise led to similar NK cell count responses in the breast cancer survivors and the controls, and that neither group experienced post-exercise decreases in NK cell counts relative to pre-exercise values. This is an important finding, as it suggests that this type of exercise, which is commonly used in exercise prescriptions for breast cancer survivors, caused a typical response as previously seen in other healthy individuals (74, 94-96, 112, 113). Additionally, NK cell counts had returned to near pre-exercise levels by 24 hours post-exercise. Furthermore, the exercise did not seem to significantly affect NKCA in the subset of breast cancer survivors. These results imply that 30 minutes of discontinuous moderate intensity aerobic exercise is safe for breast cancer survivors, and that 24 hours of recovery appears sufficient for NK cell counts to return to pre-exercise values.

The investigation also found that the breast cancer survivors had significantly higher absolute concentrations of epinephrine and cortisol at several time points, but that the magnitude of change between groups was only significantly different for epinephrine at 2 hours post-exercise . This is an important finding, as it suggests that breast cancer survivors may be experiencing higher levels of stress hormone output as an effect of their cancer treatments. It also suggests that the adrenal medulla and the adrenal cortex may each respond to the stress of exercise differently in breast cancer survivors, compared to healthy controls. For example, there were no significant differences in post-exercise percent changes in norepinephrine when comparing the study groups. However, breast cancer survivors experienced a somewhat attenuated magnitude in their epinephrine response immediately

post-exercise, and a significantly attenuated magnitude in their epinephrine response at 2 hours post-exercise. Additionally, breast cancer survivors experienced a positive percent change in cortisol immediately post-exercise whereas the controls experienced a negative percent change. These findings indicate that particularly for cortisol, the exercise intensity may have reached the threshold needed to elicit increased HPA axis activation and increased cortisol secretion in the breast cancer survivors, but that this threshold had not been reached for the controls. This finding is noteworthy, as it suggests that exercising at similar relative intensities in breast cancer survivors and healthy individuals may actually lead to different physiological responses between these groups. This could be important for exercise specialists working with breast cancer survivors, as prescribed exercise intensities may actually lead to physiological responses that are more pronounced than what might be seen in the general population, even when other markers of exertion (i.e., HR and RPE) are similar.

Regarding the relationships between changes in NK cells and changes in catecholamines and cortisol, the current study found no correlation between these parameters. Change in cortisol was found to correlate with change in NK cell counts in the control group. The general lack of correlation between NK cells and stress hormones in this study may have occurred because of the discontinuous nature of the moderate exercise session, and so changes in NK cell counts may have related more to changes in cardiac output, which can demarginalize NK cells into circulation (10, 42, 48-50). This possibility was explored, by relating changes in absolute VO_2 during the exercise (VO_2 is proportional to cardiac output) to changes in NK cell counts immediately post-exercise, although in this case too, no significant association was found. These findings suggest that while a discontinuous exercise bout may lead to significant changes in NK cell counts and some

stress hormone parameters independently, it may not provide enough of a physiological stimulus to cause both the hormonal and NK cell parameters to vary together, or that the perturbations in the stress hormone parameters were not of a sufficient quantity to drive the perturbations in the NK cell counts.

Summary of Research Hypotheses

Hypothesis 1a: There will be a significant increase in NK cell counts from pre-exercise to immediately post-exercise in both breast cancer survivors and healthy controls. Result: Supported.

Hypothesis 1b: There will be no significant difference in NK cell counts from pre-exercise to 2 hours post-exercise in both breast cancer patients and healthy controls. Result: Supported

Hypothesis 1c: There will be no significant difference in NK cell counts from pre-exercise to 24 hours post-exercise in both breast cancer patients and healthy controls. Result: Supported.

Hypothesis 1d: There will be no significant differences in NK cell counts between breast cancer survivors and healthy controls at any time point (pre-exercise, immediately post-exercise, 2 hours post-exercise, and 24 hours post-exercise). Result: Supported.

Hypothesis 2a: Changes in catecholamine levels will be positively correlated with changes in NK cell counts from pre-exercise to immediately post-exercise in both breast cancer survivors and healthy controls. Result: Rejected.

Hypothesis 2b: Changes in cortisol levels will be positively correlated with changes in NK cell counts from pre-exercise to immediately post-exercise in both breast cancer survivors and healthy controls. Result: Rejected

Hypothesis 2c: Changes in catecholamine levels will not be correlated with changes in NK cell counts from pre-exercise to 2 hours post-exercise in both breast cancer survivors and healthy controls because changes in catecholamine levels and NK cell counts may not be significantly different from pre-exercise to 2 hours post-exercise. Result: Supported.

Hypothesis 2d: Changes in cortisol levels will not be correlated with changes in NK cell counts from pre-exercise to 2 hours post-exercise in both breast cancer survivors and healthy controls because changes in cortisol levels and NK cell counts may not be significantly different from pre-exercise to 2 hours post-exercise. Result: Rejected.

Strengths and Limitations

This is the first study to investigate acute aerobic exercise responses regarding NK cells and stress hormones in breast cancer survivors, and it is one of only a few studies that have investigated acute exercise responses in any immune parameter in oncology patients (19, 25).

This study included a very homogeneous sample with regard to race, body composition, and aerobic fitness. Additionally, this study is one of the few to compare NK cell and stress hormone responses of the breast cancer survivors to those of healthy post-menopausal women. Further, this study examined these physiological responses in individuals who had a very low average aerobic fitness (< 20 mL/kg/min), as most investigations tend to examine responses in athletic and physically active individuals.

One limitation to this study included small sample sizes in each group which may have made it difficult to detect all within- or between-group differences in the parameters measured. Second, there was a significant difference in mean age between the study groups, which could have affected some hormonal parameters. Also, the study employed a

discontinuous protocol at only one exercise intensity, Additionally, some data were not available for two cancer survivor subjects (one immediately post-exercise and one 2 hours post-exercise) and one control subject at 24 hours post-exercise, either because blood samples could not be obtained, or because of limited plasma volume available from the sample. NKCA was only measured in 6 breast cancer survivors, and so comparisons with the control group could not be made. Furthermore, the period of time that elapsed between the cessation of the exercise bout and the immediate post-exercise blood draw was longer than 2-3 minutes in some subjects, which could have affected the measured catecholamine response at that time point. Lastly, while all subjects had fasted for at least 2 hours prior to all Laboratory Sessions, it is likely that some subjects had fasted for a longer period of time than others; a factor which could affect epinephrine and cortisol release, and therefore the measurement of those parameters.

Future Research

Exercise immunology and endocrinology research in oncology patients is still a relatively young field, and so there are many opportunities to expand and improve upon the current research. Exploring acute exercise response in individuals with cancer other than breast cancer, oncology patients of varying ages (pediatric, adult, and geriatric), recent survivors vs. long-term survivors, as well as patients undergoing treatment vs. patients who are post-treatment would be useful in order to determine if significant differences in exercise responses exist. These findings could ultimately lead to developing more specific exercise prescription guidelines based on cancer and treatment type, patient age, and the amount of time that has elapsed since the patient completed treatment. Examining acute exercise responses in immune parameters other than NK cells as well as investigating relationships

between exercise-induced immune responses and other biologic factors is relevant, as there are a multitude of biologic factors that contribute to anti-cancer defenses which can also be modified by exercise (9). Comparing the effect of continuous vs. discontinuous exercise of the same total duration may elucidate whether rest periods within an exercise session could lead to attenuated responses. As all exercise oncology research ultimately manifests as exercise prescription guidelines, future research should strive to understand how exercise can affect the immune and endocrine systems, so that cancer patients and survivor can reap the maximum health benefits of the exercise without putting themselves at risk for further illness

APPENDIX A

EXTENDED METHODS

Subjects

Subjects were recruited into two study groups: a breast cancer survivor group and a control group. Subjects in the breast cancer survivor group included women who had completed all planned surgery, chemotherapy, and radiation therapy in the treatment of their disease. Subjects in the control included women who did not have a history of cancer diagnosis or treatment, were sedentary (i.e., had not participated in regular organized physical activity within the past year), and were free from cardiovascular and musculoskeletal disease that would render aerobic exercise participation unsafe. Subjects in the breast cancer survivor group were recruited from the Medical Oncology clinic at the North Carolina Cancer Hospital on the campus of the University of North Carolina at Chapel Hill (UNC-Chapel Hill), the Radiation Oncology clinic at the North Carolina Cancer Hospital at UNC-Chapel Hill, and from the waitlist for the Get REAL and HEEL Breast Cancer Program in the Department of Exercise and Sport Science at UNC-Chapel Hill. Subjects in the control group were recruited from the faculty, staff, and student populations at UNC-Chapel Hill, as well as from across the Triangle region of North Carolina (Raleigh, Durham, Chapel Hill, and surrounding areas). Potential subjects were made aware of the study through fliers and mass email communication. Additionally, physicians and nurse navigators assisted in subject recruitment by speaking to potential subjects about the study and by passing along the contact information of a member of the research team.

