The Effects of a Single Bout of Resistance Exercise on Inflammatory Markers in Breast Cancer Survivors

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ABSTRACT

JACOB M. ALLEN: The Effects of a Single Bout of Resistance Exercise on Inflammatory Markers in Breast Cancer Survivors
(Under the direction of Anthony C. Hackney, Ph.D., D.Sc.)

This study examined the effect of resistance exercise (RE) on the acute responses of three inflammatory cytokines (Interleukin 6 [IL-6], Interleukin 10 [IL-10], Tumor Necrosis Factor-α [TNF-α]) in breast cancer survivors. Breast cancer survivors (BCS) (n = 4) and healthy age, matched controls (C) (n=8) completed three sets of four exercises at 70% of one-repetition max. Blood samples were collected prior (Pre), immediately after (ImP), 2 hours after (2P) and 24 hours after (24P) exercise bout. No differences were observed between groups for each cytokine at each time point after exercise. However, the RE did cause a significant upregulation in IL-6 at 2P ($p=0.027$) and a significant downregulation of TNF-α at 24P in both groups ($p=0.011$). These findings suggest that no difference exists in resistance exercise inflammatory response between BCS and C, and that RE may promote an anti-inflammatory response into recovery, as evidenced by the reduction in TNF-α at 24P.

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CHAPTER I

BASIS FOR STUDY

Introduction:

Chronic degenerative diseases, such as cancer, are often characterized by the presence of chronic low-grade inflammation (Lira et al., 2009). Cancer-related inflammation initiates the proliferation and survival of malignant cells, promotes angiogenesis and metastasis, disrupts immune responses, modifies responses to hormones, and ultimately leads to a reduced chance of survival (Mantovani et al., 2008; Pierce et al., 2009). Low-grade inflammation is also associated with numerous negative symptoms of cancer, including but not limited to: fatigue, cachexia, pain, depression and increased treatment toxicity (Seruga et al., 2008). In addition to the independent action of the disease, chemotherapy and radiation treatments are likely to augment the inflammatory response and thus contribute to the worsening of many symptoms (Balkwill & Mantovani, 2001; Pederson & Saltin, 2006; Seruga et al., 2008). Accordingly, cancer patients and survivors are in need of treatments and therapies that alleviate inflammation as well as the negative ailments associated with the disease (Battaglini et al., 2012).

Physical exercise has been presented as an acceptable and effective method for improving symptoms and possibly reducing chronic inflammation in cancer patients and survivors (Battaglini et al., 2006; Courneya et al., 2007; Seruga et al., 2008). Moreover, the ability of exercise to improve muscle strength, cardiorespiratory capacity, functional

movement and quality of life in breast cancer populations is well founded (Al-Majid & Waters, 2008; Courneya et al., 2003; Courneya et al., 2007).

While most of the current research has focused on aerobic exercise, resistance exercise has also been shown to have encouraging effects in cancer populations. Resistance training can increase lean body mass, as well as improve muscle function and strength in breast cancer survivors (Courneya et al., 2007; Schmitz et al., 2005). Additionally, studies have shown benefits of resistance exercise in halting or even reversing the effects cancer related muscle wasting (Galvao et al., 2006; Segal et al., 2003). Furthermore, resistance training may be effective to 'prevent and delay' low grade systemic inflammatory related diseases (Calle & Fernandez, 2010; Phillips et al., 2010). As a result of these encouraging studies, resistance exercise is being implemented more frequently in cancer exercise programs (Battaglini et al., 2006).

In order to optimize the use of resistance exercise in cancer survivors, it is necessary to understand the acute physiological perturbations, as uncovering these phenomena will provide a better understanding to the time course of recovery from exercise. Furthermore, the long-term effect of exercise may to some extent be attributed to the anti-inflammatory response elicited by acute exercise sessions (Mathur & Pederson, 2008). Therefore, the acute inflammatory response to resistance exercise, which has not been investigated extensively in cancer survivors, is an important concept to explore.

Acute, post-exercise inflammation is defined by intricate interactions between cytokines, catabolic hormones, acute phase proteins and immune cells which ultimately lead to variability in the internal endocrine and paracrine milieu (Nieman, 2003; Pederson, 2000). The oscillations of each of these signaling components are influential in the overall

inflammatory state. Notably, the benefits of exercise are thought to arise partially from a larger systemic anti-inflammatory rather than pro-inflammatory response (Peterson & Pederson, 2005). The initiation of an anti-inflammatory response, directed predominantly by cytokines, inhibits extensive proliferation of immune cells, impedes the production of proteins responsible for proteolysis, and ultimately establishes a more optimal milieu for recovery (Peterson & Pederson, 2006). Moreover, the acute anti-inflammatory response has been hypothesized to contribute to the long-term benefits of exercise (Peterson & Pederson, 2006).

The acute effects of exercise, however, are not always encouraging. Researchers have showed that intense exercise, prolonged exercise, or exercise in an immunosuppressed state, can induce a pro-inflammatory state, eventual immunosuppression, and thus inadequate recovery from exercise (Nieman, 1998; Smith, 2000). This phenomenon is partially described by the "open window" theory, which is understood as a period of time after exercise in which an individual may be subject to increased risk of infection due to a depression in immune function (Nieman, 1998). Hence, if subsequent exercise sessions take place during this "open window", further immunosuppression is likely to occur (Nieman, 1998). Other theories also provided support the possible negative effects of exercise. The overtraining syndrome (OTS), described by 'excessive training' and 'deteriorating results', supports that exercise in an hyper-inflamed state will initiate a 'survival' response rather an 'adaptive' response (Smith, 2000). It is hypothesized that OTS is coordinated by pro- and anti-inflammatory cytokines, and that the summation of acute interactions will propagate into lasting effects (Smith, 2000).

As a population that often experiences chronic inflammation and immunosuppression, cancer survivors are in need of programs that improve, not hinder inflammatory issues.

Therefore, deliberating the interplay between the opposing inflammatory responses in the acute state remains a principal concern in determining the overall response to exercise. In turn, exercise protocols and programs for cancer survivors can be implemented in a more comprehensible and coordinated fashion.

Purpose:

The purpose of this study was to investigate the acute inflammatory response to resistance exercise in breast cancer survivors. To accomplish this, the extent and time course of exercise induced cytokines (IL-6, TNF-α and IL-10) released into the plasma were observed. These observations will help elucidate not only isolated cytokine changes from exercise, but also the interactions amongst these proteins. To understand how the inflammatory response may differ in cancer survivors, post-exercise cytokine dynamics were compared between two groups (cancer survivors vs. healthy age-matched participants). Uncovering the acute inflammatory responses may better explain whether there is a difference between healthy controls and cancer survivors in the recovery from exercise. In turn, exercise protocols and programs for cancer survivors can be implemented in a more comprehensible and coordinated fashion. As an exploratory purpose, the effect of resistance exercise on the major catabolic hormone, cortisol, was determined. Cortisol release has also been strongly associated with the cytokine IL-6. Suitably, this study examined any correlative function that may exist between these endocrine components.

Limitations

1. The findings are applicable to post-menopausal women.
2. Cancer survivors' or healthy controls exercise training prior to treatment was not accounted for.

3. Changes in cytokines were measured only in the blood. There was no other measurements to verify local cytokine changes. (e.g. Muscle or Leukocyte mRNA)

Delimitations

1. Emotional stress levels were assessed with a questionnaire before each exercise session.
2. Subjects were asked to refrain from eating 2 hours prior to exercise session to avoid nutritional alterations in inflammation.
3. There was no strenuous activity within 24 hours of the exercise session.
4. No NSAIDS (non-steroidal anti-inflammatory), caffeine, or alcohol was consumed within 24 hours of exercise session.
5. No subjects with range of motion issues or with biomechanical deficiencies were allowed to participate in the study.

Significance of Study

This study will help to elucidate how inflammation is acutely altered after resistance exercise. Few studies to date have examined these effects in cancer survivors. This is especially significant in cancer survivors who are inclined to chronic inflammation and immunosuppression. If alterations in cytokines and cortisol are evident after the resistance exercise bout, then careful analysis of the type and time course of the inflammation will help clarify how and when the patients are recovering from the exercise session.

This is particularly significant if a pro-inflammatory state exists for an extensive time period following exercise. A chronic, low-grade pro-inflammatory state initiated by intense, acute exercise will hamper ample recovery and will likely initiate an immunosuppressed

state. Thus, if a subsequent exercise or any other strenuous activity were to be undertaken, it may hinder the ability of a participant to recover from the exercise session and/or cause further inflammation or immunosuppression. On the contrary, if an anti-inflammatory or an absence of an inflammatory response is observed, then it may be assumed that the cancer survivors are responding encouragingly to the exercise session.

Overall, understanding the acute effects of inflammation after resistance exercise will help ascertain what could be some of the necessary general guidelines for cancer exercise programs. This will be accomplished by recognizing the alterations in inflammation after exercise.

CHAPTER II

LITERATURE REVIEW

Research throughout the past two decades has demonstrated that exercise induces extensive, yet varying changes in the immune system (Nieman, 1994; Pederson & Febbraio, 2008). While moderate exercise may improve immune function, strenuous exercise elicits enhanced recruitment of immune and inflammatory mediators, which over time, may cause a suppression in immunity leading to increased susceptibility to infections (Nieman, 1994; Suzuki et al., 2002). As mediators of these phenomena, cytokines released into the circulation have been a recent emphasis for researchers. Thus, understanding the interactions between exercise and cytokines provides a unique opportunity to evaluate a portion of the underlying endocrine and immune mechanisms during and after exercise. Moreover, since this investigation was aimed to describe the effects of exercise in cancer survivors, uncovering how inflammation elicits adverse symptomology and poor prognosis in cancer survivors is an important concept to review.

Cytokines are proteins secreted by the immune system and a host of other cells that function as the key contributors to immunity and inflammation (Abbas et al., 2007; Smith, 2000). Cytokines are divided into several families: interleukins (IL), tumor necrosis factors (TNFs), interferons, growth factors, colony stimulating factors (CSFs), and cell adhesion molecules (Abbas et al., 2007). These proteins are released from immune cells in response to pathological stimuli; thus they have strong roles in up-regulation of certain immune

parameters (Abbas et al., 2007; Pederson, 2000). More recently, researchers have linked systemic cytokine production to a multitude of cell types (muscle, adipose and nervous) not associated with the immune system (Pederson & Febbraio, 2008). This extra-immune production is also important to consider when reviewing post-exercise cytokine dynamics and inflammation.

The major cytokines that are released during exercise include, but are not limited to: Interleukin-6 (IL-6), Tumor necrosis factor-α (TNF-α) and Interleukin-10 (IL-10) (Peterson & Pederson, 2005). The production and interactions of these proteins have been discussed extensively in some contexts, yet the relationships have not been thoroughly studied in exercise oncology. In order to better describe the roles of inflammation in exercise and cancer pathology, this review of literature will first attempt to unravel the actions of these signaling components in governing metabolic, endocrine and immune function. Next the effect of exercise on the cytokine dynamics will be elucidated. Finally, this review will attempt to portray these components in the context of cancer pathogenesis.

Interleukin-6

Interleukin-6 (IL-6) is a pleiotropic cytokine that has multiple roles in immunity and metabolism (Ostrowski et al., 1999; Pederson, 2000). The roles of IL-6 are sometimes conflicting. IL-6 has been implicated as both a pro and anti-inflammatory cytokine (Ostrowski et al., 1999, Peterson & Pederson 2005). Furthermore, the protein has been noted as being essential for muscle synthesis, yet directly implicated in muscle atrophy (Haddad et al., 2004; Serrano et al., 2008). For this review, IL-6 will be discussed for its role in metabolism and muscle anabolism/catabolism. In addition, IL-6 will be examined as a strong influencer of other inflammatory cytokines. Lastly, the role of IL-6 as a muscle-induced

cytokine (myokine) will be introduced. Applications of IL-6 to cancer will be discussed in subsequent sections.

IL-6 has shown to have systemic effects on metabolism as it has shown to increase glycogenesis, glucose uptake, lactate production, fatty acid uptake/oxidation and hepatic glucose production (Glund and Krook, 2008). This metabolic control is thought to arise partially from the interaction between IL-6 and glucocorticoids, namely cortisol (Steensberg et al., 2003). IL-6 can stimulate cortisol production through multiple pathways. IL-6 initiates cortisol production through a direct stimulatory effect on the hypothalamic-pituitary-adrenocortical (HPA) axis and the adrenal cortex (Schwalbe et al., 1994). Cortisol has a well-documented role as a catabolic hormone; thus has the ability to initiate the depletion of glycogen, fat and protein for use by the cell (Nieman et al., 2004). The interaction between IL-6 and cortisol is important to consider when reviewing IL-6 and its effects on metabolism.

IL-6 also has a major role in the systemic regulation of other cytokines. IL-6 has the ability to inhibit pro-inflammatory cytokines TNF-α and Interleukin-1 (IL-1) in serum (Peterson & Pederson, 2005). Additionally, IL-6 stimulates the production of anti-inflammatory cytokines, Interleukin 10 (IL-10) and Interleukin 1-receptor antagonist (IL-1ra). The ability to suppress pro-inflammatory cytokines and produce anti-inflammatory cytokines suggests IL-6 can directly affect an anti-inflammatory state (Peterson & Pederson, 2005). Starkie et al. (2003) were the first to establish that exercise-induced IL-6 may have a modulating effect on other cytokines in humans. These researchers monitored endotoxin induced TNF-α production after aerobic exercise and after infusion of recombinant IL-6. They found that groups that exercised or that were infused with recombinant IL-6 had suppressed levels of TNF-α compared to the group that rested (Starkie et al., 2003). This

initial finding suggested that IL-6 may have a governing role in immune function during and after exercise.

Before the early 2000's, the prevailing theory was that systemic production of IL-6 was a consequence of an immune cell infiltration of damaged skeletal muscle and subsequent release of cytokines into the serum (Nehlsen-Canarella et al., 1997; Nieman et al., 1998). However, others theorized that muscle itself might be responsible for the release of IL-6 (Pederson, 2000). This was confirmed when Steensberg et al. (2000) linked the increase in plasma levels of the cytokine to contracting skeletal muscle. The researchers accomplished this by comparing arterial IL-6 concentrations between an exercising and non-exercising limb. The exercising limb produced significant amounts of IL-6, while the non-exercising limb did not (Steensberg et al., 2000). Since IL-6 is synthesized and released from contracting muscles, and not from resting muscles exposed to the same endocrine changes, this validates that circulating systemic factors cannot solely explain why contracting muscles synthesize and release IL-6 (Pederson et al., 2007). Thus, it is more probable that local factors in the muscle are responsible for the systemic production of IL-6. Pederson et al. (2004) later termed IL-6 a "myokine", and suggested that muscle should be considered an endocrine organ.

In addition to its role as a myokine, IL-6 has been implicated as locally acting cytokine governing metabolic control (Pederson et al., 2007). This entails that IL-6 has the ability to initiate changes in the metabolic state in absence of other regulatory hormones. Peterson et al. (2005) demonstrated this by using recombinant IL-6 infusion techniques in healthy older individuals. The team observed increases in fat lipolysis in vivo without increases in systemic hormones such as epinephrine and glucagon. The group also induced an

IL-6 up-regulation in cultured myotubes, and again observed a significant increase in lipolysis (Peterson et al., 2005). Both of these findings suggest that IL-6 has the ability to induce metabolic events in absence of other major systemic hormones.

IL-6 has also been cited as a direct intramuscular 'energy sensor' (Pederson et al., 2004; Steensberg et al., 2003). The mechanism behind 'energy sensing' has been suggested to involve a link between IL-6 and adenosine monophosphate kinase (AMPK; Kelly et al., 2004). AMPK has the ability to inhibit malonyl-CoA production which leads to an increase in fat lipolysis and other metabolic regulations (Kelly et al., 2009). The relationship to IL-6 was elucidated by Kelly et al. (2004) when the researchers observed significant increases in AMPK after IL-6 incubation. The researchers supported this claim further when they displayed that IL6 (-/-) knockout mice had reduced activation of AMPK. This relationship between IL-6, AMPK and other energy sensing pathways suggests a novel role for IL-6 in local metabolic control (Kelly et al., 2009).

IL-6 also acts to stimulate or inhibit muscle protein synthesis. Serrano et al. (2008) found that IL-6 production can stimulate the myogenic satellite cells for repair of the muscle. In this study, IL-6 deficiency abolished satellite cell proliferation in the preexisting myofiber by impairing signal transducer of activated transcription 3 (STAT3) (Serrano et al., 2008). Conversely, Haddad et al. (2004) established that IL-6 can directly initiate muscle atrophy though the activation of a novel proteolytic pathway. With these opposing actions, it is likely that the effects of IL-6 on myoplasticity are dependent on other intra-cellular pathways or conditions (Hodge et al., 2005). Overall, however, it is currently unclear why IL-6 has differing effects on muscle growth and repair.

When discussing the production of IL-6, it is important to note that the upstream and downstream intracellular signaling pathways for IL-6 differ significantly between muscle and immune cells. IL-6 signaling in leukocytes is dependent upon activation of the nuclear factor kappa-beta (NF-kB) signaling pathway while different networks of signaling cascades are responsible for intramuscular IL-6 expression and signaling. Thus, IL-6 signaling in leukocytes induces a pro-inflammatory response linked to a preceding TNF-α signal, whereas IL-6 activation and signaling in muscle is totally independent of a preceding TNF-α response or NF-kB activation (Pederson & Febbraio, 2008). Moreover, since IL-6 is strongly linked to metabolic regulation, it can be assumed that the 'energy sensing', metabolic properties of IL-6 preside over the immune regulating properties (Pederson & Febbraio, 2012). Overall, it appears as if the intracellular activation of IL-6 in skeletal muscle is different from that in immune cells, and thus the presence of post-exercise IL-6 in serum is not indicative of a typical pathogenic immune response.

In summary, IL-6 has a strong regulatory effect on metabolic and immune processes in the human body. The ability of the cytokine to act on a systemic and local level suggests a broad and multifaceted role. This is evidenced further in the ability of IL-6 to induce both an anabolic and catabolic state in muscle. IL-6 has been termed a 'myokine' and thus substantially increases in the blood after muscular contraction.

