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The Anti-Inflammatory Effects of Vitamin D and Exercise

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THE ANTI-INFLAMMATORY EFFECTS OF VITAMIN D AND EXERCISE

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ABSTRACT

Elevated inflammation is associated with several chronic diseases, including obesity. Exercise is an established effective treatment of this condition by decreasing adiposity and independently regulating inflammatory pathways. The potential for vitamin D to confer anti-inflammatory benefits has been explored in cell culture studies, but few have explored its action at the whole body level. PURPOSE: To investigate the relationship between inflammatory markers in trained and untrained individuals with vitamin D levels either above or below a suggested optimal concentration. METHODS: College-aged females (N = 63), both trained and untrained, reported to the lab four times: to assess body size and composition, for blood collection, for a maximal aerobic test, and a test of anaerobic power. Blood was analyzed for serum 25OHD and CRP concentrations, stimulated with LPS to assess IL-6 production. Samples were prepared for FACS analysis for CD14, CD16, and TLR4 expression. RESULTS: Trained individuals presented with higher 25OHD levels, even prior to stratification into high and low groups ($p = 0.015$). VO_2peak was significantly higher ($p < 0.0001$) and fatigue during the test for anaerobic power was significantly lower ($p = 0.021$) in trained individuals. Untrained individuals had a higher average body weight ($p = 0.039$) and estimated percent body fat ($p = 0.011$) compared to trained individuals, although the average estimated percent body fat of both groups was higher than the recommended level for this age group. Additionally, measures of sun exposure were negatively correlated with measures of body size and composition, although these relationships did not exist between serum 25OHD. CONCLUSION: In this study, regular physical activity was associated with higher serum 25OHD, lower BMI, waist circumference, and estimated percent body fat as well as reduced LPS-stimulated IL-6 production. Optimal vitamin D status did not appear to provide any additional health related or anti-inflammatory benefit in those with regular physical activity

habits. However, in individuals not participating in a regular exercise program, the potential for vitamin D to mediate inflammation appeared more likely. More specifically, untrained people with optimal vitamin D status had lower numbers of total monocytes, CD14+CD16- cells, and decreased TLR4 expression on CD14+CD16+ cells; however, these differences did not translate into a change in overall cell function or markers of systemic inflammation as there was no difference between optimal and suboptimal groups with respect to LPS-stimulated IL-6 production or resting CRP concentrations. An expanded exploration of the relationship between vitamin D and inflammation may include assessing other inflammatory biomarkers, immune cell types, the vitamin D receptor, and the role of adipose tissue.

CHAPTER 1 – INTRODUCTION

Chronic, systemic inflammation is associated with diseases such as obesity, diabetes, cardiovascular disease, and others (1, 2). While there are a number of pharmaceutical anti-inflammatory treatments, many are associated with a multitude of negative side effects (3). Consequently, many experts speculate that altering the diet and increasing exercise may be the most beneficial treatment options for decreasing inflammation (4). Exercise training plays a role in mediating the inflammatory response at both an acute and chronic level, and while inflammation is peaked after an acute bout of exercise, it leads to decreased basal levels after long-term exercise training (5).

Vitamin D is most commonly known for its importance in calcium homeostasis, but new research indicates the potential for this nutrient to mediate inflammation (6, 7). Research has identified the many benefits related to adequate vitamin D status, which is commonly evaluated using serum 25-hydroxyvitamin D (25OHD), including decreasing the risk for certain types of cancer, reducing the symptoms of depression, decreasing inflammation, and altering body composition (8). Despite the ability of vitamin D to potentially ameliorate inflammation, its mechanisms of action in this process are unclear.

Because high levels of inflammatory markers such as interleukin-6 (IL-6) and C-reactive protein (CRP) are associated with chronic diseases, understanding how both vitamin D and physical activity are capable of decreasing the levels of circulating inflammatory markers could provide a potential treatment for these conditions (6, 9). Accordingly, the purpose of this study is to investigate the influence of training status and vitamin D status on circulating inflammatory markers, and monocyte number and function. Briefly, trained and untrained women with either high or low levels of serum 25OHD will be recruited in this study. Whole blood samples will be stimulated with the bacterial endotoxin lipopolysaccharide (LPS), which has been shown to elicit

an inflammatory response in monocytes through the toll-like receptor 4 (TLR4) (10). Baseline and circulating concentrations of IL-6 following stimulation, and resting CRP concentrations will be assessed using enzyme-linked immunosorbent assay (ELISA). Monocyte phenotype, classified as the classical CD14+CD16- or the non-classical CD14+CD16+, will be assessed in blood samples via flow cytometry. Data from trained and untrained subjects will be compared, as will the results from those with suboptimal 25OHD compared to those who have optimal 25OHD serum content in both trained and untrained groups.

1.1 Specific Aims

Aim 1: Are vitamin D and training status related to measures of aerobic fitness, anaerobic power, and the presence of the inflammatory biomarker, CRP?

Aim 2: Are vitamin D and training status related to the phenotype and function of monocytes?

CHAPTER 2 – LITERATURE REVIEW

Chronic, systemic, low-grade inflammation is tightly linked to the development and progression of obesity, type 2 diabetes, and cardiovascular disease (1). Inflammation can be assessed using a number of different biomarkers, including IL-6 and CRP, and a number of interventions have been evaluated in an effort to reduce the concentrations of these agents (11, 12). Significant, transient increases in these inflammatory biomarkers have been observed during and immediately following acute bouts of exercise; however chronic bouts of exercise have been shown to reduce systemic inflammation (6, 9). Vitamin D has also emerged as a significant mediator in the inflammatory process (7, 12, 13). This literature review will provide a summary of the inflammatory process associated with chronic disease, the inflammatory response to acute and chronic exercise with a focus on the role of monocytes and TLR4, and how exercise and vitamin D can modulate the inflammatory response.

2.1 Inflammation and Disease

Inflammation is an immune response elicited by the body during stress, characterized by the production of cytokines from immune and non-immune cells that mediate the inflammatory reaction (14). Inflammation can arise from a number of different stimuli, including traumatic events, sickness, or injury; alternatively, acute increases in inflammation also occur after an intense bout of exercise (14). Inflammation is also exacerbated over long periods of time in cases of obesity (1). This consistent stimulus for cell damage may lead to the progression of a number of obesity-related diseases, including insulin resistance, atherosclerosis and cardiovascular disease (CVD) (14, 15).

Adipose tissue, skeletal muscle, hepatocytes, and immune cells such as monocytes and macrophages, are stimulated via integrated pathways that results in co-activation of inflammatory pathways and increased levels of cytokines in circulation (15). Of particular

interest is the cytokine IL-6, in which increased concentrations are present in a number of chronic conditions that are associated with obesity (1). Another biomarker, CRP, is often used by clinicians to assess systemic inflammation and is most notably used as a risk factor for CVD (14).

2.1.1 Exercise and Inflammation

Exercise training presents a paradoxical situation with respect to the inflammation. That is, an acute bout of exercise can produce levels of inflammatory cytokines many times greater than resting levels (16). In some cases, exercise-induced levels of inflammatory cytokines meet or exceed the levels observed during stressors such as surgery, trauma, or sepsis (17). The marked increase in inflammation may lead many individuals to wonder about the benefits exercise would pose, when the stimulus is as stressful as other physiological catastrophic events. In most cases, the height of the response after an acute bout of exercise is proportional to exercise intensity (17). While endurance exercise is often recommended as treatment for inflammation due to its effect on weight loss, resistance training has often proven to be as effective in reducing inflammation as well (18, 19). Of additional interest are the different effects that combined resistance training and endurance training can produce in the inflammatory profile, although both modalities tend to produce successful overall results in the long term (20). However, the significant reductions in the concentration of inflammatory markers that accompany exercise training have been observed in a number of studies suggest that chronic exercise is a key mediator for this health risk (19, 21, 22). The important message from existing studies is that while the exercise stimulus causes a peak in inflammatory markers in the short term, chronic exercise of any modality may lower resting levels of inflammation and an increased ability to respond to a hyper-inflammatory state after exposure to stressful stimuli (23).

Cross-sectional studies provide beneficial insight into the inverse relationship between inflammation and exercise. To date, most studies indicate that consistent exercise leads to a reduction in resting levels of most inflammatory markers, including tumor necrosis factor-alpha (TNF-α), IL-6, and CRP (20, 24). Perhaps the biggest limitation of these studies is that they rely upon self-reported exercise habits, making associations between intensity or modality of exercise and levels of inflammatory markers difficult because of the wide interpretation and variety of physical activity habits between subjects and studies (23, 25).

While cross-sectional studies provide some clarity with respect to the correlation between inflammation and exercise, results from intervention studies are not as conclusive. There are a number of large cohort studies that indicate a strong inverse relationship between regular exercise and inflammation (25-27). This relationship is observed regardless of the population, intensity of exercise, and whether inflammation status is assessed through a single biomarker or several (23). However, observed improvement in longitudinal studies is most likely due to the enrollment of overweight or obese subjects or individuals with chronic diseases that tend to be characterized by high levels of basal inflammation, such as type 2 diabetes or CVD (23). In many cases, it is important to separate the reduction of systemic inflammation related to exercise training from the changes following a loss of body fat. Adipocytes are a major production center of many inflammatory cytokines; because exercise results in lipolysis, the capability to produce these inflammatory markers in this is decreased due to the reduction in the amount of adipose tissue (2). In a study implementing six months of exercise training in adults with type 2 diabetes, it was speculated that the changes in inflammation were modulated by the decreases in fat mass that also occurred with training (28). This study, along with several others, show improvement in markers of inflammation with changes to lifestyle habits; it is unknown as to whether this

change due to alterations in body composition independent of the stimulus of exercise training unless further analysis is carried out.

Exercise alone may not be the only method of reducing inflammation. In a study investigating the combined versus the independent effects of a hypocaloric diet or exercise intervention in obese women, the combined use of a diet and exercise intervention was capable of significantly reducing serum CRP and IL-6 concentrations (26). Interestingly, changes in diet alone were not capable of causing a significant reduction in these inflammatory markers (26). The effects of exercise on CRP concentrations are not consistently observed, although this does not indicate that training is not beneficial for overall health, especially in those suffering from chronic disease (29, 30). In a study implementing aerobic, resistance, or a combined training program in adults suffering from type 2 diabetes, none of the three treatment protocols were successful at significantly reducing CRP from baseline (31). While inflammation was not reduced, fasting glucose levels and total percent body fat were beneficially altered with exercise training, which may serve to change inflammatory markers and positively influence overall health in the future (31). Because of their significant role in reflecting progression of chronic conditions, especially those associated with obesity, understanding the role that IL-6 and CRP play in physiology and how they are altered with exercise is of ultimate importance.

2.2 Inflammatory Markers: Interleukin-6

Interleukin-6 is produced by monocytes and macrophages, as well as T and B lymphocytes, in response to elevated concentrations of other inflammatory markers (16, 32). Normal IL-6 concentrations are close to 0 pg/mL in healthy individuals, but can reach levels as high as 80 pg/mL after a prolonged bout of endurance exercise, such as after a marathon (27). This inflammatory marker is also associated with several diseases, as levels of this cytokine can be elevated 10-fold over normal resting levels in individuals who are obese (2). Interleukin-6 is

also considered a myokine, as it is produced in muscle cells during contraction (32). There is some speculation that the role of IL-6 as a myokine may be protective, as it has been shown to have inhibitory feedback on the production of tumor necrosis factor-alpha (TNF-α), and also positively influence substrate availability during endurance exercise events (33). When IL-6 is produced from monocytes through the intracellular nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, it acts in an inflammatory manner in response to production of TNF-α (34). When IL-6 is produced as a myokine, it acts independently of binding circulating inflammatory ligands and exerts its actions through calcium signaling and the mitogen activated protein kinase (MAPK) pathways (35).

2.2.1 Interleukin-6 Response to Exercise

Interleukin-6 is one of the most frequently investigated cytokines in the field of exercise immunology, because of its rapid and drastic changes with an acute bout of exercise as well as its likelihood to respond to chronic training. When plasma IL-6 levels were assessed in 15 male endurance athletes both prior to a marathon event and in 30-minute increments up to four hours following the end of the race in 15 male endurance athletes, plasma IL-6 was increased 126 times higher than resting levels immediately following the race, and remained significantly higher than normal for all time points in the four hours (27).

Although early studies investigating the use of exercise to treat elevated systemic inflammation have focused on the use of aerobic activities, resistance training may provide an alternate form of activity for individuals who are not inclined to endurance types of exercise. The peak of IL-6 following a single bout of resistance training is not as high as what might be reached during endurance exercise, which might be beneficial for individuals who are at risk for negative health events when exposed to overly stressful stimuli (18). In a study with a crossover design comparing elevated cytokines in endurance and resistance trained individuals, IL-6

peaked to an increase of 44-fold after endurance activity while it only peaked to 4-fold after resistance activity (36). However, these subjects were trained prior to enrollment in the modality in which they were tested.

Significant increases in IL-6 immediately after exercise have been consistently observed in a variety of populations and after a variety of activities; this elevation can last for up to five hours, depending on other health factors of the individual (16, 37). Levels of IL-6 increase almost immediately with the onset of exercise due to the production by both monocytes and myocytes (38). The magnitude of increase in IL-6 concentration is tightly correlated with the duration of the exercise event, although modality and intensity of exercise may also play a role in the elevation as well (37). For example, increases in IL-6 concentration may occur after one hour of running ranging from four to 30-fold, while two and a half hours of running can lead to observed increases in IL-6 concentrations of 8- to 109-fold (38). These increases can progress even higher if the duration of the activity continues longer (38). This pattern is also replicated in cycling, where one hour of cycling results in increases between 2- and 5-fold, and two hours of cycling can result in a 38-fold increase in serum IL-6 concentrations (38). It is important to note that the variability in IL-6 increases are likely due to the individual characteristics related to biomechanical efficiency or training status or the nutrition and supplementation habits of the athletes prior to the exercise event (38). The variability may also stem from the different sources of IL-6, as it comes from both stimulated monocytes and contracting muscle tissue (32).

Prolonged exercise training programs have been shown to decrease both levels of IL-6 at rest, as well as blunt the peak of IL-6 during exercise, suggesting that regular exercise produces an adaptation to the inflammatory stimuli (38). There is speculation that this decrease in IL-6 concentration with chronic exercise training is due to an increase in sensitivity to IL-6, as there is evidence that there is significant upregulation in production of IL-6 receptor mRNA following

exercise training (25, 32). Those who remain active throughout life are also prone to lower resting levels of this inflammatory marker due to the sustained and repeated exercise stimulus, which may allow the body to adapt to this stressor (39, 40). In a study comparing old and young marathoners, it was shown that those who were physically active had significantly lower levels of IL-6 compared to the control subjects, with no age by group interaction (39).

2.3 Inflammatory Markers: C-Reactive Protein

The release of IL-6 and other inflammatory cytokines is known to stimulate the liver to produce substances known as acute phase proteins, which enhance the overall immune response to a stimulus (16, 41). C-reactive protein is one such acute phase protein, released during traumatic events such as tissue injury or myocardial infarction, as well as intense bouts of exercise (16). Concentrations above 2.0 mg/L are considered elevated, although values below 1.0 mg/L are desirable and considered to be low risk for cardiovascular events (30, 42).

2.3.1 C-Reactive Protein Response to Exercise

Changes in CRP in conjunction with acute and chronic exercise are often studied because of the marked differences time course and exercise modality have on the increase in this biomarker. A long, single bout of exercise is required to stimulate an increase in CRP concentration, and depending on the intensity and duration of the exercise, CRP can remain elevated from several hours after completion of the activity to several days later (16). When measured in 90 endurance runners, CRP was significantly elevated immediately following the completion of a marathon, and remained elevated for up to 48 hours following the finish of the race (43).

Many studies have indicated that exercise training is successful in suppressing the acute phase response, leading to lower levels of CRP at rest (22). When previously untrained individuals completed nine weeks of endurance exercise training, the CRP response to a stressful

exercise stimulus peaked at a lower concentration and was attenuated more quickly than when the subjects were exposed to the stressful stimulus prior to training (44). This study did not measure changes in inflammation over time, but managed to illustrate the importance of remaining active with respect to counteracting the changes in inflammation that occur with aging (39). Another study implemented a 6-month aerobic exercise training protocol in obese adults with type 2 diabetes. After completion of the training period, CRP was decreased when compared to levels obtained prior to training (28). Similarly, in a study comparing levels of inflammatory cytokines between young and old runners to age-matched inactive controls, it was shown that CRP levels were lower in the older active individuals than the older inactive participants (39).

While endurance activities are most commonly prescribed to lower inflammation, especially in overweight individuals, resistance training has also been investigated as a potential exercise modality for decreasing CRP as well. In one study, obese post-menopausal women experienced a decrease in CRP following 12 weeks of a prescribed resistance training program (45). Similarly, when overweight individuals were prescribed a resistance training program lasting one year, there was a significant decrease in basal CRP concentrations (46). Because of the efficacy of both aerobic and resistance training in lowering inflammation, using an exercise training program that incorporates both modalities also seems to be successful. When old and young inactive participants were prescribed an exercise program of both endurance and resistance components, it was shown that serum CRP was decreased from baseline concentrations in both age groups (22). However, another study using subjects classified as either at high risk or at low risk for metabolic syndrome showed that resistance training did not alter resting levels of CRP in either risk group (47). Subjects in this study underwent a 10-week resistance training program of seven different exercises, three days per week (47). It is likely

that the inclusion of both genders without equal stratification across the treatment and risk groups may have altered the results of the study. These studies serve to highlight the fact that resistance training is a promising tool for lowering CRP, although the degree to which this biomarker is reduced by this modality alone is not understood and more research is needed in this area.

2.4 The Role of Monocytes in the Inflammatory Process

Monocytes are cells that serve as precursors for macrophages, which in addition to producing inflammatory markers, phagocytize foreign bodies found in circulation (48). After being produced in the bone marrow, monocytes enter circulation for several days, and then migrate to tissues throughout the body to carry out their protective role (48). There are several different populations of monocytes that have varying degrees of inflammatory function, and each population is characterized by different cell-surface markers (48). Monocytes are typically identified by the presence of the marker CD14 on the cell surface (5). Another cell surface marker, CD16, is present on a subset of the CD14 monocytes (5). This results in the identification of two types of monocytes: classical monocytes, CD14+CD16-, containing the CD14 but not CD16 marker with low inflammatory activity, and the non-classical CD14+CD16+ monocytes, which have high inflammatory activity (5, 48). Some have suggested that the difference in inflammatory activity stems from the difference in toll-like receptor (TLR) expression, which is present in large quantities on the non-classical CD14+CD16+ monocytes (5, 49).

2.5 The Toll-Like Receptor Pathway

Toll-like receptors are a key component of the immune function, as they are important in recognizing pathogens within the body and recruit other immune cells to the site of infection in order to protect the body (50). There are a number of TLRs, ranging from TLR1 to TLR13,

within various vertebrates that respond to different stimuli (50). Toll-like receptor 4 is one of the most recognized receptors in humans and is located on the surface of monocytes and other leukocytes, as well as non-immune cells such as endothelial cells, thyroid cells, endometrial cells, pancreatic beta cells, and adipocytes (51). The TLR4 is stimulated by LPS and produces several inflammatory markers, including IL-6 and TNF-α (50, 52). For example, when human embryonic kidney cells were transfected with the TLR4 gene and then stimulated with LPS, the NF-κB inflammatory pathway was activated (52). In this instance, NF-κB activity was assessed by measuring luciferase activity of a downstream enzyme dependent on NF-κB activation (52). When the same cells were treated with a TLR4 antagonist, this inflammatory pathway was blocked and resulting luciferase activity was negligible (52).

2.6 Monocyte and Toll-Like Receptor 4 Response to Exercise

Exercise has been demonstrated to decrease the number of inflammatory monocytes, as well as TLR4 expression on the surface of these immune cells (49). One cross-sectional study showed that a single bout of resistance exercise was capable of decreasing TNF-α production after LPS stimulation in blood samples from overweight, postmenopausal women (45). Similarly, a study using young and old physically inactive individuals showed that 12 weeks of combined resistance and aerobic exercise training was capable of reducing IL-6 production following LPS stimulation in both groups (21). Flow cytometry analysis confirmed that the exercise training reduced cell-surface expression of TLR4 on inflammatory monocytes in both young and old individuals (21). Toll-like receptors are sensitive to a variety of circulating inflammatory cytokines, so it is possible that the repeated, transient increase in inflammatory cytokines, such as IL-6, that occur with physical activity may work to downregulate the expression of TLRs (49). However, this reduction in TLR4 expression is not consistently observed. In the study that demonstrated a reduction in the percentage of CD14+CD16+ among

total monocyte population, there was not a significant reduction in the TLR4 expression on these monocytes (49). This finding may indicate that the reduction in inflammation stems from the decrease in cell number, not the receptor expression (5).