The inclusion criteria for participation in the breast cancer survivor group are presented below:

1. Confirmed diagnosis of Stage I, II, or III invasive breast cancer;
2. Received chemotherapy;
3. Completed the final treatment of surgery, chemotherapy, and/or radiation therapy within 3-6 months prior to enrollment;
4. Patients receiving adjuvant hormonal therapy or adjuvant trastuzumab were eligible;
5. No presence of metastatic disease;
6. Female, between 40 and 70 years of age;
7. Post-menopausal, or had not experienced a menstrual period for approximately 1 year prior to enrollment;
8. Not a regular user of anti-inflammatory medications.

The inclusion criteria for participation in the control group are presented below:

1. Female, between 40 and 70 years of age;
2. No history of cancer diagnosis or treatment;
3. Not involved in regular organized physical activity for at least 1 year prior to enrollment;
4. Post-menopausal (i.e., had not experienced a menstrual period) for approximately 1 year prior to enrollment;
5. Not a regular user of anti-inflammatory medications.

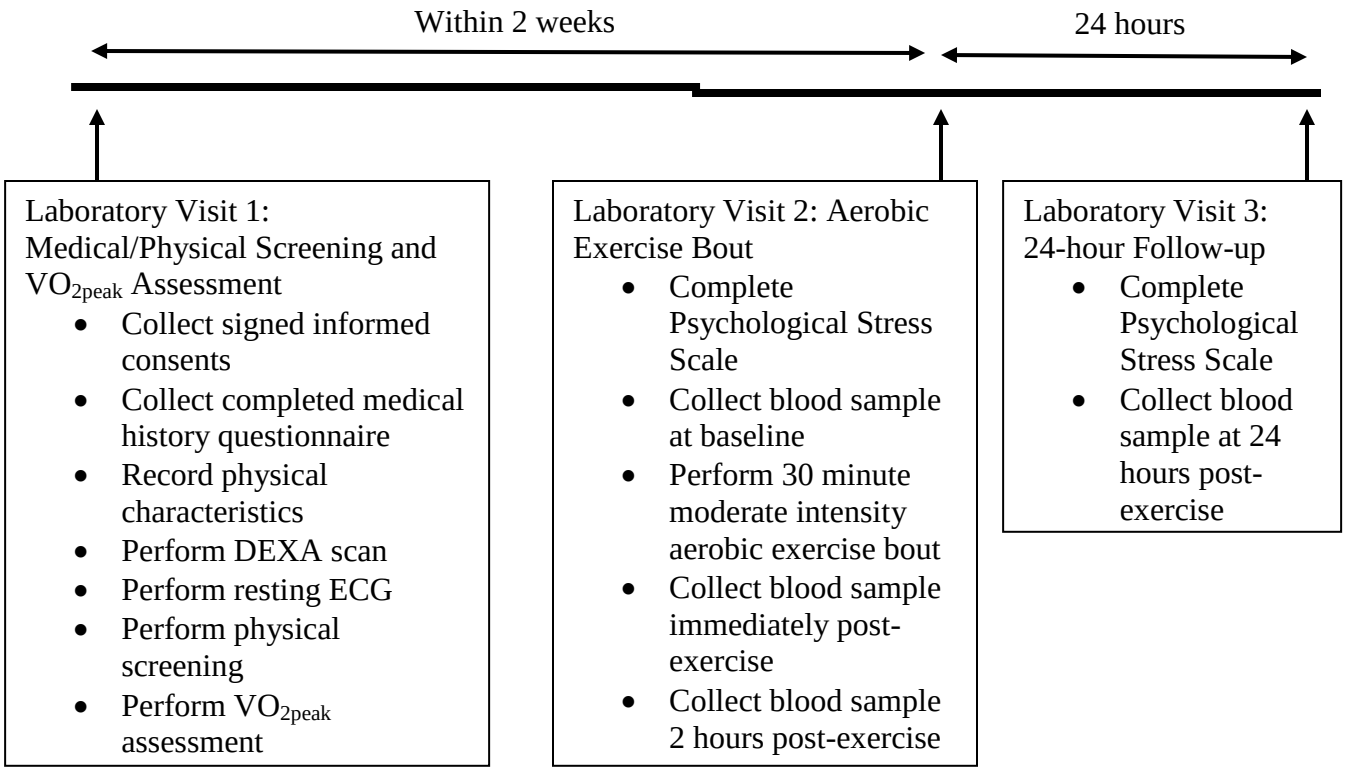
All subjects in both the breast cancer survivor group and the control group were required to complete a comprehensive medical history questionnaire, a physical screening by either a physician or other certified professional, and a 12-lead resting electrocardiogram (ECG). Subjects were screened for exclusion based on the criteria put forth by the American College of Sports Medicine (ACSM) as contraindications to exercise testing (99).

Research Design

This was a prospective cohort study with the primary goal of examining changes in NK cell count after an acute bout of moderate intensity aerobic exercise. The secondary goal was to examine the relationship between changes in NK cell count, catecholamine (epinephrine and norepinephrine), and cortisol responses after and acute bout of moderate intensity aerobic exercise. Subjects were recruited into two groups; a breast cancer survivor group and a control group as described above. Each subject visited the laboratory on three separate occasions. Laboratory Visit 1 included an orientation to the study, medical and physical screening, and the assessment of peak aerobic capacity through a VO_{2peak} test on the cycle ergometer. Physical characteristics including age, race, height, weight, and percent body fat over the past year were obtained during this visit as well. During Laboratory Visit 2, the subject performed a moderate bout of aerobic exercise on the cycle ergometer, the intensity and duration of which was approximately 60% of VO_{2peak} and 30 minutes, respectively. Blood samples were collected during this laboratory visit; immediately prior to exercise (baseline), immediately post-exercise, and 2 hours post-exercise. Laboratory Visit 3 was a resting session, during which one blood sample was collected from the subject 24 hours post-exercise. The outcome (dependent) variable for this study was NK cell count. The predictor (independent) variables for this study were catecholamines (epinephrine and norepinephrine) and cortisol. Each outcome and predictor variable was measured from the blood samples collected at the various time points outlined in Laboratory Visits 2 and 3. All Laboratory Visits occurred in the Integrative Exercise Oncology Research Laboratory (IEORL) and/or in the Applied Physiology Laboratory (APL) in the Department of Exercise and Sport Science at UNC-Chapel Hill. Approval from the Protocol Review Committee in

the Lineberger Comprehensive Cancer Center and the Institutional Review Boards in the Department of Exercise and Sport Science and School of Medicine at UNC-Chapel Hill were obtained before proceeding with subject recruitment and testing. Figure 2 illustrates a summary timeline of the major events that occurred during the Laboratory Visits.

Figure 2. Timeline of study events.



Instrumentation

The following equipment was used during Laboratory Visit 1 and 2 to measure physical characteristics, to perform the medical and physical screening, and to assess cardiorespiratory function during the VO_{2peak} assessment and aerobic exercise session:

1. Portable stadiometer (Perspective Enterprises, Portage, MI) to measure height to the nearest 0.01 cm;
2. Mechanical scale (Detecto, Webb City, MO) to measure body mass to the nearest 0.1 kg.
3. Discovery Dual Energy X-Ray Absorption (DEXA) scanner (Hologic, Inc., Bedford, MA) to assess percent body fat.
4. GE CASE Cardiosoft V. 6.6 ECG diagnostic system (General Electric, Palatine, IL) to assess cardiac function during rest and exercise;
5. Littmann® Stethoscope (3M, St. Paul, MN) to ascultate the heart and lungs during the physical screening, as well as for the measurement of blood pressure during rest and exercise;
6. Sphygomomanometer (American Diagnostics Corporation, Hauppage, NY) to measure blood pressure during rest and exercise;
7. Lode electronically-braked cycle ergometer (Lode, Gronigen, The Netherlands) as the mode of the VO_{2peak} test and the aerobic exercise session will be cycling;
8. Parvo Medics TrueMax 2400 Metabolic System (Parvo Medics, Salt Lake City, UT) to measure respiratory gases and oxygen uptake (VO_2);
9. Polar telemetry system (Polar Electro Inc., Lake Success, NY) to measure heart rate;
10. Borg's 6-20 Rate of Perceived Exertion (RPE) scale to measure RPE;

The following equipment was used during the blood sampling procedures during Laboratory Visits 2 and 3, as well as for the measurement of NK cell counts, NKCA, catecholamines, and cortisol:

1. K_3EDTA and Sodium Heparin Vaccutainer® tubes for the collection of whole blood samples;
2. IEC Centra-8R refrigerated centrifuge (International Equipment Company, Needham Heights, MA) to isolate the plasma component of the whole blood samples;
3. Sorvall Biofuge Primo R centrifuge (Thermo Fisher Scientific, Waltham, MA) to spin whole blood samples during the preparation of samples for flow cytometric analysis;
4. COULTER® Ac•T diff ™ Hematology Analyzer (Beckman Coulter, Inc., Brea, CA) to measure complete blood count and differential white blood cell count;
5. CyAn 3 laser/9 color flow cytometer (Beckman Coulter, Inc., Brea, CA) to determine NK cell proportions and NKCA;
6. Enzyme-linked immunosorbant assay (ELISA) kits (Abnova, Taipei City, Taiwan) to measure plasma catecholamines (epinephrine and norepinephrine) and cortisol;
7. Bio-tek ELX800 microplate reader (BioTek U.S., Winooski, VT) to read ELISA assays.