Interleukin-10

IL-10 is an anti-inflammatory cytokine inevitably involved in inflammation and immune function (Jankord & Jemiolo, 2004). IL-10 exerts its neutralizing effects on a variety of immune cells including B cells, T cells, natural killer cells, dendritic cells, and mast cells (Jankord & Jemiolo, 2004). IL-10 also suppresses potent inflammatory cytokines IL-1, IL-6

and TNF-α, while concurrently inducing the production of other anti-inflammatory cytokines, such as interleukin 1 receptor antagonist (IL-1-ra; Jankord & Jemiolo, 2004; Peterson & Pederson, 2005; de Waal Malefyt et al. 1991).

IL-10 regulates expression of cytokines, soluble mediators and cell surface molecules, and their ability to activate and maintain immune and inflammatory responses (Moore et al., 2001). The best-characterized IL-10 signaling pathway is the Janus Kinase/signal transducer of activated T- cell (JAK/ stat) pathway. IL-10 and its membrane receptor (IL-10R) engage tyrosine kinases and subsequent phosphorylation of 'latent' transcription factors which are thought to compete with pro-inflammatory cytokines working through similar pathways (Moore et al., 2001). Moreover, the ability of IL-10 to neutralize immune cells and cytokines lies partly in the inhibition of NF-kB pathway (Wang et al., 2005). The NF-kB pathway is involved in a multitude of intracellular and paracrine function, one of which is the induction of pro-inflammatory cytokines (Wang et al., 2005). Thus, the ability of IL-10 to inhibit this pathway is of major importance in it's anti-inflammatory actions.

IL-10 is significant in respect to the anti-inflammatory action of exercise (Peterson & Pederson, 2005). Jankord & Jemiolo (2004) displayed that higher level of physical activity was associated with higher serum levels of IL-10. Additionally, IL-10 mRNA, in concurrence with other anti-inflammatory cytokines (IL-1ra, IL-8), have shown to be significantly increased in blood leukocytes following two hours of intensive exercise training. Since the major pro-inflammatory cytokines (IL-6 and TNF-α), are released from extra-immune sources and do not appear to be upregulated in immune cells, it appears as if IL-10 and other anti-inflammatory cytokines are the major responding signals in immune cells after exercise.

Tumor Necrosis Factor-α

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine that has multiple roles in immunity, inflammation and muscle metabolism (Abbas et al., 2007; Peterson & Pederson, 2005). TNF-α is released from a variety of immune cells, muscle and adipose tissue both at rest, and in response to exercise (Mathur & Pederson, 2007). Recent evidence has emerged to suggest that chronic, low-grade levels of TNF-α have a direct role in the metabolic syndrome and cancer pathogenesis (Balkwill & Mantovani, 2001; Mathur & Pederson, 2007). Furthermore, stressed or injured muscle fibers produce TNF-α, which suggests that the cytokine has important roles in exercise recovery and adaptation.

TNF-α has been implicated in impaired glucose and triglyceride disposal (Moller, 2000). The cytokine exerts its negative effects on muscle and adipose tissue through the inactivation of receptors responsible for transporting glucose and triglycerides into the cell (Moller, 2000). As described by Hotamisligil and Spiegelman (1994), TNF-α is a 'potent inhibitor' of the insulin receptor and insulin receptor substrate. Plomgaard et al. (2005) confirmed this phenomenon by displaying that TNF-α directly infused into humans induces insulin resistance in skeletal muscle. The researchers describe impaired phosphorylation of the Akt 160 substrate, a powerful regulator of GLUT 4 signaling, as the primary trigger behind the adverse effects of TNF-α on insulin signaling (Plomgard et al., 2005). TNF-α also increases fatty acid incorporation into diacylglycerol, which may suggest another mechanism for the pathogenic effects of the cytokine (Mathur & Pederson, 2007).

TNF-α also activates proteolytic pathways that are responsible for skeletal muscle wasting (Li et al., 2003). The cytokine exerts its muscle wasting effects partially through the activation of the NF-kB pathway (Reid & Li, 2001). NF-kB activates the ubiquitin/

proteasome pathway, which enhances degradation of muscle proteins and promotes muscle wasting (Reid & Li, 2001). TNF-α is also thought to inhibit muscle growth factors responsible for anabolic processes (Reid & Li, 2001).

Overall, TNF-α is a potent inflammatory cytokine with strong associations to metabolic diseases and cancer. The cytokine acts as a powerful stimulator of muscle breakdown and inhibitor of glucose and triglyceride disposal.

Cytokine Interactions and Temporal Responses to Exercise

The post-exercise inflammatory response is coordinated by a time dependent release of cytokines into the blood. These proteins mediate the metabolism, endocrine and immune processes in the time window shortly after exercise, which reflects some of the acute exercise adjustments (Pederson et al., 2007). Furthermore, the repair of damaged skeletal muscle involves immune cells and cytokines, which mediate progression from an inflammatory response to the eventual long-term adaptive response (Tidball, 2005). Therefore, understanding the short-term dynamics of these inflammatory mediators after exercise is an important concept to consider. The response and interactions between IL-6, IL-10 and TNF-α during and after exercise will be discussed in this section.

IL-6 is the most extensively upregulated cytokine after exercise (Ostrowski et al., 1999; Peterson & Pederson, 2005). Though there is a moderate increase in the systemic concentration of many cytokines, it is clear that the appearance of IL-6 in the circulation is by far the most marked and that its appearance precedes that of the other cytokines (Pederson & Febbraio, 2008). As mentioned, the upreuglation of IL-6 after exercise has been linked to the protein's direct release from skeletal muscle and ability to 'sense' energy depletion (Pederson et al., 2004; Steensberg et al., 2001). With these roles, it is not surprising that IL-6

release is dependent on the intensity and duration of the exercise session (Febbraio et al., 2002; Pederson 2000).

Since contracting skeletal muscle is a principal source of IL-6 found in the plasma, it is not surprising that exercise involving a limited muscle mass may not initiate the cytokine's response (Hirose et al., 2004; Nosaka and Clarkson, 1996). In contrast, exercise that involves large muscle groups produces the most dramatic plasma IL-6 increases (Nehlsen-Canarella et al., 1997; Nieman et al., 2004). Additionally, Fischer (2006) displayed that exercise duration is the most important factor determining post-exercise plasma IL-6 response. In fact, more than 50% of the variation in plasma IL-6 following exercise can be explained by exercise duration alone (Fischer, 2006).

IL-6 peaks two to eight hours after both aerobic and resistance exercise and precedes that of IL-10 and TNF-α (Louis et al., 2007; Pederson, 2000). As mentioned, IL-6 has the ability to stimulate anti-inflammatory IL-10 and inhibit pro-inflammatory TNF-α after an exercise session (Steensberg et al., 2001; Steensberg et al., 2003). As the initial cytokine upregulated during exercise, IL-6 has been labeled as the possible 'true exercise factor' (Pederson, 2004).

IL-10 and other anti-inflammatory cytokines are thought to act in a regulatory fashion post-exercise (Suzuki et al., 2002). IL-10 is directly stimulated from IL-6, and thus peak production occurs after IL-6 (Peterson & Pederson, 2005; Steensberg et al., 2001). Circulating IL-10 can remain in the blood well beyond an exercise session, as far as 6 days after the cessation of exercise (Ostrowski et al., 1999; Smith et al., 2000). This suggests that exercise may induce a prolonged anti-inflammatory effect.

TNF-α is a pro-inflammatory cytokine that is also stimulated by muscle contraction (Steensberg et al., 2002). However, the cytokine shows a blunted response after exercise due to the inhibitory effects from IL-6 and IL-10 (Peterson & Pederson, 2005; Starkie et al., 2003). Therefore, the increase in TNF-α is usually minimal following exercise. This is the case unless exercise duration is prolonged or the intensity is severe (Brenner et al., 1999; Ostrowksi et al., 1999).

It is also important to review the post-exercise sources of cytokines. As discussed, IL-6 is released from skeletal muscle and its release precedes that of anti-inflammatory cytokines IL-10 and IL-ra. Researchers, however, did not find a significant increase in IL- 6 mRNA expression in blood leukocytes following 2 hours of intensive cycling (Nieman et al., 2006). Conversely, the primary anti-inflammatory cytokines do appear to be released from immune cells, as mRNA expression of IL-10 and IL-1RA are significantly upregulated post exercise in blood leukocytes (Nieman et al., 2006). This suggests that post-exercise concentrations of IL-6 may not be representative of a typical immune response, as IL-6 expression is minimal, if not absent, in immune cells following exercise (Nieman et al., 2006). Moreover, the ability of IL-6 to stimulate blood leukocytes to release anti-inflammatory cytokines indicates a strong relationship between the contraction of skeletal muscle and the post-exercise anti-inflammatory state.

In conclusion, cytokines act in a coordinated and phasic manner after exercise. IL-6 is the most extensively upregulated cytokine after exercise and its release precedes that of IL-10 and TNF-α. The interactions between multitudes of cell types make precise calculation of cytokine dynamics after exercise very difficult. Only general trends of cytokine dynamics were discussed in this section.

Acute Inflammatory Responses to Resistance Exercise

The up-regulation of cytokines following aerobic exercise is extensive (Ostrowski et al., 1999; Pederson et al., 2000). Resistance exercise also causes cytokine proliferation, but to a lesser degree (Brenner et al., 1999; Izquierdo et al., 2009). This section will discuss some of the studies that have examined how acute resistance exercise acute alters inflammation.

Brenner et al. (1999) compared cytokine changes following all-out exercise, circuit training and prolonged exercise. The investigators only found significant cytokine changes (IL-6 and TNF-α) in the prolonged exercise training. On the contrary, other researchers have found significant changes in cytokines from resistance exercise sessions in shorter resistance exercise sessions (Izquierdo et al., 2009; Louis et al., 2007; Nieman et al., 2004; Smith et al., 2000).

Smith et al. (2000) found significant increases in IL-6 following severe eccentric muscle loading 12, 24 and 72 hours post-exercise. In addition, the investigators found significant increases in IL-10 ranging from 48 to 144 hours post-exercise. The observation of serum cytokines, especially IL-10, extended well beyond the cessation of exercise, suggests this resistance exercise session may have induced a more long-term anti-inflammatory response. This is evidenced further by a concurrent reduction in the pro-inflammatory cytokine IL-1beta (Smith et al., 2000).

Toft et al. (2002) examined cytokine response to eccentric exercise in young and elderly individuals. The researches found that IL-6 increased immediately after exercise and peaked 4-5.5 hours after cessation. Furthermore, the increase in IL-6 was less pronounced in the elderly individuals compared to young. It is unclear whether behind this the amount of activated muscle or indeed, the age of the participants are responsible for the degree of IL-6

release. In support of the latter explanation, Toft et al. note that the aging immune system may be associated with impaired repair mechanisms in the muscle, thus explaining the blunted IL-6 response post-exercise in the elderly subjects (Toft et al., 2002). Others theorize that muscle fiber type may have strong influences on IL-6 production, and the blunted IL-6 response in the elderly is a result of reduced fast-twitch fiber type (Louis et al., 2007) Irrespective of the reasoning, it is clear that age does indeed have an effect on the post-exercise cytokine response.

Nieman et al. (2004) found significant increases in IL-6 and TNF-α immediately after 2 hours of heavy resistance exercise. A major finding from this study is that IL-6 release is not affected by carbohydrate ingestion. This is contrary to previous studies that note significantly greater IL-6 response in subjects that are glycogen depleted (Nieman et al., 2003; Steensberg et al., 2001). The authors also note that 'unrelenting, high- intensity' exercise workloads are more likely to induce cytokine responses than exercise regimens with multiple rest intervals (Nieman et al., 2004).

Peake et al. (2006) examined serum cytokine levels in ten healthy untrained men after completion of submaximal and maximal exercise of the elbow flexors. The submaximal session consisted of ten sets of sixty repetitions at ten percent of one repetition maximum (1-RM). The maximal protocol consisted of ten sets of three repetitions at 100 percent of 1-RM. Interestingly, serum concentrations of IL-6 increased after the submaximal workload, but not the maximal workload. The authors note that a minor glycogen depletion from the repeated submaximal contractions may have initiated the IL-6 release into the serum (Peake et al., 2006).

Izquierdo et al. (2009) examined the effects of heavy resistance training on serum IL-

6 and IL-10 changes after two loading protocols with the same relative intensity and the same absolute load, before and after a training program. Researchers found significant IL-6 responses after exercising both pre-training and post-training, but only observed significant IL-10 responses after the post-training exercise. Moreover, exercise with the same *relative* load after the training program enhanced the post-exercise IL-6 response and elicited a significant IL-10 response. This is in contrast to exercise with the same *absolute* load after the training program, in which neither IL-6 nor IL-10 were significantly upregulated (Izquierdo et al., 2009).

Louis et al. (2007) examined IL-6 and TNF-α dynamics following aerobic and resistance exercise. In contrast to other studies, these researchers observed muscle mRNA cytokine changes rather than blood concentrations. Though the absolute increases in cytokines were higher when measuring mRNA, the temporal response of cytokines, however, were not dissimilar to other studies examining serum concentrations of cytokines, as IL-6 was highest two to eight hours into recovery after resistance exercise. Concurrently, TNF-α levels peaked between four and eight hours into recovery (Louis et al., 2007).

In a study by Hirose et al. (2004), healthy, untrained males completed two bouts of eccentric action of elbow flexors (6 sets of 5 repetitions at 40 % 1-RM). The authors note that the oscillations in cytokines and other inflammatory mediators were minimal, and 'considerably smaller' than cytokine proliferation following aerobic exercise. However, the researchers found intriguing data relative to the major pro-inflammatory cytokine, TNF-α. They note a significant decrease in TNF-α (15%) 1 day, 3 days and 4 days after the exercise bout. Moreover, the study showed an acute increase in IL-10 suggesting a possible role of the anti-inflammatory cytokine in post-exercise adaptation (Hirose et al., 2004).

Pereira et al. (2012) examined the effect of an acute resistance exercise session in women with metabolic syndrome compared to healthy age matched controls. The subjects (24 sedentary, middle-aged females) performed seven different exercises at 60% of the 1RM and 10 repetitions each set. Though systemic inflammatory markers (IL-6 and TNF-α) were significantly higher or trended higher in the metabolic syndrome group at rest, resistance exercise had little effect on these cytokines in either group. This is in agreement with studies by Buford et al. (2009) and Ferreira et al. (2010) that investigated the acute effect of resistance exercise in 24 and 14 untrained women respectively, and found no increase in serum cytokine levels after each exercise session.

Prestes et al. (2009) measured the changes in cytokines (among other biomarkers) before and after a 16-week periodized resistance-training program in elderly women (mean age: 63.18 years). The researchers found that IL-6 was not elevated post-exercise and significantly decreased 48 hours post-exercise in the pre-training exercise session. Of possible significance is the down regulation of IL-6 48 hours after the bout, which could be indicative of a more long term reduction in the resting pro-inflammatory cytokine levels. This theory is supported in some degree by the significantly lower resting IL-6 levels after the 16 week exercise program (Prestes et al., 2009). This finding suggests that the chronic levels of IL-6 may be mediated by an acute exercise response. Moreover, the authors note that pre-training cytokine dynamics contrast to that of the post-training. In the exercise bout that occurred post-training, IL-6 trends upward immediately after exercise, and displays a diminished down-regulation 48 hours post-exercise compared to the pre-training exercise bout (Prestes et al., 2009).

McFarlin et al. (2004) also examined the influence of training status on inflammatory cytokine production. The researchers showed that lipopolysaccharide (LPS) stimulation of cytokines before, immediately, 2 hours after, 6 hours after and 24 hours after resistance exercise in elderly women trended higher in the untrained group vs. trained group. The authors assert that this phenomenon may be caused from a higher expression of Toll like receptor 4 (TLR4) on innate immune cells in sedentary individuals. As a primary receptor to molecular patterns expressed by pathogens and subsequent stimulating factor for pro-inflammatory cytokines by innate immune cells, TLR4s represent an important element to the pathogen-host interaction. Thus, if TL4R expression is lower, as in the case with trained individuals, the inflammatory response and immune response will be dampened. This is in agreement with Calle and Fernandez (2010) who describe the 'paradoxical' phenomenon to training adaptations, in which lower levels of pro-inflammatory cytokines are evident at rest and after exercise in the trained state relative to the pre-trained state. Therefore, it can be assumed that post-exercise cytokine dynamics is highly dependent on training status, and should be an important consideration when interpreting post-exercise inflammatory responses.

Phillips et al. (2010) used a ten-week resistance-training program to assess both the acute and chronic inflammatory milieu in sedentary elderly women. In regards to IL-6, the researchers found that the cytokine increased significantly post-exercise before returning to baseline both before and after the training program. Notably, the training program had a modulating effect on the acute cytokine response, as shown by the significantly lesser pre-exercise to post-exercise change in IL-6 after the training program (33%) as compared to before the training program (75%).

Plasma TNF-α levels were also shown to increase as a result of an acute resistance exercise bout. However, resting TNF-α levels were shown to significantly decrease (37%) after the 10 weeks of the resistance training (Phillips et al., 2010). Moreover, Phillips et al. (2010) also found a significant decrease in lipopolysaccharide (LPS) stimulated pro-inflammatory cytokine production after the training program throughout out all exercise time intervals. Overall, these findings suggest that resistance exercise training results in: a moderated resting inflammatory state, a blunted cytokine response training to acute exercise and a reduced inflammatory reactivity from a foreign pathogen; thus improving overall immune function and reducing risk of inflammatory issues associated with disease (Phillips et al., 2010).

The aforementioned study also supports earlier research by Greiwe et al. (2001) in which the authors suggest the use of resistance exercise as an effective method for alleviating the effects of muscle cachexia. The study by Greiwe et al. (2001) supports this claim by displaying that resistance could increase protein synthesis in concurrence with a decrease in systemic TNF-α levels. Phillips et al. (2010) presents a similar argument by displaying that a decrease in systemic levels of TNF-α was associated with improvements in overall strength. Though these studies do not examine cancer survivors directly, these findings are applicable to two interrelated symptoms that remain prevalent concerns in this population: chronic inflammation and muscle cachexia.