2.7 Vitamin D

Exercise is certainly a useful tool in improving overall health and decreasing inflammation, but dietary nutrients, specifically vitamin D, have emerged as potential tool to reduce inflammation as well (53). Vitamin D is well known as an important nutrient for bone health and regulating calcium levels throughout the body; however, recent studies have suggested that this compound can improve aspects of overall health and exercise performance as well (54). The benefits of increasing vitamin D intake are not limited to those interested in enhancing athletic performance, as it is believed that vitamin D may also elicit beneficial changes in body composition by decreasing adiposity (55). There are a number of health benefits associated with decreasing body fat, including the important alterations that occur in the concentrations of inflammatory cytokines. Interestingly, vitamin D is believed to directly affect levels of chronic inflammation by altering pathways within many different types of cells as well (6, 7).

2.7.1 Vitamin D Isoforms

Because there are multiple isoforms of vitamin D that occur throughout the metabolic pathway, a complete understanding of the mechanism of action of this compound in various physiological processes has not been completely elucidated (56). These structures arise from the variety of sources of the vitamin, including the complex metabolic reactions required to produce active metabolites, and the subsequent reactions needed to break down the active metabolite once it is no longer needed (57).

Vitamin D is not only a vitamin but is also considered a steroid hormone, composed of three carbon rings and a side chain (56). Modifications to the rings or the side chain through addition of hydroxyl groups, methyl groups, or double bonds, alter the potency of the compound (56). Two forms, ergocalciferol, or D_2, and cholecalciferol, or D_3, are found in the diet (56). Vitamin D_2 differs from D_3 by a double bond between carbons 22 and 23 in the side chain, and a methyl group on carbon 24; due to these differences, it has up to one-third of the biological potency that D_3 (56).

2.7.2 Vitamin D Metabolism

The metabolism of vitamin D is a complex process with many steps, all of which are intertwined and depend on concentrations of other vitamin D metabolites and nutrients in the body. This process is additionally confounded by the fact that vitamin D can also come from dietary sources. There are both individual and environmental factors that influence the metabolism and breakdown of vitamin D, making the understanding of the metabolism of vitamin D somewhat complicated.

The synthesis of vitamin D within the body involves a number of steps to activate the compound and convert it to a form that can elicit physiological actions. The pathway of vitamin D metabolism involves the production of 25OHD in the liver and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) in the kidney (57). The oxidation of $1,25(OH)_2D$ in target cells acts to mark the compound for catabolism and a secondary catabolic pathway converts both 25OHD and $1,25(OH)_2D$ to lactone products (57). Each metabolite of vitamin D has varying levels of biological activity, which is directly tied to the chemical structure of the compound and variable conditions within the body (57).

Vitamin D is considered a non-essential nutrient, because it can be made naturally in the body (56). The process begins with the activation of 7-dehydrocholesterol (7DHC), which is

synthesized from cholesterol in the diet and absorbed through the wall of the intestine and stored in large quantities in the epidermis and dermis layers of the skin (58, 59). When ultraviolet (UV) light from the sun reaches 7DHC, a conformational change occurs to form the compound known as previtamin D (58). This process occurs most efficiently at wavelengths of 290 to 315 nm, contained within the ultraviolet wavelengths that range from 10 to 400 nm (58, 60).

Previtamin D is taken up by the liver for hydroxylation, the first step in creating a biologically active isoform, 25OHD (56). Once 25OHD is produced in the liver, the hydrophobic structure is bound to a protein to allow for more stability in circulation (56). Because 25OHD and $1,25(OH)_2D$ are both insoluble in water, the active metabolites must be bound to a protein, known as D binding protein (DBP), for transportation and stability (57). Vitamin D from dietary sources, found mostly in the form of vitamin D_2 or D_3 and small amounts as 25OHD, is absorbed in the small intestine and integrated with other fat-soluble particles as part of the chylomicron (57). This allows it to be taken up by muscle and adipose tissue directly due to the action of lipoprotein lipase in these tissues (57). The vitamin D that is not taken up from the chylomicrons is absorbed by the liver in the chylomicron remnant, and subsequently bound to DBP. Dietary vitamin D has a low affinity for DBP, so while some is transferred to the protein in circulation, this process happens much more slowly than as if it were absorbed from the chylomicrons and transported to the liver (57). Almost all of the vitamin D that is synthesized from 7DHC in the skin is bound to DBP once it enters circulation (57). 25-hydroxyvitamin D is the form of vitamin D found in circulation that is used to establish vitamin D status (56). When 25OHD reaches the kidney, it is hydroxylated again to produce the biologically active metabolite, $1,25(OH)_2D$ (56, 58).

After the body no longer needs $1,25(OH)_2D$ for physiological processes, the compound must be broken down into a number of byproducts before it can be removed from the body (57).

The enzyme responsible for inactivating $1,25(OH)_2D$ is also capable of inactivating 25OHD; even though 25OHD is not responsible for eliciting actions throughout the body, it must also be inactivated so that it is no longer converted to $1,25(OH)_2D$ and levels can continue to decrease. 1,25-dihyrdoxyvitamin D is hydroxylated to produce $1,24,25(OH)_3D$; the new hydroxyl group is ketonized; another hydroxyl group is added to carbon 23, and then the molecule is cleaved to produce $1,23(OH)_2D$ and calcitroic acid, which are both water soluble and can be excreted in the bile (56). Even though this pathway is the primary method of breakdown for the active metabolites, there is also a secondary pathway that is far less understood, which results in the production of lactone products that are marked for excretion (57).

2.7.3 Influences on Vitamin D Status

Given the complexity of vitamin D metabolism, it is not surprising that there are a number of factors that can augment the amount of vitamin D available to be used within the body. These vitamin D-altering factors are linked to individual qualities such as skin pigmentation, the presence of disease, and environmental factors such as location and season (60, 61). Because of the number and complexity of each of these variables involved in determining the bioavailability of vitamin D, it is difficult to establish recommendations related to dietary intake and sun exposure for the general public.

2.7.4 Environmental Influences on Vitamin D Concentrations

There are a number of environmental factors that drastically decrease the amount of $1,25(OH)_2D$ that is produced from 7DHC. Conversion of 7DHC occurs most rapidly at UV wavelengths between 290 and 315 nm, although these wavelengths are not likely to reach the earth's surface in significant amounts due to pollution or at latitudes further away from the equator (58). Even in the absence of pollution, oxygen and nitrogen molecules in the ozone can interfere with UV radiation, which by some measures, can decrease vitamin D production to only

1% of optimal levels (58). In addition, both time of day and season can influence the overall amount of UV light that reaches the surface of the earth. Maximal ultraviolet radiation occurs in the middle of the day during the summer (58). However, while the UV light is sufficient to produce at least some 25OHD all year long at latitudes below 40°N, equivalent to the location of Philadelphia, Pennsylvania, vitamin D production via UV radiation ceases for at least some portion of the year in a large portion of the world (58). For instance, a study by Close et al. examining the 25OHD status in athletes showed that 60 out of 91 total subjects had serum 25OHD levels below 20 ng/mL during the winter in the UK (54). On the other hand, living at lower latitudes where UV light is sufficient all year does not exclude individuals in these regions from insufficient levels of 25OHD, as individual factors must also be considered (13, 62). Because season and geographic location are related to a significant amount of 25OHD variation, it is nearly impossible to estimate the amount of vitamin D that could be endogenously produced for any given amount of time in a single population (58).

2.7.5 Individual Factors Influencing Vitamin D Status

Individual factors and behaviors also influence endogenous production of vitamin D. Production through UV radiation can be attenuated through the use of sunscreen and melanin levels in the skin (58, 60). A number of studies have investigated the efficacy of various levels of protection from sunscreen, with the results indicating that even when applied incorrectly, sunscreen with an SPF as low as 8 is capable of blocking most endogenous production of vitamin D (58).

Melanin, a compound produced by the melanocytes in the skin that results in pigmentation, also competes with 7DHC for UV radiation (58). Different ethnic groups with higher levels of melanin, specifically African-American and Hispanic individuals, are more at risk for insufficient or deficient levels of vitamin D because of the increased levels of melanin

that occurs naturally in these populations (60, 63, 64). Melanin decreases production of vitamin D by absorbing UV radiation at a wider spectrum of wavelengths when compared to 7DHC (58). The inverse relationship between skin pigmentation and 7DHC leads to a decrease in the potential of endogenous vitamin D production (63, 64). In fact, several studies have investigated the relationship between ethnicity and prevalence of low levels of vitamin D, showing that minorities tend to have lower serum 25OHD than Caucasian individuals (63, 65). Also, aging and damage to the skin from burns or scars can decrease the amount of 7DHC stored in the skin, which decreases the potential amount of vitamin D that can be produced (15).

Synthesis of 25OHD rarely exceeds 10 to 15% of the conversion of 7DHC, which may be an evolutionary mechanism to prevent toxicity (58). Because of this reason, toxicity due to excess UV exposure has never been observed (60). Previtamin D and vitamin D are also able to absorb UV light, which converts them to the biologically inactive byproducts lumestrol and tachysterols that remain in circulation for later conversion back to vitamin D, if necessary (58, 60). Excessive UV radiation can also degrade vitamin D, resulting in the formation of suprasterol, which cannot be converted back to vitamin D (60). For this reason, even those who spend sufficient or even excessive time in the sun are still at risk for suboptimal serum levels of 25OHD. Furthermore, the conversion of previtamin D to vitamin D is positively correlated to skin temperature; while this thermal reaction usually occurs efficiently because skin temperature is increased at times when UV exposure is also high, the temperature of skin can vary widely between individuals and location, which causes fluctuations in vitamin D synthesis as well (60).

2.7.6 Dietary Influences on Vitamin D Concentrations

Vitamin D is obtained from the diet in addition to the endogenous production through sunlight. However, recent reports suggest that dietary intake can be considered almost negligible because such small quantities are consumed (58). While vitamin D is classified as a hormone

because of the capability of the body to produce it endogenously, it received its title as a vitamin due to its need for growth in the body and ability to be obtained from a variety of dietary sources. Small amounts of vitamin D are found in a western diet and natural production of vitamin D in the skin accounts for the majority of this compound in the body (58). While the endogenous metabolism of vitamin D requires many more conversions in order to produce the active form from the precursor that is found in the epidermis, dietary sources are able to bypass these reactions and enter the same metabolic pathway (58). Vitamin D from the diet is ingested and is then transported to the liver either in chylomicrons or bound to DBP to create 25OHD. From there, it is subject to the same hydroxylation reactions as 25OHD that results from endogenous production (58).

Vitamin D is naturally occurring in a variety of foods (66). Dietary sources are composed of both vitamin D_2 and D_3. Vitamin D_2 is derived from invertebrates, fungi, and plant sources, and D_3 comes from vertebrate sources, found in products such as dairy products and fatty fish (58). Both vitamin D_2 and D_3 have been used to treat osteomalacia, rickets and overall suboptimal status, although D_3 is more effective in raising serum levels of 25OHD (58, 67). The US, Canada and several European countries require that certain fruit juices, dairy sources, and whole grain products be fortified with vitamin D (63). Even though vitamin D_3-fortified foods are available to consumers in industrialized countries, the relative amount of vitamin D present in these sources and consumption of these foods relative to other sources is low (63). For this reason, some nutritionists suggest that because dietary consumption of vitamin D is so small, the contribution of these sources to overall vitamin D levels in the body should be considered negligible (60). For example, 3.5 ounces of cooked salmon provides only about 250 International Units (IU) of vitamin D (63). This value decreases by about 50% for every type of fish when it is fried (63). Also, just one 8-ounce glass of fortified whole milk provides just

around 100 IU of vitamin D_3. Interestingly, one study found that the variability in vitamin D_3 content present in fortified milk can range from 10% to 300% of the reported value on the label, with several samples containing no detectable vitamin D (63, 64).

2.7.7 Vitamin D Requirements

With the recent surge in interest of exploring the benefits of vitamin D, it is expected that a debate concerning the optimal status of this hormone would occur. In fact, there is a discrepancy between the current intake and optimal serum levels recommended by the Institute of Medicine (IOM), and levels believed to be optimal by experts in the field (60). The variability in serum levels caused by exogenous intake and endogenous production only adds to the uncertainty of the exact amount of vitamin D to be included in the diet, how much UV exposure is absolutely necessary, and optimal serum 25OHD for different populations (58).

While vitamin D is consumed in the diet, most clinicians agree that the amounts are so small that food and drink should not be considered as a primary means for increasing serum levels in the body (60, 61). Dietary sources were not needed many years ago, as individuals spent maximal time in sunlight and produced most of the vitamin D needed through the ultraviolet activation of 7DHC in the skin (60). The recommended intake value established in 1989 was determined before serum 25OHD could be measured in the general population. Proponents of increasing vitamin D intake requirements argue that because there is an increase in the prevalence of 25OHD deficiency, there should be an increase in the Daily Recommended Intake (DRI) (60). There is also a movement for the DRI of vitamin D to become more modernized to accommodate current lifestyles, as many individuals are reducing the amount of time they spend in the sunlight to decrease their risk of skin cancer or demands placed on their schedule because of their occupations (60).

The IOM is the governing body in the field of nutrition, responsible for establishing the recommended guidelines of intake of various nutrients (68). The IOM published new recommendations in 2010 as emerging studies began to establish potential new benefits for vitamin D. Indeed, the recommended intake was increased from 400 IU per day to 600 IU, and the recommendation was established as a Recommended Daily Allowance (RDA) instead of the previous Adequate Intake (AI) published in 1997. While it may seem insignificant, the change in designation to an RDA indicates that there was more evidence to prove that vitamin D would provide the benefits that were published (68). Interestingly, the vitamin D Dietary Reference Intake (DRI) published in 1997 was based solely on the well-established, direct relationship between bone health and serum 25OHD levels, which caused some controversy with nutritionists (60, 68). The IOM investigated indicators beyond bone health including calcium absorption, 25OHD and parathyroid hormone (PTH) interactions, risk of cancers and neoplasms, cardiovascular disease, hypertension, diabetes and metabolic syndrome, immune dysfunction, infectious diseases, pregnancy disorders, neurological dysfunction, as well as reduced exercise performance and risk of falls. However, after considering all of these potential outcomes, the IOM felt there were no consistent results that would warrant a further increase in the DRI (31). The IOM correctly noted that there are, to date, very few randomized control trials or clinical trials showing a dose-response and causal relationships concerning outcomes other than the effect of vitamin D on bone density (31). While more conclusive evidence supporting the use of vitamin D in the treatment of these diseases has surfaced in the past few years, it is clear that the IOM established such a low intake level because the recommendation was based on the only reliable and convincing results that were available at that time.

Another factor related to the lack of an increase in the IOM recommended intake levels of vitamin D is centered on the reported levels of insufficiency. Many studies in a wide variety

of populations have revealed an increased proportion of subjects with low levels of 25OHD (65, 66, 69, 70). However, the IOM cites that insufficiency is likely over-reported in these studies, due to the lack of standards defining serum levels establishing insufficiency and deficiency (68). Again, this is a glaring lack of understanding in the area of vitamin D research, and the IOM could not recommend a more radical increase in optimal intake when the levels are meant to apply to the public at large (68). This fact only highlights the importance for an increase in understanding as to how best establish recommendations for optimal vitamin D intake.

Of course, there are two sides to the vitamin D intake controversy. While the IOM has taken a more conservative approach to establishing recommended intake levels, many nutritionists would appreciate recommendations based on current lifestyles with a more modernized intake and optimal serum level (60). "Normal" serum levels were once determined by taking repeated samples and plotting the distribution with the mean of the population used to establish as normal values (60). It is argued that optimal levels should not be defined by average serum 25OHD content of whole populations, because the inclusion of individuals with impaired vitamin D metabolism due to disease or lifestyle would significantly reduce overall levels (60). Those in favor of increasing the ideal serum 25OHD concentration argue that humans evolved by spending significant periods of time in the sunlight and produced thousands of IU per day; therefore, recommendations should account for the fact that more modern lifestyles do not allow for this much time outdoors (60, 61). If humans evolved in the presence of thousands of IU produced endogenously, yet are not currently produced in the same quantity, the DRI should be increased far above 600 IU per day to compensate for the lack of UV exposure (60).

Yet another point of contention is centered on the amount of vitamin D produced or consumed that will translate to optimal serum concentrations of 25OHD. Those living in sun-rich environments with no blocking to UV exposure routinely present with 54 to 90 ng/mL

25OHD, although this range is obviously highly variable based on season and geographical location (58, 60, 61). There is also considerable variability in the amount of vitamin D ingested and the resulting increase in serum 25OHD (58). Research has shown that negative feedback can occur between serum 25OHD levels prior to supplementation and the amount included in the supplementation regimen. That is, those with higher baseline 25OHD prior to supplementation will show a lower rate of improvement in serum 25OHD concentrations, and those with more impaired baseline 25OHD status will respond better to vitamin D treatment (58). This is most likely due to decreased 25-hydroxylase activity in those with higher baseline 25OHD. Based on existing research, basal 25OHD levels below 20 ng/mL will increase approximately 0.48 ng/mL with every 40 IU per day and those suffering from severe deficiency with levels below 4 ng/mL will see an increase in 1.38 ng/mL for every 40 IU per day. However, those with serum levels above 28 ng/mL prior to supplementation only raise serum levels an average 0.28 ng/mL for every 40 IU contained in the daily supplement (58, 60).

The key to increasing recommended intake and optimal serum levels is centered on the incidence of injury and illness that occurs when intake and serum levels are maintained at those set forth by the IOM. Because of the inverse relationship between 25OHD and PTH concentrations, secondary hyperparathyroidism can occur when 25OHD levels are low. In fact, deficient elderly individuals often present with hyperparathyroidism when serum levels are below 30 ng/mL, and the condition is resolved when serum levels reach at least 32 ng/mL (60). This is an important piece of evidence supporting the official increase vitamin D intake recommendations, because calcium absorption is impaired when 25OHD serum levels fall below 32 ng/mL, the same concentration that prevents hyperparathyroidism (60). The National Health and Nutrition Examination Survey (NHANES) III indicated the relationship between 25OHD and bone mineral density, confirming there was a clear optimization of calcium levels and bone

mineral density (BMD) when 25OHD reached 32 ng/mL (60). Further, there is a distinct association with severely low BMD and high incidence of fractures in individuals with serum levels in the range to be considered "normal" by the IOM of 10 to 15 ng/mL (60). Data indicates that no known harmful effects occur with serum 25OHD levels greater than 100 ng/mL, but there are serious risks related to keeping serum levels below 32 ng/mL, especially for clinical populations (60).

2.7.8 Vitamin D and Health Outcomes in Older Populations

Vitamin D is most recognized for improving bone health by regulating calcium concentrations (71). However, relationships first emerged between improvements in vitamin D status and exercise performance as early as the 1940s, when the Germans discovered that athletes who received more ultraviolet exposure had faster 100-meter sprint times (71, 72). These findings were largely ignored until researchers began to explore the usefulness of using calcium supplementation in older adults to increase BMD (73). It was through this research that a significant portion of older adults was found with deficient serum levels of vitamin D. Vitamin D may also be involved in regulating many other physiological processes outside of increasing bone density (74).

Aging is associated with sarcopenia and bone loss (75). The changes that occur with insufficient levels of vitamin D and relationship of this hormone to the aging process seem to have been of particular interest over the course of the last several years. Suboptimal vitamin D levels in older individuals can lead to changes that include decreased BMD and the development of sarcopenia, which is the loss of muscle mass that occurs naturally over time with increased age (76, 77). Diets low in vitamin D content and decreased sun exposure, often observed in the lifestyles of older individuals, are major factors in the observed vitamin D insufficiencies. The

7DHC content present in the epidermis declines as a result of aging, leading to a decreased ability to produce vitamin D and overall progression of poor bone and muscle health (75).

Decreased muscle strength and bone density cause older populations to be at significant risk for health-related consequences including increased falls risk, fractures, and decreased quality of life (74, 77). One study showed a significant inverse relationship between 25OHD and body sway in community-dwelling women with an average age of 63 years (78). Other significant relationships emerged among body sway, incidence of falls and fractures (78). Because some have speculated that the relationships between falls and fractures are mediated by 25OHD, increasing the status of these individuals may be a key to preventing serious injury (77, 78).

Older populations are at risk for poor nutrition, and this condition can directly fuel the progression of many chronic conditions. Consequently, these individuals are often used in supplementation studies involving one or more nutrients. This includes a fairly large body of work investigating the effects of calcium supplementation, either alone or paired with vitamin D, and the changes in BMD and activities of daily living (74). Work in this area shows that increased calcium intake results in a healthier skeletal system, fewer falls, more independence and an overall greater quality of life.

Parathyroid hormone is important in the regulation of calcium levels, and becomes elevated as 25OHD levels drop (79). When calcium levels drop and PTH levels increase, softening of the bone tissue known as osteomalacia occurs. Osteomalacia is related to low levels of vitamin D, which leads to the release of calcium from the bone matrix and softening of the bones. In most cases, sarcopenia is associated with osteomalacia. Some have suggested that the drop in 25OHD and associated increase in PTH may accelerate the development of sarcopenia associated with osteomalacia. As observed in some studies, using supplements to increase

calcium concentrations cause PTH levels to return to closer to normal, which has helped ameliorate the effects of loss of sarcopenia associated with osteomalacia (79).