Data Collection Procedures

Laboratory Visit 1: Medical/Physical Screening and VO_{2peak} Assessment

During the first visit to the laboratory, the subject received an explanation of the study protocol as well as information regarding the potential risks of participation in the study. Prior to arriving to the laboratory, all subjects were asked to follow a set of pre-assessment guidelines, presented below. Subjects were also asked about their adherence to these pre-assessment guidelines during the laboratory visit.

1. No eating for at least 2 hours prior to testing;

2. No exercise for at least 12 hours prior to testing;
3. Subjects should maintain adequate hydration status;
4. Subjects should refrain from caffeine use at last 12 hours prior to testing;
5. Subjects should refrain from alcohol use at least 48 hours prior to testing;
6. Subjects should wear clothing (including shoes) that is comfortable for exercise.

Once the subject had had all questions answered regarding study participation, the informed consent documents were signed. The subject then underwent a medical and physical screening to determine if she was free of any contraindication to performing an exercise test as described previously. During the medical and physical screening, the following procedures were performed:

1. Completion of a comprehensive medical questionnaire (Appendix B);
2. 12-lead resting ECG;
3. Auscultation of carotid, radial, and pedal pulses;
4. Auscultation of heart and lungs;
5. Assessment of resting blood pressure in the supine, sitting, and standing positions.

Upon clearance to participate in the study, the subject's physical characteristics were measured and/or recorded. Height, weight, and percent body fat were assessed using equipment housed in the laboratory, as described in the above section. Age and race were obtained by verbally asking the subject. Other physical characteristics such as menopausal status, physical activity participation, and cancer treatment type (for subjects in the breast cancer survivor group) were assessed from the subject's responses to the comprehensive medical questionnaire.

The final component of Laboratory Visit 1 included a cardiopulmonary exercise test to measure VO_{2peak}. The purpose of measuring VO_{2peak} was to assess peak aerobic capacity and to determine the workload that the subject would perform during Laboratory Visit 2.

The VO_{2peak} test was performed on an electronically-braked cycle ergometer, using the Astrand Cycle Ergometer Maximal Test Protocol (100) (Appendix C). The protocol is a multi-stage protocol with incremental increases in workload after every 3 minutes. The subject was seated on the cycle ergometer and fitted with the mask that was connected to the metabolic system, through which expired gases were collected and analyzed. The subject sat quietly on the cycle ergometer for 3 minutes while resting metabolic data was collected. The subject began the first 3-minute stage of the test by cycling at 50 Watts. At the end of the stage, the workload was increased by 25 Watts every 3 minutes until volitional fatigue. Heart rate, Rating of Perceived Exertion (RPE) (101), expired gases, and 12-lead ECG monitoring were performed throughout the test. Heart rate and RPE were recorded at the end of every minute. The test was administered by three individuals, one of whom was an exercise physiology faculty member of the Department of Exercise and Sport Science at UNC-Chapel Hill.

The highest VO_2 measured by the metabolic system during the last stage of the test was recorded at the subject's VO_{2peak}, and the corresponding workload on the cycle ergometer was recorded as the subject's peak workload. Once the VO_{2peak} test was complete, the subject entered an active cool-down period where the subject pedaled at a very low workload (<20 Watts) on the cycle ergometer. The subject was allowed to remove the mask, drink fluids ad libitum, and dismount the cycle ergometer. ECG monitoring continued for at

least 5 minutes post-exercise and until heart rate and blood pressure had returned to near-baseline levels (102).

Laboratory Visit 2: Acute Aerobic Exercise Session

Laboratory Visit 2 occurred within 2 weeks after Laboratory Visit 1. During Laboratory Visit 2, the subject performed 1 acute bout of moderate aerobic exercise on the cycle ergometer at a workload corresponding to 60% of the subject's VO_{2peak} for a duration of 30 minutes. The purpose for choosing an intensity of 60% of VO_{2peak} and a duration of 30 minutes was twofold. Firstly, 30-minute exercise bouts at 60% of VO_{2peak} are commonly used in many studies examining the effects of aerobic exercise on physical functioning in breast cancer patients, and therefore represent an intensity and duration widely used in exercise prescriptions for this population (3, 4, 6, 17, 39,102-107). Secondly, an exercise intensity of 50-60% of VO_{2peak} and a duration of 30 minutes is necessary in order to elicit a response in cortisol, which is one of the independent variables in this study (57). Additionally, previous pilot testing in the laboratory with breast cancer survivors newly enrolled in an exercise and recreation therapy rehabilitation program demonstrated that exercising for 30 minutes at an intensity of 60% of VO_{2peak} is feasible. However, to ensure that all subjects will be able to complete the exercise session, a discontinuous protocol was employed such that the subject alternated ten 3-minute intervals of exercise with 1.5 minutes of rest, for a total of 30 minutes of exercise in a 43.5-minute period. Two individuals supervised the exercise session and all subjects started the exercise session between 7:00-10:00 am in order to control for daily variations in the study variables.

As was the case with Laboratory Visit 1, subjects were asked to follow the set of pre-assessment guidelines before arriving for Laboratory Visit 2. Upon arrival to the laboratory,

the subject was asked about adherence to the pre-assessment guidelines. The subject was then asked to complete the Perceived Stress Scale (PSS), which is a widely-used psychological instrument used tor measuring the perception of stress (125, 126) (Appendix D). The purpose for administering the PSS was to assess the subject's perception of the stressors in her daily life, as these stressors could potentially impact resting values of the stress hormone variables in this study (epinephrine, norepinephrine, and cortisol). After completing the PSS, the subject rested in the supine position for approximately 20 minutes while a catheter was inserted into an antecubital vein in the arm for blood sampling. Catheter insertion was performed by an individual who was trained in phlebotomy techniques. Blood was drawn into two 3-mL K_3EDTA Vacutainer® tubes. For a subset of subjects, blood was also drawn into a 3-mL sodium heparin Vacutainer® tube. The blood that was drawn into the K_3EDTA Vacutainer® tubes was to be used for the measurement of NK cell counts, catecholamine, and cortisol levels. The blood that was drawn into the sodium heparin Vacutainer® tube was to be used for the measurement of NKCA. This blood sample was drawn before the exercise session (pre-exercise).

The subject performed a warm-up period, consisting of 4-5 minutes of light pedaling at about 30 Watts on the cycle ergometer. The subject then dismounted the cycle ergometer and was given the opportunity to stress the lower body in a manner that was most comfortable for the subject. The subject re-mounted the cycle ergometer and was fitted with the mouthpiece that was connected to the metabolic system, through which expired gases were collected and analyzed. The subject sat quietly on the cycle ergometer for 3 minutes while resting metabolic data was collected. The subject then began the exercise bout, which involved cycling at the prescribed workload for 30 minutes. As described previously, a

discontinuous protocol was employed such that the subject alternated ten 3-minute intervals of exercise with 1.5 minutes of rest. Heart rate and RPE were recorded at the end of every 3-minute period of exercise. Expired gases were monitored during the first, third, seventh, and tenth exercise interval. If necessary, adjustments in workload were made to ensure that the subject was exercising at 60% of VO_{2peak}. Immediately upon completion of the exercise session, the subject dismounted the cycle ergometer and returned to the supine position. Blood sampling immediately post-exercise was performed in the same manner as described for the pre-exercise sample.

The subject was then allowed to rest comfortably in the laboratory. The subject was allowed to drink water ad libitum; however, no food or other beverage could be ingested. A final blood sample was obtained at 2 hours post-exercise in the same manner as the previous two samples. The catheter was then removed and any necessary bandaging was performed.