As discussed, age and training status have important effects on the exercise-induced cytokine response. Post-exercise inflammation is also directly related to the intensity, duration and mode of the exercise task (Brenner et al., 1999; Nieman et al., 2004; Phillips et al., 2010). Moreover, the cytokine response may vary according to the recovery between

exercise bouts, as well as substrate storage and utilization (Pereira et al., 2012). In regards to these aforementioned concepts, it is important to note that the differences between type of muscle and the amount of muscle mass that was exercised differed between the studies. For instance, Louis et al. (2007) used a lower body, isolated, concentric action of the quadriceps muscle, as compared to Smith et al. (2000) that used upper and lower body, multi-jointed, and eccentric action of multiple muscle groups. Furthermore, some researchers used eccentric muscle loading as the exercise protocol (Hirose et al., 2004; McIntyre et al., 2000; Smith et al., 2000; Toft et al., 2002). These differences in resistance exercise protocols will undoubtedly have an effect on inflammation. As described by Paulsen and Peake (2013) the inflammatory response to resistance exercise is heavily dependent on the specificity of the exercise program. Applying heavy loads with few repetitions and long rest intervals are likely to produce small to moderate changes in hormonal response. However, circuit training, with higher repetitions and short rest intervals are likely to induce changes more similar to aerobic exercise (Paulsen & Peake, 2013). Additionally, the hormonal and inflammatory response is dependent the type and the size of the exercised muscle or muscle groups. For example, exercise that emphasizes large muscle groups and 'multi-jointed, dynamic movement patterns' are likely to induce a greater systemic inflammatory response than 'isolated concentric muscle contractions' (Paulsen & Peake, 2013).

Substrate availability, which is dependent mainly on exercise duration and nutrition, is also likely to have influential effects of serum cytokine levels. As noted by Nieman et al. (2004) IL-6 upregulation during and after exercise is heavily dependent on carbohydrate intake and glycogen storage. Therefore, in the studies by Brenner et al. (1999) Nieman et al. (2004) and Peake et al. (2006), it is possible that duration of the contractions had an effect on

the production of IL-6. Overall, it is apparent that protocols differed between groups when measuring cytokines; and that duration, mode and intensity are important factors to consider when interpreting the results.

Inflammation and Cancer

In 1863, Rudolph Virchow first proposed that there was a significant link between immunity, inflammation and cancer when he observed leukocyte infiltration in tumor cells (Balkwill & Mantovani, 2001). Recently Virchow's theory has resurfaced, as cancer has been shown to produce a network of pro and anti-inflammatory cytokines that collaborate in cancer progression (Balkwill & Mantovani, 2001). In addition, adverse symptoms have been linked to cancer-related inflammation and the production of cytokines (Seruga et al., 2008). Increased circulating cytokines have been associated with cachexia, fatigue, depression and pain in cancer patients (Bower et al. 2002, Seruga et al. 2008). The likely cause of these symptoms lies in the tendency of the tumor microenvironment to exhibit a pro-inflammatory state (Balkwill & Mantovani, 2001; Bower et al. 2002). Overall, chronic, low grade production of cytokines including TNF-α, and IL-6 is associated with negative symptoms and poor prognosis (Balkwill & Mantovani, 2001; Bower et al., 2002; Karin & Greten, 2005; Seruga et al., 2008).

TNF-α has an especially integrative role in cancer-induced inflammation (Al-Majid & Waters, 2003; Balkwill & Mantovani, 2001). At high-levels, the cytokine induces an apoptotic response in cells (Reid & Li, 2001). Paradoxically, however, chronic low-grade levels of TNF-α can actually initiate tumor progression (Balkwill & Mantovani, 2001; Karin & Greten, 2005). A direct role of TNF-α in cancer progression was found when TNF-α deficient mice were resistant to skin carcinogens (Moore et al., 1999). Chronic, low-grade

levels of TNF-α can also act as a ‘double-edge sword’; acting in tumor progression while simultaneously initiating apoptosis in other parts of the body (e.g. muscle; Balkwill & Mantovani, 2001, Figueras et al., 2005, Karin & Greten, 2005).

Chronic levels of IL-6 have also been associated in the progression of diseases such as rheumatoid arthritis, diabetes and cancer (Chung et al., 1999; Pradhan et al., 2001). The role of IL-6 in cancer biology is complex. As described by Chung et al. (1999), IL-6 appears to change roles from a ‘paracrine growth inhibitor to an autocrine growth stimulator’ during the progression of cancer. Giri et al. (2001) supported this claim, citing that IL-6 is upregulated 18-fold in pancreatic cancer cells. This group noted that autocrine signaling, through upregulation of particular receptors and intracellular signaling molecules, are likely the cause of this phenomenon (Giri et al., 2001). Others have argued that IL-6 is not a always a problematic cytokine; and that the elevated levels of IL-6 are only a result of an unbalanced cytokine network (Pederson et al., 2007). This is especially the case in respect to exercise, in which acute elevation of IL-6 concentrations are not associated with poor prognosis (Pederson et al., 2006.) Pederson’s group goes further by arguing that the post-exercise internal milieu of cytokines, including IL-6 and other myokines, are involved in mediating the beneficial effects of exercise.

Overall, systemic, chronic-low grade levels of TNF-α and IL-6 have shown to illicit complications in cancer populations. The immune system recognition of the tumor as a pathogen or a disruption to the internal milieu of autocrine, paracrine and endocrine signals are likely causes for the alterations in serum cytokine levels (Balkwill & Mantovani, 2001). Though the chronic stimulation of these cytokines prove problematic in cancer pathogenesis,

the production and release of these components after exercise may present an optimal state for adaptation to external stress.

The Inflammatory Response to Exercise in Cancer Survivors

The amount of research examining the effect of effects exercise on cytokine changes is limited in cancer survivors. Studies that have focused on exercise and cytokines in cancer groups have focused on the changes over an extended training period. On the contrary, the acute cytokine dynamics in cancer populations has not been observed thoroughly. This section will examine some of these studies in further detail to further understand the role of exercise on cytokines and inflammation in cancer survivors.

Fairey et al. (2005) was one of the first groups to look at the effects of a long-term exercise intervention on cancer subjects. The researchers examined the effect of an eight-week (three sessions per week) exercise program on cytokines and other hematological variables. Cytokines remained unchanged in these subjects throughout the exercise intervention.

Jones et al. (2012) analyzed changes in IL-6, TNF-α and C-reactive protein before and after a 6-month exercise intervention in breast cancer survivors. The team found no changes in any cytokines. It is important to note, however, that the exercise prescription was outside a controlled setting. When adherence was accounted for (> 80%), the researchers found a significant decrease in IL-6 as a result of the exercise program.

In a study examining the effects of an exercise intervention on sleep quality and duration, Sprod et al. (2010) determined that exercise can play a beneficial role in mediating long term cytokine dynamics in breast cancer survivors. Though IL-6 and TNF-α increased in both groups (exercise and control) over the four-week intervention, the exercise group

showed a significantly lesser cytokine proliferation than the sedentary controls (Sprod et al., 2010).

Hutnick et al. (2005) examined the effect of three and six month exercise program on the change in lymphocyte and IL-6 activation (among other immune markers). The exercise protocol consisted of aerobic and resistance training. In contrast to other studies, in which IL-6 tended to decrease across a training intervention, this group found that exercise induced higher (though non-significant) levels of IL-6 throughout the training protocol. This suggests that IL-6 may be chronically elevated in certain circumstances or under certain exercise programs. It should be noted, however, that unlike Sprod et al. (2010), the study by Hutnick et al. (2005) did not include a control group.

Other research has found no significant changes in cytokines post-training in cancer subjects. Jones et al. (2009) observed effects of pre-surgical exercise training on systemic inflammatory markers among patients with malignant lung lesions. The team found no changes in IL-6, Il-10 or TNF-α (Jones et al., 2009). Gomez et al. (2011) examined the effects of an eight week aerobic and resistance exercise program on a numbers of cytokines in breast cancer survivors. The researchers found no significant change in IL-6 or TNF-α.

One known study, to date, has examined the acute cytokine dynamics in cancer survivors. Galvao et al. (2008) examined both the long term and acute immune and endocrine responses to resistance exercise in prostate cancer patients. With respect to the chronic changes, the researches found that markers of inflammation (IL-6, TNF-α and IL-1ra) did not change over the 20-week resistance protocol. The authors note that the lack of significant change may be derived from the difference in protocol between their study and other studies examining long-term cytokine dynamics. The study did display significant acute alterations

in cytokines, as both IL-6 and TNF-α increased significantly immediately after four sets of eight isotonic exercises at 6-repetition max. Rest between sets was 1-1.5 minutes, with 2-4 minutes between exercises.

Overall, there is no clear consensus that exercise interventions have a discernable effect on systemic cytokine levels. Sprod et al. (2010) and Greiwe et al. (2001) found significant changes while other studies (Fairey et al., 2005; Galvao et al., 2008; Hutnick et al., 2005; Jones et al., 2009; Jones et al., 2012) did not. It should be noted, however, that some studies showed significant alteration in critical immune parameters from the exercise intervention; Fairey et al. (2005) detected increases in cytotoxic NK activity, and Hutnick et al. (2005) observed rises in CD4+ lymphocyte activity. Thus, it appears as if more studies, perhaps exhibiting longer exercise interventions, need to be compiled to improve the understanding of the effect of extended exercise programs on the long-term immune and inflammatory regulation in cancer survivors. Moreover, the study by Galvao et al. (2008) displays intriguing but still partial results with respect to the acute post-exercise cytokine dynamics in cancer survivors.

Summary

Three particular cytokines that are linked to cancer and exercise were examined in the study. IL-6, which is most extensively upregulated post-exercise, assumes essential roles in metabolic function and immunity in healthy and cancer patients alike. IL-10 is an immunosuppressive, anti-inflammatory cytokine that maintains inhibitory action on other inflammatory signals, while concurrently neutralizing immune cell mobilization. TNF-α is a potent inflammatory cytokine that is upregulated after intense exercise; linked to impaired metabolic, endocrine and immune function; and when chronically stimulated, induces tumor

progression. Cortisol will be examined as a secondary observation in this study. IL-6 and cortisol are linked in important metabolic, endocrine and immune functions, thus defining the correlative function between these two factors during and after exercise is desirable.

Exercise has shown to modulate cytokines in a fairly predictable fashion. IL-6 increases most extensively and it's release precedes that of IL-10 or TNF-α. IL-6 and its role as a modulatory factor for IL-10 and other anti-inflammatory cytokines, suggests that this cytokine may be the crucial signal in suppressing inflammation. TNF-α is not as extensively upregulatedafter exercise, but if exercise is severe, and immunosuppression is present (e.g. cancer survivors), this cytokine could prove problematic in promoting proper recovery.

The interrelationship between inflammation and immunity on cancer prognosis is a prevalent concern when examining prevention, treatment and recovery from this disease. Since exercise has a prominent role in modulation both of these factors, it appears that close monitoring of the acute effects of exercise may play a vital role in determining long-term outcomes for exercise therapy. In particular, resistance exercise has displayed a recent resurgence in the exercise oncology field, yet studies examining immune and inflammatory effects are limited and results are inconsistent.

CHAPTER III

METHODOLOGY

Subjects

Participants of this study consisted of 4 female breast cancer survivors and 8 healthy age matched (± 5 years) women. The breast cancer survivor group was comprised of post-menopausal females, 40 - 72 years old. Subjects with stage 1, 2 or 3 invasive breast cancers and within 1-year post treatment were eligible to participate. At the time of the experiment, subjects had no presence of metastatic disease and were in post-menopausal status for at least 1 year. Patients receiving adjuvant hormonal or adjuvant therapy were also eligible. Subjects were not taking any anti-inflammatory medications, and had medical clearance from their oncologist to participate in the study.

The control group consisted of healthy females of 40 to 72 years of age. The control group was also post-menopausal, but had no history of cancer diagnosis or treatment and no limitations in range of motion that would impede upon the ability to perform the prescribed resistance training exercises safely.

Exclusion criteria were set forth by the ACSM guidelines to determine contraindications to exercise participation (Whaley, Brubaker, and Otto, 2006). Information regarding the exclusion criteria was obtained from each subject based on their responses to their medical history form, from a medical and physical screening, and from their medical records.

All participants filled out a Medical History Questionnaire and an informed consent, both of which were reviewed prior to participation in the study.

Recruitment

Participants for the breast cancer survivor group were recruited from the Get REAL & HEEL Breast Cancer Program at the University of North Carolina, Chapel Hill. Participants for the control group were recruited by the posting of flyers at various locations throughout the campus of the University of North Carolina, Chapel Hill, as well as via informational emails sent out to the campus community at the University of North Carolina, Chapel Hill.

Instrumentation

Anthropometric Measures: Resting cardiac function was assessed using a GE CASE Cardiosoft V. 6.6 ECG diagnostic system (Palatine, IL). Height was taken using a Perspective Enterprises Stadiometer (Portage, MI). Weight was attained via a Detecto Scale (Webb City, MO). A Discovery Dual Energy X-Ray Absorption (DEXA) scanner (Bedford, MA) was used to assess body composition.

Exercise Testing: Blood pressure was assessed before and after the exercise protocol using a sphygmomanometer (American Diagnostics Corporation, Hauppage, NY) and a Littmann® Stethoscope (St. Paul, MN). Heart rate before and throughout the exercise protocol was assessed using a Polar telemetry system (Lake Success, NY). The warm-up period was performed on a Lode® electronically-braked cycle ergometer (Gronigen, The Netherlands). The exercise components was performed on a seated leg press (Cybex, Medway, MA), latissimus dorsi pull down machine (Cybex, Medway, MA), seated leg extension (Life Fitness, Schiller Park, IL), and a seated row (Life Fitness, Schiller Park, IL).

Blood Analyses: Blood was taken at 4 time intervals throughout the study. The first three were done using a 22 gage Protectiv Plus catheter (Ethicon Endo-Surgery Inc., Cincinnati, OH). The fourth was done using standard venipuncture techniques. Blood was collected in 10.8 mg K_2EDTA BD Vacutainers ® (Franklin Lakes, NJ). To analyze for IL-6, IL-10 and TNF-α enzyme linked immunosorbent assays (ELISA) were conducted using Biolegend (San Diego, USA) ELISA kits. Plasma volume shift measurements were performed to account in blood volume changes from pre-exercise to post-exercise. The changes were assessed using the Dill and Costill (1974) Method. Blood cortisol values were determined using a cortisol ELISA Kit from Abnova (Tapei City, Taiwan).

General Procedures:

Familiarization Session: Medical history forms, Physical Activity Readiness Questionnaires (PAR-Q's), and pre-assessment guidelines were administered to subjects prior to their first visit to the Integrative Exercise Oncology Research Laboratory (IEORL) at the University of North Carolina at Chapel Hill (APL). Upon arrival, forms were reviewed for any pre-testing health contraindications. Subjects then signed an informed consent form detailing the experimental protocol and potential risks and benefits associated with participation in the study. Next, subjects underwent a medical and physical screening to determine if there are any contraindications to exercise. After receiving clearance to participate in the study, adherence to pre-assessment guidelines was reviewed. Height and weight were taken, and subjects were fitted with a heart rate monitor. They then rested in the seated position for 10 minutes, at which time resting heart rate and blood pressure was recorded. If either of these values were abnormal, the subject will be asked to return to the laboratory on a different day as a safety precaution. Subjects underwent a 5-minute warm-up

consisting of unloaded cycling on a cycle ergometer. Heart rate remained below 40% of heart rate reserve (HRR).

Following the warm-up, subjects were familiarized with the resistance training equipment that was in the experimental protocol by rehearsing each exercise using minimal resistance. Following initial exposure to each of the resistance exercise machines in the same order in which they were used during the experimental protocol, each subject's one-repetition- maximum (1-RM) values was obtained, using the protocol from Levinger et al. (2009). The order of exercises during the experimental protocol was as follows: leg press, latissimus dorsi pull down, leg extension, and seated row. Heart rate was monitored continuously throughout the exercise session.

Upon the completion of the familiarization trial, subjects were asked to rest for 10 minutes, after which heart rate and blood pressure were taken again. If abnormally elevated, subjects were to remain seated until the values decreased. If within normal values, subjects were released from the IEORL.

Experimental Session: Subjects returned to the IEORL between 2 and 14 days following the familiarization session. The extended time window between sessions was to ensure adequate recovery from the previous exercise session. Upon arrival, subjects rested in the seated position for 10 minutes, during which time adherence to the pre-assessment guidelines was reviewed. A Psychological Stress Scale (PSS) evaluation was administered to determine the subjects' level of psychological stress, which could affect hormone levels during and after the exercise session.

After the initial review of consent, subjects underwent catheter placement into the antecubital vein in the arm for blood sampling by a phlebotomy-trained faculty member from

the Department of Exercise and Sport Science. Subjects then rested for ten minutes after which blood pressure and heart rate were taken. After the placement of catheter and rest, subjects underwent a warm-up session on an unloaded cycle ergometer for 5 minutes. Heart rate was kept below 40 % of HRR. Following the warm up, the resistance training protocol began. Subjects performed10 repetitions at 70% of 1-RM of each exercise (leg press, lateral pull down, leg extension, and seated row, respectively) with 30-45 seconds of rest in between subsequent exercise and 90-120 seconds of rest between each set. The entire circuit was completed three times for a total of 120 repetitions. Rhythm and timing of each repetition was set at 4 seconds (2 seconds concentric, 2 seconds eccentric) and was kept consistent throughout the exercise protocol and between subjects. Heart rate and RPE was taken following each set to ensure subject safety and monitor their conditions. Subjects were encouraged to consume water at any point during and following the experimental protocol.

Upon completion of the third exercise circuit, subjects were seated and the immediately-post exercise sample was taken. Subjects were then sequestered for the next 2 hours to the APL or the exercise oncology laboratory. Throughout this time period, subjects were allowed to perform a brief series of light stretches and drink water. Following the resting session, the 2 hour post-exercise blood sample was taken. After blood was drawn, the catheter was removed and any necessary bandaging was administered. The subjects were reminded not to perform any kind of strenuous activity over the next 24 hours, and to adhere, once again, to the pre-assessment guidelines before their third and final laboratory visit. Specifically, subjects were asked to consume similar food and beverage and get equal amount of sleep as they did prior to the initial exercise session.