When comparing the studies in this area, there are several confounding variables that make researching a consensus difficult. The most complicated aspect of this area of research is centered on the variable dosage amounts of vitamin D and age of the subjects between studies. Furthermore, the pathology of individuals considered to have entered old age but are still relatively healthy is drastically different from those who are elderly and suffer from a range of debilitating diseases. This disparity may lead to a difference in both baseline measures and an ability to detect a response (79).

Vitamin D-mediated improvements in strength and power output in older adults are one of the most promising areas of research (73, 77, 79). Several studies show similar positive relationships between 25OHD and markers of muscular health that result in improvements in functional ability, including body sway, balance, 8-foot walk tests, sit-to-stand times, and reaction times (74). A cross-sectional study indicated power, as measured by leg extension, declined with age and was positively correlated with, a less common indicator of vitamin D status, $1,25(OH)_2D$, in both men and women between ages 64 and 99 years (77). Of further interest was the significantly lower power for those who were considered deficient, which was defined for this study as serum 25OHD levels below 12 ng/mL (77). A separate, longitudinal study showed that there was a positive association between 25OHD levels and grip strength, as well as the loss of muscle mass over time (76). Given these relationships, the authors concluded that higher levels of 25OHD acted to protect against the signs of sarcopenia over the three years of study observation (76). This included a strong association between serum 25OHD and grip strength when subjects were divided into categories based on 25OHD concentrations, with individuals being considered deficient with serum levels below 10 ng/mL and sufficient when

serum levels were above 20 ng/mL (76). Those with serum 25OHD below 10 ng/mL were 2.14 times more likely to develop sarcopenia when based on grip strength, and 2.59 times more likely when based on skeletal muscle mass (76). The relative importance of this particular study is that it connects the observational and cross-sectional studies with those that supplement individuals and observe increases in strength (76).

2.7.9 Vitamin D and Exercise Performance

Recent reports suggest that vitamin D may have the potential to alter muscle tissue physiology, leading to improved strength and power measures in younger, healthy populations (74). Interestingly, these studies revealed an unexpected lack of vitamin D intake and low serum 25OHD concentrations in this population as well (54, 60). To date, the results of these studies are inconclusive, but suggest that increasing intake of vitamin D in younger populations may lead to beneficial changes in muscle physiology. However, several recent findings have piqued the interest in vitamin D and muscle function in younger individuals. This includes the discovery that many otherwise healthy individuals may suffer from low levels of vitamin D, possibly due to being overweight (79). There is also the potential of vitamin D to increase athletic performance in those that are of healthy body weight and without the presence of significant illness (79, 80).

Vitamin D is important in maintaining bone density, increasing muscle synthesis and immune function in athletes (9). Unfortunately, most have only speculated that increasing vitamin D status in these individuals may improve athletic performance and never explicitly explored this hypothesis. Some of the first studies investigating the effects of vitamin D and athletic performance were carried out in Germany in the 1940s and 50s, when it was determined that individuals with increased UV exposure time routinely experienced improvements in athletic performance (72). In one study, thirty-two students underwent irradiation from a sun lamp twice

a week for six weeks, and experienced improved performance on a cycle ergometer test compared to the unexposed control subjects (9). More recent research indicates that athletes who train indoors or during the winter are at increased risk for deficient 25OHD levels and decreased performance (54). One such study showed that gymnasts had significantly lower serum 25OHD levels, and 37% of participants' levels were in the range for potential osteomalacia and 45% had symptoms of hypocalcemia (81). While these findings were not correlated directly with a decrease in performance, the risk associated with low levels of calcium, such as softened bone tissue and grand mal seizures (as observed in the study), certainly lead to unsafe conditions for athletic performance (81).

Although there has been consistent interest in the relationship between vitamin D and muscular strength and power, there is comparatively little information on the potential relationship between vitamin D and aerobic performance. To date, only a handful of studies have observed cross-sectional relationships between 25OHD and various aerobic outcome measures, while no studies have investigated the long-term effects of increasing vitamin D status and potential improvements in aerobic performance (53). Several cross sectional studies show a positive relationship between 25OHD status and aerobic performance (11, 53, 82). These studies also show that athletes of all modalities are at the same risk for vitamin D insufficiency or deficiency when compared to the population at large, indicating that increasing these individuals' status is equally important not only for overall health, but improving athletic ability as well (53).

Two studies that were part of the Cooper Center Longitudinal Study established a positive relationship between serum 25OHD levels and cardiorespiratory fitness (CRF), detected independently in both men and women. In these studies, CRF was determined via maximal treadmill testing (11, 82). Statistical analysis showed that there was a significant positive relationship between CRF and 25OHD (11, 82). Some have speculated that those who are more

aerobically fit tend to spend more time outdoors and consume healthier diets with higher vitamin D content, driving the observed relationship between these three variables (11, 82). However, it is not outside the realm of possibility that 25OHD may play a role in increasing CRF to some degree.

Results from a cross sectional study conducted in our lab are consistent with the studies above. A total of 39 subjects, both male and female, reported to our laboratory for various athletic performance measurements. Subjects were split into groups for analysis based on whether their serum 25OHD concentrations fell above or below 35 ng/mL, as this level has been recommended as optimal 25OHD levels for all healthy individuals, especially those who are physically active (53, 72). Analysis indicated that males with 25OHD levels above 35 ng/mL had significantly higher VO_2max levels compared to males whose serum levels were below 35 ng/mL (83).

2.7.10 Vitamin D and Inflammation

To date, a number of cases have suggested that there is an inverse relationship between serum 25OHD and concentrations of inflammatory markers such as CRP, TNF-α, and IL-6 (6, 12, 13, 69). It appears that changes in inflammation with vitamin D supplementation, with or without a parallel exercise training program, depend on the vitamin D status of the subjects prior to initiation of treatment (6). In some cases, it may be that individuals respond differently to increased vitamin D status; although the outcome measure of a given research project may not be considered significantly changed, another health outcome not analyzed in the project may have improved.

Vitamin D supplementation has the potential to alter the concentrations of inflammatory biomarkers but the relationship is not consistent, nor is the mechanism behind this observation understood. One study, using active adults with insufficient 25OHD levels, investigated the

relationship between serum 25OHD and concentrations of inflammatory cytokines and peak power output; it was observed that there were no differences in power output based on 25OHD status (6). Nonetheless, levels of the inflammatory cytokines interleukin-2 (IL-2), interferon-gamma (IFN-γ), TNF-α, and interleukin-1 beta (IL-1β) were all significantly elevated in individuals with serum 25OHD levels below 32 ng/mL (6). Further, 25OHD concentrations were significantly inversely related to IL-1β and IFN-γ, and positively correlated with peak power output determined via single-leg jump heights (6). Based on the high levels of inflammatory cytokines with no associated changes in the anti-inflammatory cytokine interleukin-10 (IL-10), as well as the relationship with peak power output observed in this study, it is possible that lower levels of 25OHD could mediate the inflammatory cascade without completely affecting the capacity of skeletal muscle in insufficient adults (6). This reasoning could also lead to an explanation as to the inconsistent results when investigating the relationship between vitamin D and athletic performance outcomes.

Relationships between 25OHD and inflammatory cytokines have also been observed in endurance athletes; however, the evidence is inconclusive. For example, when serum 25OHD and TNF-α, IFN-γ, IL-4, and IL-10 were measured in a total of 19 endurance runners, the only significant correlation that was observed was the inverse relationship between 25OHD and TNF-α (13). Although the mean serum 25OHD concentrations were considered sufficient, with males (average 25OHD = 33.8 ng/mL) and females (average 25OHD = 43.1 ng/mL), eight of the 19 subjects had levels below 32 ng/mL and two more subjects had serum levels below 20 ng/mL (13). As with other studies, this study proves further investigation is warranted in a larger population with variables such as physical activity and modality, as well as body composition or diet being more controlled.

In a longitudinal study with overweight and obese adults (average baseline serum 25OHD concentration of 19.3 ng/mL) supplemented with either 4000 IU of vitamin D per day or a matching placebo, participated in a resistance training program for 12 weeks (84). Although no changes in CRP, IL-6, or TNF-α were observed over time, there was a significant correlation between 25OHD and CRP following the 12-week treatment when treatment groups were combined, indicating that the effects of decreased inflammation were mediated in part due to the resistance training program and altered body composition, not necessarily due to the changes in 25OHD (84). Blood samples from subjects were also treated with lipopolysaccharide (LPS) to elicit an inflammatory response (84). Even though both vitamin D and placebo groups experienced a reduction in TNF-α levels at the end of the treatment period, only the samples from the placebo group experienced an increase in LPS-stimulated TNF-α levels, which suggests that vitamin D blunts the acute inflammatory response (84).

CHAPTER 3 – METHODS

3.1 Study Design

The purpose of this study was to investigate the relationship between vitamin D status and exercise habits and their relationship with markers of inflammation, immune cell response, and monocyte phenotypes. Healthy females, who were either regularly physically active (PA) or did not have a history of regular physical activity (NPA), were recruited for this project. Subjects were further stratified into groups based on their vitamin D status (high vitamin D status, HD; or low vitamin D status, LD). Four groups were formed based on the stratification strategy above: active individuals with high vitamin D levels (PA-H), active individuals with low vitamin D levels (PA-L), inactive individuals with high vitamin D levels (NPA-H), and inactive subjects with low levels of vitamin D (NPA-L). This project was approved by the Louisiana State University Institutional Review Board.

3.2 Subjects

Female subjects, with no apparent chronic illness and between the ages of 19 and 35 years, were placed into one of four groups based on activity level, either trained (PA) or untrained (NPA), and vitamin D status, either below (LD) or above (HD) a given optimal serum concentration. For the purpose of this study, optimal levels of serum 25OHD were considered 32 ng/mL for the trained group and 20 ng/mL for the untrained group. These levels have been proposed by several different governing bodies in the areas of nutrition and athletic performance as the optimal levels for these populations (68, 72, 85). The IOM determined in 2010 that 20 ng/mL is a sufficient concentration of 25OHD in a healthy population (68, 85). However, those in the area of sport nutrition believe that there is evidence that concentrations higher than the IOM recommendation may be beneficial for those who are physically active (72). Those in PA reported following a consistent exercise training regimen for at least the past three months,

consisting of a minimum of 150 minutes per week of moderate to vigorous activity. Those in NPA did not report engaging in a regular exercise program. Groups were further subdivided using both exercise habits and vitamin D status: physically active subjects with high levels of vitamin D (PA-H), active subjects with low levels of vitamin D (PA-L), inactive subjects with high levels of vitamin D (NPA-H), and untrained subjects with low levels of vitamin D (NPA-L).

There were several criteria that would exclude a potential subject from participating. Any individual reporting any history of smoking, regardless of frequency or if they had quit habitual or recreational use, was excluded from participation. Additionally, subjects without regular menstrual cycles were not recruited. Subjects on pharmaceutical birth control or using birth control methods that delayed a monthly cycle were allowed to participate. Any subject reporting a change in body weight greater than 5% in the past three months was also excluded from participation.

3.3 Study Visit Description

Subjects reported to the lab for four visits: first, to sign the consent forms and take anthropometric measures; second, for blood collection; third, for aerobic capacity assessment; and fourth, for anaerobic power evaluation.

In the first visit, subjects were presented with the consent form and were disclosed of all pertinent information relating to the study. Subjects also completed an extensive medical health history form. This questionnaire required the subject to document use of prescription medications, family history of significant medical conditions, and history of major medical condition diagnosis. The form also included an obstetric and gynecological portion concerning the subject's history of pregnancy, birth control use, and hysterectomy. The date of the subject's last menstrual period was recorded, and the investigator made verbal confirmation that the subject experienced a regular schedule and/or was on birth control. There were also questions

concerning alcohol and tobacco use. After the subject read and signed the informed consent and completed the medical health history form, she filled out a questionnaire known as the Physical Activity Readiness Questionnaire (PARQ), which evaluated the individual's overall health and ability to participate in exercise testing (86). Any "yes" answer to questions on the PARQ resulted in exclusion from the study. Participants also filled out an additional questionnaire assessing physical activity habits. The International Physical Activity Questionnaire (IPAQ)-Short Format is used to obtain internationally comparable estimates of physical activity with adults aged 18-65 years (87). It also is designed to assess health-related aspects of physical activity and sedentary behaviors. The short version contains four items (seven questions) targeting time spent in vigorous- and moderate-intensity activity, walking, and in sedentary activity (87). Appendix 1 contains all forms presented in the first visit.

Height and weight were measured on a traditional stadiometer, and used to calculate body mass index (BMI). Waist and hip circumference were measured using a Guillick tension tape, and used to calculate waist-to-hip ratio (W:H). Skinfold measurements were taken at seven sites across the body with a skinfold caliper: triceps, subscapular, midaxillary, chest, abdomen, suprailiac, and thigh (88). Measurements were repeated three times and averaged. The sum of the averages was then used to determine body density, which was applied to an equation to estimate body fat percent (88).

Subjects were then instructed to complete two forms during the week prior to blood collection to characterize dietary intake of vitamin D and sun exposure. The diet log required subjects to list all food they ate for two weekdays and one weekend day. Each item was then analyzed for total vitamin D content from the USDA database, which provides amounts of vitamin D, both vitamin D_2 and D_3, for many foods (89). Amounts of vitamin D, in total IU, were totaled for the three days. Sun exposure logs characterized time spent in the sunlight for

the week prior to blood collection. In addition to the total time spent outdoors each day, subjects were also asked to list what type of clothing they wore that day or "exposure" their body received. This method, proposed by Hanwell et al, allows for total quantification of UV exposure (90). Time outdoors and exposure are each given a numerical score; these scores are multiplied for the day and added for the week. Scoring is as follows:

Time Spent Outdoors	Exposure
< 5 minutes – 0 points	Face and Hands Only = 1 point
5-30 minutes – 1 point	Face, Hands, and Arms = 2 points
> 30 minutes – 2 points	Face, Hands, Arms, and Legs = 3 points
	Bathing Suit = 4 points

This allows for a scoring range between 0 and 56 points for the weekly total. Habitual use of sunscreen and vitamin D or multivitamin supplements were noted at this time.

Subjects reported to the laboratory for the second time for blood collection by a certified Emergency Medical Technician. Samples were collected between 6 and 7:30 am following a 10-hour fast, during which time subjects were instructed to drink only water and avoid food and other drinks. Blood collection was conducted during days 5 to 7 of the menstrual cycle. Subjects were asked to refrain from alcohol for 48 hours prior and vigorous exercise for 72 hours prior to testing.

The third visit involved a test of cardiorespiratory fitness, or a VO_2peak test. While there are a number of different protocols for VO_2peak testing, the Bruce Ramp protocol, tested using a treadmill (ProForm Treadmills, Logan, UT) and standard metabolic system (AEI Technologies, Pittsburgh, PA), was used for this study (91). This protocol uses both walking and running

speeds, allowing the same protocol to be used on a subject pool with varying levels of fitness (91). Briefly, subjects wear a mouthpiece that is connected to a metabolic cart for the entirety of the test, which analyzes the amount and composition of inhaled and exhaled gases. In the Bruce Ramp protocol, the initial stage is one minute in duration, where the subject walks at 1 mile per hour with no incline in order to check the placement of the metabolic cart and mask as well as familiarize the subject with walking on the treadmill. Each stage following the warm-up is three minutes in length, increasing the speed by 0.3-0.4 miles per hour and incline by 2-3% at the end of each stage. The subject continued to walk or run until they could no longer continue, which varied for each participant depending on her aerobic capacity (88). Subjects also wore a heart rate monitor and asked to report their rating of perceived exertion (RPE), which is a scale ranging from 6 to 20 and used to assess subjective workload. Heart rate, RPE, and VO_2 were recorded at the end of each three minute stage. VO_2peak was considered the highest recorded VO_2 during the course of the test.

Results from the VO_2peak test will be used to categorize the subjects into fitness levels, as determined by the American College of Sports Medicine (ACSM). These classifications are based on percentiles of VO_2max values obtained from large populations of individuals and are divided by gender and age group. Percentile values for maximal aerobic capacity are provided by ACSM for these gender and age groups, with classifications of "superior," "excellent," "good," "fair," "poor," and "very poor" that correspond to increments of the percentile values. Reference values for obtained VO_2max from females ages 20-29 are listed below (88).

Percentile	Classification	VO_2max (mL/kg/min)
95th	Superior	50.2
80th	Excellent	44.0
60th	Good	39.5
40th	Fair	35.5
20th	Poor	31.6
1st	Very Poor	22.6

The fourth and final visit involved an evaluation of anaerobic power using a Wingate test, carried out on a cycle ergometer (Monark Exercise AB, Vansboro, Sweden). Prior to the start of the actual test, subjects were allowed a warm-up period at 50 watts, during which they adjusted the height of the seat and became acclimatized to the cycle ergometer. Once the subjects were ready to begin, they pedaled at an all-out effort for 30 seconds against a given resistance based on their body weight. Revolutions were counted over the course of the 30 seconds and in five-second intervals. This information was then used to calculate peak power output and anaerobic capacity, relative peak power and anaerobic capacity based on body weight, and fatigue index (92).

3.4 Blood Analysis

One resting blood sample of 30 mL was collected for analyses. Samples were collected in three 10 mL tubes, containing 1) no additive, 2) sodium heparin, or 3) ethylenediaminetetraacetic acid (EDTA), resulting in a total sample of approximately 30 mL. First, serum was isolated from blood collection tubes with no additive (Beckton Dickinson, East Rutherford, NJ) for determination of serum 25OHD and CRP concentrations. Samples were allowed to cool immediately after collection at 8°C for up to two hours. Following cooling, samples were centrifuged at 1000 rcf for 20 minutes at 10°C. Serum was then aliquoted and

frozen at -80°C until analysis. Serum 25OHD and CRP were assessed using enzyme-linked immunosorbent assays (ELISA; Alpco Diagnostics, Salem, NH). These kits are commercially available and provide a reliable assessment of a variety of circulating compounds in the blood. Samples were prepared in a 1:100 dilution prior to CRP analysis, according to manufacturers' instructions.

Blood samples from tubes treated with sodium heparin (Becton Dickinson, East Rutherford, NJ) were cultured with lipopolysaccharide (LPS) to assess the production of IL-6. Roswell Park Memorial Institute (RPMI) cell culture media (Sigma Aldrich, St. Louis, MO) was prepared in a 1:100 dilution with L-glutamine, streptomycin, and penicillin (Sigma Aldrich, St. Louis, MO). Samples were then prepared in a 1:10 dilution in the prepared media. Samples were plated in 2 mL volumes and treated with 50 µL of 1 mg/1 mL LPS (*S. enteriditis*; Sigma Aldrich, St. Louis, MO), for a final concentration of 25 µL. Control wells were treated with 50 µL of media. After 24 hours of incubation at 37°C and 5% CO2, plates were centrifuged for 8 minutes at 800 rcf. Supernatants were harvested, aliquoted, and stored at -80°C until analysis. Stimulated samples were diluted 1:1000 prior to analysis with ELISA kits (Alpco Diagnostics, Salem, NH).

Whole blood samples from EDTA-treated blood collection tubes (Beckton Dickinson, East Rutherford, NJ) were incubated with fluorescent-labeled antibodies for the CD14 (anti-human CD14-FITC), CD16 (anti-human CD16-PE), and TLR4 receptors (anti-human CD284 (TLR4)-APC) (eBioscience, San Diego, CA). Matching isotype control samples were also prepared (mouse IgG1 iso control-FITC, mouse IgG1 iso control-PE, mouse IgG2a iso control-APC; eBioscience, San Diego, CA). Samples were then analyzed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) utilizing a 488 nm argon-ion laser and a 635 nm red diode laser configured for FITC, PE, and APC measurements with log amplification and

analyzed with CellQuest Pro Software (BD Biosciences, San Jose, CA). Gates were set to analyze monocytes in each sample for the presence of each of the three receptors. Cell counts were provided for total monocytes and CD14+CD16-, $CD14+^{dim}CD16+^{bright}$, $CD14+^{bright}CD16+^{dim}$ populations, while mean fluorescence channel (MFC) was provided for TLR4 presence in both the CD14+CD16- and CD14+CD16+ subsets.

3.5 Statistical Analysis

All statistical analysis was carried out in JMP Pro 11 (SAS Software, Cary, NC). Group means and standard deviations were calculated for all descriptive and outcome variables. Pearson's correlations between all outcome variables were determined for the overall data set, trained and untrained groups, and each of the four subgroups, and were considered significant at the $\alpha = 0.05$ level. Additionally, a two-by-two group ANOVA was used to compare the differences in outcome variables between each of the four groups. Student's t-tests were performed post hoc for any significant differences detected at the $\alpha = 0.05$ level. Concentrations of CRP were log transformed to adjust for normality for statistical analysis.