Laboratory Visit 3: 24-hour Follow-up Session

Laboratory Visit 3 occurred 24 hours after Laboratory Visit 2. As was the case for Laboratory Visits 1 and 2, the subject was asked to follow the pre-assessment guidelines before arriving to the laboratory. Upon arrival to the laboratory, the subject was asked about adherence to the pre-assessment guidelines. The subject was allowed to use the restroom if needed. The subject will sat quietly in a phlebotomy chair and a blood sample was drawn from an antecubital vein in the arm using a standard venipuncture technique by an individual trained in phlebotomy techniques. The blood was drawn into the Vaccutainer® tubes in the same manner as was done for the previous three blood samplings. The timing of the blood draw occurred as close as possible to 24 hours after completion of the moderate aerobic

exercise session. However, a window of 30 minutes before or after the 24-hour post-exercise mark was deemed acceptable.

Data Processing and Reduction

Oxygen Uptake (VO_2) and Metabolic Data

Oxygen uptake (VO_2) and metabolic data was collected during Laboratory Visits 1 and 2, using the Parvo Medics TrueMax 2400 Metabolic System. The subject breathed into a mask which was connected to the metabolic system via plastic tubes. Since the metabolic system is computerized, the computer software was able to analyze a variety of parameters including the fraction of expired oxygen (FeO_2), fraction of expired carbon dioxide ($FeCO_2$), minute ventilation (V_E), oxygen uptake (VO_2), carbon dioxide output (VCO_2), and respiratory exchange ratio (RER). Data were recorded continuously, but was displayed as 15-second averages. These data were printed from the computer in text form, which was then transcribed into a Microsoft Excel workbook for easier manipulation.

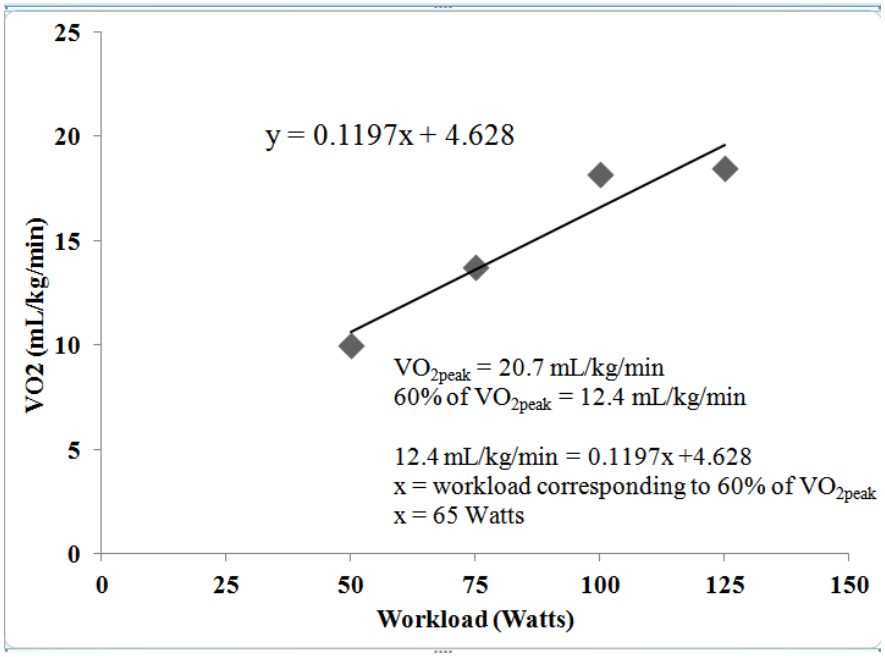
Calculation of VO_{2peak} and Exercise Workload

Peak oxygen consumption (VO_{2peak}) was determined from data collected during Laboratory Visit 1. VO_{2peak} was calculated by the metabolic system, and was simply the highest VO_2 value recorded during the Astrand Cycle Ergometer Maximal Test Protocol. The peak exercise workload was the workload on the cycle ergometer at which the subject was exercising when she achieved VO_{2peak}.

During Laboratory Visit 2, the subject exercised at approximately 60% of VO_{2peak}. This intensity was taken as 60% of the value that corresponded to the subject's VO_{2peak} (i.e., if the subject's VO_{2peak} was 20 mL/kg/min, then a 60% exercise intensity would be 12 mL/kg/min). The workload corresponding to 60% of VO_{2peak} was determined from a

regression analysis where VO_2 values for each stage of the VO_{2peak} test were averaged and plotted against the workload for that stage. Figure 3 shows the regression analysis for one subject as an example of how the workload corresponding to 60% of VO_{2peak} was calculated.

Figure 3. Example regression for determining workload corresponding to 60% of VO_{2peak}.



Determination of NK Cell Counts

Complete blood count (CBC) and differential white blood cell count were obtained from the whole blood samples collected at each of the four study time points into the K_3EDTA Vacutainer® tubes. These blood samples were analyzed using a COULTER® Ac•T diff ™ Hematology Analyzer. Total lymphocyte count was noted for each subject at each time point, as NK cells are a subset of lymphocytes.

The whole blood samples were stained using fluorescently-labeled monoclonal antibodies for the cell surface markers CD3, CD16, and CD56. The specific fluorescent labels used in this study were fluorescein isothyocyanate (FITC) for the CD3 marker,

phycoerythrin (PE) for the CD16 marker, and allophycocyanin (APC) for the CD56 marker. Fluorescent staining was done in order to identify the lymphocytes that express the $CD3^-$ $CD16^+CD56^+$ phenotype which is characteristic of NK cells. Stained samples were further prepared for flow cytometric analysis according to procedures adapted from protocols made available by Becton Dickinson (109). The specific procedures used in this study to prepare stained blood samples for flow cytometric analysis of NK cell counts are listed in Appendix E. Samples were analyzed within 24 hours of preparation.

The percentage of NK cells in the lymphocyte population was acquired from these blood samples using a CyAn 3 laser/9 color flow cytometer. For each subject, a total of 8 samples were prepared: one unstained control sample, 3 single color control samples (anti-CD3-FITC, anti-CD16-PE, and anti-CD56-APC), and the stained samples for each study time point (baseline, immediately post-exercise, 2 hours post-exercise, and 24 hours post-exercise). The unstained control sample and single color control samples were used to adjust the voltage settings on the flow cytometer so that individual cell populations would be in view, as well as to identify the positive and negative cell populations for each of the 3 cell surface markers. A region was drawn around the overall lymphocyte population on the side-scatter (SS) vs. forward-scatter (FS) plot, which was used to set a gate on the SS vs. anti-CD3-FITC plot. This was done in order to specifically identify which lymphocytes from the SS vs. FS plot were negative for the CD3 cell surface marker ($CD3^-$) and which lymphocytes were positive for the CD3 cell surface marker ($CD3^+$). A region was then drawn around the $CD3^-$ lymphocyte population in the SS vs. anti-CD3-FITC plot, which was then used to set a gate on the anti-CD56-APC vs. anti-CD16-PE plot. This was done in order to specifically identify which $CD3^-$ lymphocytes from the SS vs. anti-CD3-FITC plot were either positive

and/or negative for the CD16 and CD56 cell surface markers. Auto-compensation of the single color control samples was performed in order to mathematically eliminate spectral overlap between fluorophores. Flow cytometry data was viewed and analyzed using Summit 4.3 software (Dako North America, Inc., Carpinteria, CA).

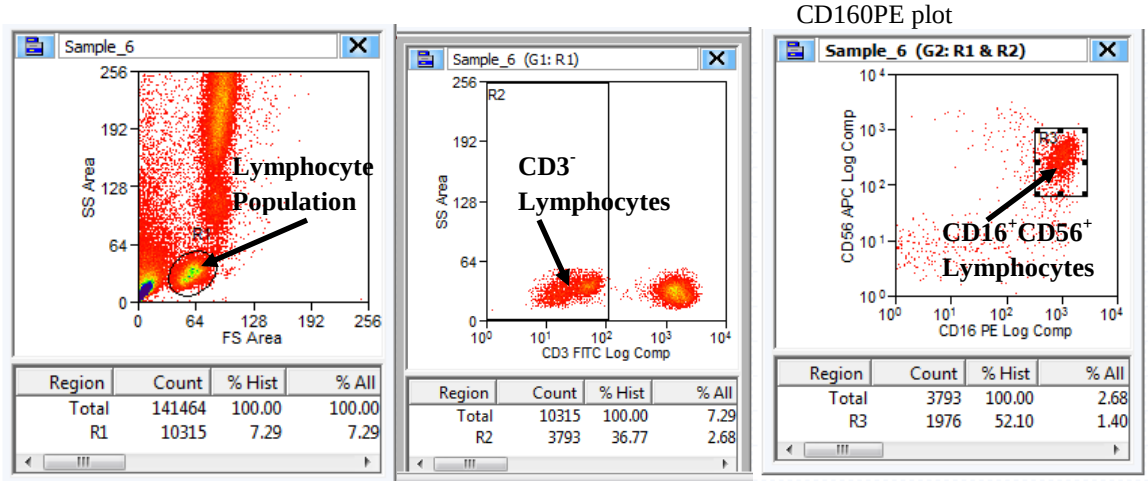
Figure 4 (Panels 4a-4c) shows the flow cytometry output for one subject's sample as a means to illustrate how these cell populations appear. Panel 4a shows the region R1 highlighting the overall lymphocyte population on the SS vs. FS plot. Panel 4b shows the region R2 highlighting the $CD3^-$ lymphocytes on the SS vs. anti-CD3-FITC plot. Panel 4c shows the region R3 highlighting the $CD3^-$ lymphocytes that are positive for the CD16 and CD56 cell surface markers. By convention, cell populations that are negative for a cell surface marker plotted on the x-axis appear on the left side of the plot, while cell populations that are positive for that cell surface marker appear on the right. Similarly, cell populations that are negative for a cell surface marker plotted on the y-axis appear on the bottom of the plot, while cell populations that are positive for that cell surface marker appear on the top.