24-Hour Blood Draw: Subjects returned to the IEORL for the final blood draw 24 hours (± 30 minutes) following the commencement of the resistance exercise session. Upon arrival to the laboratory, pre-assessment guidelines were reviewed, and the blood sample was taken using a 22 gage needle. After the 24-hour post exercise blood draw subjects had completed the experimental procedure. The blood-draw timeline is depicted in Figure 2

Statistical analysis-

All data was analyzed using SPSS version 17.0 (Chicago, IL). For comparison of cytokine (IL-6, Il-10 and TNF-α) and cortisol changes between the cancer group and healthy controls, four separate (2X4) mixed-model analysis of variance (ANOVAs) were performed. Statistical significance was set a priori at an α-level of $p \leq 0.05$. The independent variables for this analysis include subject condition (breast cancer survivor and healthy control) and the resistance training intervention. The dependent variables were the baseline values and changes in cytokines: IL-6, IL-10, TNF-α and also cortisol, throughout the time intervals (immediately post-exercise, 2 hours post-exercise, and 24 hours post-exercise). For any significant results, a follow-up Bonferroni post-hoc analysis was used to determine any between or within group differences.

To establish if or to what degree a relationship exists between IL-6 and cortisol during and after exercise, a Spearman rho correlation was performed. The analysis compared the peak (%) changes (±) of IL-6 and cortisol. This analysis was exploratory in nature due to the overall small sample size.

Hypotheses:

H_1: IL-6 will be significantly upregulated from baseline in the cancer survivors and healthy controls immediately-post exercise and 2 hours post-exercise and return to baseline 24 hours post exercise

H_2: IL-10 will be significantly upregulated from baseline at 2 hours post-exercise and 24 hours post-exercise in healthy controls but not in the cancer survivors.

H_3: TNF-α will be significantly upregulated 2 hours post exercise and 24 hours post exercise in the cancer survivors but not in the healthy controls.

H_4: Peak (%) change (±) in IL-6 will correlate significantly with peak (%) change (±) of cortisol.

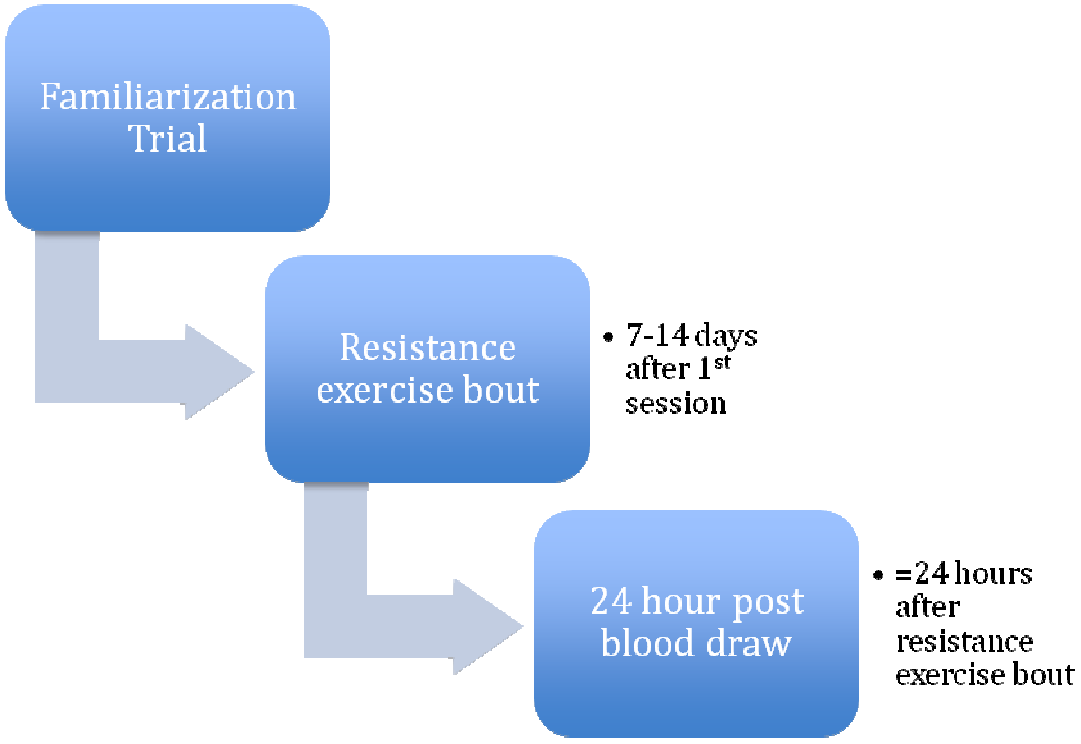

Figure 1- Timeline of lab visits.

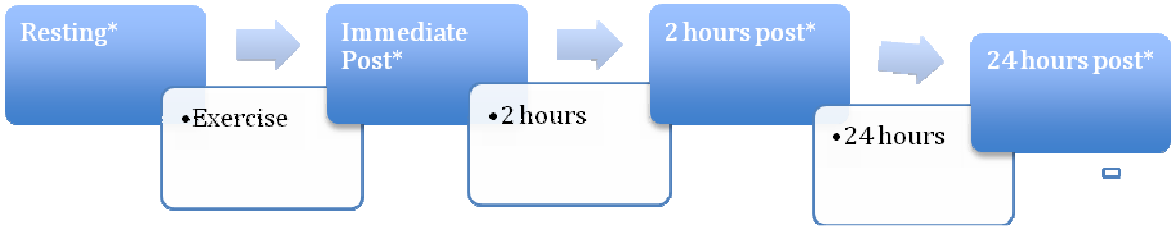

Figure 2- Timeline of blood draws.

CHAPTER IV

RESULTS

Subject Characteristics:

Four middle-aged female breast cancer survivors and eight, age-matched healthy controls participated in this study (See Table 1 for subject characteristics). Due to recruitment and scheduling difficulties, the number of participants to participate in the study that was proposed at the beginning of the study was not obtainable, leaving the sample size relatively small. Therefore, the results should be interpreted with such limitations in mind. Moreover, difficulties in obtaining blood specimens or adequate amounts of specimen occurred in each study group, resulting in a limited ability to evaluate all biomarkers. Due to this limitation in blood sample amount, only *six out of the eight* subjects' blood samples were used for the analysis of cytokines.

Table 1: Subject Characteristics (Mean ± SD)

	Age (years)	**Height (cm)**	**Mass (kg)**	**Body Fat (%)**
Healthy Controls (n=8)	56 ± 6.7	163.6 ± 6.8	67.1 ± 6.3	37.6 ± 4.5
Breast Cancer Survivors (n=4)	60 ± 12.2	168.5 ± 3.1	75.6 ± 13.4	37.6 ± 10.3

Strength Assessments

Table 2 displays the 1- RM assessments for both groups. No differences in strength were observed between breast cancer survivors and healthy controls throughout all exercises.

Table 2: Strength Assessment Measures: One-Repetition Maximum (kg) (Mean ± SD)

	Leg Press	**Lat Pulldown**	**Leg Extension**	**Seated Row**
Healthy Controls (n=8)	79.4 ± 14.4	35.7 ± 6.8	60.2 ± 12.5	38.8 ± 3.9
Breast Cancer Survivors (n=4)	81.6 ± 20.0	33.18± 5.5	58.32 ± 15.1	34.0 ± 5.3

Plasma Volume Shifts

Plasma volume (PV) shifts as a result of the experimental trials were calculated from mean Hemoglobin (Hb) and Hematocrit (Hct) values for each time point. The greatest mean PV shifts occurred from pre-trial to post-trial. No differences in PV shift were observed between groups. See *Table 3* for PV shifts.

Table 3: Plasma volume changes (**Δ** % changes) from pre-exercise to immediate post-exercise; pre-exercise to 2-hour post-exercise and pre exercise to 24 hour post. All values are expressed as mean ± standard deviation (SD).

Trial	**Δ Pre- to Post**	**Δ Pre- to 2 hours Post**	**Δ Pre- to 24 hours Post**
Healthy Controls (n=8)	-10.53 ± 5.26	1.59 ± 6.49	-2.95 ± 7.46
Breast Cancer Survivors (n=4)	-3.58 ± 5.22	2.57 ± 1.39	0.91 ± 3.69

Plasma Cytokine Concentrations

Table 4 displays the plasma cytokine concentrations for both groups (healthy controls, cancer survivors) and each time interval after PV shift was accounted for. For the analysis, four separate 2X4 mixed-model analysis of variance (ANOVAs) were performed. A

Bonferroni post-hoc was administered to assess any within or between group differences. Significance was set a priori at $P<0.05$.

Table 4: Plasma cytokine concentrations (pg/ml) (Mean ± SD).

Trial		Pre- Exercise	Immediate-Post Exercise	2 Hours-Post Exercise	24 Hours-Post Exercise
IL-6	Healthy Controls (n=6)	6.24 ± 3.26	4.95 ± 1.07	20.89 ± 17.63$^{\delta}$	6.36 ± 2.66
	Cancer Survivors (n=4)	4.86 ± 2.77	6.12± 3.33	8.51 ± 2.45$^{\delta}$	6.45 ± 3.14
IL-10	Healthy Controls (n=6)	3.66 ± 1.95	7.28 ± 7.58	12.88 ± 13.73	6.23 ± 8.62
	Cancer Survivors (n=4)	4.75 ± 4.31	7.77 ± 4.45	9.90± 9.94	6.10± 0.99
TNF-α	Healthy Controls (n=6)	10.33 ± 3.37	10.48 ± 4.34	12.08 ± 4.70	7.44 ± 3.15$^{\delta}$
	Cancer Survivors (n=4)	8.52 ± 1.91	8.21 ± 5.10	8.43 ± 2.66	5.22 ± 2.44$^{\delta}$

δ *P<0.05 vs. Pre-exercise (main-effect of time)*

IL-6: The analysis revealed a significant main effect of time (p=0.015) when examining the changes in IL-6 over the time periods. A Bonferoni post-hoc revealed that there was a significant increase of IL-6 in both groups from pre-exercise to 2 hours-post exercise (p=0.027). No differences were observed between groups (p=0.341).

IL-10: The analysis revealed no differences main effect of time (p=0.285) nor differences between groups (p=0.397).

TNF-α: The analysis revealed a significant main effect of time (p=0.023) when examining the changes in TNF-α over the time periods. A Bonferroni post-hoc analysis revealed a

significant downregulation of TNF-α from pre-exercise to 24 hours-post exercise (p=0.011). No differences existed between groups (p=0.213)

Cortisol

Table 5. Plasma Cortisol Concentrations (ng/mL). All values are expressed as mean ± standard deviation (SD).

Trial		**Pre- Exercise**	**Immediate Post Exercise**	**2 Hours Post Exercise**	**24 Hours Post Exercise**
Cortisol	Healthy Controls (n=6)	101.8 ± 26.0	87.9± 42.8	126.5± 128.5	127.2 ± 97.1
	Cancer Survivors (n=4)	91.7 ± 8.8	61.2 ± 8.5	47.6 ± 11.3	55.3 ± 14.6

*The cortisol responses were not statistically analyzed for significant changes between groups or over time, since this measure was not a primary outcome variable in this study.

Cortisol vs. IL-6

Table 6 displays the peak percent change of IL-6 and cortisol from pre-exercise to 2-hours post exercise. A Spearman's rho correlation was used to calculate any correlation that may exist between peak percent change of cortisol and IL-6.

Table 6. Average peak percent (%) change for cortisol and Interleukin 6 (Pre-exercise to 2 hours post-exercise). All values expressed as mean ± standard deviation (SD).

Trial	Cortisol	IL-6
Healthy Controls (n=6)	-53.7 ± 207.1	364.9 ± 6.4
Breast Cancer Survivors (n=4)	-199.2 ± 49.9	202.4 ± 85.6

Cortisol vs. IL-6: There was no relationship (Spearman rho) between peak percent change of cortisol and IL-6 (p=0.751).

CHAPTER V

DISCUSSION

Introduction

The primary purpose of this study was to explore the effects of an acute bout of moderate intensity resistance exercise on inflammatory markers in breast cancer survivors. Contrary to the proposed hypothesis, breast cancer survivors responded similarly to the exercise session compared to the healthy controls. The theorized inflammatory response in cancer survivors, which would have been represented by greater increases in pro-inflammatory cytokines and a reduced upregulation of anti-inflammatory cytokines relative to the healthy controls, was not evident. To the contrary, the resistance exercise session was shown to reduce the inflammation in both groups, evident by a reduction in TNF-α 24 hours post exercise. Furthermore, and irrespective to any differences between the groups, the exercise session elicited an inflammatory response that was moderately consistent with what has been reported in previous literature (Galvao et al., 2008; Phillips et al., 2010; Toft et al., 2002).

In order to describe the main findings from this study in more detail, this discussion will be separated into different sections. First, each cytokine will be discussed in isolation, both in relation to the group response and the time of measurement. Next, the complete cytokine response will be examined in an effort to portray any interactions that may exist between the cytokines, deliberate what type of inflammatory effect is present (if at all) at

each time point, and to present more clearly what the inflammatory response may represent in terms of recovery and adaptation from exercise. Lastly, this discussion will explore the questions that arise from this study, and how those inquiries may be applied to cancer exercise therapy and future research.

Interleukin-6

The response of Interleukin-6 (IL-6) to the resistance exercise session did not differ between groups ($p=0.341$) and the temporal response was similar to patterns that were displayed in previous literature (Peake et al., 2006; Phillips et al., 2010; Toft et al., 2002). Of particular note, is that IL-6 was shown to be upregulated at 2 hours post-exercise ($p=0.027$). This is in agreement with other studies that also displayed the peak upregualtion of IL-6 between two and six hours into recovery (MacIntyre et al., 2000; Peake et al., 2006; Toft et al., 2002).

This pattern of IL-6 dynamics is consistent with the idea that IL-6 is a cytokine that is released directly from muscle, and that complete diffusion out of the muscle will likely occur sometime after the end of the exercise session (Pederson & Febbraio, 2008). Moreover, the degree of IL-6 proliferation is also an important aspect to examine, as variation in IL-6 responses have been reported based on the mode and duration of the exercise task (Nieman et al., 2004; Paulsen & Peake, 2013; Toft et al., 2002). In this present study IL-6 was upregulated approximately 3-fold from baseline values. Toft et al. (2002) and Phillips et al. (2010) displayed similar results, displaying IL-6 increases of approximately 2-fold and 3-fold respectively. It is important to note, however, that unlike some studies (Phillips et al., 2010; Toft et al., 2002), the present study did not display an upregulation of IL-6 immediately post-exercise. A possible explanation lies in the resistance exercise modality used in this study.

More specifically, the exercise was administered in a circuit fashion and in a short time window (less than 30 minutes), thus it is possible that the IL-6 produced by the contracting muscle failed to enter into the blood before the immediately post-exercise blood draw.

Before continuing further with the discussion of IL-6, it is important to note that the source of post-exercise IL-6 production carries some debate amongst researchers. The prevailing theory before the early 2000's, was that systemic production of IL-6 was a consequence of an immune cell infiltration of damaged skeletal muscle and subsequent release of cytokines into the serum (Izqueirdo et al., 2010; Nehlsen-Canarella et al., 1997; Toft et al., 2002). Conversely, others have hypothesized that the post-exercise IL-6 response is mainly dependent on muscular contraction with the main factors determining response being the duration of the exercise and substrate availability (Nieman et al., 2002; Pederson & Febbraio, 2008). To the former point, Izquierdo et al. (2010) explained that the amplified delayed onset muscle soreness (DOMS) that occurred after the exercise session is directly related to the systemic IL-6 production. Smith et al. (2000) and Suzuki et al. (2002) derived similar conclusions, explaining the muscle damage and the production of IL-6 are inexorably linked. To the latter point, Fischer et al. (2006) explains that 50% of the variation in plasma IL-6 following exercise can be explained by exercise duration alone. Moreover, a study by Nieman et al. (2004), which consisted of 2 hours of resistance exercise, induced the most significant IL-6 upregulation (~5-fold) of the reviewed studies. In regards to these opinions, it cannot be ruled out that the difference in observations may be representative of separate phenomena, and that post-exercise IL-6 may be a result of varying stimuli and a byproduct from diverse group of sources (e.g., muscle, immune cells, satellite cells).

The present study displayed significant IL-6 changes after relatively short exercise session (<30 minutes), therefore exercise duration is unlikely to be a major factor in the IL-6 production. In regards to this concept, other factors of the exercise protocol may have aided in the release of IL-6 from the muscle. As noted by Nieman et al. (2004) IL-6 upregulation during and after exercise is heavily dependent on fuel utilization, particularly on carbohydrate intake and glycogen storage. Since the present study was administered in a circuit fashion rest intervals were relatively short (30-45 seconds), it is likely that carbohydrate utilization was much higher relative to a protocol that allows for longer rest intervals. As described by Paulsen and Peake (2013), applying heavy loads with few repetitions and long rest intervals are like to produce small to moderate changes in hormonal and inflammatory response. However, circuit training, with higher repetitions and short rest intervals are likely to induce changes more similar to aerobic exercise (Paulsen & Peake, 2013). Therefore, the present study displays changes in IL-6 that are most likely indicative of continuous muscle contraction and resultant glycogen depletion.

Some of the differences in IL-6 production after exercise noted above may also be related to the type of muscle contraction used in the exercise program. Eccentric muscle contraction is likely to induce more moderate increases in IL-6, yet promote an extended low-grade production of the cytokine well into recovery (Hirose et al., 2004; Smith et al., 2000). Conversely, exercise sessions comprised mainly of concentric contraction are likely to induce a more significant increase in IL-6 immediately after exercise, followed by a return to baseline shortly within the recovery period (Hirose et al., 2004). The present study reveals a trend more similar to the latter phenomenon. Twenty-four hours after the completion of the exercise session, IL-6 returned to baseline values in both groups, suggesting that this exercise

session did not induce a prolonged inflammatory effect or severe muscle damage. This is not surprising, as eccentric action was not a main component of the exercise session; thus it is unlikely that muscle damage was too severe. Moreover, the IL-6 response into recovery is consistent with other studies using similar protocols (Ferreira et al., 2010; Phillips et al., 2010; Prestes et al., 2009).