CHAPTER 4 – RESULTS

Females (N = 63) were allocated into one of four groups based on physical activity habits and serum 25OHD level (physically active & low 25OHD, PA-L (n = 15); physically active & high 25OHD, PA-H (n = 15); not physically active & low 25OHD, NPA-L (n = 14); not physically active & high 25OHD, NPA-H (n = 19)). When subgroups were combined based on activity habits (PA-L with PA-H vs. NPA-L with NPA-H), there were 30 subjects in the trained category (PA) and 33 subjects in the inactive group (NPA). Physically active subjects (PA) with serum levels below 32 ng/mL were considered to be in the low group, while 20 ng/mL was used for NPA. When subgroups were combined into high and low 25OHD groups (PA-L with NPA-L vs. PA-H with NPA-H), there were 29 individuals with serum 25OHD concentrations below optimal (LD) and 34 subjects with levels above optimal (HD).

4.1 Descriptive Measures

Age, height, and waist to hip ratio (W:H) were not significantly different between PA and NPA. Three subjects were African-American, while the remaining subjects were Caucasian. Weight ranged from 103 to 163.5 lbs in PA, and 103.75 to 255 in NPA, and the average body weight in PA was significantly lower than NPA ($p = 0.039$) (Table 1). Body mass index and estimated percent body fat were also significantly lower in PA compared to NPA ($p = 0.015$, $p = 0.011$) (Table 1). Additionally, PA had significantly higher serum 25OHD compared to NPA, even before being stratified into PA-L or PA-H and NPA-L or NPA-H ($p = 0.015$). Average values for all descriptive measures are provided in Table 1.

4.2 Vitamin D Status: Measures of Intake and Serum Content

The mean dietary intake for all subjects was 466.75 IU over the course of three days, which was not related to serum 25OHD content ($r = -0.186$, $p = 0.144$) (Table 2). Additionally, there were no significant relationships between sun exposure and 25OHD, measured either as the

Table 1 – Descriptive Measures. * indicates a significant difference between paired groups at $p < 0.05$, reported as mean ± SD. PA = physically active, NPA = not physically active, LD = low vitamin D, HD = high vitamin D. PA-L = physically active & low vitamin D, PA-H = physically active & high vitamin D, NPA-L = not physically active & low vitamin D, NPA-H = not physically active & high vitamin D. BMI = body mass index.

	Total	PA	NPA	LD	HD
n	63	30	33	29	34
Age	21.9 ± 2.8	22.1 ± 2.7	21.8 ± 2.9	22.1 ± 2.9	21.8 ± 2.8
Weight (lb)	139.49 ± 29.6	131.46 ± 14.4*	146.79 ± 37.3	145.15 ± 39.3	134.66 ± 16.6
BMI	24.00 ± 4.56	22.55 ± 2.02*	25.31 ± 5.74	25.03 ± 5.87	23.11 ± 2.86
Waist (in)	27.54 ± 3.33	28.80 ± 1.57	28.22 ± 4.27	28.36 ± 4.23	26.85 ± 2.13
Waist:Hip	0.800 ± 0.05	0.798 ± 0.05	0.801 ± 0.05	0.813 ± 0.05	0.788 ± 0.04*
Body Fat (%)	34.4 ± 7.8	31.8 ± 4.5*	36.7 ± 9.3	35.9 ± 8.5	33.1 ± 6.9
	Total	PA-L	PA-H	NPA-L	NPA-H
n	63	15	15	14	19
Age	21.9 ± 2.8	22.5 ± 3.2	21.7 ± 2.1	21.7 ± 2.6	21.8 ± 3.3
Weight (lb)	139.49 ± 29.6	127.56 ± 12.6	135.35 ± 15.5	164.00 ± 49.2	134.11 ± 17.8
BMI	24.00 ± 4.56	22.49 ± 1.72	22.60 ± 2.35	27.75 ± 6.46	23.52 ± 3.21
Waist (in)	27.54 ± 3.33	26.63 ± 1.59	26.97 ± 1.60	30.21 ± 5.37	26.75 ± 2.52
Waist:Hip	0.800 ± 0.05	0.806 ± 0.05	0.790 ± 0.05	0.820 ± 0.05	0.787 ± 0.04
Body Fat (%)	34.4 ± 7.8	31.8 ± 4.4	31.8 ± 4.7	40.3 ± 9.8	34.1 ± 8.2

composite score from the survey or as total minutes spent outdoors per week ($r = -0.022$, $p = 0.865$; $r = -0.096$, $p = 0.463$). Six of the 63 subjects met the Estimated Average Requirement (EAR), which is the intake level estimated to meet the requirement of half of the population. For this age group, the EAR is 400 IU per day. The scores of the survey ranged from 19 to 46 in the current study (Table 2). While there were no correlations between either the sun exposure score

Table 2 = Vitamin D Status and Measures of Intake. Differences calculated between paired groups, reported as mean ± SD. Pairs not connected by a common letter are significantly different ($p < 0.05$). PA = physically active, NPA = not physically active, LD = low vitamin D, HD = high vitamin D. PA-L = physically active & low vitamin D, PA-H = physically active & high vitamin D, NPA-L = not physically active & low vitamin D, NPA-H = not physically active & high vitamin D. 25OHD = serum 25-hydroxyvitamin D, IU = international units.

	Total	PA	NPA	LD	HD
n	63	30	33	29	34
25OHD (ng/mL)	30.79 ± 17.8	36.42 ± 18.2[a]	25.67 ± 15.9	17.05 ± 7.4	42.52 ± 15.5[a]
Time Outdoors (min/week)	580.20 ± 361.5	519.07 ± 304.2	639.36 ± 405.6	521.48 ± 365.6	627.62 ± 344.9
Survey Score	33.36 ± 7.5	34.47 ± 6.9	32.39 ± 7.9	33.97 ± 7.6	32.59 ± 7.4
Dietary Intake (IU/day)	155.04 ± 181.9	136.34 ± 131.6	172.04 ± 218.6	181.35 ± 230.7	132.61 ± 126.0
	Total	PA-L	PA-H	NPA-L	NPA-H
n	63	15	15	14	19
25OHD (ng/mL)	30.79 ± 17.8	22.15 ± 5.4[b]	50.70 ± 14.9[a,b]	11.58 ± 4.9	36.06 ± 12.9[a]
Time Outdoors (min/week)	580.20 ± 361.5	473.33 ± 295.5	564.80 ± 315.9	574.85 ± 451.3	685.94 ± 375.5
Survey Score	33.36 ± 7.5	34.67 ± 7.9	34.27 ± 6.0	33.08 ± 7.7	31.7 ± 8.2
Dietary Intake (IU/day)	155.04 ± 181.9	158.24 ± 163.4	114.44 ± 90.4	206.09 ± 290.9	146.95 ± 149.2

or time spent outdoors and serum 25OHD in the overall data set (all subjects analyzed together), sun exposure scores and time spent outdoors were correlated with each other ($r = 0.528$, $p < 0.0001$). There were no significant differences between PA and NPA in either the sun exposure survey scores or time spent outdoors (Table 2).

There were several observed relationships between sun exposure survey scores and measures of body size and composition. Weight (r = -0.288, p = 0.022), BMI (r = -0.335, p = 0.007), and waist circumference (r = -0.270, p = 0.032) were inversely related to sun exposure scores in the overall data set. The relationship between sun exposure survey score and weight (r = -0.355, p = 0.042) and BMI (r = -0.385, p = 0.027) was also significant in NPA. Furthermore, weight (r = -0.661, p = 0.010), BMI (r = -0.673, p = 0.008), waist circumference (r = -0.665, p = 0.010), and estimated percent body fat (r = -0.594, p = 0.025) were all inversely related to the sun exposure survey in NPA-L. No significant relationships emerged between sun exposure scores and body size and composition in PA, or the subgroups NPA-H, PA-H, or PA-L. Additionally, while these relationships emerged between sun exposure survey scores, there were no observed relationships between serum 25OHD concentrations and measures of body composition across any groups.

4.3 Exercise Performance Measures

Aerobic capacity, as assessed by VO_2peak, was significantly different between PA and NPA ($p < 0.0001$) (Table 3). There were no untrained individuals with VO_2peak values falling higher than 39.4 mL/kg/min and no trained individuals with values lower than 35.5 mL/kg/min. Fifteen subjects in NPA had VO_2peak values in the "good" category, while the remaining subjects fell in either the "fair" or "poor" groups of the norms set forth by ACSM. All individuals in PA had VO_2peak values higher than the classification of "good" (88). These classifications further serve to indicate the difference in aerobic capacity between the trained and untrained groups.

There were no significant differences in peak power output or anaerobic capacity between PA and NPA. However, relative peak power and fatigue index were significantly different between the training groups (p = 0.029, p = 0.021) (Table 3). Relative peak power

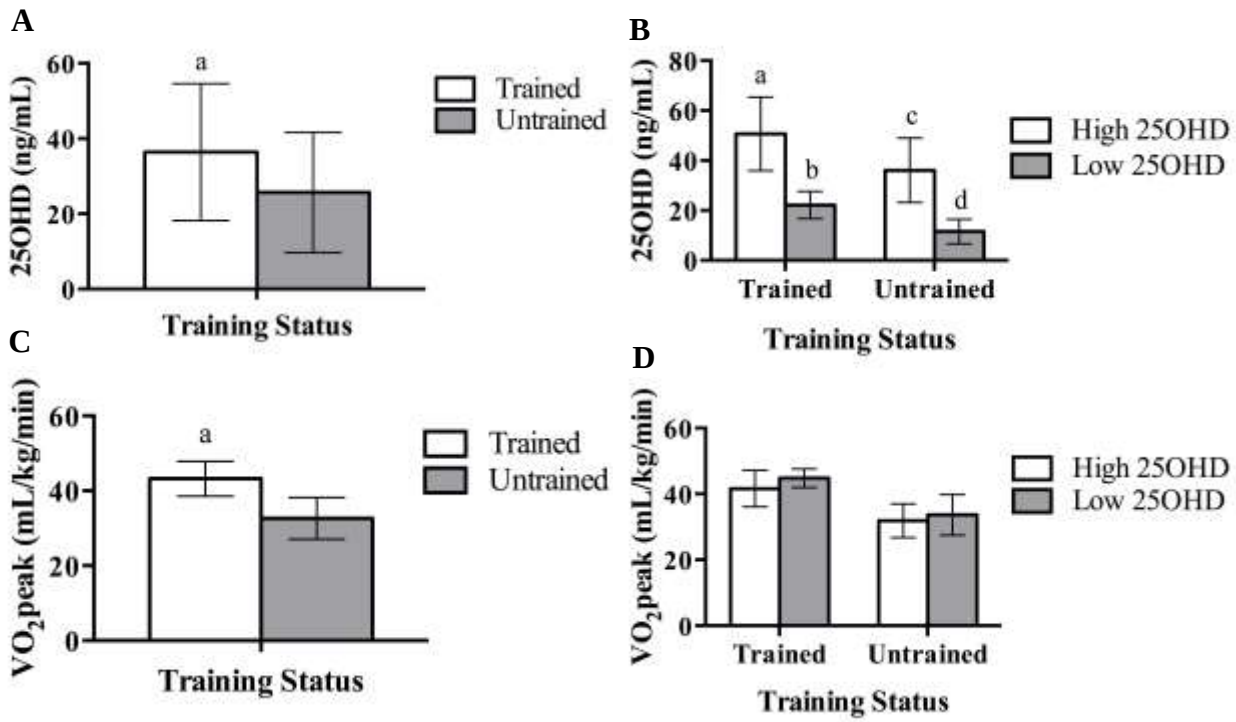

Figure 1 – Fitness and Vitamin D Status of Subgroups. Differences calculated between paired groups. Pairs not connected by a common letter are significantly different ($p < 0.05$). Graphs A and C show vitamin D status and aerobic fitness by training group. Graphs B and D show vitamin D status and aerobic fitness by four subgroups. 25OHD = serum 25-hydroxyvitamin D concentration.

standardizes the peak power output by dividing power output by the subjects' body weights, allowing values to be compared across different body sizes. Fatigue index is a measure of the subjects' ability to sustain the same level of power output throughout the duration of the test, and was significantly lower in PA compared to NPA (Table 3). There were significant differences in both relative peak power ($p = 0.030$) and relative anaerobic capacity ($p = 0.024$) in NPA-L and NPA-H (Table 3).

4.4 Serum CRP and Stimulated Cytokine Production

There were no significant differences between PA and NPA with respect to CRP concentrations, or in the four subgroups (Table 4). When considering the overall data set, there was an inverse relationship between CRP and VO_2peak (r = -0.265, $p = 0.036$) (Figure 2). There

Table 3 – Exercise Performance Measures. Differences calculated between paired groups. Pairs not connected by a common letter are significantly different ($p < 0.05$), reported as mean ± SD**.** PA = physically active, NPA = not physically active, LD = low vitamin D, HD = high vitamin D. PA-L = physically active & low vitamin D, PA-H = physically active & high vitamin D, NPA-L = not physically active & low vitamin D, NPA-H = not physically active & high vitamin D.

	Total	PA	NPA	LD	HD
n	63	30	33	29	34
VO_2peak (mL/kg/min)	37.68 ± 7.38	43.23 ± 4.63[a]	32.63 ± 5.55	39.44 ± 7.34[a]	36.18 ± 7.18
Peak Power (w)	394.06 ± 87.55	402.76 ± 83.01	386.16 ± 92.04	379.00 ± 98.38	406.91 ± 76.28
Relative Peak Power (w/kg)	6.36 ± 1.4	6.76 ± 1.2[a]	6.00 ± 1.5	5.98 ± 1.6	6.70 ± 1.1[a]
Anaerobic Capacity (kJ)	9.13 ± 1.9	9.52 ± 1.7	8.78 ± 2.1	8.86 ± 2.0	9.37 ± 1.89
Relative Anaerobic Capacity (kJ/kg)	0.160 ± 0.09	0.161 ± 0.02	0.158 ± 0.12	0.167 ± 0.13	0.154 ± 0.30
Fatigue Index (%)	40.4 ± 12.1	36.8 ± 13.6[a]	43.8 ± 9.6	38.6 ± 12.6	42.0 ± 11.4

were also positive correlations between CRP and BMI (r = 0.774, $p < 0.0001$) and estimated percent body fat (r = 0.324, $p = 0.010$).

While baseline IL-6 concentrations were not significantly different between PA and NPA, IL-6 concentrations following LPS stimulation were significantly lower in PA compared to NPA ($p = 0.016$) (Table 4). The IL-6 production, defined as the difference between stimulated and unstimulated IL-6 concentrations, was also significantly lower in PA than NPA ($p = 0.016$) (Figure 3). There was a positive relationship between IL-6 production and time spent outdoors in NPA-L (r = 0.6153, $p = 0.0192$). When IL-6 production was expressed per monocyte, the

Table 3 – Exercise Performance Measures (continued).

	Total	PA-L	PA-H	NPA-L	NPA-H
n	63	15	15	14	19
VO_2peak (mL/kg/min)	37.68 ± 7.38	44.83 ± 2.83	41.63 ± 5.55	33.66 ± 6.14	31.87 ± 5.12
Peak Power (w)	394.06 ± 87.55	385.89 ± 85.8	419.62 ± 79.4	371.62 ± 113.2	396.87 ± 74.3
Relative Peak Power (w/kg)	6.36 ± 1.4	6.70 ± 1.4	6.82 ± 1.0	5.20 ± 1.5	6.60 ± 1.2[a]
Anaerobic Capacity (kJ)	9.13 ± 1.9	9.27 ± 1.4	9.77 ± 1.9	8.41 ± 2.4	9.05 ± 1.8
Relative Anaerobic Capacity (kJ/kg)	0.160 ± 0.09	0.164 ± 0.02	0.159 ± 0.02	0.170 ± 0.18[a]	0.150 ± 0.03
Fatigue Index (%)	40.4 ± 12.1	34.5 ± 13.1	39.0 ± 14.2	43.1 ± 11.4	44.3 ± 8.2

difference between PA and NPA was trending towards significance (p = 0.063), but no differences were detected among the four subgroups or HD and LD.

4.5 Monocyte Phenotype and TLR4 Surface Expression

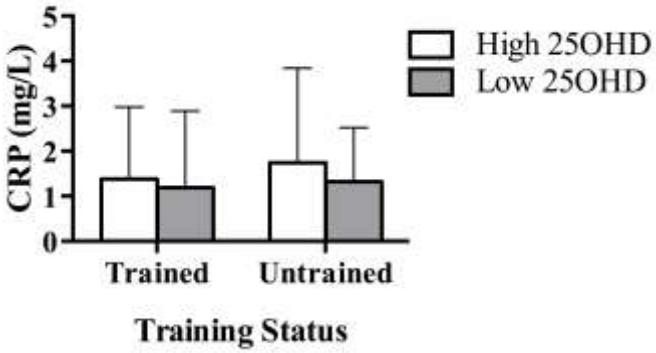

Figure 2 – Serum C-Reactive Protein Concentrations by Subgroup. 25OHD = serum 25-hydroxyvitamin D.

There were no significant differences in total monocyte numbers, or in counts of the monocyte subpopulations CD14+CD16-, CD14++CD16+, or CD14+CD16++ between PA and NPA (Table 4). Subjects in NPA-H had significantly lower numbers of total monocytes and the CD14+CD16- subset when compared to NPA-L ($p = 0.016$, $p = 0.037$) (Table 4). Additionally, there was no significant difference between the relative expression of CD14+CD16- or CD14+CD16+ monocytes expressing TLR4 between PA and NPA, although the NPA-L had a significantly higher expression of TLR4 (reported as MFC) on the CD14+CD16- subset compared to NPA-H (Table 4).

There were several correlations in the overall data between body size and composition and monocyte phenotypes. Total monocyte count was positively correlated with weight (r = 0.338, $p = 0.007$), BMI (r = 0.372, $p = 0.003$), waist circumference (r = 0.382, $p = 0.002$), hip circumference (r = 0.329, $p = 0.009$), and estimated percent body fat (r = 0.284, $p = 0.024$). Additionally, CD14+CD16- monocyte counts were positively correlated with weight (r = 0.368, $p = 0.003$), BMI (r = 0.399, $p = 0.001$), waist circumference (r = 0.412, $p = 0.001$), hip circumference (r = 0.345, $p = 0.006$), and estimated percent body fat (r = 0.291, $p = 0.021$) in the overall data set. Total monocytes were also correlated with weight (r = 0.522, $p = 0.002$), BMI

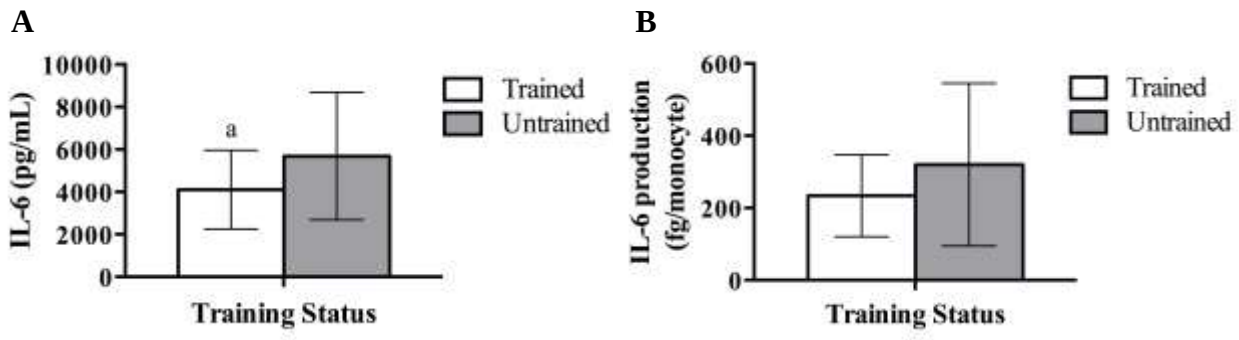

Figure 3 – IL-6 Production by Training Group, as absolute concentration and per monocyte. Differences calculated between paired groups. [a] indicates a significant difference ($p < 0.05$). Graph A shows stimulated IL-6 production by training group, Graph B shows IL-6 production per monocyte by training group.

(r = 0.489, p = 0.004), waist circumference (r = 0.515, p = 0.002), and hip circumference (r = 0.506, p = 0.003) in NPA. CD14+CD16- was also related to weight (r = 0.533, p = 0.001), BMI (r = 0.514, p = 0.002), waist circumference (r = 0.534, p = 0.001), and hip circumference (r = 0.494, p = 0.004) in NPA. Within NPA-L, total monocytes were related to weight (r = 0.620, p = 0.018), BMI (r = 0.587, p = 0.027), waist circumference (r = 0.594, p = 0.025) and hip circumference (r = 0.585, p = 0.028). CD14+CD16- was also related to weight (r = 0.680, p = 0.007), BMI (r = 0.672, p = 0.009), waist circumference (r = 0.655, p = 0.011), and hip circumference (r = 0.618, p = 0.019) in the NPA-L subgroup. Even though no monocyte subpopulation was correlated with body composition or size measures in NPA-H, CD14+CD16+ monocytes expressing TLR4 were positively related to weight (r = 0.583, p = 0.009), BMI (r = 0.523, p = 0.022), waist circumference (r = 0.496, p = 0.031), hip circumference (r = 0.631, p = 0.004), and estimated percent body fat (r = 0.587, p = 0.008). Interestingly, the monocyte subset CD14++CD16+ was positively correlated with W:H in PA (r = 0.410, p = 0.025), although this relationship was not present in either PA-H or PA-L. Within the overall data set, most monocyte populations were correlated with each other (Table 5).