Figure 4. Illustration of the overall lymphocyte, CD3, CD16, and CD56 cell populations using flow cytometry.

Figure 4a. Lymphocyte population on the SS vs. FS plot

Figure 4b. CD3 lymphocytes on the SS vs. anti-CD3-FITC plot

Figure 4c. $CD3^-$ lymphocytes with the $CD16^+CD56^+$ phenotype on the anti-CD56-APC vs. anti-CD160PE plot



NK cell count was determined by multiplying the total lymphocyte count by the proportion of NK cells in the lymphocyte population. Proportion of NK cells was determined by multiplying the proportion of $CD3^-$ lymphocytes (denoted as % Hist of R2 in Panel 4b) by the proportion of $CD16^+CD56^+$) lymphocytes (denoted as % Hist of R3 in Panel 4c).

Determination of NK Cell Activity

NKCA was determined for a subset of subjects (1 control and 6 breast cancer survivors) using a whole-blood flow cytometric assay adapted from Fondell et al. (110). The specific protocol used in this study for sample preparation is listed in Appendix F. K562 human erythroleukimic cells were cultured in a medium containing 89% Iscove's Dulbecco's Medium, 10% fetal bovine serum, and 1% penicillin-streptomycin. The cell culture was kept in an air incubator at 37°C and 5% CO_2. Culture medium was changed every 2-3 days and cell density was maintained between 10^5 and 10^6 cells per mL. The K562 cells were used as the target cells in the NKCA assay.

For each subject, 14 samples were prepared: two samples with target cells only, four samples with whole blood only (one for each study time point), and eight tubes with target cells and whole blood mixed together (two for each study time point). Samples were stained with FITC-labeled anti-CD71, as this fluorescently-labeled monoclonal antibody only stains the K562 target cells. Samples were acquired by within 2 hours of preparation using a CyAn 3 laser/9 color flow cytometer, and data was viewed and analyzed using Summit 4.3 software. NKCA, measured as % NK cytolysis, was calculated using the following equation:

$$\%\text{NK cytotox} = 100*[\text{target cells} - (\text{mixed cells} - \text{effector cells})] / \text{target cells}$$

where the "target cells" refers to the number of K562 cells measured in the samples with target cells only and "mixed cells" refers to the number of remaining K562 cells in the samples with a mixture of whole blood and K652 cells. "Effector cells" refers to cells in the samples with whole blood only which may be present in the region for $CD71^+$ K562 cells.

Calculation of Plasma Volume Shifts

Plasma volume shifts (changes in plasma volume due to exercise) were calculated according to the equation by Dill and Costill (111). The hematocrit and hemoglobin values that were used in these calculations were obtained from the CBC data at baseline, immediately post-exercise, 2 hours post-exercise, and 24 hours post-exercise. Plasma volume shifts were reported in order to indicate the effect that exercise may have on fluid shifts, which may affect concentrations of white blood cell populations (lymphocytes, monocytes, and granulocytes), catecholamines and cortisol.

Determination of Plasma Levels of Catecholamines and Cortisol

Catecholamines (epinephrine, norepinephrine) and cortisol were measured from stored plasma via enzyme-linked immunosorbent assay (ELISA) procedures. All samples were measured in duplicate to ensure quality control, except for two control subject samples which were run in singlet due to limited plasma volume. Catecholamines were measured using a competitive binding, single antibody, solid phase epinephrine/norepinephrine ELISA kit with analytical sensitivities of 10 pg/mL and 50 pg/mL for epinephrine and norepinephrine, respectively. Cortisol was measured using a competitive binding, double antibody, combination solid and mobile phase ELISA kit with a sensitivity of 0.44 ng/mL. The reported mean intra-assay coefficients of variation for epinephrine range from 6.9-15.8%, and the reported mean inter-assay coefficients of variation range from 13.2-18.2%. The reported mean intra-assay precisions coefficients of variation for norepinephrine range from 9.8-16.1%, and the reported mean inter-assay coefficients of variation range from 8.5-15.0%. The reported mean intra-assay coeffcients of variation for the cortisol ELISA range from 6.2-9.4%, and the reported mean inter-assay coefficients of variation range from 8.6-15.0%.

Concentration of plasma levels of catecholamines in samples were calculated using a calibration curve, constructed from plotting the natural log of the standard concentrations against the corresponding absorbance readings Second-order polynomial regression models were used to fit the standard curve, the equation from which was used to calculate the concentrations of plasma epinephrine and norepinephrine. Concentrations of plasma levels of cortisol were calculated in the same manner.

Data Analysis

Sample Size Estimation

The primary purpose of this study was to examine the effect of the moderate intensity aerobic exercise bout on changes in NK cell counts from baseline to post-exercise in breast cancer survivors and healthy controls. The secondary purpose of this study was to examine the relationships between changes in catecholamines and cortisol with changes in NK cell counts from baseline to post-exercise in response to the moderate intensity acute aerobic exercise bout in breast cancer survivors and healthy controls. Exploratory analyses of this study aimed to examine the effect of the moderate intensity aerobic exercise bout on changes in catecholamine and cortisol levels from baseline to post-exercise in breast cancer survivors and healthy controls, and to examine the effect of the moderate intensity aerobic exercise bout on changes in NKCA from baseline to post-exercise in breast cancer survivors and healthy controls.

The sample size estimation was based on the primary purpose of evaluating the change in NK cell count from pre-exercise to immediately post-exercise in the breast cancer survivor group. For this study, the desired sample size was the number of subjects per group needed to detect statistical significance when the percent difference between NK cell counts at baseline and immediately post-exercise was at least 50%. To determine this, the fold change was calculated (NK cell count immediately post-exercise divided by the NK cell count at baseline) and tested to see if it was significantly different from 1. A one group t-test with a two sided 0.05 significance level has 80% power to detect a difference between a null hypothesis of 1 and an alternative hypothesis of 1.5, assuming a standard deviation of 0.5

with 10 patients. To allow for similar analyses, 10 controls were to be enrolled as well, bringing the total goal sample size to 20 subjects for this study.

Statistical Analysis

All statistical analyses were performed using SPSS Statistics version 18.0. The alpha level was set *a priori* at 0.05.

Aim #1: Evaluation of Changes in NK Cell Counts

Hypotheses 1a: There will be a significant increase in NK cell counts from pre-exercise to immediately post-exercise in both breast cancer survivors and healthy controls.

Hypothesis 1b: There will be no significant difference in NK cell counts from pre-exercise to 2 hours post-exercise in both breast cancer patients and healthy controls.

Hypothesis 1c: There will be no significant difference in NK cell counts from pre-exercise to 24 hours post-exercise in both breast cancer patients and healthy controls.

Hypothesis 1d: There will be no significant differences in NK cell counts between breast cancer survivors and healthy controls at any time point (pre-exercise, immediately post-exercise, 2 hours post-exercise, and 24 hours post-exercise).

A 2x4 mixed model ANOVA was used to compare NK cell concentrations between the two study groups across the four time points. Pairwise comparisons between groups were made using independent samples t-tests. Percent changes in NK cell counts calculated as [(post-exercise NK cell count – pre-exercise NK cell count) / pre-exercise NK cell count)]*100 were compared between groups for each of the three post-exercise time points using independent samples t-tests.

Aim #2: Evaluation of Correlations between Changes in Catecholamine and Cortisol Responses with Changes in NK Cell Counts

Hypothesis 2a: Changes in catecholamine levels will be positively correlated with changes in NK cell counts from pre-exercise to immediately post-exercise in both breast cancer survivors and healthy controls. The fold changes in catecholamine responses from baseline to immediately post-exercise were correlated with the fold changes in NK cell count from baseline to immediately post-exercise using Spearman's Rank Correlation coefficients. This analysis was performed for both the breast cancer survivor group and the healthy control group.