Interleukin-10

The resistance exercise bout did not induce any significant changes in the major anti-inflammatory cytokine, Interleukin-10 (IL-10), in either group at each time interval ($p=0.226$), nor were there any differences between groups ($p=0.810$). IL-10 did, however, show a trend increasing two hours into recovery with respect to all subjects (main effect). This is revealed when examining individual responses through a Wilcoxon non-parametric statistical test, as eight out of the ten subjects displayed an upregulation of IL-10 in the plasma two hours into recovery compared to baseline ($p=0.037$). Though only exploratory in nature, the trend found in this analysis is consistent with other literature examining IL-10 responses, in which this major anti-inflammatory cytokine is upregulated 45 minutes to 6 hours after resistance exercise (Hirose et al., 2004; Izquierdo et al., 2010).

In regards to these aforementioned studies, it is important to note that Hirose et al. (2004) and Izquierdo et al. (2010) only found significant increases in acute IL-10 production after a prolonged resistance exercise-training period. This leads to the notion that training may have an effect on the IL-10 response, and consequently the anti-inflammatory effect of exercise. Put differently, the acute production of IL-10 may be representative of an enhanced adaptive response after exercise training. In regards to the present study, the high variation in

IL-10 response (see Results Table 4) may be due, in some part, to the varying levels of training status among the participants.

Overall, a major function of IL-10 is to suppress the production of the major pro-inflammatory cytokines (TNF-α, IL-6 and IL-1). Furthermore, others propose that IL-10 is a key component in the repair of muscle damage, and subsequent post-exercise adaptation (Malm et al. 2000). The current data displays that IL-10 concentration trended upward within a short time-window following the cessation of the exercise bout, suggesting that the response may reflect a role for IL-10 in suppression of inflammation and muscle recovery. However, this last point is speculation since statistical significance in the primary analysis was not found.

Tumor Necrosis Factor-α

Tumor Necrosis Factor-α (TNF-α), the major pro-inflammatory cytokine examined in this study, remained unchanged immediately after exercise and two-hours into recovery, but was significantly downregulated twenty-four hours into recovery in both groups ($p=0.011$; main effect of time). No differences were observed between groups ($p=0.213$).

The twenty-four hours post-exercise downregulation of TNF-α was an unexpected finding from this study. Researchers have shown consistently that exercise can initiate an anti-inflammatory response over an extended training period (Greiwe et al., 2001; Pederson & Febbraio, 2008; Phillips et al., 2010). However, the acute downregulation of a significant pro-inflammatory cytokine has not been shown consistently. Only one known study, Hirose et al. (2004), displayed a similar decrease in TNF-α (15%) 1 day, 2 days, and 4 days after a resistance exercise bout.

Differences, however, do exist between the Hirose et al. (2004) and the current study. Most significantly, the protocol from the current study consisted of exercises that focused on multiple lower and upper body muscle groups with dynamic movement patterns, while the protocol from Hirose et al. (2004) consisted of eccentric action of the elbow flexors with isolated movement patterns. As explained by Paulsen and Peake (2013) the hormonal and inflammatory response is dependent on the type and the size of the exercised muscle or muscle groups. For example, exercise that emphasizes large muscle groups and 'multi-jointed, dynamic movement patterns' are likely to induce a greater systemic inflammatory response than 'isolated muscle contractions' (Paulsen & Peake, 2013). Moreover, the reduction in TNF-α in the study by Hirose et al. (2004) was independent of an increase in IL-6, while in the current study it is likely that the upregulation of IL-6 had some effects on the systemic reduction in TNF-α (see *Cytokine Interactions and Inflammatory effects of Resistance Exercise* section). Regardless of the differences in these two studies, it remains intriguing that some degree of an anti-inflammatory effect persisted after the bouts of exercise.

Exploring a broader concept, the acute down regulation of TNF-α in this study and the study by Hirose et al. (2004) may be indicative of a more long-term anti-inflammatory response. In support of this theory, researchers that have displayed that resistance exercise can reduce TNF-α over an extended training period (Greiwe et al., 2001; Phillps et al., 2010). The study by Greiwe et al. (2001) supports this claim by displaying that resistance training could increase protein synthesis in concurrence with as decrease in systemic TNF-α levels. Phillips et al. (2010) present a similar argument by displaying that a decrease in systemic levels of TNF-α was associated with improvements in overall strength. In addition, and as

previously discussed, the study by Hirose et al. (2004) displays a significant downregulation of TNF-α extended to 4 days following the resistance exercise session.

To the contrary, the post-exercise reduction of TNF-α evident in this study is not in agreement with all studies examining resistance exercise and inflammation, as other researchers have displayed either small increases (Louis et al., 2007) or no changes (Periera et al., 2012; Prestes et al., 2009) in TNF-α after a resistance exercise session. However, important differences exist between the present and the aforementioned studies. For instance, Louis et al. (2007) used muscle mRNA to observe cytokine dynamics, thus the acute changes of the TNF-α after exercise may represent a local, intramuscular response, as compared to the present study that examined the cytokine in the systemic circulation. Moreover, the findings from Periera et al. (2012) and Prestes et al. (2009) display no changes in IL-6 or IL-10 following the exercise session. This is noteworthy, as IL-6 and IL-10 act as major suppressors of TNF-α, thus it is likely that TNF-α will remained unchanged without changes in these signaling components (Pederson & Febbraio, 2008). This present study did show a significant increase in IL-6 post-exercise and a trend towards an upregulation of IL-10, thus it is plausible that these cytokines had modulating effects on TNF-α at twenty-four hours post exercise time point. A more detailed explanation of cytokine interactions will be discussed in the next section of this chapter.

Cytokine Interactions and the Inflammatory Effects of Resistance Exercise

The interactions of IL-6, IL-10 and TNF-α are important to consider when reviewing the inflammatory response to resistance exercise. These cytokines (along with others) are thought to mediate progression from an inflammatory response to the eventual long-term adaptive response (Tidball, 2005). This section will investigate some of these interactions in

an effort to propose a physiological reasoning behind the findings from this study. The discussion, however, should be reviewed as speculative, as definitive interactions are not fully substantiated due to the limited sample size.

IL-6 is the most extensively upregulated cytokine after exercise (Ostrowski et al., 1999; Peterson & Pederson, 2005). Though there is a moderate increase in the systemic concentration of many cytokines, it is clear that the appearance of IL-6 in the circulation is by far the most marked and that its appearance precedes that of the other cytokines (Pederson & Febbraio, 2008). This is again evident in the present study, as IL-6 is the only cytokine measured that significantly increases over baseline at any post-exercise time points ($p=0.027$). As discussed, the upregulation of IL-6 from this particular exercise session is likely due to its direct release from skeletal muscle and its ability to 'sense' energy depletion (Pederson et al., 2004; Steensberg et al., 2001).

The present study also displays an upward trend in IL-10 at the 2-hours post-exercise time interval at similar to that of IL-6. The concurrent increase in these two cytokines points to a theory which states that IL-10 is directly stimulated by IL-6 (Peterson & Pederson, 2005). Thus, the peak production of IL-10 should occur in a period during or right after the peak production of IL-6 (Peterson & Pederson, 2005). This phenomenon is present in this study and others. For example, Izquierdo et al. (2010) showed that IL-10 increases (2-fold) in concurrence with an upregulation of IL-6 (3-fold) at 45 minutes after exercise. Additionally, Hirose et al. (2004) displayed that increases in IL-6 precede that of IL-10. More broadly, Peterson and Pederson (2005) explain that the IL-6 production drives the anti-inflammatory effect after exercise, which is represented in some degree by an increase in circulating IL-10.

With respect to this discussion, the data from this study support (to some degree) the notion that IL-6 can act as stimulating factor for the anti-inflammatory cytokine, IL-10.

The anti-inflammatory effects of IL-6 and IL-10 may also be related to the suppression of pro-inflammatory cytokines (Peterson & Pederson, 2005). In the present study, though the theory remains speculative, the 2-hour post exercise upregulation in IL-6 and trending increase in IL-10 may have had a role in the suppression of TNF-α at 24 hours-post-exercise (See Figure 3). This observation is in agreement with a study completed by Starkie et al. (2003) in which infused recombinant IL-6 decreased the systemic levels of endotoxin induced TNF-α. Concurrently, others suggest that IL-10 directly suppresses the production in TNF-α through the inhibition of the NF-kb pathway in macrophages and other immune cells (Wang et al., 2005). With these dual inhibitory actions, it is possible that the inflammatory state 2-hours after resistance exercise, as evident in this study, may have a prolonged effect on the recovery period 24 hours after the exercise session.

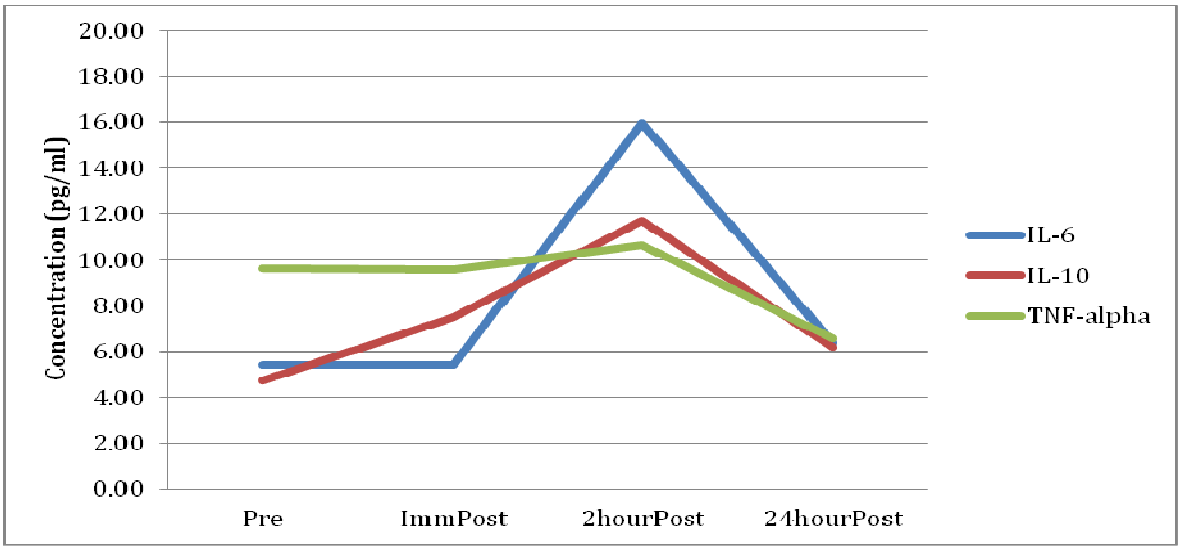

Figure 3: Cytokine responses (IL-6, IL-10 and TNF-α) at pre, immediately-post, 2 hours-post, and 24 hours-post exercise.

Implications for Cancer Survivors and Future Directions

As discussed above, the current study displayed no differences between healthy controls and cancer survivors with regards to inflammation after an acute bout of resistance exercise. These findings are encouraging, as the observed temporal response of cytokines after resistance exercise is representative of a possible anti-inflammatory effect. This may be especially important for cancer survivors suffering from chronic, low-grade inflammation and associated symptoms. Although, it is recognized that the above interpretation must be tempered by the fact the sample size in this study is so limited.

This study is not the first to present the acute effects of resistance training on inflammation and the immune system in cancer survivors. Galvao et al. (2008) also displayed that resistance exercise had similar immune and inflammatory effects in prostate cancer survivors compared to healthy controls. Comparable to the current study, IL-6 was upregulated as a result of exercise and TNF-α remained unchanged immediately after exercise. However, unlike the current study Galvao et al. did not observe the cytokine responses into recovery, and they did not examine responses of IL-10. Galvao and colleagues also used a different protocol with longer rest intervals (2-4 minutes) compared to the present study (30-45 seconds). Even with these slight protocol differences, Galvao et al. (2008) reported similar, encouraging findings, as no pro-inflammatory response persisted after the exercise bout, while subjects saw improvements in the hormonal milieu evidenced by increases in growth hormone and insulin like growth-factor following the exercise bout. Described in a broader perspective the researchers also noted the long-term benefits of resistance exercise training: reduced treatment related side effects, enhanced the hormonal response to exercise, and possibly improved immune function.

It should be noted, that the study by Galvao et al. (2008) examined the effects of resistance exercise in prostate cancer survivors (males) undergoing Androgen Deprivation Therapy (ADT). This contrasts from the current study, which observed the effects of exercise on breast cancer survivors (females) undergoing various treatment protocols. The differences in these studies, both the protocols and types of cancers, highlights more clearly that more studies need to be compiled in this area. Nevertheless, immunosuppression and chronic, low-grade inflammation are associated with both diseases; which suggests these studies are comparable to some degree (Al-Majid & Waters, 2008; Backwill and Matovani, 2001).

Resistance exercise may also improve symptoms associated with chronic low-grade inflammation in cancer survivors. As reported by Al-Majid and Waters (2008) resistance training may diminish cancer-related skeletal muscle wasting by downregulating the activity of pro-inflammatory cytokines. The author's note that TNF-α and IL-6 are chronically elevated in many types of cancer and that a reduction of these cytokines in the blood will lead improved strength and muscle function. Moreover, Battaglini et al. (2012) derived a theoretical model (Exercise Anti-Cachectic Hypothetical—EACH) that suggests that exercise can improve the inflammatory state in cancer patients and survivors, which will ultimately lead to enhanced functionality and improved quality of life in these individuals. Additionally, these authors propose that resistance exercise, and the subsequent reduction in pro-inflammatory cytokines, will generate a more balanced protein turnover in cancer patients and survivors (Battaglini et al., 2012). These concepts are confirmed by research that shows that resistance trained cancer survivors have lower resting plasma IL-6 levels compared to sedentary controls (Jones et al., 2012; Sprod et al., 2010) while other studies display that resting TNF-α can be reduced over a training period with a concurrent increase in muscle

mass and strength (Greiwe et al., 2001).

The findings of these studies lead one to ask the question of whether the acute inflammatory response may have effects on the eventual, long-term adaptive response. Offering support to this idea, Hirose et al. (2004) displayed decreases in TNF-α on day 1, day 3, and day 4 days follwoing the cessation of exercise, Smith et al. (2000) showed that anti-inflammatory IL-10 can be upregulated up to 6 days beyond the cessation of an exercise session, while Prestes et al. (2009) showed that IL-6 can be downregulated up to 48 hours after the resistance exercise bout. Without question, the long-term adaptive response also requires slower, less noticeable changes in metabolic state and endocrine/inflammatory milieu; however the aforementioned studies give some acknowledgment to the concept that the acute inflammatory state after exercise may direct, to some degree, the long-term adaptive response.

These inflammatory responses to exercise, both long and short-term, are important for cancer survivors using exercise therapy. As proposed by Battaglini et al. (2012), 'incorporation of exercise training as a part of the therapeutic regime of cancer treatment can result in anti-inflammatory cytokines mitigating some of the effects of the pro-inflammatory cytokines'. More specifically, if the acute inflammatory state directs (to any degree) the long-term response to exercise, it remains vital for researchers to observe these responses in order to design safe, efficient and effective exercise programs. The current study provides some encouraging data in regards to these concepts, yet more studies, exhibiting more in-depth exploration of the acute inflammatory state after exercise, are needed.

Limitations of Study

There are several potential limitations and confounding factors in this study, which may have impacted the results and potentially limited the reliability and validity of the findings. First, the small sample size (n=12; cancer survivor=4; healthy controls=8) made it difficult to generalize the findings to cancer populations and the general public alike. The recruitment and scheduling of the subjects in a short time window was the likely cause of this shortcoming. Furthermore, the training status of the participants was not entirely accounted for. As discussed in previous sections, training status has important effects of post-exercise cytokine production, thus this uncontrolled variable may introduce error into the study.

The type and timing of cancer treatment may also have had effects on the results. Namely, this study allowed for participants with a relatively wide-range of treatments to participate. Additionally, the relatively large time-window after treatment (3 months to 1 year) in the cancer survivor group will reduce the reliability and validity of the current study. Also, the truthfulness of the participants in providing background information and adhering to pre-assessment guidelines is a potential confounder within the data.

The collection of blood samples also may have introduced sources of error into the study. Namely, the venipuncture and catheter placement procedures may have elicited stressful responses in some individuals, leading to misguided interpretations of hormonal and inflammatory responses. Additionally, there was one missing blood sample collection at the 24-hour post exercise time point due to scheduling conflicts. In order to account for the missing sample, the statistical procedure of mean substitution was used.

It is important to note that the researchers tried to control some of these limitations through efficient planning and scheduling, discussion with the participants, and data collection in a controlled environment, yet errors and oversights may still have occurred.

Chapter VI

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Despite the limitations of this study, valuable insights can be gained from these findings. First, the inflammatory response to resistance exercise does not appear to differ between healthy controls and breast cancer survivors. This is evident by no significant difference between the groups when comparing circulating levels of cytokines (IL-6, IL-10, and TNF-α) at each time point after exercise (Immediately post, 2-hours post, 24-hours post). Additionally, this study reveals intriguing data in regards to the post-exercise dynamics of cytokines. In both groups, the resistance exercise session elicited an upregulation in IL-6 2 hours-post exercise, and a downregulation of TNF-α 24 hours post-exercise. The response of IL-6 was consistent with other studies using similar protocols (Phillips et al., 2010), while the response of TNF-α was more unexpected. These findings, with particular focus on the reduction in TNF-α, indicate that resistance exercise may alter the inflammatory state post-exercise and into recovery through a shift in pro and anti-inflammatory cytokines. More specifically, it appears as if resistance exercise at a moderate intensity may promote an overall anti-inflammatory state 24 hours into recovery relative to baseline measures.

With regards to cancer survivors, these results are encouraging. Firstly, the cancer survivors respond similarly to exercise relative to their healthy counterparts, providing support that moderate intensity resistance exercise does not elicit any major adverse outcomes. Moreover, a pro-inflammatory state did not persist in the blood after exercise, thus

it can be assumed that the cancer survivors are responding encouragingly to the exercise session. In fact, an acute reduction in inflammation was evident through a downregulation in circulating TNF-α. As discussed previously, TNF-α remains as one of the leading contributors to chronic inflammation in many types of cancer, which may ultimately lead to worsening of symptoms and the progression of the tumor (Balkwill & Mantovani, 2001). Overall, there is data from this study, and others previously (Battaglini et al., 2012; Galvao et al., 2008) to suggest that resistance exercise does not introduce a harmful inflammatory response and that it remains as a safe and effective therapy for cancer survivors.