Within NPA, there were several interesting relationships that emerged between vitamin D status and monocyte phenotypes. Total monocyte numbers (r = -0.428, p = 0.013), CD14+CD16- (r = -0.367, p = 0.036), and CD14+CD16++ (r = -0.405, p = 0.020) were inversely related to 25OHD serum concentrations. These correlations were not observed in PA or the overall data set. When further divided into NPA-L and NPA-H, the differences were not observed in NPA-H, but the relationship between CD14+CD16- and serum 25OHD continued to remain significant in NPA-L (r = 0.544, p = 0.045). Interestingly, there was a positive relationship observed between CD14++CD16+ and time spent outdoors in NPA (r = 0.451, p = 0.008), as well as NPA-L (r = 0.757, p = 0.002).

Table 4 – Inflammatory Measures. Differences calculated between paired groups. * indicates a significant difference from its paired group ($p < 0.05$), reported as mean ± SD. PA = physically active, NPA = not physically active, LD = low vitamin D, HD = high vitamin D. PA-L = physically active & low vitamin D, PA-H = physically active & high vitamin D, NPA-L = not physically active & low vitamin D, NPA-H = not physically active & high vitamin D. IL-6 CTRL = resting concentrations, IL-6 STIM = concentrations following LPS stimulation. CRP = C-reactive protein resting concentrations. Total monocytes, CD14+CD16-, CD14++CD16+, CD14+CD16++, and CD14+CD16+ = total cell numbers. MFC = median fluorescence channel.

	Total	PA	NPA	LD	HD
n	63	30	33	29	34
IL-6 CTRL (pg/mL)	49.99 ± 63.4	37.00 ± 35.7	61.81 ± 89.2	51.03 ± 62.1	49.12 ± 65.3
IL-6 STIM (pg/mL)	4980.70 ± 2653.9	4145.12 ± 1858.9*	5740.31 ± 3043.9	4777.19 ± 2306.4	5154.27 ± 2941.4
CRP (mg/L)	1.43 ± 1.7	1.29 ± 0.5	1.56 ± 1.7	1.25 ± 1.5	1.58 ± 1.8
Total Monocytes	19,123.46 ± 5988.5	18,690.30 ± 5664.1	19,571.24 ± 6330.3	20,493.03 ± 5895.0	17.955.29 ± 5902.2
CD14+CD16-	15,882.81 ± 5497.1	15,500.60 ± 4967.7	16,230.37 ± 5993.5	17,066.14 ± 5405.0	14,873.50 ± 5450.3
CD14++CD16+	776.24 ± 991.5	777.20 ± 1026.5	775.36 ± 974.5	978.55 ± 1244.1	603.77 ± 683.2
CD14+CD16++	1352.43 ± 859.3	1528.70 ± 968.8	1192.18 ± 724.1	1539.21 ± 971.7	1193.11 ± 727.5
CD14+CD16+	2128.67 ± 1484.0	2305.90 ± 1518.5	1967.55 ± 1456.4	2517.76 ± 1723.0	1796.79 ± 1171.5
CD14+CD16- TLR4+ (MFC)	11.61 ± 6.1	11.85 ± 1.6	11.39 ± 1.6	11.69 ± 1.7	11.53 ± 1.5
CD14+CD16+ TLR4+ (MFC)	15.34 ± 4.3	15.52 ± 4.2	15.14 ± 4.5	15.91 ± 5.0	14.85 ± 3.7

Table 4 – Inflammatory Measures (continued).

	Total	PA-L	PA-H	NPA-L	NPA-H
n	63	15	15	14	19
IL-6 CTRL (pg/mL)	49.99 ± 63.4	33.42 ± 27.1	40.58 ± 24.8	69.89 ± 82.4	55.86 ± 85.1
IL-6 STIM (pg/mL)	4980.70 ± 2653.9	4097.23 ± 2121.0	4193.01 ± 1629.2	5505.71 ± 2346.8	5913.17 ± 3524.2
CRP (mg/L)	1.43 ± 1.7	1.19 ± 1.7	1.38 ± 1.6	1.32 ± 1.2	1.74 ± 2.1
Total Monocytes	19,123.46 ± 5988.5	18,429.47 ± 4788.1	18,951.13 ± 6586.6	22,704.00 ± 6323.2	17,169.11 ± 5352.1[a]
CD14+CD16-	15,882.81 ± 5497.1	15,264.93 ± 4293.6	15,736.27 ± 5706.5	18,996.00 ± 5942.7	14,192.37 ± 5293.6[a]
CD14++CD16+	776.24 ± 991.5	823.73 ± 1077.9	730.67 ± 1008.0	1144.43 ± 1423.1	503.42 ± 203.7
CD14+CD16++	1352.43 ± 859.3	1598.80 ± 1126.0	1458.60 ± 816.0	1475.36 ± 812.3	983.53 ± 589.4
CD14+CD16+	2128.67 ± 1484.0	2422.53 ± 1529.9	2189.27 ± 1551.2	2619.79 ± 1962.5	1486.95 ± 644.1
CD14+CD16- TLR4+ (MFC)	11.61 ± 6.1	11.54 ± 1.5	12.16 ± 1.7	11.86 ± 1.9	11.04 ± 1.2
CD14+CD16+ TLR4+ (MFC)	15.34 ± 4.3	14.76 ± 3.4	16.35 ± 4.8	17.14 ± 6.1	13.67 ± 1.7[a]

Table 5 – Relationships Between Monocyte Populations. Relationships between all monocyte phenotypes reported. Total monocytes, CD14+CD16-, CD14++CD16+, and CD14+CD16++ = total cell counts. MFC = median fluorescence channel. * denotes significant correlations ($p < 0.05$).

	Total Monocytes	**CD14+CD16-**	**CD14++CD16+**	**CD14+CD16++**	**CD14+CD16- TLR4+ (MFC)**	**CD14+CD16+ TLR4+ (MFC)**
Total Monocytes		r = 0.9634 $p < 0.0001$*	r = 0.3263 $p = 0.0091$*	r = 0.4709 $p < 0.0001$*	r = 0.0496 $p = 0.6996$	r = -0.1020 $p = 0.4264$
CD14+CD16-	r = 0.9634 $p < 0.0001$*		r = 0.1605 $p = 0.2090$	r = 0.3575 $p = 0.0040$*	r = -0.0047 $p = 0.9711$	r = -0.1230 $p = 0.3367$
CD14++CD16+	r = 0.3263 $p = 0.0091$*	r = 0.1605 $p = 0.2090$		r = 0.2823 $p = 0.0205$*	r = 0.1259 p =0.3255	r = 0.0375 $p = 0.7705$
CD14+CD16++	r = 0.4709 $p < 0.0001$*	r = 0.3575 $p = 0.0040$*	r = 0.2823 $p = 0.0205$*		r = 0.0738 $p = 0.5652$	r = -0.1228 $p = 0.3376$
CD14+CD16- TLR4+	r = 0.0496 $p = 0.6996$	r = -0.0047 $p = 0.9711$	r = 0.1259 p =0.3255	r = 0.0738 $p = 0.5652$		r = 0.5304 $p < 0.0001$*
CD14+CD16+ TLR4+	r = -0.1020 $p = 0.4264$	r = -0.1230 $p = 0.3367$	r = 0.0375 $p = 0.7705$	r = -0.1228 $p = 0.3376$	r = 0.5304 $p < 0.0001$*	

CHAPTER 5 – DISCUSSION

5.1 Chapter Overview

Exercise has been well established as an effective intervention in decreasing inflammation (33). Recent studies have implicated the role of vitamin D in mediating inflammation as well (7). However, research examining the effects of exercise training in conjunction with vitamin D status on markers is lacking, especially in young and healthy individuals. Accordingly, the purpose of this study was to investigate whether vitamin D and physical activity status were associated with measures of fitness and overall inflammation, as well as shifts in specific monocyte populations and cell function.

In this chapter, the results of the study will be compared to findings in the literature, I will discuss limitations of the study design and interpretation of results, and identify future directions of research in this area. This chapter will highlight descriptive measures of the overall population, measures of vitamin D intake and serum content, exercise performance measures, serum CRP and stimulated cytokine production, and finally monocyte phenotype and TLR4 expression.

5.2 Descriptive Measures of the Overall Population

Average body size and composition measures were one of the most clinically relevant outcomes of the current study. Subjects weighed between 103 to 225 lbs in the overall data set (mean = 139.45 lbs), which is comparable to the average weight for this age group according to NHANES (National Health and Nutrition Examination Survey) data (mean = 139 lbs) (93). However, estimated percent body fat averaged 34.4% in this study (range 12.8% to 58.7%), and was higher compared to the average for this population (mean = 30.8%) (93). In fact, only six of the 63 subjects were below 25% estimated body fat, which is the current recommendation for this age group (94). When groups were divided by physical activity status, weight and estimated

percent body fat were below the NHANES reported averages in PA but were much higher in NPA. Although PA had healthier anthropometric measures compared to NPA, the differences in these outcome variables were not statistically significant, suggesting that regular exercise may not be the only factor mediating healthy body composition.

Awareness of the obesity epidemic in young adults has become increasingly marked in the general public, and considerable efforts have been made to improve overall health in this age group (95). However, the findings of this study show that young individuals are still likely to have unhealthy body size and composition even if they report regular physical activity. Furthermore, the unhealthy body size and composition measures observed in this study may have been associated with insalubrious drinking habits, with many subjects disclosing high consumption of alcoholic beverages on the weekends. Consequently, individuals in this age group should understand the relationship between increased weight and adiposity and overall health, especially considering the potential for these factors to exacerbate the detrimental effects of the aging process. Lifestyle choices developed around this age are often kept for most of the individuals' life and emphasis should be placed on maintaining a healthy body composition through vigorous activity and healthy eating habits (94, 96).

5.3 Vitamin D Status: Measures of Intake and Serum Content

Despite the lack of significant relationships in PA, sun exposure data, particularly in NPA, provided additional insight related to the impact of lifestyle habits on serum 25OHD concentration and inflammatory markers. Sun exposure survey scores were negatively correlated with body weight, BMI, waist circumference, and estimated percent body fat in NPA. The relationships between sun exposure survey scores and body composition persisted in NPA-L as well. These observations are thought provoking, as several studies suggest a potential for vitamin D to modulate overall body composition and adipocyte biology (97-99). Although there

were no observed relationships between serum 25OHD and body size and composition variables in the present study, there is a possibility that because the relationships reported above existed exclusively in NPA and NPA-L, vitamin D made through the endogenous pathway may have been sequestered in adipose tissue as opposed to being present in circulation (55). Vitamin D is a fat-soluble vitamin, and therefore has a greater affinity to be retained in adipocytes rather than enter the circulation and elicit physiological changes; those who have higher amounts of adipose tissue therefore have greater "reserves" in which vitamin D would reside (55, 58). In the present study, NPA and NPA-L had higher average weight and estimated percent body fat than their group counterparts (NPA = 36.7%, NPA-L = 35.9% vs. PA = 31.7%, NPA-H = 33.1%). These differences may be clinically significant although not statistically different due to high variability.

Dietary analysis in the current study supports the emerging trend of lack of vitamin D in the average American diet. Recommended intake for the age group used in this study is 600 IU/day (68), and reported vitamin D intake in this study averaged 466 IU for three days (average = 155.33 IU/day). This trend has been observed in the average adult population (60, 100); and was previously reported in our lab with a similar population (83). Although some have suggested that supplement use is the only way to reach a healthy vitamin D status, only three subjects out of the 63 reported habitual use of a multivitamin or vitamin D supplement. Two of these subjects were in NPA-H and had sufficient serum 25OHD levels, and one person fell in PA-L and did not have sufficient serum 25OHD concentration despite their use of a multivitamin. Many studies have noted that it is very difficult to obtain the recommended intake of vitamin D from the diet, and the results of this study confirm this notion (60). This trend is likely observed for several reasons. First, those who are highly trained may tend to indulge in unhealthy eating habits, which may be due to disordered eating to maintain weight, or a diet

higher in fatty foods considered a "reward" for a difficult training day (101, 102). Additionally, it is possible that those who are untrained do not place as much inherent value on a regularly healthy diet, thereby limiting their vitamin D intake (103).

Another important relationship that emerged was the disparity between serum 25OHD concentrations in PA and NPA. Subjects in PA had 29.5% higher concentrations of 25OHD compared to NPA, which was statistically significant. This difference serves to support a common theory about the relationship between vitamin D status and physical activity level. Studies from the Cooper Center support the notion that those individuals who are physically active are more likely to spend increased time outdoors and have healthier diets, and therefore have higher circulating levels of 25OHD and lower levels of body fat (11, 82). The implication of these studies is that while being physically active will in fact keep levels of body fat lower, vitamin D may be an independent factor with the potential to modulate changes in body composition. The negative relationships observed in NPA between sun exposure survey scores and body weight, BMI, waist circumference, and estimated percent body fat certainly support the notion that vitamin D could mediate beneficial changes in adiposity. This finding supports our initial hypothesis that vitamin D status would be related measures of fitness.

5.4 Exercise Performance Measures

The International Physical Activity Questionnaire was used to assess physical activity habits of the subjects upon enrollment into the study. Successful screening and group designation was confirmed by the 24.5% difference in VO_2peak between PA and NPA (Table 3). The mean VO_2peak observed for all subjects in this study is consistent with trends reported in the literature concerning this population. For example, a study characterizing the fitness habits and body composition of college students reported an average VO_2max of 34 mL/kg/min (104). This value is very close to the overall average of 37.68 mL/kg/min in the overall data set of the

present study. Studies using other protocols or submaximal estimate protocols also provide comparable values for this population (105, 106).

The observed differences in relative anaerobic capacity and fatigue index in PA and NPA also accurately reflected the training status of the groups (Table 3). Subjects in NPA appear to have produced the same level of power output as those in PA at the start of the test, but fatigued much more quickly and to a greater degree. This response may be driven by the fact that on average, subjects in NPA were required to work against higher resistance; the prescribed force setting is determined by body weight, which was significantly higher in NPA. Consequently, it is not surprising that they did not produce as much power over the course of the entire test. Interestingly, fatigue index was inversely related to serum 25OHD concentrations in the PA-H, indicating the potential that vitamin D may play an additional role in generating and sustaining muscular power output beyond the effects of regular exercise training. Even though causality cannot be determined due to the nature of this study, this finding supports our hypothesis that there would be a link between vitamin D status and exercise outcome variables.

While no studies have explicitly measured significant relationships between anaerobic power indices in the Wingate protocol and serum 25OHD, other studies have noted positive correlations between vitamin D and muscular power assessed through other means (107, 108). In a study investigating the effect of 25OHD concentrations in adolescent females, two-legged jump height was used to assess muscular power and jump height and velocity. Significant positive relationships emerged between vitamin D and the major outcome variables (107). Values obtained in the Wingate test in the current study were comparable to those obtained in a previous study conducted by our lab using a similar population (83).

5.5 Serum CRP and Stimulated Cytokine Production

5.5.1 Resting CRP Concentrations

C-reactive protein is an inflammatory marker produced by the liver in response to circulating inflammatory cytokines (109). Serum CRP concentrations are used to assess systemic inflammation with respect to a number of chronic diseases, most commonly used to assess an individual's risk for CVD (109). Concentrations of CRP less than 1 mg/L indicate a low risk category, 1-3 mg/L indicate moderate risk, and greater than 3 mg/L indicates a high cardiovascular risk (42). In this study, the average CRP concentration was 1.43 mg/L, with seven of the 63 subjects reporting with concentrations in the high-risk category (all values less than 5.621 mg/L), three of whom were in PA. There is a chance that subjects reported with high CRP concentrations due to engaging in stressful activities the few days prior to blood collection, or had an undisclosed acute sickness, leading to uncharacteristically elevated values.

There were no significant differences in CRP concentrations between PA and NPA, or between HD and LD or among any of the four subgroups. The lack of relationship between CRP and training status was unexpected, although still supported by some of the literature (110). However, there was a negative relationship between CRP and VO_2peak in the overall data set, which is consistent with previous findings (24). Exercise has significant anti-inflammatory properties, but some studies have suggested that this response is mediated to a greater degree by body composition (110, 111). There were also positive relationships between CRP and BMI and estimated percent body fat (69). While subjects were recruited into "trained" or "untrained" groups based on physical activity patterns, it is important to note that subjects in NPA were not sedentary nor were they unhealthy. No untrained subject reported overt chronic illness, and although some subjects were overweight, there were also several subjects in this group with very low levels of body fat. The lack of relationship between CRP and serum 25OHD is actually

supported by the literature, as correlations between these variables only seem to appear in cases of chronic disease or when serum 25OHD levels are extremely low (112-114). However, this finding disproves our initial hypothesis that there would be a link between vitamin D status and overall inflammation.

5.5.2 Stimulated IL-6 Production

When whole blood samples were cultured with LPS, PA produced significantly less IL-6 compared to NPA (-34.8%); other studies have found similar responses with exercise training (33, 115). This response is provocative; however, expressing IL-6 production per monocyte allows for comparison of monocyte inflammatory capability, indicating that the individual monocytes of NPA were more responsive to an inflammatory stimulus than PA. Values of LPS-stimulated IL-6 production in this study (average IL-6 production = 4930.7 pg/mL; average IL-6 production per monocyte = 279.0 fg/monocyte) were similar to other values using the same protocol published in the literature (45). For example, in a study by Phillips et al, trained individuals produced and average of 308.7 fg per monocyte and untrained individuals produced 550.2 fg per monocyte following LPS stimulation. In the current study, PA produced an average of 233.9 fg/monocyte and NPA 320.1 fg/monocyte following the same LPS stimulation protocol.

There were no significant differences in IL-6 production among the four subgroups, reducing the possibility that vitamin D modulates this inflammatory pathway in a physiological model, which does not support our original hypothesis that vitamin D might lead to lower inflammation following LPS stimulation. Although the studies using a cell culture model suggest that vitamin D plays an anti-inflammatory role by downregulating the MAPK-1 pathway, it is possible that there were no differences observed in the present study due to the lack of a supraphysiological dose of vitamin D that was utilized in the cell culture model (7). In the study by Zhang et al, whole blood samples were cultured with an additional amount of

25OHD, ranging from 0 to 70 ng/mL beyond what was already in the blood (7). This additional additive was required to stimulate an anti-inflammatory reaction against a response to the LPS. In fact, another study that explored vitamin D mediated changes in whole blood LPS stimulated response yielded a slightly different outcome. When overweight subjects participated in a 12-week resistance training program while taking either a 4000 IU daily supplement or matching placebo, LPS-stimulated TNF-α production was significantly higher in the placebo group compared to the group receiving the supplement (84). While IL-6 was not assessed in this situation, this study serves to illustrate the potential of vitamin D to inhibit LPS-induced inflammation.

Interestingly, there was a positive correlation between IL-6 production and time spent outdoors in NPA-L. These subjects had serum 25OHD concentrations lower than 20 ng/mL, the concentration considered to be healthy by the IOM. It is possible that although subjects were increasing the potential for endogenous vitamin D production by being outside, they were possibly engaging in activities that may have blunted these anti-inflammatory effects as demonstrated by the higher levels of IL-6 production in NPA and NPA-L following the LPS stimulation (Table 4). Subject recruitment and data collection took place during late summer and early fall at a large university with a significant cultural importance placed on football and tailgating. Because these subjects were primarily college students and participated in this study during football season, it is possible that they were engaging in tailgating activities and consuming a high amount of alcoholic beverages while still spending long periods of time outdoors without engaging in physical activity. Therefore, it is likely that these unhealthy behaviors were blunting the anti-inflammatory effects of vitamin D.

IL-6 has been shown to bind to the promoter region of the CRP gene to induce transcription. Interestingly, there was no significant relationship between LPS-stimulated IL-6

production and serum CRP in the overall data set ($p = 0.1997$), suggesting that whole blood production of IL-6 may only partially contribute to overall changes in CRP in the body. This response is supported by other studies, which suggests that IL-6 can be produced in many other tissues in the body including adipose tissue (116). This hypothesis is supported by our data, which revealed a positive correlation between body fat percent and CRP ($r = 0.3239$; $p = 0.0096$).