Hypothesis 2b: Changes in cortisol levels will be positively correlated with changes in NK cell counts from pre-exercise to immediately post-exercise in both breast cancer survivors and healthy controls. The fold changes in cortisol responses from baseline to immediately post-exercise was correlated with the fold changes in NK cell count from baseline to immediately post-exercise using Spearman's Rank Correlation coefficients. This analysis was performed for both the breast cancer survivor group and the healthy control group.

Hypothesis 2c: Changes in catecholamine levels will not be correlated with changes in NK cell counts from pre-exercise to 2 hours post-exercise in both breast cancer survivors and healthy controls because changes in catecholamine levels and NK cell counts may not be significantly different from pre-exercise to 2 hours post-exercise. The fold changes in catecholamine responses from baseline to 2 hours post-exercise were correlated with the fold changes in NK cell count from baseline to 2 hours post-exercise

using Spearman's Rank Correlation coefficients. This analysis was performed for both the breast cancer survivor group and the healthy control group.

Hypothesis 2d: Changes in cortisol levels will not be correlated with changes in NK cell counts from pre-exercise to 2 hours post-exercise in both breast cancer survivors and healthy controls because changes in cortisol levels and NK cell counts may not be significantly different from pre-exercise to 2 hours post-exercise. The fold changes in cortisol responses from baseline to 2 hours post-exercise were correlated with the fold changes in NK cell count from baseline to 2 hours post-exercise using Spearman's Rank Correlation coefficients. This analysis was performed for both the breast cancer survivor group and the healthy control group.

Exploratory Analyses

Statistical procedures for the exploratory analyses of this study were the as those described above for Research Aim #1. Catecholamine levels and cortisol levels were compared between the groups across the four study time points using 2x4 mixed model ANOVAs, and pair-wise comparisons were made between groups using independent samples t-tests NKCA was examined across the four study time points using a repeated-measures ANOVA. Percent changes in post-exercise catecholamines, cortisol, and NKCA were also compared between the groups using independent samples t-tests.

APPENDIX B

MEDICAL HISTORY QUESTIONNAIRE

Department of Exercise and Sport Science
Medical History

Subject: _____ ID: _____ Telephone:_____

Address:_____

Occupation:_____ Age:_____

Race:_____

YES NO

Patient History

1. How would you describe your general health at present?

Excellent_____ Good_____ Fair_____ Poor_____

2. Do you have any health problems at the present time? _____ _____
3. If yes, please describe:_____
_____
4. Have you ever been told you have heart trouble? _____ _____
5. If yes, please describe:_____
_____
6. Do you ever get pain in your chest? _____ _____
7. Do you ever feel light-headed or have you ever fainted? _____ _____
8. If yes, please describe:_____
_____
9. Have you ever been told that your blood pressure has been elevated? _____ _____
10. If yes, please describe:_____
_____
11. Have you ever had difficulty breathing either at rest or with exertion? _____ _____
12. If yes, please describe:_____
_____
13. Are you now, or have you been in the past 5 years, under a doctor's care for any reason?
_____ _____
14. If yes for what reason?_____
_____
_____
15. Have you been in the hospital in the past 5 years? _____ _____
16. If yes, for what reason?_____
_____
17. Have you ever experienced an epileptic seizure or been informed that you have epilepsy?
_____ _____

18. Have you ever been treated for infectious mononucleosis, hepatitis, pneumonia, or another infectious disease during the past year? _____ _____
19. If yes, name the disease:_____
20. Have you ever been treated for or told you might have diabetes? _____ _____
21. Have you ever been treated for or told you might or low blood sugar? _____ _____
22. Do you have any known allergies to drugs? _____ _____
23. If so, what?_____

_____

24. Have you ever been "knocked-out" or experienced a concussion? _____ _____
25. If yes, have you been "knocked-out" more than once? _____ _____
26. Have you ever experienced heat stroke or heat exhaustion? _____ _____
27. If yes, when?_____

_____

28. Have you ever had any additional illnesses or operations? (Other than childhood diseases)

_____ _____

29. If yes, please indicate specific illness or operations:_____

_____

_____

_____

30. Are you now taking any pills or medications? _____ _____
31. If yes, please list:_____

_____

32. Have you had any recent (within 1 year) difficulties with your:
    a. Feet _____ _____
    b. Legs _____ _____
    c. Back _____ _____

Family History

33. Has anyone in your family (grandparent, father, mother, and/or sibling) experienced any of the following?
    a. Sudden death _____ _____
    b. Cardiac disease _____ _____
    c. Marfan's syndrome _____ _____

Mental History

34. Have you ever experienced depression? _____ _____
35. If yes, did you seek the advice of a doctor? _____ _____
36. Have you ever been told you have or has a doctor diagnosed you with panic disorder, obsessive-compulsive disorder, clinical depression, bipolar disorder, or any other psychological disease? _____ _____
37. If yes, please list condition and if you are currently taking any medication.

Condition Medication

_____

_____

_____

_____

Bone and Joint History

34. Have you ever been treated for Osgood-Schlatter's disease? _____ _____
35. Have you ever had any injury to your neck involving nerves or vertebrae? _____ _____
36. Have you ever had a shoulder dislocation, separation, or other injury of the shoulder that incapacitated you for a week or longer? _____ _____
37. Have you ever been advised to or have you had surgery to correct a shoulder condition? _____ _____
38. Have you ever experienced any injury to your arms, elbows, or wrists?_____ _____
39. If yes, indicate location and type of injury:_____

_____

40. Do you experience pain in your back? _____ _____
41. Have you ever had an injury to your back? _____ _____
42. If yes, did you seek the advice of a doctor? _____ _____
43. Have you ever been told that you injured the ligaments or cartilage of either knee joint? _____ _____
44. Do you think you have a trick knee? _____ _____
45. Do you have a pin, screw, or plate somewhere in your body as the result of bone or joint surgery that presently limits your physical capacity? _____ ____
46. If yes, indicate where:_____

_____

47. Have you ever had a bone graft or spinal fusion? _____ _____

Activity History

48. During your early childhood (to age 12) would you say you were:
    Very active ____ Quite active____ Moderately active____ Seldom active____
49. During your adolescent years (age 13-18) would you say you were:
    Very active ____ Quite active____ Moderately active____ Seldom active____
50. Did you participate in:
    a. Intramural school sports? _____ _____
    b. Community sponsored sports? _____ _____
    c. Varsity school sports? _____ _____
    d. Active family recreation? _____ _____
51. Since leaving high school, how active have you been?
    Very active ____ Quite active____ Active____ Inactive____
52. Do you participate in any vigorous activity at present? _____ _____
53. If yes, please list:

Activity	Frequency	Duration	Intensity

54. How would you describe your present state of fitness?
Excellent_____ Good_____ Fair_____ Poor_____
55. Please list the type(s) of work you have been doing for the previous ten years:
Year Work Indoor/Outdoor Location (city/state)

_____

_____

Menstrual Cycle History

56. Have you been post-menopausal for the past year, in that you have not experienced a menstrual period for at least 1 year? _____ _____

Emergency Contact Information

57. Whom shall we notify in case of emergency?
Name:_____
Phone: (Home)_____ (Work)_____
Address:_____
58. Name and address of personal physician:_____

_____

_____

FOR SUBJECTS IN THE BREAST CANCER SURVIVOR GROUP ONLY

59. Please indicate which type(s) of treatment you received/are receiving for your cancer
Surgery____ Chemotherapy____ Radiation Therapy____ Hormonal Therapy____
Trastuzumab_____

60. How long ago did you finish receiving surgery, chemotherapy, and/or radiation therapy?

_____

61. If you received surgery, which type did you receive?
Mastectomy ____ Lumpectomy____

62. If you received chemotherapy, please list the names of the drugs that were included in your treatment.

_____

_____

63. If you are receiving hormonal therapy, please list the names of the medications that you are taking and how long you have been taking them.

_____

_____

64. If you are receiving trastuzumab, please list how long you have been taking this medication.

_____

65. Please list any other medication(s) that you have taken/are currently taking that is/are directly related to your cancer treatment.

_____

_____

All of the above questions have been answered completely and truthfully to the best of my knowledge.

Signature:_____ Date:_____

APPENDIX C

ASTRAND CYCLE ERGOMETER MAXIMAL TEST PROTOCOL

Instructions:

1. Attach 12-lead ECG to subject.
2. Adjust cycle ergometer seat to the appropriate height for subject.
3. Fit subject with mask while subject is seated on cycle ergometer.
4. Collect resting metabolic data (Test Stage: Rest)
5. Start VO_{2peak} test (Test Stage: 1).
    a. Test Stage 1 workload is 50 Watts.
    b. Each Test Stage lasts for 3 minutes.
    c. Increase workload by 25 W from Test Stage 1 to 2. Use this same increment for all subsequent increases in workload as the test progresses.
    d. Monitor ECG and expired gases continuously.
    e. Record HR and RPE at the end of each minute.
    f. Record BP at the end of Minute 2 of each stage.
6. Subject will progress through test until volitional fatigue or symptom-limited indication*.
7. Record VO_{2peak} as the highest VO_2 reached during the test.
8. Record Peak Workload as the highest workload used during the test.
9. Record total test time.
10. Allow subject to cool down on cycle ergometer (pedal at very light workload). Subject may remove mask and drink fluids during this time.
11. Continue ECG monitoring for at least 5 minutes (longer if needed).
12. Assessment is concluded when HR, ECG, and BP return to pre-test levels.
13. Disconnect ECG leads and remove electrodes from subject.