As mentioned, this study has various shortcomings and possible sources of error. Consequently, any findings this study should be reviewed with some degree of caution. Nevertheless, results from this study may help direct future researchers towards a more thorough exploration into the acute immune and inflammatory responses to exercise in cancer survivors.

Conclusions:

Research Hypothesis #1: *IL-6 will be significantly upregulated from baseline in the cancer survivors and healthy controls immediately-post exercise and 2 hours post-exercise and return to baseline 24 hours post exercise.* This hypothesis was rejected for the immediately-post exercise IL-6 response, but accepted for the 2-hours post-exercise ($p=0.027$).

Research Hypothesis #2*: IL-10 will be significantly upregulated from baseline at 2 hours post-exercise and 24 hours post-exercise in healthy controls but not in the cancer survivors.* This hypothesis was rejected since circulating IL-10 did not change as a result of exercise in either group.

Research Hypothesis #3: *TNF-α will be significantly upregulated 2 hours post exercise and 24 hours post exercise in the cancer survivors but not in the healthy controls*. This hypothesis was also rejected, as plasma TNF-*α* did not change at 2 hours post-exercise and was significantly reduced at 24 hours post exercise in both groups (p=0.011).

Research Hypothesis # 4 (Exploratory): *Peak (%) change (+) in IL-6 will correlate significantly with peak (%) change (+) of cortisol.* This hypothesis was rejected, as no correlation was evident between IL-6 and cortisol at any time point after exercise.

Appendix A

University of North Carolina-Chapel Hill
Consent to Participate in a Research Study
Adult Subjects: Breast Cancer Survivor Group
Biomedical Form

IRB Study # 11-1405
Consent Form Version Date: January 10, 2013

Title of Study: The Impact of Acute Aerobic and Resistance Exercise on Natural Killer Cell, Catecholamine, and Cortisol Responses in Breast Cancer Survivors

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Funding Source and/or Sponsor: NA

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Study Contact email: rgraff1@email.unc.edu or jmallen@email.unc.edu

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What are some general things you should know about research studies?

You are being asked to take part in a research study. To join the study is voluntary. You may refuse to join, or you may withdraw your consent to be in the study, for any reason.

Research studies are designed to obtain new knowledge that may help other people in the future. You may not receive any direct benefit from being in the research study. There also may be risks to being in research studies.

Deciding not to be in the study or leaving the study before it is done will not affect your relationship with the researcher, your health care provider, or the University of North Carolina-Chapel Hill. If you are a patient with an illness, you do not have to be in the research study in order to receive health care.

Details about this study are discussed below. It is important that you understand this information so that you can make an informed choice about being in this research study. You will be given a copy of this consent form. You should ask the researchers named above, or staff members who may assist them, any questions you have about this study at any time.

What is the purpose of this study?

The purpose of this research study is to learn about the effect of one session of moderate intensity aerobic or resistance exercise on the immune system and endocrine system of breast cancer survivors. The immune system helps to protect the body from getting sick. The endocrine system includes the hormones that the body makes, some of which can affect the immune system. We will also be examining how the same type of aerobic or resistance exercise affects age-matched women who have never experienced cancer treatment.

Over the past 20 years, regular aerobic and resistance exercise has been shown to improve the health and well-being of breast cancer survivors in many different ways. Regular participation in exercise can help decrease feelings of fatigue, anxiety, and depression. Regular aerobic and resistance exercise can also help improve physical fitness, build strong bones and muscles, and lower the amount of fat stored in the body. Regular moderate aerobic or resistance exercise may also improve the immune system's ability to fight infections, and it may also lower the body's production of stress hormones. However, researchers still have many questions to answer regarding how aerobic exercise and resistance exercise affect the immune system and stress hormone levels in breast cancer survivors, especially when compared to healthy age-matched women who have never experienced treatment for cancer.

The primary aim of this study is to examine the effects of a moderate-intensity, 30-minute aerobic exercise bout or a moderate-intensity resistance exercise bout on the natural killer (NK) cell. The NK cell is an important part of the immune system that helps to destroy viruses and tumor cells. Secondly, the affect of these exercise bouts on the stress hormones epinephrine, norepinephrine, and cortisol will be examined. Thirdly, the relationships between the response of the NK cell and the response of the stress hormones will be measured. The levels of these markers will be measured immediately before exercise, immediately after exercise, 2 hours post-exercise, and 24 hours post-exercise via blood samples.

Why are you being asked to participate in this study?

You are being asked to be in the study because you are a woman who has been diagnosed with breast cancer, you have completed surgery, chemotherapy, and radiation therapy within the past 3-6 months, and you are between the ages of 40 and 70 years. The full list of inclusion criteria for participation in this study are listed below:

3.1.1. Confirmed diagnosis of Stage I, II, or III invasive breast cancer;
3.1.2. Must have received chemotherapy;

3.1.3. Must have completed surgery, chemotherapy, and radiation therapy 3-6 months prior to enrollment;
3.1.4. Patients receiving adjuvant hormonal therapy or adjuvant trastuzumab are eligible;
3.1.5. No presence of metastatic disease;
3.1.6. Female, between 40 and 70 years of age;
3.1.7. Not involved in regular organized physical activity for at least 1 year prior to enrollment, meaning that you exercise less than 3 times per week and that you do not engage in activities of moderate or vigorous intensity.
3.1.8. Not taking any anti-inflammatory medications;
3.1.9. Clearance from your oncologist to participate in exercise.

Are there any reasons you should not be in this study?
You should not be in this study if you have any type of disease that affects your heart, lungs, bones, or muscles which would make it very difficult for you to exercise. You should not be in this study if you have uncontrolled diabetes or thyroid disease. You should not be in this study if you have had a fever, body aches, and/or swollen glands within the past 6 weeks or if you have a chronic infectious disease such as hepatitis or AIDS. You should not be in this study if you have abnormally low values for certain blood markers, including potassium, magnesium, white blood cells, hematocrit, and platelets. Also, you should not be in this study if you are currently using an anti-inflammatory medication such as non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids, or you have any other mental or physical impairment that would prevent you from being able to exercise.

How many people will take part in this study?
If you decide to be in this study, you will be one of approximately 40 people in this research study.

How long will your part in this study last?
You will be asked to come to the laboratory for 3 separate visits. Laboratory Visit 1 will last approximately 2 hours, Laboratory Visit 2 will last approximately 3 hours, and Laboratory Visit 3 will last approximately 30 minutes. The overall length of time that you will participate in the study will be approximately 1-2 weeks, but may be shorter.

As this study involves drawing blood, some of your blood samples may be frozen and stored indefinitely for future analyses. You will be given a separate consent form describing storage of the blood samples.

What will happen if you take part in the study?
Participation in this study will require you to report to the laboratory for 3 separate visits. All Laboratory Visits will take place in the Integrative Exercise Oncology Research Laboratory (IEORL), which is located in Fetzer Hall, in the Department of Exercise and Sport Science at UNC-Chapel Hill. All study procedures are a requirement of participation in the study.

Laboratory Visit 1: Orientation / Familiarization to the Study and 1-RM Assessment (Resistance Exercise Group Only)

The purpose of Laboratory Visit 1 is to provide you with an overview of the study, give you an opportunity to ask questions about your participation, sign this informed consent, gather some demographic information about you, and to assess your muscular strength. The specific procedures that will be performed are listed below:

1. Sign informed consent documents
2. Fill out a medical history questionnaire
3. Undergo a brief medical and physical screening, including a resting electrocardiogram (ECG) to measure heart function
4. Undergo body composition analysis to assess percent body fat using a DEXA
5. Familiarization with equipment used during exercise testing
6. Undergo an exercise test to assess muscular strength

The purposes of items 2, 3, and 4, are to determine if it is safe for you to participate in exercise and to gain some demographic information about you, including your age, height, weight, percent body fat, race, physical activity participation over the past year, and type of cancer treatments you have received and are currently receiving. The machine that will be used to measure your percent body fat in item 4 is called a DEXA. DEXA stands for Dual Energy X-ray Absorptiometry. The DEXA uses two x-ray beams to measure differences in the composition of different tissues in your body, such as bones, soft tissue, and fat. The scan is performed while you rest on your back, and takes approximately 6 minutes.

The purpose of item 6 is to perform an exercise assessment that will allow the researchers to determine your muscular strength, or the greatest amount of weight that certain muscle groups can lift one time. This exercise assessment will involve performing 4 different exercises. They are: leg press, lateral pull down, leg extension, and seated row. The exercise assessment will involve a series of one-repetition trials with increasing workloads until you decide that the given weight is the most that you can lift with proper form. This will be done for each of the 4 exercises. The duration of the exercise assessment will likely last no more than 30 minutes.

Laboratory Visit 2: Acute Resistance Exercise Session (Resistance Exercise Group Only)

Laboratory Visit 2 will occur between 7-14 days of Laboratory Visit 1. During this Visit, you will be performing 1 session of moderate-intensity resistance exercise on the same 4 pieces of equipment used in Laboratory Visit 1 (leg press, lateral pull down, leg extension, and seated row). This will be the exercise bout that will be used to elicit a response in your immune cells, inflammatory markers, and stress hormones. The specific procedures that will be performed are listed below:

1. Fill out a psychological stress questionnaire
2. Pre-exercise blood sample
3. Moderate-intensity resistance exercise session
4. Post-exercise blood sample (immediately post-exercise)
5. Post-exercise blood sample (2 hours post-exercise)

The purpose of item 1 is to determine if you are feeling any emotional or psychological stress on the day of testing. This is important information as the levels of stress hormones in your blood may be influenced by emotional or psychological stress.

During item 3, you will be performing an approximately 30-minute bout of moderate-intensity resistance exercise on the leg press, lateral pull down, leg extension, and seated row. A moderate intensity level of exercise will make you feel like your muscles are working, but you are able to carry on a conversation. You will alternate between each of the four resistance exercises, performing 10 repetitions of each followed by 1.5 minutes of rest before continuing on to the next exercise. You will move from the leg press to the lateral pull down to the leg extension to the seated row, with the same number of repetitions and amount of rest until you have completed each exercise three times.

The 3 blood samples to be obtained during items 2, 4, and 5 will be performed by using a small catheter inserted into a vein in your arm. This blood sampling procedure is very similar to a blood sampling procedure that is used in routine medical clinic settings. Approximately 1 tablespoon of blood will be collected for each item. Some of this blood will be used during the course of this study to measure the number of NK cells in your blood, the activity of the NK cells (i.e., how well they destroy other cells), and the levels of stress hormones and cytokines in your blood.

Laboratory Visit 3: 24-hour Follow-up Session

Laboratory Visit 3 will occur 24 hours after Laboratory Visit 2. During this Visit, you will answer the same psychological stress questionnaire as you did in the previous laboratory visit. You will also have 1 blood sample drawn, which will also be used to the number of NK cells in your blood, the activity of the NK cells (i.e., how well they destroy other cells), and the levels of stress hormones and cytokines in your blood. The procedure for obtaining the blood sample is the same as described above, except that the blood will be drawn through a small needle instead of a catheter.

What are the possible benefits from being in this study?

Research is designed to benefit society by gaining new knowledge. The benefits to you from being in this study may be the opportunity to have your various aspects of your health assessed during Laboratory Visit 1. This would include looking at how well your heart is working by using the ECG and by listening to your heart with a stethoscope. Additionally, you will have your muscular strength assessed, as well as your percent body fat. This information may be useful if you are be thinking of beginning an exercise program, and you may elect to provide this information to an exercise specialist who could construct and supervise an individualized exercise program for you.

What are the possible risks or discomforts involved with being in this study?

The risk of physiological and psychological harm is very minimal and would not cost you physical or emotional loss. However, any research study does carry with it some potential for risk or discomfort, listed below:

1. During Laboratory Visit 1, you will be performing an exercise test to determine your muscular strength. The test will start with an easy warm-up and movement rehearsal for your muscles. As the one repetition maximum trials begin, you will find that the assessment will become more difficult. It is possible that increased stress will be placed on your musculoskeletal system. To ensure your safety, your heart rate and your lifting technique will be monitored continuously throughout the various trials to verify that there are no movement compensations. Your heart rate will continue to be monitored for several minutes after the conclusion of the one repetition maximum assessments. At the conclusion of the test, you will be allowed to cool down, stretch, and drink water at liberty.

2. During Laboratory Visit 1, you will be exposed to radiation during the DEXA scan. However, the scan itself is very safe, and the amount of x-ray radiation that you will receive during the scan is 0.8 mrems, which is extremely small. To put this in perspective, this amount of radiation is about the same amount of radiation that you would receive from natural background sources in one day.

3. During Laboratory Visits 1 and 2, it is possible that you could experience slight muscle or joint soreness after the exercise. However, this soreness would not be any worse than after doing any other type of exercise. To minimize the amount of soreness you might feel, you will be allowed to stretch before and after exercise, and you will be allowed to do a light warm-up and cool-down before and after exercise.

4. During Laboratory Visits 2 and 3, it is possible that you could experience some mild bruising or discomfort while having your blood drawn. This discomfort would not be any more significant than having your blood drawn at the doctor's office. The blood sampling procedures will be performed by research team members who have drawn blood many times before, and proper bandaging will be performed to minimize bleeding.

In addition, there may be uncommon or previously unknown risks that might occur. You should report any problems to the researchers.

What if we learn about new findings or information during the study?
You will be given any new information gained during the course of the study that might affect your willingness to continue your participation.

How will your privacy be protected?
You will not be identified in any report or publication about this study. Although every effort will be made to keep research records private, there may be times when federal

or state law requires the disclosure of such records, including personal information. This is very unlikely, but if disclosure is ever required, UNC-Chapel Hill will take steps allowable by law to protect the privacy of personal information. In some cases, your information in this research study could be reviewed by representatives of the University, research sponsors, or government agencies (for example, the FDA) for purposes such as quality control or safety.

All data obtained from you during this study will be coded using a research ID number, and only the members of the research team will have access to these codes. Study databases and logs will not have any information that will allow for subjects to be identified by anyone other than a research team member.

A copy of this consent form will go in to your medical record. This will allow the doctors caring for you to know what study tests you may be receiving as a part of the study and know how to take care of you if you have other health problems or needs during the study.

What will happen if you are injured by this research?
All research involves a chance that something bad might happen to you. This may include the risk of personal injury. In spite of all safety measures, you might develop a reaction or injury from being in this study. If such problems occur, the researchers will help you get medical care, but any costs for the medical care will be billed to you and/or your insurance company. The University of North Carolina at Chapel Hill has not set aside funds to pay you for any such reactions or injuries, or for the related medical care. However, by signing this form, you do not give up any of your legal rights.

What if you want to stop before your part in the study is complete?
You can withdraw from this study at any time, without penalty. The investigators also have the right to stop your participation at any time. This could be because you have had an unexpected reaction, you become ill, you use a medication that may affect the outcome of the study variables being measured, you are not able to complete the exercise testing sessions, you refuse to have your blood drawn, you miss a Laboratory Visit, or because the entire study has been stopped. If you decide to withdraw from the study, all data collected from you will be destroyed and will not be used in any data analyses that may result from this study.

Will you receive anything for being in this study?
You will be provided with a written report of your fitness level and percent body fat, both of which will be measured during Laboratory Visit 1.

Will it cost you anything to be in this study?
It will not cost you anything to participate in this study.

What if you are a UNC student?
You may choose not to be in the study or to stop being in the study before it is over at any time. This will not affect your class standing or grades at UNC-Chapel Hill. You will

not be offered or receive any special consideration if you take part in this research.

What if you are a UNC employee?
Taking part in this research is not a part of your University duties, and refusing will not affect your job. You will not be offered or receive any special job-related consideration if you take part in this research.

What if you have questions about this study?
You have the right to ask, and have answered, any questions you may have about this research. If you have questions, complaints, concerns, or if a research-related injury occurs, you should contact the researchers listed on the first page of this form.

What if you have questions about your rights as a research subject?
All research on human volunteers is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research subject, or if you would like to obtain information or offer input, you may contact the Institutional Review Board at 919-966-3113 or by email to IRB_subjects@unc.edu.

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Title of Study: The Impact of Acute Aerobic and Resistance Exercise on Natural Killer Cell, Catecholamine, and Cortisol Responses in Breast Cancer Survivors

Principal Investigator: Claudio L. Battaglini, PhD

Subject's Agreement:

I have read the information provided above. I have asked all the questions I have at this time. I voluntarily agree to participate in this research study.

_____ _____
Signature of Research Subject Date

_____
Printed Name of Research Subject

_____ _____
Signature of Research Team Member Obtaining Consent Date

_____
Printed Name of Research Team Member Obtaining Consent

Appendix B

University of North Carolina-Chapel Hill
Consent to Participate in a Research Study
Adult Subjects: Healthy Control Group
Biomedical Form

IRB Study # 11-1405
Consent Form Version Date: January 10, 2013

Title of Study: The Impact of Acute Aerobic and Resistance Exercise on Natural Killer Cell, Catecholamine, and Cortisol Responses in Breast Cancer Survivors

Principal Investigator: Claudio L. Battaglini, PhD
UNC-Chapel Hill Department: Exercise & Sport Science
UNC-Chapel Hill Phone number: 843-6045
Email Address: claudio@email.unc.edu
Co-Investigators: Elizabeth S. Evans, MA; Anthony C. Hackney, PhD, DSc; Robert G. McMurray, PhD; Hyman B. Muss, MD; Scott H. Randell, PhD; Rachel Graff, BS; Jacob Allen, BA; Miles Bartlett, MA; Nathan Berry, MA; Jamie Pearson, BS; Charlotte Shatten MA; Ryan Vanhoy, BS; Jeremiah Boles, MD; Mingqing Li, MD; Amy Lucas, MD; Autumn Mcree, MD; Payal Desai, MD; Christine Lin, MD; Marshall Mazepa, MD; Micah Mooberry, MD; Ryan Raddin, MD; Tyler Buckner, MD; Andrea Dean, MD; Satish Gopal, MD; Emily Jenkins, MD; Adam Kuykendal, MD; Keeran Sampat, MD

Funding Source and/or Sponsor: NA

Study Contact telephone number: (516) 633-5235 or (919) 621-1322
Study Contact email: rgraff1@email.unc.edu or jmallen@email.unc.edu

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What are some general things you should know about research studies?

You are being asked to take part in a research study. To join the study is voluntary. You may refuse to join, or you may withdraw your consent to be in the study, for any reason.