5.6 Monocyte Phenotype and TLR4 Expression

Classical monocytes are considered CD14+CD16- and possess low inflammatory capability, compared to the non-classical monocytes with high inflammatory activity and classified as CD14+CD16+ (48). In this study, three specific gates were generated based on the parabolic shape of CD14 and CD16 analysis within monocytes to categorize each monocyte more specifically. Traditional FACS methodology only provides quadrant analysis based on whether the receptor is present or not. Consequently, this particular evaluation allowed for the identification of two subpopulations with high inflammatory capability. They are classified as CD14++CD16+, also referred to as $CD14+^{bright}CD16+$ and CD14+CD16++, or $CD14+^{dim}CD16+^{bright}$ subpopulations (48). This approach also allows the researcher to quantify the receptors on the cell surface rather than simply whether that receptor is present or not. Additionally, TLR4 receptor analysis was provided as a histogram reflecting light intensity, where median fluorescent channel (MFC) is reported as opposed to a traditional cell count approach. This analysis allows for the quantification of TLR4 receptor density on the monocyte cell surface instead of simply a number of cells possessing the receptor or not. Although reporting the number of TLR4+ cells is commonly seen in older studies, using MFC provides more descriptive data and ensures that only monocytes expressing this receptor were included for analysis (117).

While overall numbers of each monocyte phenotype are important, it is also critical to consider the percentages of each monocyte phenotype within the total monocyte population. For example, an individual may have a considerably higher number of total monocytes, but the percentage of quiescent and activated monocytes may still be comparable to someone who has normal numbers of total monocytes. In fact, this phenomenon was observed in the current study. Although not statistically significant, PA individuals had lower monocyte numbers in all measured phenotypes compared to NPA. However, the percentages of the CD14+CD16- (PA 82.75% vs. NPA 82.48%), CD14++CD16+ (PA 3.92% vs. NPA 3.85%), and CD14+CD16++ (8.15% vs. 6.27%) within the total monocyte population were virtually identical between PA and NPA. This does not support our initial hypothesis, as we believed that trained individuals would have a lower percentage of CD14+CD16+ monocytes compared to untrained individuals. In fact, several studies have indicated that the relative balance between populations of monocytes can be altered by events that might activate the innate immune response. In some cases, the decreased presence of CD14++CD16+ relative to CD14+CD16++ may indicate the change in receptor expression within the same proinflammatory monocyte population in response to a stimulus, such as acute infections (118). This means that there is a shift in the relative percent of monocytes expressing these receptors and the degree to which they are expressed (119). In most cases, an increase in the number of monocytes expressing the CD16 receptor is correlated with an increase in inflammatory cytokine production, such as TNF-α and IL-6 (118). There is also evidence that physical activity has the ability to significantly alter proportion of specific monocyte subpopulations (5, 119). For example, in a study comparing monocyte phenotypes before, immediately after, and one hour after the cessation of 45 minutes of running at 75% VO_2max, the proportion of CD14+CD16+ was 49% higher compared to baseline (resting) values immediately following exercise and fell an additional 24% below the resting values at one hour

following exercise (119). Similarly, in a longitudinal study, 12 weeks of combined resistance and aerobic training led to an increase in absolute numbers of CD14+CD16-, while CD14++CD16+ and CD14+CD16++ were reduced, as well a downward shift the percentage of the CD14+CD16+ phenotype (5). However, in the present study, no differences were observed in total monocyte numbers or any of the subpopulations between PA and NPA.

The lack of an observed difference between the relative percentages of monocyte phenotypes in the present study may be explained by several variables. First, the average estimated percent body fat in PA, while significantly lower than NPA, is higher than the recommended level for this age group. Second, because the recruited subjects were mostly college students, the intensity of their regular exercise may not reach levels consistently high enough to elicit changes in monocyte phenotypes. Finally, there is a chance that although the subjects were engaging in regular exercise, they may have also been participating in activities detrimental to overall health, such as consuming alcoholic beverages or maintaining unhealthy diets, that would counteract the anti-inflammatory effects of exercise. Although subjects in this study were stratified into groups based on exercise training habits, it is very likely that no subject was entirely sedentary. Furthermore, it was required that all subjects were healthy and did not have any blatant chronic illness. Therefore, the lack of difference in monocyte populations between PA and NPA may be due to the fact that subjects in NPA still spent time outdoors doing recreational activities, such as walking to and from class. Finally, it is possible that shifts in monocyte populations could potentially be occurring compartmentally, such that CD16 expression was changing in the adipose tissue and these changes were not accurately reflected in the plasma (120).

Correlational analysis indicating the relationship between body size and composition measures and overall monocyte numbers and phenotypes are consistent with the consensus that

exists in the literature. Individuals with higher adiposity are known to have higher numbers of monocytes, particularly of the CD14+CD16+ phenotype (121); correlations in the overall data set between total monocyte number and weight, BMI, waist circumference, hip circumference, and estimated percent body fat support previously established findings. In a study comparing overweight patients with controlled diabetes mellitus to normal controls, there were no differences in CD14+CD16- absolute numbers or percent of total monocytes between the normal and obese individuals (122). The CD14+CD16+ monocytes accounted for 9% of the total monocyte population, which is comparable to the 11% in the overall data set, 12% in PA, and 10% in NPA observed in this study (122). Additionally, the number of CD14+CD16- monocytes was correlated with these measures as well. Interestingly, these relationships persisted in NPA and NPA-L, but not NPA-H or PA or either PA-L or PA-H. This suggests that when exercise training is not a mediating cofactor, serum 25OHD concentrations below optimal produce a stressful environment leading to increased monocyte numbers.

The relationship between monocyte numbers and measures of body composition may be regulated by the increased adiposity that is commonly associated with lack of regular physical activity (123). Adipose tissue is known to produce the chemokine monocyte chemoattractant protein-1 (MCP-1) (124, 125). This protein regulates migration of monocytes and mature macrophages when their immune response is needed (125). This occurs during times of stress or infection, but research has also indicated MCP-1 secretion and monocyte infiltration in adipose tissue (124). When body fat is in excess, MCP-1 is produced at higher levels and would lead to increased numbers of monocytes in these individuals (124). Interestingly, exercise is known to decrease MCP-1 independently of a decrease in body fat (126). The suggestion that MCP-1 levels are different between trained and untrained individuals, as well as in those with higher levels of adiposity, may provide an explanation as to why there was an observed positive

relationship between total monocyte numbers and the number of cells that are CD14+CD16- in NPA and NPA-L which did not persist in the PA or any of the other subgroups.

Although there were beneficial relationships between sun exposure scores and body composition measures in NPA, these correlations were not reflected between sun exposure and monocyte populations. Additionally, no correlations appeared between 25OHD and the monocyte subpopulations, which allows us to reject our original hypothesis that vitamin D may alter the balance between monocyte phenotypes. Given the previously described relationship between time outdoors and LPS stimulated IL-6 production, positive relationships between time outdoors and 1) the numbers of total monocytes, and 2) the CD14++CD16+ monocytes subset, it is possible that although untrained individuals were spending requisite time outdoors and there were beneficial relationships observed between sun exposure and body composition, these individuals were engaging in activities that elicited a stressful environment for the immune system. This suggestion is further warranted by the lack of observed relationship in PA and either PA-L or PA-H.

With respect to TLR+ cells, there were a number of significant positive relationships between the numbers of CD14+CD16+ TLR4+ cells and measures of body size and composition in NPA-H. It is possible that this was likely mediated by the untrained status and levels of adiposity in this subgroup, rather than the fact that these individuals met the required serum 25OHD requirement set forth by the IOM. An emerging body of research indicates a relationship between TLR4 and $1,25(OH)_2D$, whereby TLR4 regulates the action of vitamin D metabolites rather than 25OHD or 1,25(OH)2D eliciting actions on this pathway (127). There were no observed relationships between cells expressing TLR4 and serum 25OHD concentration in the overall data set or any of the subgroups. This finding allows us to reject our initial hypothesis that vitamin D may alter monocyte populations and TLR4 expression. Finally, there

were no significant relationships between the number of TLR4+ cells and IL-6 production in the overall data set, which may be due to the high variability of the IL-6 measure between subjects.

5.7 Limitations

There are several limitations of this study that should be mentioned. First, because this study was cross-sectional in nature, no causal relationships could be established. The balance between inflammation and vitamin D levels or exercise training status does stress the importance of including this nutrient in the diet or remaining physically active, but the results of this study do not allow the freedom to say that exercise training or vitamin D cause a shift in inflammation or phenotypes of monocytes. Future studies including exercise training with or without concurrent vitamin D supplementation interventions would certainly be interesting, and would expose a potential cause-and-effect relationship.

A second limitation of the current study was the lack of cutting-edge technology used throughout the project. Body composition was determined via skinfold calipers, which are certainly not the standard of measurement in this area. Dual-energy x-ray absorptiometry (DXA) is a newer technology that allows exact determination of bone, fat, and fat-free mass of an individual. Because radiation is used to assess these measures, albeit at very low levels, there is an inherent risk to a female subject who may not know she is pregnant. Therefore, the cost of this measure was outside of the proposed budget. Because body composition was not an outcome variable of the study and only used as an anthropometric measure to characterize the different populations, the safer method of skinfold measurements was used instead. Any future work in this area should consider the use of DXA technology. Additionally, it should be noted that many researchers believe the use of HPLC is the best technique to measure serum 25OHD (128). However, many studies have indicated that the use of ELISA protocols produce reliable data as well, and are much easier to carry out without producing significant error or variation in

the results (129). Early studies assessing vitamin D status also used RIA kits, but ELISA plates do not require the use of radiation and are therefore a safer alternative to the investigator (129).

The sun exposure survey used in the study by Hanwell et al was used as a comprehensive measure of subjects' exposure to UV light. However, outside of the original study in which this survey was developed and used, this survey is not widely used in other research projects. Total time spent outdoors is more commonly used as a way to quantify the potential for endogenous vitamin D production. The survey proposed by Hanwell et al allows for a more comprehensive quantification of exposure to UV light, and therefore, potential vitamin D production. Scores in the current study ranged from 19 to 63; for reference, scores in the original study ranged from 11 to 52 (90). While the use of this survey limits the ability of the results of this study to other studies in which time outdoors is related to vitamin D production and other outcome variables, it is in fact more detailed than simply adding time spent outdoors. The use of this survey was validated by the correlation between sun exposure and time spent outdoors, which was significant across the overall data set as well as all subgroup analyses ($p < 0.0001$).

The population included in this study also proposed potential limitations in interpretation of the findings. Only females were recruited for the study, which allowed for the use of an unstudied population. Females are more likely to have disordered eating habits (130), allowing for the development of a subgroup of those who are very physically active and more likely to have lower levels of vitamin D due to lack of proper nutrition. However, in the future, the use of both males and females in longitudinal studies would allow for a better understanding of the anti-inflammatory effects of vitamin D and exercise.

Finally, the identification between optimal and suboptimal vitamin D serum levels posed a difficult problem for analysis. The initial study design for this project proposed that both trained and untrained groups be further divided into optimal and suboptimal vitamin D groups,

based on a set point of 32 ng/mL. However, after initial subject recruitment and vitamin D determination, it was apparent that untrained individuals were not likely to reach serum levels of 32 ng/mL. There are several likely reasons for this phenomenon. First, untrained individuals are not as likely to spend time outdoors in the sun and also more likely to have a less healthy diet than those who are trained. Therefore, these individuals are not receiving exposure to the UV light to produce vitamin D endogenously, nor are they ingesting the proper levels from the diet. In this study, it was observed that untrained individuals spent as much time outdoors as trained individuals, eliminating this proposed discrepancy as a potential explanation for the difference in serum 25OHD concentrations. There was also no difference between the amount of vitamin D consumed in the diet, likely due to the fact that most of the subjects were not meeting the required level of vitamin D intake. Second, untrained individuals are more likely to have higher amounts of fat than trained individuals (123). Because vitamin D is a fat soluble vitamin, these individuals may be receiving proper nutrition but the vitamin is being stored in the excess adipose tissue rather than circulating in the serum (55, 58). As noted above, there is the small subset of untrained individuals who have low levels of body fat due to disordered eating habits; these individuals would also have low circulating levels of vitamin D because of improper nutrition.

5.8 Future Directions

Vitamin D is implicated in increasing health in a variety of areas, such as decreasing the risk of cancer, treatment of existing cancer, decreasing the symptoms of anxiety and depression, as well as the areas of muscle physiology and inflammation investigated in this study (131-133). Because of its significant potential, a large body of work has determined correlations among a wide range of outcome variables. However, there are very few studies investigating the effects of vitamin D supplementation and changes in overall health. As it relates to the present study,

the next logical step would include a supplementation study with concurrent exercise training. Overweight or obese subjects might be most desirable in a study of this nature due to their propensity for vitamin D deficiency, but any changes in outcomes such as increases in fitness, body composition, or inflammation might be confounded due to changes in adiposity that would accompany consistent exercise training. Therefore, use of normal weight individuals should also be considered. TNF-α and the VDR also represent likely targets for future investigation.

5.8.1 Tumor Necrosis Factor Alpha

Tumor necrosis factor-alpha is produced by several different types of leukocytes, including monocytes and macrophages (16). Normal, resting levels of TNF-α are around 2 pg/mL (27). These levels can become elevated in overweight and obese individuals, as increases in adipose tissue leads to the prevalence of macrophages that are capable of producing TNF-α (134). Once secreted, TNF-α can elicit a number of effects, such as binding to receptors present in a wide variety of tissues and leading to the production of other inflammatory cytokines (135). Activation of the NF-κB pathway increases the production of cytokines including IL-1β, IL-6, and IL-8 (135, 136). The general consensus is that training exercise modality and volume, as well as existing tissue damage from a particularly difficult training bout prior to blood collection, may influence an individual's capacity to produce TNF-α (16).

Because TNF-α acts as an inflammatory marker and also independently elicits the production of other inflammatory biomarkers, changes in its concentration are often studied after acute exercise training bouts (27). This cytokine also has a unique time course in its changes in response to the exercise stimulus compared to other traditional markers of inflammation such as IL-6 (36). Levels of TNF-α may not increase during or immediately following acute bouts of exercise, but may become increased in plasma between one and two hours after completion of the activity (16). Changes in TNF-α levels with chronic exercise training have also been

documented, although there are mixed results. Regular training results in lower resting levels of TNF-α, as well as the increased ability to return levels to normal after exposure to a stimulus, compared to those who are not physically active (20). In a study comparing concentrations of TNF-α in both older (average age of 64 years) and younger (average age 24 years) and age-matched sedentary controls, concentrations of were significantly different between older trained and older untrained subjects, although this difference did not exist in the younger subjects (39). The results were clinically relevant, as inflammation is normally a condition that develops and persists with increasing age, and this study highlighted the potential for exercise to slow the development of this condition (39). Because changes in concentrations of this inflammatory cytokine have been observed in several studies, measuring the response of this variable in response to a stimulus should be considered for future projects.

5.8.2 Vitamin D Receptor

The vitamin D receptor (VDR) adds yet another complex facet to the understanding of the role vitamin D plays in mediating the inflammatory response. Vitamin D works traditionally through an endocrine mechanism and controls genetic expression (137). The VDR is located in the nucleus and binds with its ligand, $1,25(OH)_2D$, and forms a heterodimer with the retinoic X receptor in. Once this heterodimer is formed, transcription of the genetic material proceeds (137). The VDR is present in nearly every cell type in the body (137).

Modulation by the VDR of the inflammatory response in immune cells is one of the areas of vitamin D research that gives promise of understanding a mechanism. When monocytes were incubated with varying concentrations of either 25OHD or $1,25(OH)_2D$ and stimulated with lipopolysaccharide (LPS), production of IL-6 and TNF-α was inhibited in a dose-dependent manner for both isoforms of vitamin D treatment (7). To date, there have been no studies investigating the relationship between vitamin D status, with or without the influence of exercise

training status, on the presence and amount of VDR in a human model after the TLR pathway is stimulated.

The effect of exercise training on expression of the VDR has not been well documented. In a study with exercise-trained rats on vitamin D supplement or a matching placebo, it was determined that those rats on the vitamin D supplement and exercise training program had higher expression of the VDR in skeletal muscle compared to those rats on the placebo treatment (138). Rats were exposed to the same exercise treatment protocol and performance under varying exercise conditions was not assessed. However, the inflammatory profiles of rats on the vitamin D supplement were significantly more favorable compared to those that were on the placebo, even though all rats underwent the same exercise stimulus (138). Some work has focused on bone density in humans with different polymorphisms of the VDR, and speculated that those with polymorphisms that favor higher bone resorption may not perform as well in athletic events, although this line of research has not been pursued (139).

Relative presence of the VDR in states of vitamin D sufficiency and insufficiency is also another area of research that has presented conflicting results. Some projects offer a positive relationship between VDR content and $1,25(OH)_2D$ concentrations; this seems logical, as $1,25(OH)_2D$ is the ligand for the receptor (140). As long as the ligand is present, the receptor remains present in order to elicit its action. However, the relationship between 25OHD and $1,25(OH)_2D$ makes interpretation of this relationship difficult. When the body is deficient of 25OHD, $1,25(OH)_2D$ continues to be produced, and may reach higher than normal levels in order for normal physiological processes to continue (141). This means that in studies investigating the relationship between vitamin D status and the presence of the vitamin D receptor, those individuals that have suboptimal vitamin D status, as assessed by 25OHD concentration, may actually be expressing higher levels of VDR because of the inverse

relationship between the vitamin D biomarker used to establish vitamin D status and the vitamin D ligand used to elicit responses throughout the body. Conversely, other studies have shown that individuals who are deficient in vitamin D still have decreased presence of VDR, even when $1,25(OH)_2D$ is present in elevated concentrations (142).

Considering its many implications in changes in inflammation and muscular health, work investigating VDR content and these variables is lacking. Future work should focus on VDR content, particularly in trained and untrained individuals. Using this methodology in a longitudinal training study would also provide insight into changes that exercise training might induce in VDR content and a potential mechanism by which vitamin D would elicit these changes. Drawing correlations between VDR levels and monocyte subpopulations would also push the boundaries in the area of immune function research and increase the understanding vitamin D and VDR play in decreasing inflammation.

5.9 Conclusion

Exercise training has been shown to have anti-inflammatory effects with some studies suggesting that the intervention may reduce inflammatory monocyte numbers with concomitant reductions in the inflammatory capacity and overall levels of systemic inflammation in the body. The anti-inflammatory actions of vitamin D in the body are not as well defined. In this study, regular physical activity was associated with higher levels of serum 25OHD, lower BMI and waist circumference and percent body fat as well as reduced LPS-stimulated IL-6 production. Optimal vitamin D status did not appear to confer any additional health related or anti-inflammatory benefit in those engaging in regular exercise. However, in individuals not participating in a regular exercise program, the potential for vitamin D to mediate inflammation appeared more likely. More specifically, untrained people with optimal vitamin D status had lower numbers of monocytes, CD14+CD16- and TLR4 expression on CD14+CD16+ cells;

however, these differences did not translate into a change in overall cell function or markers of systemic inflammation as there was no difference between optimal and suboptimal groups with respect to LPS-stimulated IL-6 production and CRP. An expanded exploration of the relationship between vitamin D and inflammation may include other inflammatory biomarkers, immune cell types, the vitamin D receptor and the role of adipose tissue.

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APPENDIX 1 – CONSENT FORMS

1.1 LSU IRB Approval

ACTION ON PROTOCOL APPROVAL REQUEST

Institutional Review Board
Dr. Robert Mathews, Chair
130 David Boyd Hall
Baton Rouge, LA 70803
P: 225.578.8692
F: 225.578.5983
irb@lsu.edu | lsu.edu/irb

TO: Laura Stewart
Kinesiology

FROM: Robert C. Mathews
Chair, Institutional Review Board

DATE: February 17, 2014
RE: **IRB#** 3458

TITLE: Vitamin D and Training Status in the TLR Inflammatory Pathway

New Protocol/Modification/Continuation: New Protocol

Review type: Full _X_ Expedited ___ **Review date:** 2/14/2014

Risk Factor: Minimal _____ Uncertain ___X___ Greater Than Minimal_____

Approved* ____X____ **Disapproved** _____

Approval Date: 2/14/2014 **Approval Expiration Date:** 2/13/2015

Re-review frequency: (annual unless otherwise stated)

Number of subjects approved: 80

Protocol Matches Scope of Work in Grant proposal: (if applicable) _____

***Approval Note:**

By: Robert C. Mathews, Chairman Robert C Mathews

PRINCIPAL INVESTIGATOR: PLEASE READ THE FOLLOWING –
Continuing approval is CONDITIONAL on:

1. Adherence to the approved protocol, familiarity with, and adherence to the ethical standards of the Belmont Report, and LSU's Assurance of Compliance with DHHS regulations for the protection of human subjects*
2. Prior approval of a change in protocol, including revision of the consent documents or an increase in the number of subjects over that approved.
3. Obtaining renewed approval (or submittal of a termination report), prior to the approval expiration date, upon request by the IRB office (irrespective of when the project actually begins); notification of project termination.
4. Retention of documentation of informed consent and study records for at least 3 years after the study ends.
5. Continuing attention to the physical and psychological well-being and informed consent of the individual participants including notification of new information that might affect consent.
6. A prompt report to the IRB of any adverse event affecting a participant potentially arising from the study.
7. Notification of the IRB of a serious compliance failure.
8. SPECIAL NOTE:

**All investigators and support staff have access to copies of the Belmont Report, LSU's Assurance with DHHS, DHHS (45 CFR 46) and FDA regulations governing use of human subjects, and other relevant documents in print in this office or on our World Wide Web site at http://www.fas.lsu.edu/osp/irb*

Application for Approval of Projects Which Use Human Subjects

This application is used for projects/studies that cannot be reviewed through the exemption process.