Test Stage	Stage Duration	Workload (Watts)	HR (bpm)	RPE	BP (mmHg)
Rest	3 minutes	NA	NA	NA	NA
1	3 minutes	50	Min 1: Min 2: Min 3:	Min 1: Min 2: Min 3:	Min 2:
2	3 minutes	75	Min 1: Min 2: Min 3:	Min 1: Min 2: Min 3:	Min 2:
3	3 minutes	100	Min 1: Min 2: Min 3:	Min 1: Min 2: Min 3:	Min 2:
4	3 minutes	125	Min 1: Min 2: Min 3:	Min 1: Min 2: Min 3:	Min 2:
5	3 minutes	150	Min 1: Min 2: Min 3:	Min 1: Min 2: Min 3:	Min 2:

VO_{2peak} _____
Peak workload _____
Total test time _____

Have HR, BP, and ECG returned to pre-assessment levels before leaving lab? YES NO
HR _____
BP _____

*Symptom-limited indications for stopping a VO_{2peak} test

1. Drop in systolic blood pressure of >10 mmHg from baseline blood pressure, despite an increase in workload;
2. Hypertensive response, characterized by systolic blood pressure >250 mmHg and/or a diastolic blood pressure of >115 mmHg;
3. Moderately severe angina (defined as 3 on standard scale) or increasing chest pain;
4. Wheezing, shortness of breath, leg cramps, dizziness, or near syncope;
5. Signs of poor perfusion, such as cyanosis or pallor;
6. Arrrythmias including sustained ventricular tachycardia, multifocal PVCs, triplets of PVCs, supraventricular tachycardia, heart block, or bradyarrhythmias;
7. Development of bundle branch block or intraventricular conduction delay that cannot be distinguished from ventricular tachycardia;
8. ST elevation (+1.0 mm) in ECG leads without diagnostic Q-waves (other than V_1 or aVR)
9. ST or QRS changes such as excessive ST depression (> 2 mm horizontal or downsloping ST-segment depression) or marked axis shift.

APPENDIX D

PERCEIVED STRESS SCALE (PSS)

PERCEIVED STRESS SCALE

Sheldon Cohen

The *Perceived Stress Scale* (PSS) is the most widely used psychological instrument for measuring the perception of stress. It is a measure of the degree to which situations in one's life are appraised as stressful. Items were designed to tap how unpredictable, uncontrollable, and overloaded respondents find their lives. The scale also includes a number of direct queries about current levels of experienced stress. The PSS was designed for use in community samples with at least a junior high school education. The items are easy to understand, and the response alternatives are simple to grasp. Moreover, the questions are of a general nature and hence are relatively free of content specific to any subpopulation group. The questions in the PSS ask about feelings and thoughts during the last month. In each case, respondents are asked how often they felt a certain way.

Evidence for Validity: Higher PSS scores were associated with (for example):

- failure to quit smoking
- failure among diabetics to control blood sugar levels
- greater vulnerability to stressful life-event-elicited depressive symptoms
- more colds

Health status relationship to PSS: Cohen et al. (1988) show correlations with PSS and: Stress Measures, Self-Reported Health and Health Services Measures, Health Behavior Measures, Smoking Status, Help Seeking Behavior.

Temporal Nature: Because levels of appraised stress should be influenced by daily hassles, major events, and changes in coping resources, predictive validity of the PSS is expected to fall off rapidly after four to eight weeks.

Scoring: PSS scores are obtained by reversing responses (e.g., 0 = 4, 1 = 3, 2 = 2, 3 = 1 & 4 = 0) to the four positively stated items (items 4, 5, 7, & 8) and then summing across all scale items. A short 4 item scale can be made from questions 2, 4, 5 and 10 of the PSS 10 item scale.

Norm Groups: L. Harris Poll gathered information on 2,387 respondents in the U.S.

Norm Table for the PSS 10 item inventory

Category	N	Mean	S.D.
Gender			
Male	926	12.1	5.9
Female	1406	13.7	6.6
Age			
18-29	645	14.2	6.2
30-44	750	13.0	6.2
45-54	285	12.6	6.1
55-64	282	11.9	6.9
65 & older	296	12.0	6.3
Race			
white	1924	12.8	6.2
Hispanic	98	14.0	6.9
black	176	14.7	7.2
other minority	50	14.1	5.0

Perceived Stress Scale

The questions in this scale ask you about your feelings and thoughts **during the last month**. In each case, you will be asked to indicate by circling *how often* you felt or thought a certain way.

Name _____ Date _____

Age _____ Gender (*Circle*): **M F** Other _____

	0 = Never	1 = Almost Never	2 = Sometimes	3 = Fairly Often	4 = Very Often
1. In the last month, how often have you been upset because of something that happened unexpectedly?	0	1	2	3	4
2. In the last month, how often have you felt that you were unable to control the important things in your life?	0	1	2	3	4
3. In the last month, how often have you felt nervous and "stressed"?	0	1	2	3	4
4. In the last month, how often have you felt confident about your ability to handle your personal problems?	0	1	2	3	4
5. In the last month, how often have you felt that things were going your way?	0	1	2	3	4
6. In the last month, how often have you found that you could not cope with all the things that you had to do?	0	1	2	3	4
7. In the last month, how often have you been able to control irritations in your life?	0	1	2	3	4
8. In the last month, how often have you felt that you were on top of things?	0	1	2	3	4
9. In the last month, how often have you been angered because of things that were outside of your control?	0	1	2	3	4
10. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?	0	1	2	3	4

Please feel free to use the *Perceived Stress Scale* for your research.

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The PSS Scale is reprinted with permission of the American Sociological Association, from Cohen, S., Kamarck, T., and Mermelstein, R. (1983). A global measure of perceived stress. *Journal of Health and Social Behavior, 24,* 386-396.

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APPENDIX E

SAMPLE PREPARATION FOR NK CELL COUNTS

The following are the procedures for preparing whole blood samples for flow cytometric analysis of NK cell counts. This protocol is adapted from Becton Dickinson's protocol for direct immunofluorescence staining of whole blood using a lyse/wash procedure: (http://www.bdbiosciences.com/support/resources/protocols/stain_lyse_wash.jsp)

Materials

1. FITC Mouse Anti-Human CD3 Antibody (BD Catalog # 555339)
2. PE Mouse Anti-Human CD16 Antibody (BD Catalog # 555407)
3. APC Mouse Anti-Human CD56 Antibody (BD Catalog # 555518)
4. 10X FACS Lysing Solution (BD Catalog # 349202)
5. Dulbecco's PBS (pH = 7.2 ± 0.2; not containing calcium, magnesium, or phenol red (Invitrogen Catalog # 14190-136)
6. Paraformaldehyde solution (Invitrogen Catalog # FB001)
7. 0.2 µm filter (Fisher Scientific Catalog #09 740 24A)
8. K_3EDTA Vacutainer tubes
9. 12x75 mm polypropylene test tubes (BD Catalog # 352063)*

*The specific type of tube is of importance depending on which type of flow cytometer is being used. The CyAn flow cytometer used in this study requires polypropylene tubes.

Blood Collection

1. Collect blood into K_3EDTA Vaccutainer® (Lavender top) tube.
2. Keep anticoagulated blood at room temperature (20-25°C) until ready for staining. Blood samples should be stained within 6 hours of collection.

Preparation of Reagents

Before performing the staining and fixing procedures below, some steps will need to be performed ahead of time. These include a 1X dilution of the 10X FACS Lysing Solution and filtering of the PBS wash buffer. See below for details.

1. Preparation of 1X dilution of the 10X FACS Lysing Solution (used in Step 8)
    a. Mix 10X FACS Lysing Solution with room temperature de-ionized water in a 1:10 ratio. For example, 10 mL of the FACS Solution can be mixed with 90 mL de-ionized water.
    b. Store prepared solution in a glass container at room temperature for up to 1 month.
2. Preparation of the PBS wash buffer (used in Steps 3-5, 11 and 13)
    a. Filter PBS solution through a 0.2 µm filter prior to use. This can be done in bulk (i.e., 250 mL at a time) and stored at room temperature.