Research studies are designed to obtain new knowledge that may help other people in the future. You may not receive any direct benefit from being in the research study. There also may be risks to being in research studies.

Deciding not to be in the study or leaving the study before it is done will not affect your relationship with the researcher, your health care provider, or the University of North Carolina-Chapel Hill. If you are a patient with an illness, you do not have to be in the research study in order to receive health care.

Details about this study are discussed below. It is important that you understand this information so that you can make an informed choice about being in this research study. You will be given a copy of this consent form. You should ask the researchers named above, or staff members who may assist them, any questions you have about this study at any time.

What is the purpose of this study?

The purpose of this research study is to learn about the effect of one session of moderate intensity aerobic or resistance exercise on the immune system and endocrine system of breast cancer survivors. The immune system helps to protect the body from getting sick. The endocrine system includes the hormones that the body makes, some of which can affect the immune system. We will also be examining how the same type of aerobic or resistance exercise affects age-matched women who have never experienced cancer treatment.

Over the past 20 years, regular aerobic and resistance exercise has been shown to improve the health and well-being of breast cancer survivors in many different ways. Regular participation in exercise can help decrease feelings of fatigue, anxiety, and depression. Regular aerobic and resistance exercise can also help improve physical fitness, build strong bones and muscles, and lower the amount of fat stored in the body. Regular moderate aerobic or resistance exercise may also improve the immune system's ability to fight infections, and it may also lower the body's production of stress hormones. However, researchers still have many questions to answer regarding how aerobic exercise and resistance exercise affect the immune system and stress hormone levels in breast cancer survivors, especially when compared to healthy age-matched women who have never experienced treatment for cancer.

The primary aim of this study is to examine the effects of a moderate-intensity, 30-minute aerobic exercise bout or a moderate-intensity resistance exercise bout on the natural killer (NK) cell. The NK cell is an important part of the immune system that helps to destroy viruses and tumor cells. Secondly, the affect of these exercise bouts on the stress hormones epinephrine, norepinephrine, and cortisol will be examined. Thirdly, the relationships between the response of the NK cell and the response of the stress hormones will be measured. The levels of these markers will be measured immediately before exercise, immediately after exercise, 2 hours post-exercise, and 24 hours post-exercise via blood samples.

Why are you being asked to participate in this study?

You are being asked to be in the study because you are a woman who has never been diagnosed with or treated for cancer, you are post-menopausal, you are sedentary, and you are between the ages of 40 and 70 years. The full list of inclusion criteria for participation in this study are listed below:

3.1.10. Female, between 40 and 70 years of age;
3.1.11. No history of cancer diagnosis or treatment;
3.1.12. No presence of any bone, muscle, or other medical condition that would prevent you from being able to participate in resistance exercise;

3.1.13. Not taking anti-inflammatory medications such as non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids;
3.1.14. Not involved in regular organized physical activity for at least 1 year prior to enrollment, meaning that you exercise less than 3 times per week and that you do not engage in activities of moderate or vigorous intensity.
3.1.15. Clearance from your primary physician, if you are age 65 years or older.

Are there any reasons you should not be in this study?
You should not be in this study if you have any type of disease that affects your heart, lungs, bones, or muscles which would make it very difficult for you to exercise. You should not be in this study if you have uncontrolled diabetes or thyroid disease. You should not be in this study if you have had a fever, body aches, and/or swollen glands within the past 6 weeks or if you have a chronic infectious disease such as hepatitis or AIDS. You should not be in this study if you have abnormally low values for certain blood markers, including potassium, magnesium, white blood cells, hematocrit, and platelets. Also, you should not be in this study if you are currently using an anti-inflammatory medication such as non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids, or you have any other mental or physical impairment that would prevent you from being able to exercise.

How many people will take part in this study?
If you decide to be in this study, you will be one of approximately 40 people in this research study.

How long will your part in this study last?
You will be asked to come to the laboratory for 3 separate visits. Laboratory Visit 1 will last approximately 2 hours, Laboratory Visit 2 will last approximately 3 hours, and Laboratory Visit 3 will last approximately 30 minutes. The overall length of time that you will participate in the study will be approximately 1-2 weeks, but may be shorter.

As this study involves drawing blood, some of your blood samples may be frozen and stored indefinitely for future analyses. You will be given a separate consent form describing storage of the blood samples.

What will happen if you take part in the study?
Participation in this study will require you to report to the laboratory for 3 separate visits. All Laboratory Visits will take place in the Integrative Exercise Oncology Research Laboratory (IEORL), which is located in Fetzer Hall, in the Department of Exercise and Sport Science at UNC-Chapel Hill. All study procedures are a requirement of participation in the study.

Laboratory Visit 1: Orientation / Familiarization to the Study and 1-RM Assessment (Resistance Exercise Group Only)
The purpose of Laboratory Visit 1 is to provide you with an overview of the study, give you an opportunity to ask questions about your participation, sign this informed consent, gather some demographic information about you, and to assess your muscular

strength. The specific procedures that will be performed are listed below:

7. Sign informed consent documents
8. Fill out a medical history questionnaire
9. Undergo a brief medical and physical screening, including a resting electrocardiogram (ECG) to measure heart function
10. Undergo body composition analysis to assess percent body fat using a DEXA
11. Familiarization with equipment used during exercise testing
12. Undergo an exercise test to assess muscular strength

The purposes of items 2, 3, and 4, are to determine if it is safe for you to participate in exercise and to gain some demographic information about you, including your age, height, weight, percent body fat, race, physical activity participation over the past year, and type of cancer treatments you have received and are currently receiving. The machine that will be used to measure your percent body fat in item 4 is called a DEXA. DEXA stands for Dual Energy X-ray Absorptiometry. The DEXA uses two x-ray beams to measure differences in the composition of different tissues in your body, such as bones, soft tissue, and fat. The scan is performed while you rest on your back, and takes approximately 6 minutes.

The purpose of item 6 is to perform an exercise assessment that will allow the researchers to determine your muscular strength, or the greatest amount of weight that certain muscle groups can lift one time. This exercise assessment will involve performing 4 different exercises. They are: leg press, lateral pull down, leg extension, and seated row. The exercise assessment will involve a series of one-repetition trials with increasing workloads until you decide that the given weight is the most that you can lift with proper form. This will be done for each of the 4 exercises. The duration of the exercise assessment will likely last no more than 30 minutes.

<u>Laboratory Visit 2: Acute Resistance Exercise Session (Resistance Exercise Group Only)</u>
Laboratory Visit 2 will occur between 7-14 days of Laboratory Visit 1. During this Visit, you will be performing 1 session of moderate-intensity resistance exercise on the same 4 pieces of equipment used in Laboratory Visit 1 (leg press, lateral pull down, leg extension, and seated row). This will be the exercise bout that will be used to elicit a response in your immune cells, inflammatory markers, and stress hormones. The specific procedures that will be performed are listed below:

6. Fill out a psychological stress questionnaire
7. Pre-exercise blood sample
8. Moderate-intensity resistance exercise session
9. Post-exercise blood sample (immediately post-exercise)
10. Post-exercise blood sample (2 hours post-exercise)

The purpose of item 1 is to determine if you are feeling any emotional or psychological stress on the day of testing. This is important information as the levels of stress hormones in your blood may be influenced by emotional or psychological stress.

During item 3, you will be performing an approximately 30-minute bout of moderate-intensity resistance exercise on the leg press, lateral pull down, leg extension, and seated row. A moderate intensity level of exercise will make you feel like your muscles are working, but you are able to carry on a conversation. You will alternate between each of the four resistance exercises, performing 10 repetitions of each followed by 1.5 minutes of rest before continuing on to the next exercise. You will move from the leg press to the lateral pull down to the leg extension to the seated row, with the same number of repetitions and amount of rest until you have completed each exercise three times.

The 3 blood samples to be obtained during items 2, 4, and 5 will be performed by using a small catheter inserted into a vein in your arm. This blood sampling procedure is very similar to a blood sampling procedure that is used in routine medical clinic settings. Approximately 1 tablespoon of blood will be collected for each item. Some of this blood will be used during the course of this study to measure the number of NK cells in your blood, the activity of the NK cells (i.e., how well they destroy other cells), and the levels of stress hormones and cytokines in your blood.

Laboratory Visit 3: 24-hour Follow-up Session

Laboratory Visit 3 will occur 24 hours after Laboratory Visit 2. During this Visit, you will answer the same psychological stress questionnaire as you did in the previous laboratory visit. You will also have 1 blood sample drawn, which will also be used to the number of NK cells in your blood, the activity of the NK cells (i.e., how well they destroy other cells), and the levels of stress hormones and cytokines in your blood. The procedure for obtaining the blood sample is the same as described above, except that the blood will be drawn through a small needle instead of a catheter.

What are the possible benefits from being in this study?

Research is designed to benefit society by gaining new knowledge. The benefits to you from being in this study may be the opportunity to have your various aspects of your health assessed during Laboratory Visit 1. This would include looking at how well your heart is working by using the ECG and by listening to your heart with a stethoscope. Additionally, you will have your muscular strength assessed, as well as your percent body fat. This information may be useful if you are be thinking of beginning an exercise program, and you may elect to provide this information to an exercise specialist who could construct and supervise an individualized exercise program for you.

What are the possible risks or discomforts involved with being in this study?

The risk of physiological and psychological harm is very minimal and would not cost you physical or emotional loss. However, any research study does carry with it some potential for risk or discomfort, listed below:

5. During Laboratory Visit 1, you will be performing an exercise test to determine your muscular strength. The test will start with an easy warm-up and

movement rehearsal for your muscles. As the one repetition maximum trials begin, you will find that the assessment will become more difficult. It is possible that increased stress will be placed on your musculoskeletal system. To ensure your safety, your heart rate and your lifting technique will be monitored continuously throughout the various trials to verify that there are no movement compensations. Your heart rate will continue to be monitored for several minutes after the conclusion of the one repetition maximum assessments. At the conclusion of the test, you will be allowed to cool down, stretch, and drink water at liberty.

6. During Laboratory Visit 1, you will be exposed to radiation during the DEXA scan. However, the scan itself is very safe, and the amount of x-ray radiation that you will receive during the scan is 0.8 mrems, which is extremely small. To put this in perspective, this amount of radiation is about the same amount of radiation that you would receive from natural background sources in one day.

7. During Laboratory Visits 1 and 2, it is possible that you could experience slight muscle or joint soreness after the exercise. However, this soreness would not be any worse than after doing any other type of exercise. To minimize the amount of soreness you might feel, you will be allowed to stretch before and after exercise, and you will be allowed to do a light warm-up and cool-down before and after exercise.

8. During Laboratory Visits 2 and 3, it is possible that you could experience some mild bruising or discomfort while having your blood drawn. This discomfort would not be any more significant than having your blood drawn at the doctor's office. The blood sampling procedures will be performed by research team members who have drawn blood many times before, and proper bandaging will be performed to minimize bleeding.

In addition, there may be uncommon or previously unknown risks that might occur. You should report any problems to the researchers.

What if we learn about new findings or information during the study?
You will be given any new information gained during the course of the study that might affect your willingness to continue your participation.

How will your privacy be protected?
You will not be identified in any report or publication about this study. Although every effort will be made to keep research records private, there may be times when federal or state law requires the disclosure of such records, including personal information. This is very unlikely, but if disclosure is ever required, UNC-Chapel Hill will take steps allowable by law to protect the privacy of personal information. In some cases, your information in this research study could be reviewed by representatives of the University, research sponsors, or government agencies (for example, the FDA) for purposes such as quality control or safety.

All data obtained from you during this study will be coded using a research ID number, and only the members of the research team will have access to these codes. Study databases and logs will not have any information that will allow for subjects to be identified by anyone other than a research team member.

A copy of this consent form will go in to your medical record. This will allow the doctors caring for you to know what study tests you may be receiving as a part of the study and know how to take care of you if you have other health problems or needs during the study.

What will happen if you are injured by this research?
All research involves a chance that something bad might happen to you. This may include the risk of personal injury. In spite of all safety measures, you might develop a reaction or injury from being in this study. If such problems occur, the researchers will help you get medical care, but any costs for the medical care will be billed to you and/or your insurance company. The University of North Carolina at Chapel Hill has not set aside funds to pay you for any such reactions or injuries, or for the related medical care. However, by signing this form, you do not give up any of your legal rights.

What if you want to stop before your part in the study is complete?
You can withdraw from this study at any time, without penalty. The investigators also have the right to stop your participation at any time. This could be because you have had an unexpected reaction, you become ill, you use a medication that may affect the outcome of the study variables being measured, you are not able to complete the exercise testing sessions, you refuse to have your blood drawn, you miss a Laboratory Visit, or because the entire study has been stopped. If you decide to withdraw from the study, all data collected from you will be destroyed and will not be used in any data analyses that may result from this study.

Will you receive anything for being in this study?
You will be provided with a written report of your fitness level and percent body fat, both of which will be measured during Laboratory Visit 1.

Will it cost you anything to be in this study?
It will not cost you anything to participate in this study.

What if you are a UNC student?
You may choose not to be in the study or to stop being in the study before it is over at any time. This will not affect your class standing or grades at UNC-Chapel Hill. You will not be offered or receive any special consideration if you take part in this research.

What if you are a UNC employee?
Taking part in this research is not a part of your University duties, and refusing will not affect your job. You will not be offered or receive any special job-related consideration if you take part in this research.

What if you have questions about this study?
You have the right to ask, and have answered, any questions you may have about this research. If you have questions, complaints, concerns, or if a research-related injury occurs, you should contact the researchers listed on the first page of this form.

What if you have questions about your rights as a research subject?
All research on human volunteers is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research subject, or if you would like to obtain information or offer input, you may contact the Institutional Review Board at 919-966-3113 or by email to IRB_subjects@unc.edu.

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Title of Study: The Impact of Acute Aerobic and Resistance Exercise on Natural Killer Cell, Catecholamine, and Cortisol Responses in Breast Cancer Survivors

Principal Investigator: Claudio L. Battaglini, PhD

Subject's Agreement:

I have read the information provided above. I have asked all the questions I have at this time. I voluntarily agree to participate in this research study.

____ ____________
Signature of Research Subject Date

____
Printed Name of Research Subject

____ ____________
Signature of Research Team Member Obtaining Consent Date

____
Printed Name of Research Team Member Obtaining Consent

Appendix C (not copyrighted)

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 _____ _____

<u>Family History</u>
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 _____ _____

<u>Mental History</u>
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 D

University of North Carolina-Chapel Hill
HIPAA Authorization for Use and Disclosure of Health Information for Research Purposes

IRB Study #11-1405

Title of Study: The Impact of Resistance Exercise on Cellular Immune System Responses, Stress Hormones, and Cytokines in Breast Cancer Survivors

Principal Investigator: Claudio L. Battaglini, PhD
Mailing Address for UNC-Chapel Hill Department: Department of Exercise & Sport Science, CB #8700

This is a permission called a "HIPAA authorization." It is required by the "Health Insurance Portability and Accountability Act of 1996" (known as "HIPAA") in order for us to get information from your medical records or health insurance records to use in this research study.

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1. If you sign this HIPAA authorization form, you are giving your permission for the following people or groups to give the researchers certain information about you (described below):

Any health care providers or health care professionals or health plans that have provided health services, treatment, or payment for you such as physicians, clinics, hospitals, home health agencies, diagnostics centers, laboratories, treatment or surgical centers, including but not limited to the UNC Health Care System, health insurance plans, and government health agencies.

2. If you sign this form, this is the health information about you that the people or groups listed in #1 may give to the researchers to use in this research study any information in your medical records that relates to your participation in this research. These records might include information about your age, race, height, weight, menopausal status, physical activity participation over the past year, breast cancer stage at diagnosis, types of cancer therapies and drugs you have received, medications you may be taking, and presence of any other diseases that could preclude your ability to exercise, including cardiovascular disease, pulmonary disease, orthopedic disease, diabetes, thyroid disease, and chronic infectious disease including hepatitis or AIDS.

3. The HIPAA protections that apply to your medical records will not apply to your information when it is in the research study records. Your information in the research study records may also be shared with, used by or seen by collaborating researchers, the sponsor of the research study, the sponsor's representatives, and certain employees

of the university or government agencies (like the FDA) if needed to oversee the research study.

HIPAA rules do not usually apply to those people or groups. If any of these people or groups reviews your research record, they may also need to review portions of your original medical record relevant to the situation. The informed consent document describes the procedures in this research study that will be used to protect your personal information. You can also ask the researchers any questions about what they will do with your personal information and how they will protect your personal information in this research study.

4. If this research study creates medical information about you that will go into your medical record, you may not be able to see the research study information in your medical record until the entire research study is over.

5. If you want to participate in this research study, you must sign this HIPAA authorization form to allow the people or groups listed in #1on this form to give access to the information about you that is listed in #2. If you do not want to sign this HIPAA authorization form, you cannot participate in this research study. However, not signing the authorization form will not change your right to treatment, payment, enrollment or eligibility for medical services outside of this research study.

6. This HIPAA authorization will not stop unless you stop it in writing.

7. You have the right to stop this HIPAA authorization at any time. You must do that in writing. You may give your written stop of this HIPAA authorization directly to Principal Investigator or researcher or you may mail it to the department mailing address listed at the top of this form, or you may give it to one of the researchers in this study and tell the researcher to send it to any person or group the researcher has given a copy of this HIPAA authorization. Stopping this HIPAA authorization will not stop information sharing that has already happened.

8. You will be given a copy of this signed HIPAA authorization.

_____ _____
Signature of Research Subject Date

_____
Print Name of Research Subject

For Personal Representative of the Research Participant (<u>if applicable</u>)

Print Name of Personal Representative: _____

Please explain your authority to act on behalf of this Research Subject:

_____

I am giving this permission by signing this HIPAA Authorization on behalf of the Research Participant.