Institutional Review Board
Dr. Robert Mathews, Chair
130 David Boyd Hall
Baton Rouge, LA 70803
P: 225.578.8692
F: 225.578.5983
irb@lsu.edu | lsu.edu/irb

– Applicant, Please fill out the application in its entirety and include two copies of the competed application as well as parts A-E, listed below. Once the application is completed, please submit to the IRB Office for review and please allow ample time for the application to be reviewed. Expedited reviews usually takes 2 weeks. Carefully completed applications should be submitted 3 weeks before a meeting to ensure a prompt decision.

– A Complete Application Includes All of the Following:

(A) Two copies of this completed form and two copies of part B thru F.
(B) A brief project description (adequate to evaluate risks to subjects and to explain your responses to Parts 1&2)
(C) Copies of all instruments to be used.
*If this proposal is part of a grant proposal, include a copy of the proposal and all recruitment material.
(D) The consent form that you will use in the study (see part 3 for more information.)
(E) Certificate of Completion of Human Subjects Protection Training for all personnel involved in the project, including students who are involved with testing or handling data, unless already on file with the IRB. Training link: (http://phrp.nihtraining.com/users/login.php)
(F) IRB Security of Data Agreement: (https://sites01.lsu.edu/wp/ored/files/2013/07/Security-of-Data-Agreement.pdf)

1) Principal Investigator*: Laura K. Stewart **Rank** Associate Professor

*PI **must be** an LSU Faculty Member

Dept: Kinesiology **Ph:** 578-3549 **E-mail:** stewart6@lsu.edu

2) Co Investigator(s): please include department, rank, phone and e-mail for each

Laura A. Forney, Department of Kinesiology, PhD Student, 859-285-9192, lforne2@tigers.lsu.edu

IRB# 3458 LSU Proposal #

☑ Full ◉ Expedited

☑ Complete Application

☑ Human Subjects Training

☑ IRB Security of Data Agreement

3) Project Title: Vitamin D and Training Status in the TLR Inflammatory Pathway

4) Proposal Start Date: March 2014 **5) Proposed Duration Months:** 12 months

6) Number of Subjects Requested: 80 **7) LSU Proposal #:**

8) Funding Sought From: LSU

STUDY APPROVED BY:
Dr. Robert C. Mathews, Chairman
Institutional Review Board
Louisiana State University
130 David Boyd Hall
225-578-8692 / www.lsu.edu/irb
Approval Expires: 2/13/2015

ASSURANCE OF PRINCIPAL INVESTIGATOR named above

I accept personal responsibility for the conduct of this study (including ensuring compliance of co-investigators/co-workers) in accordance with the documents submitted herewith and the following guidelines for human subject protection: The Belmont Report, LSU's Assurance (FWA00003892) with OHRP and 45 CFR 46 (available from http://www.lsu.edu/irb). I also understand that copies of all consent forms **must be maintained at LSU for three years after the completion of the project**. If I leave LSU before that time, the consent forms should be preserved in the Departmental Office.

Signature of PI Laura K. Stewart Date 01/28/2014

ASSURANCE OF STUDENT/PROJECT COORDINATOR named above. If multiple Co-Investigators, please create a "signature page" for all Co-Investigators to sign. Attach the "signature page" to the application.

I agree to adhere to the terms of this document and am familiar with the documents referenced above.

Signature of Co-PI (s) Laura A. Forney Date 01/28/2014

1. Study Title: Vitamin D and Training Status in the TLR Inflammatory Pathway

2. Performance Site: Louisiana State University
   Baton Rouge, Louisiana 70803

3. Investigators: The following investigators will be available for questions about this study Monday – Friday 8am – 8pm.
   Principal Investigator: Laura K. Stewart, Ph.D., 225.578.3549
   Co-investigator: Laura A. Forney Bobart, 859.285.9192

4. Purpose of the Study:
   The purpose of this study is to establish vitamin D status in individuals who are either athletically trained or untrained. Subjects with optimal vitamin D status will be compared to those who have suboptimal vitamin D status among and between both trained and untrained groups. Serum will be analyzed for vitamin D and inflammatory markers associated with risk of cardiovascular disease and diabetes. Whole blood samples will also be stimulated and assessed for the presence of the vitamin D receptor and other immune cells.

   Subject inclusion: Participants must be healthy females and between 18-40 years of age. Subjects included in the "trained" group will have been engaging in regular (at least 3 days per week for at least 40-60 min per bout) physical activity for at least three months prior to the start of the study. Those who have a mostly sedentary lifestyle will be included in the "untrained" group. Subjects will have had a consistent body weight (within 5%) for the three months prior to testing. Females will need to have regular menstrual cycles, and no pregnant subjects will be included in the study. Additionally, those who smoke or use other tobacco products will be excluded from the study.

5. Study Procedures: You will report to the lab for testing four times. In the first visit, you will be given the informed consent and health and physical activity assessments, questionnaires assessing lifestyle habits that may impact vitamin D status, and heart rate, blood pressure, height, weight, waist and hip circumference, and skinfold measurements will be taken. You will also be instructed to fill out a diet log during the three days prior to the blood collection visit (visit 2). In the second visit, a licensed phlebotomist or nurse will take a blood sample (40 mL), and the three-day dietary logs will be collected. In the third visit, you will complete an aerobic fitness assessment, and two surveys assessing overall mood and training burnout status. Finally, in the fourth visit, you will complete an anaerobic power assessment. You will be asked to refrain from heavy exercise for 72 hours prior to each testing procedure, and refrain from alcohol or caffeine for 24 hour prior to blood collection.

6. Benefits: While no guarantee of benefits can be made, you will be given measures of anaerobic and aerobic power, as well as strength test results and a body composition analysis at no cost to you. These measures are a valid assessment of physical fitness and health status. Vitamin D is becoming more widely accepted as a valuable nutrient, so knowing your vitamin D status can help improve your health.

7. Risks/Discomforts:

Exercise Testing: Because of the nature of the testing procedures, there is a remote risk of a heart attack or stroke and in very rare cases, death. Precautions to minimize this risk have been taken by requiring completion of a health history questionnaire. Your honest answers in completing the health history form will decrease this risk. As with any exercise program, there is a chance that you will experience muscle soreness, fatigue, or even injuries such as sprains or strains.

Blood Sampling: There is a risk of bruising and a remote risk of infection with the blood sampling techniques. You may also become lightheaded and faint during these procedures. These risks will be minimized by having trained technicians using sterile, single-use supplies for blood sampling. You will be seated during blood sampling, but you should tell us if you feel dizzy or faint.

Skinfold Measurements: Since a slight pinching of the skin is required to measure subcutaneous fat through calipers, you may experience mild bruising in the measurement sites (chest, midaxillary, triceps, subscapular, abdomen, suprailiac, and thigh).

In addition to the risks listed above, you may experience a previously unknown risk or side effect.

Injury/Illness: In the unlikely event of injury or medical illness resulting from the above procedures, contact **Laura Stewart, Ph.D., 225-578-3549**. You will be referred for treatment, but the expense of medical treatment will be your responsibility. No compensation is available in case of study-related illness or injury.

8. Right to Refuse: You may choose not to participate or to withdraw from the study at any time without penalty or loss of any benefit to which you might otherwise be entitled.

9. Privacy: Your identity will remain confidential unless disclosure is required by law. In other words, data will be kept confidential unless release is legally compelled. All data collection will be handled only by the investigators and kept in a secure location. Results of the study may be published using group means only and names or identifying information will not be included in the publication.

10. Financial Information: These tests are provided at no cost to you, nor is there any compensation for participating in the study outside of the results of your personal tests.

11. Signatures: The study has been discussed with me and all my questions have been answered. I may direct additional questions regarding study specifics to the investigators. If I have any questions about subjects' rights or other concerns, I can contact Robert C. Matthews, Institutional Review Board at 225.578.8692. I agree to participate in the study described above and acknowledge the investigators' obligation to provide me with a signed copy of this consent form.

_____ _____

Participant's Signature Date

The study subject has indicated that s/he is unable to read. I certify that I have read this consent form to the subject and explained that by completing the signature line above, the subject has agreed to participate.

_____ _____

Reader's Signature Date

STUDY APPROVED BY:
Dr. Robert C. Mathews, Chairman
Institutional Review Board
Louisiana State University
130 David Boyd Hall
225-578-8692 / www.lsu.edu/irb
Approval Expires: 2/13/2015

1.2 Medical History Form

Medical History Information	Place Patient Identification Sticker here

ACROSTIC: _____

Please complete the following questionnaire as completely and accurately as possible. All of your information will be kept CONFIDENTIAL and will be used by the researchers to ensure your safety.

If you have questions, please ask a staff member for assistance.

CURRENT MEDICAL STATUS			
1.	**PRESENT Medical Problems:** Do you have any known significant medical problems <u>at the present time</u>? (including any problems that require ongoing medical treatment or problems that cause you to miss work.) ☐ Yes ☐ No If yes, please list the medical condition and the date of onset.		
	CONDITION	DATE OF ONSET	COMMENTS
1a.			
1b.			
1c.			
1d.			
1e.			

PAST MEDICAL HISTORY			
2.	**Significant PAST Illnesses**: Have you had any other significant illnesses <u>in the past</u> (including any illnesses requiring hospitalization or ongoing Medical Treatment and excluding common illnesses such as Chicken Pox or strep throat)? ☐ Yes ☐ No If yes, please list the illness and the year(s) it occurred.		
	ILLNESS	YEAR(S)	COMMENTS
2a.			
2b.			
2c.			
2d.			
2e.			

Medical History Information	Place Patient Identification Sticker here

3.	Past Surgery: Have you had any surgeries? ☐ Yes ☐ No If yes, please list the surgeries in chronological order and the year(s) the surgery was performed.		
	TYPE OF SURGERY	YEAR(S)	COMMENTS
3a.			
3b.			
3c.			
3d.			

4.	Diagnostic Procedures: Please indicate whether or not you have undergone any of the following diagnostic procedures. Please indicate the year(s) the procedure(s) was performed.					
		YES	NO	UNSURE	YEAR(S)	COMMENTS
4a.	ECG or EKG (Electrocardiography)	☐	☐	☐		
4b.	Exercise Stress Test	☐	☐	☐		
4c.	Ultrasound or echocardiogram examination of the heart	☐	☐	☐		
4d.	Heart Catherization (Dye test of heart vessels)	☐	☐	☐		
4e.	MRI	☐	☐	☐		
4f.	CAT Scan	☐	☐	☐		

Medical History Information	Place Patient Identification Sticker here

REVIEW OF MEDICAL HISTORY						
5.	**Review of Medical History**: Please indicate whether you have ever been **DIAGNOSED** with any of the symptoms or conditions listed below. If yes, list the year of onset.					
		YES	NO	UNSURE	YEAR(S)	COMMENTS
5a.	Heart attack	☐	☐	☐		
5b.	High blood pressure	☐	☐	☐		
5c.	Heart valve disease	☐	☐	☐		
5d.	Calf pain with exercise	☐	☐	☐		
5e.	Stroke or TIA	☐	☐	☐		
5f.	High blood cholesterol	☐	☐	☐		
5g.	High blood triglycerides	☐	☐	☐		
5h.	Any type of cancer	☐	☐	☐		
5i.	Thyroid disease	☐	☐	☐		
5j.	High blood sugar	☐	☐	☐		
5k.	Type 2 Diabetes	☐	☐	☐		
5l.	Asthma	☐	☐	☐		
5m.	Ulcer disease	☐	☐	☐		
5n.	HIV Positive / AIDS/Tuberculosis	☐	☐	☐		
5o.	Arthritis	☐	☐	☐		
5p.	Seizures or epilepsy	☐	☐	☐		
5q.	Hospitalization for psychiatric or psychological disorder	☐	☐	☐		
5r.	Treatment for Depression	☐	☐	☐		
5s.	Bleeding or clotting disorder	☐	☐	☐		

Medical History Information	Place Patient Identification Sticker here

REVIEW OF MEDICAL HISTORY CONTINUED			
6.	**Review of Medical History Continued**: Please indicate whether you have ever been **DIAGNOSED** with any of the symptoms or conditions listed below.		
		YES	NO
6a.	Are you currently physically active for 3 or more days each week for 20 minutes or more each time?	☐	☐
6b.	Are you currently using insulin?	☐	☐
6c.	Have you been hospitalized for depression in last 6 months?	☐	☐
6d.	Have you lost 20 pounds or more in the last year?	☐	☐
6e.	Are you currently using weight loss medications?	☐	☐
6f.	Have you been diagnosed with schizophrenia or bipolar disorder?	☐	☐
6g.	Are members of your household participating in DOMS?	☐	☐
6h.	Have you had bariatric surgery (stomach stapling, gastric bypass)?	☐	☐
6i.	Are you pregnant/lactating?	☐	☐
6j.	Do you have a history of arrhythmias (abnormal heart rhythms), cardiomyopathy (enlarged heart), congestive heart failure (heart does not pump enough blood to the rest of your body), or aortic aneurysm (weakened and bulging area in a large blood vessel near your heart)?	☐	☐
6k.	Do you have renal (kidney) disease, or are you currently receiving dialysis?	☐	☐
6L.	Have you had a heart transplant?	☐	☐
6m.	Do you have chronic obstructive lung disease (chronic bronchitis and emphysema), peripheral vascular disease (disease of the blood vessels outside the heart and brain) or angina (chest pain) that limits your ability to exercise?	☐	☐
6n.	Do you have advanced neuropathy (nerve damage) or retinopathy (retinal damage)?	☐	☐

Medical History Information	Place Patient Identification Sticker here

REVIEW OF MEDICAL SYMPTOMS								
7.	**Review of Medical Symptoms**: Please indicate whether you have ever had a **SIGNIFICANT PROBLEM** with any of the symptoms or conditions listed below. Note: If yes, list year of onset.							
		YES	NO	UNSURE	YEAR	IS THIS STILL A PROBLEM?		COMMENTS
						YES	NO	
7a.	Chest pain or pressure	☐	☐	☐		☐	☐	
7b.	Chest pain with exertion	☐	☐	☐		☐	☐	
7c.	Rapid or irregular heartbeats	☐	☐	☐		☐	☐	
7d.	Fainting or lightheadedness	☐	☐	☐		☐	☐	
7e.	Calf pain with exertion	☐	☐	☐		☐	☐	
7f.	Unexplained shortness of breath while sleeping	☐	☐	☐		☐	☐	
7g.	Unexplained shortness of breath while sitting	☐	☐	☐		☐	☐	
7h.	Unexplained shortness of breath with physical activity	☐	☐	☐		☐	☐	
7i.	Frequent heartburn	☐	☐	☐		☐	☐	
7j.	Chronic joint or muscle pain	☐	☐	☐		☐	☐	
7k.	Low back pain	☐	☐	☐		☐	☐	
7l.	Anxiety	☐	☐	☐		☐	☐	
7m.	Thoughts of suicide	☐	☐	☐		☐	☐	

Medical History Information	Place Patient Identification Sticker here

OBSTETRIC AND GYNECOLOGIC HISTORY (WOMEN ONLY)

Obstetric and Gynecologic History (WOMEN ONLY): Please answer the following questions.

		YES	NO	DURATION MONTHS	DURATION YEARS
8.	Have you ever been pregnant? **If no**, skip to question 9.	☐	☐		
	If yes, how many pregnancies have you had? _____				
9.	Have you ever taken birth control pills? **If no**, skip to question **10**.	☐	☐		
	If yes, what is the total length of time you have used birth control?				
	Do you currently take birth control pills?	☐	☐		
10.	Have you had a hysterectomy (surgery to remove your uterus)?	☐	☐		
11.	Have you had a surgery to remove both ovaries?	☐	☐		
12.	Have you ever taken estrogen pills or hormone replacement therapy? **If no**, skip to question **13.**	☐	☐		
	If yes, what is the total length of time you used hormone replacement therapy?				
13.	Are you currently taking hormone replacement therapy?	☐	☐		

FAMILY MEDICAL HISTORY

14. **Parents:** Provide information for biological parents.

		AGE ONLY IF LIVING	AGE AT DEATH	WORST HEALTH PROBLEM OR CAUSE OF DEATH (Mark all that apply)		If other, Describe.
14a.	Biological Father			☐ Diabetes	☐ High Cholesterol	
				☐ Heart Attack	☐ Colon Cancer	
				☐ Stroke	☐ Other	
				☐ Hypertension		
14b.	Biological Mother			☐ Diabetes	☐ High Cholesterol	
				☐ Heart Attack	☐ Breast Cancer	
				☐ Stroke	☐ Colon Cancer	
				☐ Hypertension	☐ Other	

FAMILY MEDICAL HISTORY

Medical History Information	Place Patient Identification Sticker here

15.	Brothers / Sisters: Provide information for all biological siblings					
		AGE ONLY IF LIVING	AGE AT DEATH	WORST HEALTH PROBLEM OR CAUSE OF DEATH (Mark all that apply)		If other, Describe.
15a	☐ Brother ☐ Sister			☐ Diabetes	☐ High Cholesterol	
				☐ Heart Attack	☐ Breast Cancer	
				☐ Stroke	☐ Colon Cancer	
				☐ Hypertension	☐ Other	
15b	☐ Brother ☐ Sister			☐ Diabetes	☐ High Cholesterol	
				☐ Heart Attack	☐ Breast Cancer	
				☐ Stroke	☐ Colon Cancer	
				☐ Hypertension	☐ Other	
15c.	☐ Brother ☐ Sister			☐ Diabetes	☐ High Cholesterol	
				☐ Heart Attack	☐ Breast Cancer	
				☐ Stroke	☐ Colon Cancer	
				☐ Hypertension	☐ Other	
15d.	☐ Brother ☐ Sister			☐ Diabetes	☐ High Cholesterol	
				☐ Heart Attack	☐ Breast Cancer	
				☐ Stroke	☐ Colon Cancer	
				☐ Hypertension	☐ Other	
15e.	☐ Brother ☐ Sister			☐ Diabetes	☐ High Cholesterol	
				☐ Heart Attack	☐ Breast Cancer	
				☐ Stroke	☐ Colon Cancer	
				☐ Hypertension	☐ Other	
15f.	☐ Brother ☐ Sister			☐ Diabetes	☐ High Cholesterol	
				☐ Heart Attack	☐ Breast Cancer	
				☐ Stroke	☐ Colon Cancer	
				☐ Hypertension	☐ Other	
15g.	☐ Brother ☐ Sister			☐ Diabetes	☐ High Cholesterol	
				☐ Heart Attack	☐ Breast Cancer	
				☐ Stroke	☐ Colon Cancer	
				☐ Hypertension	☐ Other	

PERSONAL HABITS

Medical History Information	Place Patient Identification Sticker here

	Tobacco: Please answer the following questions.					
		YES	NO	DURATION		NUMBER OF CIGARETTES PER DAY
				MONTHS	YEARS	
16.	Do you currently use tobacco? **If no, skip to question 17.**	☐	☐			
	If yes: How many cigarettes do you smoke per day?					
	How long have you been smoking?					
17.	Have you used cigarettes in the past, but do not use them now? **If no, skip to question 18.**	☐	☐			
	If yes: How many cigarettes per day did you smoke?					
	How long did you smoke cigarettes?					

	Alcohol: Please answer the following questions to the best of your knowledge.					
		YES	NO	DURATION		NUMBER OF DRINKS PER WEEK
				MONTHS	YEARS	
18.	Do you drink alcoholic beverages? **If no, skip to question 19.**	☐	☐			
	If yes, how many drinks per week? 18a. Beer (12 oz)					
	18b. Wine (5 oz)					
	18c. Hard Liquor (1.5 oz)					
19.	Have you used alcohol in the past but subsequently quit?	☐	☐			
20.	Do you now have or have you ever had problems with excessive alcohol use?	☐	☐			

1.3 Physical Activity Readiness Questionnaire

Physical Activity Readiness
Questionnaire - PAR-Q
(revised 2002)

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

YES	NO		
☐	☐	1.	**Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?**
☐	☐	2.	**Do you feel pain in your chest when you do physical activity?**
☐	☐	3.	**In the past month, have you had chest pain when you were not doing physical activity?**
☐	☐	4.	**Do you lose your balance because of dizziness or do you ever lose consciousness?**
☐	☐	5.	**Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?**
☐	☐	6.	**Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?**
☐	☐	7.	**Do you know of any other reason why you should not do physical activity?**

If you answered

YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:

- start becoming much more physically active – begin slowly and build up gradually. This is the safest and easiest way to go.
- take part in a fitness appraisal – this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

DELAY BECOMING MUCH MORE ACTIVE:

- if you are not feeling well because of a temporary illness such as a cold or a fever – wait until you feel better; or
- if you are or may be pregnant – talk to your doctor before you start becoming more active.