Staining and Fixing Cells

1. Label test tubes as follows for each experiment:
    Tube 1: blood + CD3 antibody (single color control for FITC CD3)
    Tube 2: blood + CD16 antibody (single color control for PE CD16)
    Tube 3: blood + CD56 antibody (single color control for APC CD56)
    Tube 4: blood + CD3 antibody + CD16 antibody + CD56 antibody (the actual sample)*

*Note that depending on how many different blood collections have been done, there will be multiple Tube 4's (i.e., one for pre-exercise and one for each post-exercise time point). For the single color controls, only one tube of each is needed, and it doesn't matter which blood is used.

2. Add 100 µL of whole blood to each tube.
3. Add 8 µL of CD3 antibody and 12 µL of PBS wash buffer to Tubes 1 and 4.
4. Add 5 µL of CD16 antibody and 15 µL of PBS wash buffer to Tubes 2 and 4.
5. Add 8 µL of CD56 antibody and 12 µL of PBS wash buffer to Tubes 3 and 4.
6. Vortex tubes gently and incubate in the dark at room temperature (20-25°C) for 30 minutes.
7. Prepare another tube with 100 µL of whole blood only. This can be Tube 5. It will serve as the unstained control for the experiment.
8. Add 2 mL of the 1X FACS Lysing Solution to each tube.
9. Vortex tubes gently and incubate in the dark at room temperature for 10 minutes.
10. After the tubes have finished incubating, centrifuge at 500 x g for 5 minutes. Remove the supernatant.
11. Add 2-3 mL of PBS wash buffer to each tube.
12. Centrifuge tubes at 500 x g for 5 minutes. Remove the supernatant.
13. Add 250 µL of the paraformaldehyde solution and 250 µL of the PBS wash buffer to each tube and mix thoroughly. Let sit for 20 minutes.
14. Cap tubes and store in the dark at 2-8°C until ready to analyze on the flow cytometer. Samples should be analyzed within 24 hours.

APPENDIX F

SAMPLE PREPARATION FOR NKCA ASSAY

Materials

1. Target Cells: K562 cells (a NK-sensitive human Caucasian chronic myelogenous leukemia cell line)
2. Effector Cells: whole blood, collected into sodium-heparin Vacutainer® tubes
3. Monoclonal antibody for labeling target cells: FITC Mouse Anti-Human CD71 Antibody (BD Catalog # 555536)
4. Lysing Solution: 10X FACS Lysing Solution (BD Catalog # 349202), diluted to 1X concentration
5. Test Preparation Tubes: 12x75 mm polypropylene test tubes (BD Catalog # 352063)*
6. Sodium Heparin Vacutainer® tubes
7. Dulbecco's PBS (pH = 7.2 ± 0.2; not containing calcium, magnesium, or phenol red (Invitrogen Catalog # 14190-136)
8. Dilution Buffer: GIBCO® RPMI 1640 w/Glutamax-1 + heat inactivated FBS + penicillin-streptomycin (Invitrogen Catalog # 61870-036, 16140-071, and 15140-148)
9. Culture Medium for maintaining K562 cells: Iscove's Modified Dulbecco's Medium (ATCC Catalog # 30-2005) + heat inactivated FBS + penicillin-streptomycin

*The specific type of tube is of importance depending on which type of flow cytometer is being used. The CyAn flow cytometer used in this study requires polypropylene tubes.

Preparation of Dilution Buffer

Mix 250 mL GIBCO® RPMI 1640 w/Glutamax-1, 25 mL heat-inactivated FBS, and 1.25 mL penicillin-streptomycin. To ensure that the mixture is sterile, filter through a 0.2 µm filter. Store prepared Dilution Buffer at 2-8°C.

Preparation of 1X dilution of the 10X FACS Lysing Solution

Mix 10X FACS Lysing Solution with room temperature de-ionized water in a 1:10 ratio. For example, 10 mL of the FACS Solution can be mixed with 90 mL de-ionized water. Store prepared solution in a glass container at room temperature for up to 1 month.

Preparation of PBS Wash Buffer

Filter PBS solution through a 0.2 µm filter prior to use. This can bedone in bulk (i.e., 250 mL at a time) and stored at room temperature.

Maintenance of K562 Cell Culture

1. Prepare the Culture Medium, which consists of 89% Iscove's Modified Dulbecco's Medium, 10% FBS, and 1% penicillin-streptomycin. This can be made in bulk (i.e., quantities of 250 mL or 500 mL). To ensure that the Culture Medium is sterile, filter through a 0.2 µm filter. Store prepared Culture Medium at 2-8°C.

2. Maintain the K562 cells in the Culture Medium. Cultures should be maintained at a density of 10^5-10^6 cells/mL and kept in an air incubator at 37°C in 5% CO_2. Fresh Culture Medium should be added every 2-3 days depending on cell density.

Assay Protocol

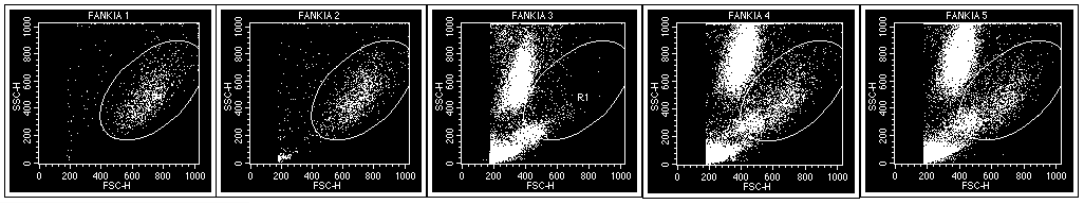
1. Label test tubes as follows for each experiment
   - Tube 1: target cells only
   - Tube 2: target cells only
   - Tube 3: effector cells only*
   - Tube 4: target cells + effector cells*
   - Tube 5: target cells + effector cells*

*Note that depending on how many different blood collections have been done, there will be multiple Tube 3's, 4's, and 5's (i.e., ones for pre-exercise and ones for each post-exercise time point).

2. Count freshly prepared K562 cells with a high viability (>95%) and adjust to a concentration of $2x10^5$ viable cells per mL.
3. Add 500 µL of K562 cells to Tubes 1, 2, 4, and 5.
4. Add 500 µL of Dilution Buffer to Tubes 1, 2, and 3.
5. Add 500 µL of whole blood to Tubes 3, 4, and 5.
6. Centrifuge tubes at 200 x g for 1 minute.
7. Incubate tubes at 37°C in 5% CO_2 for 2 hours.
8. Add 10 µL of CD71 antibody to each tube. Mix gently.
9. Incubate tubes at room temperature in the dark for 15 minutes.
10. Add 2 mL of 1X FACS Lysing Solution to each tube. Mix gently.
11. Incubate tubes at room temperature in the dark for 20 minutes.
12. Centrifuge tubes at 500 x g for 5 minutes. Remove the supernatant.
13. Re-suspend cells in 500 µL PBS wash buffer.
14. Cap tubes and and store in the dark at 2-8°C until ready to analyze on the flow cytometer. Samples should be analyzed within 2 hours.

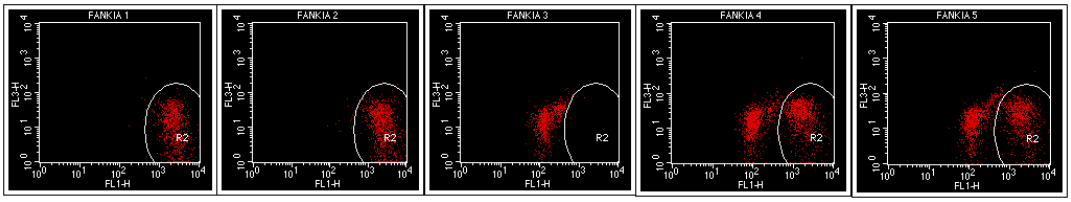
Analysis of NKCA on the flow cytometer

1. Use the same flow rate when acquiring data for each sample. Additionally, each acquisition should last for the same amount of time for each sample so that the same volume is analyzed for each sample. A 1 minute acquisition time is recommended.
2. Use the side scatter (SS) vs. forward scatter (FS) plot to draw a region R1 around the targets as shown below:



Tube 1 Tube 2 Tube 3 Tube 4 Tube 5

3. Use this region to set a gate in the FL3 vs. FL1 plot. Draw a region R2 around the $CD71^{+}$ target cells (see below). This will allow for separation of labeled target cells from unlabeled cells in the whole blood that may overlap the K562 region in the SS vs. FS plot.



Tube 1 Tube 2 Tube 3 Tube 4 Tube 5

4. The numbers of cells in regions R2 are the ones that are used in the equation to calculate NKCA (% NK cytotox).

% NK cytotox = 100*[target cells – (mixed cells – effector cells)] / target cells
% NK cytotox = 100*[(Tube 3 + Tube 3 + Tube 1 + Tube 2 – Tube 4 – Tube 5) / (Tube 1 + Tube 2)]

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