_____ _____

Signature of Personal Representative Date

Appendix E

University of North Carolina at Chapel Hill
Consent for Storing Biological Specimens <u>With</u> Identifying Information

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IRB Study # 11-1405
Consent Form Version Date: November 2, 2012

Title of Study: The Impact of Resistance Exercise on Cellular Immune System Responses, Stress Hormones, and Cytokines in Breast Cancer Survivors

Principal Investigator: Claudio L. Battaglini, PhD
Principal Co-Investigator: Anthony C. Hackney, PhD, DSc
UNC-Chapel Hill Department: Exercise & Sport Science
UNC-Chapel Hill Phone number: 843-6045
Email Address: claudio@email.unc.edu
Co-Investigators: Lisa A. Carey; Hyman B. Muss, MD; Eric D. Ryan, PhD; Elizabeth Evans, MA; Rachel Graff BA; Jacob Allen, BA; Dustin Buttars, BS; Robert Mills, BA; Sarah Fultz, BS; and Mary Woessner, B.A.

Funding Source and/or Sponsor: Lineberger Comprehensive Cancer Center

Study Contact telephone number: (516) 633-5235 or (919) 621-1322
Study Contact email: rgraff1@email.unc.edu or jmallen@email.unc.edu

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<u>What are some general things you should know about research?</u>
Research is designed to gain scientific information that may help other people in the future. You may not receive any direct benefit from participating. There also may be risks.

You may refuse to take part in research. If you are a patient with an illness, you do not have to be in research in order to receive treatment.

Details are discussed below. It is important that you understand this information so that you can make an informed choice. You will be given a copy of this consent form. You should ask the researchers named above, or staff members who may assist them, any questions you have about this study at any time.

<u>What is the purpose of this specimen repository or "biobank?"</u>
Research with blood, tissue or body fluids (specimens) can help researchers understand how the human body works. Research can also answer other questions by using specimens. Researchers may develop new tests to find diseases, or new ways to treat diseases. In the future, research may help to develop new products, such as drugs. Specimens are commonly used for genetic research. Sometimes researchers collect and

store many specimens together and use them for different kinds of research, or share them with other scientists; this is called a specimen repository or "biobank."

The purpose of this particular repository or biobank is to allow researchers to conduct future analysis of blood samples that will be obtained during the course of this study. For this particular study, blood samples will be collected in order to measure immune and inflammatory markers in the blood in response to one session of moderate-intensity resistance exercise. Additionally, levels of the stress hormone cortisol will be measured. Future analyses that may be performed on these blood samples may include measuring the levels of other hormones as well as the levels of other components of the immune system that may be affected by resistance exercise.

How will the specimens be collected?
Blood samples are collected as part of the main study. We would like to store any samples remaining for future use.

What will happen to the specimens?
Once your blood samples are collected, they will be frozen and stored in a freezer located in the Applied Physiology Laboratory, Room 25 Fetzer Hall, in the Department of Exercise and Sport Science at UNC-Chapel Hill. The freezer is kept within a self-contained section of the laboratory that is accessible only by a keypad combination on a closed door. The only individuals with access to the freezer are faculty of the Applied Physiology Laboratory and their graduate students. Blood samples will be kept in the freezer indefinitely until it is apparent that an insufficient amount exists needed to perform analysis. The only other investigators besides those on the research team who would have access to your blood samples and accompanying data would be future graduate students of the Principal Investigator.

What are the possible benefits to you?
Benefits to you are unlikely. Studies that use specimens from this repository may provide additional information that will be helpful in understanding the effect of acute resistance exercise on hormone responses and immune system responses in breast cancer survivors and healthy women who have never been diagnosed with or received treatment for cancer.

What are the possible risks or discomforts involved with the use of your specimens?
The risk of physiological and psychological harm related to specimen collection is very minimal and would not cost you physical or emotional loss. However, any research study does carry with it some potential for risk or discomfort. Additionally, there is a risk of breach of confidentiality; however, your stored blood samples will be coded using a research ID number that will not be linked with any information that would personally identify you.

Will there be any cost to you for storage of the specimens?

There will be no cost to you for the storage and use of the specimens for research purposes.

Will you receive anything for the use of your specimens?
You will not receive anything for taking part in this research.

Who owns the specimens?
Any blood, body fluids, or tissue specimens obtained for this purpose become the exclusive property of the University of North Carolina at Chapel Hill. This organization may retain, preserve or dispose of these specimens and may use these specimens for research that may result in commercial applications. There are no plans to compensate you for any future commercial use of these specimens.

How will your privacy be protected?
All data obtained from you during this study will be coded using a research ID number, and only the members of the research team will have access to these codes. Study databases and logs will not have any information that will allow for subjects to be identified by anyone other than a research team member.

Information from your medical records may be stored along with your specimens(s). You will be asked to sign a separate form ("HIPAA authorization") to allow researchers to review your medical records.

You will not be identified in any report or publication about research using your specimens. Although every effort will be made to keep research records private, there may be times when federal or state law requires the disclosure of such records, including personal information. This is very unlikely, but if disclosure is ever required, UNC-Chapel Hill will take steps allowable by law to protect the privacy of personal information. In some cases, your information in this research could be reviewed by representatives of the University, research sponsors, or government agencies for purposes such as quality control or safety.

Will researchers seek approval from you to do future studies involving the specimens?
By signing this consent form, you are giving your permission for researchers to use your specimens as described above. Current and future research is overseen by a committee called the Institutional Review Board (IRB). The role of the IRB is to protect the rights and welfare of research participants. In some cases, the IRB may require that you be re-contacted and asked for your consent to use your specimens in a specific research study. You have the right, at that future time, not to participate in any research study for which your consent is sought. Refusal to participate will not affect your medical care or result in loss of benefits to which you are entitled.

Will you receive results from research involving your specimens?

Most research with your specimens is not expected to yield new information that would be meaningful to share with you personally. There are no plans to re-contact you or other subjects with information about research results.

Can you withdraw the specimens from the research repository?
If you decide that you no longer wish for the specimens to be stored, you should contact the researchers on the front page of this form. It is best to make your request in writing.

Any analysis in progress at the time of your request or already performed prior to your request being received by the researcher will continue to be used as part of the research study. Once the researchers have been notified, your remaining specimens would be destroyed. If you do not make such a request, the specimens may be stored forever. The researchers may choose to destroy the specimens at any time.

What will happen if you are injured by this research?
NA

What if you have questions about this research?
You have the right to ask, and have answered, any questions you may have about this research. If you have questions, you should contact the researchers listed on the first page of this form.

What if you have questions about your rights as a research subject?
All research on human volunteers is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research subject you may contact, anonymously if you wish, the Institutional Review Board at 919-966-3113 or by e-mail to IRB_ subjects@unc.edu.

IRB Study # 11-1405: The Impact of Resistance Exercise on Cellular Immune System Responses, Stress Hormones, and Cytokines in Breast Cancer Survivors

Subject's Agreement:

I have read the information provided above. I have asked all the questions I have at this time. I voluntarily agree to participate. I agree to my specimen(s) being stored with the identifying code(s).

_____ _____

Signature of Research Subject Date

_____

Printed Name of Research Subject

_____ _____

Signature of Research Team Member Obtaining Consent Date

_____

Printed Name of Research Team Member Obtaining Consent

Appendix F

Laboratory Visit Appointment Reminder

Thank you for agreeing to participate in this study entitled "The Impact of Acute Aerobic and Resistance Exercise on Natural Killer Cell, Catecholamine, and Cortisol Responses in Breast Cancer Survivors." Your time and participation is greatly appreciated!

This is an appointment reminder for Laboratory Visit (circle one): 1 2 3

Your next Laboratory Visit is scheduled for _____ at _____.
Date Time

Your Laboratory Visit will take place in the Integrative Exercise Oncology Research Laboratory (IEORL), which is room 125 Fetzer Hall (corner of South Rd and Raleigh Rd).

If you are driving into campus, here are the directions for arriving to Fetzer Hall and parking. These directions are written assuming you are coming from the direction of UNC Hospitals.

1. Drive south on Columbia Street until you come to the intersection of South Rd. and Columbia.
2. Turn right onto South Rd.
3. Drive a short distance on South Rd. and turn right onto Stadium Drive. The bell tower will be on your right.
4. Drive a very short distance on Stadium Drive and take your first left. The football stadium will be on your right, and there will be several crosswalk lines painted on the asphalt.
5. You are now in the small visitor parking lot at the junction of Fetzer Hall, the outdoor pool, and the Stallings-Evans Sports Medicine Facility. Park in any of the available visitor parking spaces.
6. One of us (Rachel Graff or Jacob Allen) will meet you in the parking lot with your pink visitor parking pass.

*****Please follow these pre-assessment guidelines prior to reporting to your Laboratory Visit*****

1. No eating for at least 2 hours prior to your appointment. We do suggest that you eat a little something before you come to the lab, even if it is early in the morning.
2. No exercise for at least 12 hours prior to your appointment
3. Drink water throughout the day to keep yourself hydrated.
4. No caffeine use for at last 12 hours prior to your appointment
5. No alcohol use for at least 48 hours prior to your appointment

What to bring to your Laboratory Visit:

1. Clothing (including shoes) that are comfortable for exercise.
2. Water bottle.
3. Filled-out copies of the informed consents and medical history form.

If you have any questions at any time, please do not hesitate to contact Rachel Graff or Jacob Allen at (516) 633-5235 or (919) 621-1322 or rgraff1@email.unc.edu or jmallen@email.unc.edu.

Appendix G (not copyrighted)

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.

Mind Garden, Inc.
info@mindgarden.com
www.mindgarden.com

References

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.

Cohen, S. and Williamson, G. Perceived Stress in a Probability Sample of the United States. Spacapan, S. and Oskamp, S. (Eds.) *The Social Psychology of Health*. Newbury Park, CA: Sage, 1988.

Appendix H

Data Collection Form: Visit 1

Subject:

ID:_____

Height:_____ (cm)

Weight:_____(kg)

Resting HR_____ (BPM) Heart rate reserve (50%)_____(HRR)

Resting BP_____ (mmHg)

Body fat (%): _____

One Repetition Max:

Leg Press:_____ (lbs) **Machine Settings:**_____

Lateral Pull Down:_____ (lbs) **Machine Settings**:_____

Leg Extension :_____ (lbs) **Machine Settings**:_____

Seated Row:_____ (lbs) **Machine Settings**:_____

Post-Exercise Measures:

Final HR_____ (BPM)

Final BP_____ (mmHg)

Appendix I

Data Collection Form: Visit 2

Subject:

ID:_____

Resting HR_____ (BPM) Heart rate reserve (50%)_____(HRR)

Resting BP_____ (mmHg)

Time of Exercise Onset:_____

Resistance Training Protocol:

Leg Press_____(lbs) Lat Pull Down_____(lbs) Leg Extension_____ (lbs) Seated Row_____(lbs)

1st Set:	HR:_____	HR:_____	HR:_____	HR:_____
	RPE:_____	RPE:_____	RPE:_____	RPE:_____
	Rest Time:_____	Rest Time:_____	Rest Time:_____	Rest Time:_____
2nd Set:	HR:_____	HR:_____	HR:_____	HR:_____
	RPE:_____	RPE:_____	RPE:_____	RPE:_____
	Rest Time:_____	Rest Time:_____	Rest Time:_____	Rest Time:_____
3rd Set:	HR:_____	HR:_____	HR:_____	HR:_____
	RPE:_____	RPE:_____	RPE:_____	RPE:_____
	Rest Time:_____	Rest Time:_____	Rest Time:_____	Rest Time:_____

Post-Exercise Measures:

Final HR_____ (BPM)

Final BP_____ (mmHg)

Time of Exercise Completion: _____

APPENDIX J (not copyrighted)

IL-6 Assay Procedures:

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.

2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.

3. Prepare 500 µL of the 500 pg/mL top standard by diluting 12.5 µL of the standard stock solution in 487.5 µL of Assay Buffer A. Perform six two-fold serial dilutions of the 500 pg/mL top standard in separate tubes using Assay Buffer A as the diluent. Thus, the human IL-6 standard concentrations in the tubes are 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, and 7.8 pg/mL, respectively. Assay Buffer A serves as the zero standard (0 pg/mL).

4. Wash the plate 4 times with at least 300 µL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes should be performed similarly.

5. Add 50 µL of Assay Buffer A to each well that will contain either standard dilutions or samples.

6. Add 50 µL of standard dilutions or samples to the appropriate wells

7. Seal the plate with a Plate Sealer included in the kit and incubate the plate at room temperature for 2 hours while shaking at 200 rpm.

8. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

9. Add 100 µL of Human IL-6 Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour while shaking.

10. Discard the contents of the plate into a sink, then wash the plate 4 times Tel: 858-768-5800 LEGEND MAX™ Human IL-6 ELISA Kit 6

with 1X Wash Buffer as in step 4.

11. Add 100 µL of Avidin-HRP A solution to each well, seal the plate and incubate at room temperature for 30 minutes while shaking.

12. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 4. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

13. Add 100 µL of Substrate Solution F to each well and incubate for 15 minutes in the dark. Wells containing human IL-6 should turn blue in color with an intensity proportional to its concentration. It is not necessary to seal the plate during this step.

14. Stop the reaction by adding 100 µL of Stop Solution to each well. The solution color should change from blue to yellow.

15. Read absorbance at 450 nm within 30 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

Calculations:

1. The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm.
2. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with cytokine concentration on the X-axis and absorbance on the Y-axis.
3. Draw a best fit line through the standard points.
4. To determine the unknown cytokine concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve.
5. At the point of intersection, draw a vertical line to the X-axis and read the cytokine concentration.

IL-10 Assay Procedures:

1. Bring all reagents to room temperature prior to use. It is strongly recom- mended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.

2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.

3. Prepare 500 µL of the 250 pg/mL top standard by diluting 6.25 µL of the standard stock solution in 493.75 µL of Assay Buffer A. Perform six two-fold serial dilutions of the 250 pg/mL top standard in separate tubes using Assay Buffer A as the diluent. Thus, the human IL-10 standard concentrations in the tubes are 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL and 3.9 pg/mL, respectively. Assay Buffer A serves as the zero standard (0 pg/mL). □6.25 µL 250 µL 250 µL 250 µL 250 µL 250 µL 250 µL □493.75 µL.

4. Wash the plate 4 times with at least 300 µL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on absor- bent paper. All subsequent washes should be performed similarly.

5. Add 50 µL of Assay Buffer A to each well that will contain either standard dilutions or samples.

6. Add 50 µL of standard dilutions or samples to the appropriate wells

7. Seal the plate with a Plate Sealer included in the kit and incubate the plate at room temperature for 2 hours while shaking at 200 rpm.

8. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

9. Add 100 µL of Human IL-10 Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour while shaking.

10. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

11. Add 100 µL of Avidin-HRP A solution to each well, seal the plate and incubate at room temperature for 30 minutes while shaking.

12. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 4. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

13. Add 100 µL of Substrate Solution F to each well and incubate for 20 minutes in the

dark. Wells containing human IL-10 should turn blue in color with an intensity proportional to its concentration. It is not necessary to seal the plate during this step.

14. Stop the reaction by adding 100 μL of Stop Solution to each well. The solution color should change from blue to yellow.

15. Read absorbance at 450 nm within 30 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

Calculations:

1. The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm.

2. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with cytokine concentration on the X-axis and absorbance on the Y-axis.

3. Draw a best fit line through the standard points.

4. To determine the unknown cytokine concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve.

5. At the point of intersection, draw a vertical line to the X-axis and read the cytokine concentration.

TNF-A Assay Procedures:

1. Bring all reagents to room temperature prior to use. It is strongly recom- mended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.

2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.

3. Prepare 500 µL of the 1,000 pg/mL top standard by diluting 25 µL of the standard stock solution in 475 µL of Assay Buffer A. Perform six two-fold serial dilutions of the 1,000 pg/mL top standard in separate tubes using Assay Buffer A as the diluent. Thus, the human TNF-α standard concentra- tions in the tubes are 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL and 15.6 pg/mL, respectively. Assay Buffer A serves as the zero standard (0 pg/mL). □25 µL 250 µL 250 µL 250 µL 250 µL 250 µL 250 µL**.**

4. Wash the plate 4 times with at least 300 µL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on absor- bent paper. All subsequent washes should be performed similarly.

5. Add 50 µL of Assay Buffer A to each well that will contain either standard dilutions or samples.

6. Add 50 µL of standard dilutions or samples to the appropriate wells

7. Seal the plate with a Plate Sealer included in the kit and incubate the plate at room temperature for 2 hours while shaking at 200 rpm.

8. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

9. Add 100 µL of Human TNF-α Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour while shaking.

10. Discard the contents of the plate into a sink, then wash the plate 4 times □with 1X Wash Buffer as in step 4.

11. Add 100 µL of Avidin-HRP B solution to each well, seal the plate and incu- bate at room temperature for 30 minutes while shaking.

12. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 4. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

13. Add 100 μL of Substrate Solution D to each well and incubate for 15 minutes in the dark. Wells containing human TNF-α should turn blue in color with an intensity proportional to its concentration. It is not necessary to seal the plate during this step.

14. Stop the reaction by adding 100 μL of Stop Solution to each well. The solution color should change from blue to yellow.

15. Read absorbance at 450 nm within 30 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

Calculations:

1. The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm.

2. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with cytokine concentration on the X-axis and absorbance on the Y-axis.

3. Draw a best fit line through the standard points.

4. To determine the unknown cytokine concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve.

5. At the point of intersection, draw a vertical line to the X-axis and read the cytokine concentration.

Cortisol Assay Procedures:

1. Place the desired number of coated strips into the holder.
2. Pipette 25 µl of Cortisol standards, control and patient's sera.
3. Add 200 µl of Cortisol Enzyme Conjugate to all wells.
4. Thoroughly mix for 10 seconds.
5. Incubate for 60 minutes at room temperature (18-26°C).
6. Remove liquid from all wells. Wash wells three times with 300 µl of 1X wash buffer.
7. Blot on absorbent paper towels.
8. Add 100 µl of TMB substrate to all wells.
9. Incubate for 15 minutes at room temperature (18-26°C).
10. Add 50 µl of stop solution to all wells. Shake the plate gently to mix the solution.
11. Read absorbance on ELISA Reader at 450 nm within 20 minutes after adding the stop solution.

Calculations:

1. Check Cortisol standard value on each standard vial. This value might vary from lot to lot. Make sure you check the value on every kit. See example of the standard attached.
2. To construct the standard curve, plot the absorbance for Cortisol standards (vertical axis) versus Cortisol
3. Standard concentrations (horizontal axis) on a linear graph paper. Draw the best curve through the points.
4. Read the absorbance for controls and each unknown sample from the curve.
5. Record the value for each control or unknown sample

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