PLEASE NOTE: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Informed Use of the PAR-Q: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

NOTE: If the PAR-Q is being given to a person before he or she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

NAME _____

SIGNATURE _____ DATE _____

SIGNATURE OF PARENT _____ WITNESS _____
or GUARDIAN (for participants under the age of majority)

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.

Supported by: Health Canada Santé Canada

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1.4 International Physical Activity Questionnaire

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

   _____ **days per week**

   ☐ No vigorous physical activities → ***Skip to question 3***

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

   _____ **hours per day**

   _____ **minutes per day**

   ☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

   _____ **days per week**

   ☐ No moderate physical activities → ***Skip to question 5***

SHORT LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised August 2002.

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

SHORT LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised August 2002.

1.5 Sun Exposure Survey

Sun Exposure Log

ID:_____ Dates: From _____ to _____

	Time	Exposure	Total
Monday			
Sunday			
Saturday			
Friday			
Thursday			
Wednesday			
Tuesday			

Time	Exposure
<5 min/day = 0 points	Face and hands only = 1 point
5-30 min = 1 point	Face, hands, arms = 2 points
>30 min = 2 points	Face, hands, arms, legs = 3 points
	Bathing suit = 4 points

1.6 Diet Log

Diet Log

Use the following to track what you ate over the course of a week. Be sure to be as detailed as possible – don't just put "cereal," put what type of cereal and how much you had. (Ex. If you had a glass of milk, was it 2% or skim? 8 oz or more? Be aware the serving size listed on the box is usually much smaller than what you would normally eat.)

	Breakfast	Lunch	Dinner	Snacks
Monday				
Tuesday				
Wednesday				
Thursday				
Friday				
Saturday				
Sunday				

APPENDIX 2 – RAW DATA

Age

Trained/Low	Age	Trained/High	Age
T01	21	T03	21
T02	21	T04	26
T06	26	T05	21
T09	21	T07	20
T11	21	T08	27
T12	20	T10	23
T13	21	T15	21
T14	32	T16	21
T17	24	T18	20
T21	25	T20	21
T26	20	T23	22
U03	21	T25	21
U14	21	T27	20
U16	21	T28	21
U26	22	U02	21

Untrained/Low	Age	Untrained/High	Age
U05	22	T19	21
U06	22	T30	21
U10	21	U01	22
U11	21	U04	35
U13	30	U07	22
U17	21	U08	21
U19	21	U09	21
U20	21	U12	21
U22	23	U15	21
U25	20	U18	22
U27	21	U21	21
U28	21	U23	21
U29	19	U30	20
U38	21	U31	21
		U33	22
		U34	20
		U35	21
		U37	21
		U39	20

Weight (lbs)

Trained/Low	Weight	Trained/High	Weight
T01	132.25	T03	148
T02	126	T04	123
T06	143	T05	130
T09	123.75	T07	142.25
T11	107.25	T08	155.5
T12	112.5	T10	149.25
T13	123.75	T15	124
T14	136	T16	144
T17	132.5	T18	124.5
T21	145	T20	125.5
T26	124.5	T23	147
U03	103	T25	111.75
U14	134	T27	163.5
U16	142.75	T28	125.75
U26	127	U02	116.25

Untrained/Low	Weight	Untrained/High	Weight
U05	108	T19	166.25
U06	169.5	T30	144
U10	232.5	U01	145
U11	136	U04	126.75
U13	255	U07	150
U17	130.25	U08	114.5
U19	132	U09	126.25
U20	138	U12	128.75
U22	126.5	U15	144.5
U25	117.5	U18	124.5
U27	200	U21	152.25
U28	223.75	U23	103.75
U29	204	U30	122.75
U38	123	U31	114.25
		U33	119.5
		U34	165.75
		U35	150.75
		U37	128.25
		U39	120.25

Height (in)

Trained/Low	Height	Trained/High	Height
T01	61	T03	63
T02	63.5	T04	64
T06	66	T05	64.75
T09	60.25	T07	65.5
T11	61.5	T08	66.25
T12	60	T10	67.25
T13	65.25	T15	59
T14	64	T16	67.5
T17	60	T18	66
T21	68	T20	60.25
T26	63.25	T23	64.5
U03	58.5	T25	65.5
U14	63.5	T27	69.5
U16	65.75	T28	65.75
U26	66.5	U02	65

Untrained/Low	Height	Untrained/High	Height
U05	62.5	T19	65.5
U06	64.75	T30	64
U10	67.5	U01	67
U11	64.5	U04	60
U13	68.25	U07	61
U17	63	U08	63
U19	62	U09	63
U20	67.25	U12	61.25
U22	66	U15	64
U25	63	U18	64.5
U27	61	U21	63.5
U28	64	U23	62
U29	65	U30	65.5
U38	61	U31	63
		U33	62.5
		U34	62
		U35	63
		U37	64.75
		U39	64

BMI

Trained/Low	BMI	Trained/High	BMI
T01	25.00	T03	26.21
T02	21.97	T04	21.11
T06	23.08	T05	21.80
T09	23.98	T07	23.30
T11	19.93	T08	24.92
T12	21.97	T10	23.19
T13	20.43	T15	25.04
T14	23.34	T16	22.22
T17	25.87	T18	20.09
T21	22.04	T20	24.30
T26	21.88	T23	24.84
U03	21.16	T25	18.31
U14	23.36	T27	23.80
U16	23.21	T28	20.45
U26	20.19	U02	19.35

Untrained/Low	BMI	Untrained/High	BMI
U05	19.44	T19	27.24
U06	28.42	T30	24.71
U10	35.87	U01	22.71
U11	22.98	U04	24.75
U13	38.48	U07	28.34
U17	23.06	U08	20.28
U19	24.14	U09	22.36
U20	21.45	U12	24.13
U22	20.42	U15	24.80
U25	20.81	U18	21.04
U27	37.79	U21	26.54
U28	38.40	U23	18.97
U29	33.94	U30	20.11
U38	23.24	U31	20.24
		U33	21.51
		U34	30.31
		U35	26.70
		U37	21.50
		U39	20.64

Waist Circumference (in)

Trained/Low	Waist	Trained/High	Waist
T01	26.5	T03	28
T02	26.5	T04	26
T06	28	T05	28
T09	25.5	T07	27
T11	23.5	T08	28
T12	25	T10	29
T13	28.5	T15	28
T14	28	T16	27.5
T17	28	T18	24
T21	27.5	T20	27.5
T26	25	T23	29
U03	26	T25	24
U14	28.5	T27	27
U16	28.5	T28	25
U26	25	U02	26.5

Untrained/Low	Waist	Untrained/High	Waist
U05	23	T19	30
U06	32.5	T30	29
U10	38	U01	27.25
U11	25.5	U04	26
U13	40	U07	28.5
U17	27	U08	26
U19	27.5	U09	25.5
U20	27.5	U12	27.5
U22	26	U15	27
U25	26	U18	25.5
U27	34	U21	30
U28	35	U23	24
U29	35	U30	23
U38	26	U31	24.5
		U33	24.5
		U34	33
		U35	27.5
		U37	25
		U39	24.5

Hip Circumference (in)

Trained/Low	Hip	Trained/High	Hip
T01	36	T03	33.5
T02	32.5	T04	32
T06	37.5	T05	34
T09	30	T07	35
T11	30.5	T08	35.5
T12	31	T10	36
T13	32.5	T15	32.5
T14	33	T16	35
T17	34	T18	34
T21	35.5	T20	32.5
T26	32	T23	35
U03	31	T25	32
U14	33	T27	37
U16	35	T28	34
U26	33	U02	34.5

Untrained/Low	Hip	Untrained/High	Hip
U05	31	T19	37.5
U06	36.75	T30	35
U10	42.5	U01	35.0
U11	32	U04	33
U13	51	U07	35.5
U17	33	U08	33
U19	36	U09	33
U20	36	U12	32
U22	33	U15	34
U25	31	U18	35
U27	40	U21	37
U28	42	U23	31
U29	39	U30	31
U38	32	U31	32
		U33	32
		U34	38
		U35	37.5
		U37	32.5
		U39	31

Waist:Hip Ratio

Trained/Low	W:H	Trained/High	W:H
T01	0.736	T03	0.836
T02	0.815	T04	0.813
T06	0.733	T05	0.824
T09	0.850	T07	0.771
T11	0.770	T08	0.789
T12	0.806	T10	0.806
T13	0.877	T15	0.862
T14	0.848	T16	0.786
T17	0.824	T18	0.706
T21	0.775	T20	0.846
T26	0.781	T23	0.829
U03	0.839	T25	0.750
U14	0.864	T27	0.730
U16	0.814	T28	0.735
U26	0.758	U02	0.768

Untrained/Low	W:H	Untrained/High	W:H
U05	0.742	T19	0.800
U06	0.884	T30	0.829
U10	0.894	U01	0.779
U11	0.797	U04	0.788
U13	0.784	U07	0.803
U17	0.818	U08	0.788
U19	0.764	U09	0.773
U20	0.775	U12	0.859
U22	0.788	U15	0.794
U25	0.839	U18	0.729
U27	0.850	U21	0.811
U28	0.833	U23	0.768
U29	0.897	U30	0.742
U38	0.813	U31	0.766
		U33	0.766
		U34	0.868
		U35	0.733
		U37	0.769
		U39	0.790

Estimated Body Fat (%)

Trained/Low	Body Fat	Trained/High	Body Fat
T01	30.9	T03	28.6
T02	25.2	T04	24.6
T06	30.5	T05	33.9
T09	31.6	T07	30.3
T11	28.8	T08	39.5
T12	27.6	T10	35.4
T13	34.5	T15	32.6
T14	39.0	T16	34.5
T17	32.9	T18	32.1
T21	34.1	T20	34.1
T26	28.9	T23	38.8
U03	24.7	T25	23.1
U14	35.6	T27	33.3
U16	39.3	T28	28.5
U26	33.1	U02	28.1

Untrained/Low	Body Fat	Untrained/High	Body Fat
U05	29.4	T19	40.4
U06	42.9	T30	39.6
U10	41.1	U01	29.8
U11	27.7	U04	37.9
U13	58.7	U07	39.1
U17	35.0	U08	26.9
U19	41.9	U09	12.8
U20	34.3	U12	32.2
U22	39.4	U15	35.9
U25	24.2	U18	32.8
U27	51.3	U21	44.9
U28	51.2	U23	30.8
U29	47.6	U30	24.0
U38	39.8	U31	27.5
		U33	37.9
		U34	49.5
		U35	40.7
		U37	31.2
		U39	33.7

Serum 25OHD Concentration (ng/mL)

Trained/Low	25OHD	Trained/High	25OHD
T01	26.964	T03	61.986
T02	27.270	T04	61.215
T06	30.248	T05	74.638
T09	22.920	T07	42.648
T11	23.330	T08	84.955
T12	20.160	T10	33.555
T13	19.555	T15	33.619
T14	22.810	T16	41.041
T17	24.654	T18	54.474
T21	30.565	T20	48.247
T26	16.215	T23	39.255
U03	23.117	T25	39.278
U14	17.062	T27	54.634
U16	14.752	T28	48.290
U26	12.572	U02	42.609

Untrained/Low	25OHD	Untrained/High	25OHD
U05	14.657	T19	29.149
U06	10.647	T30	35.910
U10	14.665	U01	61.319
U11	19.693	U04	24.990
U13	13.920	U07	21.936
U17	12.572	U08	20.560
U19	6.495	U09	22.466
U20	1.990	U12	49.184
U22	6.229	U15	61.478
U25	5.542	U18	44.025
U27	13.804	U21	45.665
U28	15.932	U23	35.288
U29	14.912	U30	34.068
U38	11.092	U31	25.620
		U33	21.079
		U34	33.550
		U35	46.203
		U37	27.848
		U39	44.730

Sun Exposure Survey Scores

Trained/Low	Score	Trained/High	Score
T01	44	T03	24
T02	26	T04	42
T06	27	T05	30
T09	38	T07	35
T11	48	T08	37
T12	38	T10	36
T13	34	T15	39
T14	34	T16	42
T17	24	T18	30
T21	44	T20	30
T26	30	T23	34
U03	26	T25	35
U14	30	T27	30
U16	46	T28	44
U26	31	U02	26

Untrained/Low	Score	Untrained/High	Score
U05	42	T19	20
U06	28	T30	32
U10	30	U01	38
U11	32	U04	30
U13	24	U07	24
U17	42	U08	46
U19	24	U09	21
U20	42	U12	30
U22	44	U15	28
U25	36	U18	30
U27	36	U21	39
U28	23	U23	39
U29	27	U30	42
U38	35	U31	40
		U33	19
		U34	38
		U35	22
		U37	33
		U39	23

Time Spent Outdoors (min/week)

Trained/Low	Time	Trained/High	Time
T01	1260	T03	260
T02	120	T04	270
T06	480	T05	195
T09	330	T07	540
T11	780	T08	395
T12	395	T10	1380
T13	395	T15	745
T14	550	T16	945
T17	240	T18	450
T21	660	T20	480
T26	315	T23	590
U03	170	T25	540
U14	260	T27	472
U16	375	T28	900
U26	770	U02	310

Untrained/Low	Time	Untrained/High	Time
U05	630	T19	660
U06	603	T30	650
U10	875	U01	420
U11	550	U04	540
U13	275	U07	592
U17	685	U08	795
U19	135	U09	255
U20	510	U12	740
U22	1900	U15	315
U25	465	U18	635
U27	400	U21	870
U28	185	U23	1650
U29	260	U30	1050
U38	550	U31	1445
		U33	360
		U34	550
		U35	360
		U37	460
		U39	520

Dietary Intake (IU/3 days)

Trained/Low	IU	Trained/High	IU
T01	436	T03	479
T02	522	T04	58
T06	162	T05	119
T09	322	T07	752
T11	187	T08	189
T12	453	T10	293
T13	66	T15	669
T14	517	T16	178
T17	216	T18	224
T21	717	T20	249
T26	1754	T23	978
U03	1388	T25	480
U14	261	T27	95
U16	59	T28	222
U26	61	U02	165

Untrained/Low	IU	Untrained/High	IU
U05	265	T19	266
U06	74	T30	1785
U10	134	U01	303
U11	452	U04	108
U13	452	U07	441
U17	225	U08	117
U19	223	U09	42
U20	269	U12	181
U22	471	U15	635
U25	3111	U18	279
U27	2082	U21	450
U28	89	U23	412
U29	347	U30	1017
U38	462	U31	54
		U33	162
		U34	450
		U35	135
		U37	1203
		U39	336

VO_2peak (mL/kg/min)

Trained/Low	VO_2	Trained/High	VO_2
T01	48.5	T03	54.7
T02	46.3	T04	48.7
T06	46.2	T05	39.0
T09	45.3	T07	42.6
T11	45.4	T08	43.6
T12	45.4	T10	39.7
T13	37.9	T15	49.8
T14	46.6	T16	35.7
T17	40.6	T18	40.1
T21	47.0	T20	37.9
T26	46.2	T23	36.4
U03	41.9	T25	35.5
U14	46.5	T27	38.2
U16	42.6	T28	41.7
U26	46.1	U02	40.9

Untrained/Low	VO_2	Untrained/High	VO_2
U05	39.2	T19	33.9
U06	37.7	T30	36.0
U10	30.3	U01	26.3
U11	37.6	U04	38.4
U13	23.0	U07	36.4
U17	38.2	U08	29.3
U19	39.4	U09	27.5
U20	37.0	U12	36.7
U22	38.8	U15	35.5
U25	27.0	U18	37.6
U27	31.5	U21	26.9
U28	21.2	U23	31.7
U29	33.8	U30	38.6
U38	36.5	U31	37.4
		U33	28.7
		U34	22.9
		U35	25.6
		U37	29.0
		U39	27.1

Peak Power Output (w)

Trained/Low	w	Trained/High	w
T01	370.27	T03	474.01
T02	403.50	T04	344.24
T06	567.95	T05	311.86
T09	494.97	T07	454.72
T11	299.85	T08	497.69
T12	405.33	T10	417.65
T13	395.82	T15	396.62
T14	326.25	T16	460.59
T17	476.78	T18	348.44
T21	289.87	T20	351.20
T26	298.66	T23	528.96
U03	329.88	T25	268.07
U14	482.18	T27	522.96
U16	342.44	T28	452.49
U26	304.66	U02	464.84

Untrained/Low	w	Untrained/High	w
U05	344.57	T19	531.72
U06	232.52	T30	460.59
U10	650.69	U01	289.87
U11	326.25	U04	304.06
U13	509.73	U07	480.32
U17	416.45	U08	366.22
U19	316.66	U09	403.78
U20	441.40	U12	514.26
U22	252.88	U15	462.15
U25	234.89	U18	448.00
U27	319.85	U21	304.36
U28	355.47	U23	290.37
U29	407.81	U30	392.62
U38	393.42	U31	365.47
		U33	430.00
		U34	331.32
		U35	421.91
		U37	358.94
		U39	384.62

Relative Peak Power Output (w/kg)

Trained/Low	w	Trained/High	w
T01	6.17	T03	7.06
T02	7.06	T04	6.18
T06	8.76	T05	5.30
T09	8.83	T07	7.05
T11	6.17	T08	7.06
T12	7.94	T10	6.17
T13	7.06	T15	7.06
T14	5.30	T16	7.06
T17	7.94	T18	6.18
T21	4.41	T20	6.18
T26	5.30	T23	7.94
U03	7.06	T25	5.30
U14	7.95	T27	7.06
U16	5.29	T28	7.94
U26	5.30	U02	8.82

Untrained/Low	w	Untrained/High	w
U05	7.04	T19	7.06
U06	3.03	T30	7.06
U10	6.18	U01	4.41
U11	5.30	U04	5.30
U13	4.41	U07	7.06
U17	7.06	U08	7.06
U19	5.29	U09	7.06
U20	7.06	U12	8.82
U22	4.41	U15	7.06
U25	4.41	U18	7.94
U27	3.53	U21	4.41
U28	3.53	U23	6.18
U29	4.41	U30	7.06
U38	7.06	U31	7.06
		U33	7.94
		U34	4.41
		U35	6.18
		U37	6.18
		U39	7.06

Anaerobic Capacity (kJ)

Trained/Low	kJ	Trained/High	kJ
T01	9.77	T03	10.96
T02	10.34	T04	6.88
T06	12.56	T05	9.10
T09	11.14	T07	10.52
T11	7.71	T08	13.69
T12	7.71	T10	9.84
T13	8.91	T15	9.67
T14	8.97	T16	9.50
T17	10.60	T18	9.21
T21	8.41	T20	8.53
T26	7.96	T23	10.87
U03	8.65	T25	5.36
U14	9.91	T27	11.44
U16	8.85	T28	10.56
U26	7.62	U02	10.46

Untrained/Low	kJ	Untrained/High	kJ
U05	7.11	T19	13.29
U06	5.15	T30	10.08
U10	14.41	U01	7.54
U11	8.97	U04	7.09
U13	11.21	U07	10.51
U17	10.15	U08	6.87
U19	7.12	U09	10.10
U20	9.38	U12	11.57
U22	6.07	U15	10.40
U25	5.87	U18	9.71
U27	7.20	U21	7.00
U28	8.00	U23	6.43
U29	7.75	U30	8.83
U38	9.34	U31	7.54
		U33	10.51
		U34	7.29
		U35	9.34
		U37	8.72
		U39	9.14

Relative Anaerobic Capacity (kJ/kg)

Trained/Low	kJ/kg	Trained/High	kJ/kg
T01	0.163	T03	0.163
T02	0.181	T04	0.124
T06	0.194	T05	0.154
T09	0.199	T07	0.163
T11	0.159	T08	0.194
T12	0.194	T10	0.146
T13	0.159	T15	0.172
T14	0.146	T16	0.146
T17	0.177	T18	0.163
T21	0.128	T20	0.150
T26	0.141	T23	0.163
U03	0.185	T25	0.106
U14	0.163	T27	0.155
U16	0.137	T28	0.185
U26	0.132	U02	0.199

Untrained/Low	kJ/kg	Untrained/High	kJ/kg
U05	0.145	T19	0.177
U06	0.067	T30	0.155
U10	0.137	U01	0.115
U11	0.146	U04	0.124
U13	0.097	U07	0.154
U17	0.172	U08	0.132
U19	0.119	U09	0.177
U20	0.150	U12	0.198
U22	0.106	U15	0.159
U25	0.110	U18	0.172
U27	0.079	U21	0.102
U28	0.794	U23	0.137
U29	0.084	U30	0.159
U38	0.168	U31	0.146
		U33	0.194
		U34	0.097
		U35	0.137
		U37	0.150
		U39	0.168

Fatigue Index (%)

Trained/Low	kJ/kg	Trained/High	kJ/kg
T01	42.9	T03	37.5
T02	25.0	T04	17.4
T06	25.0	T05	16.7
T09	40.0	T07	50.0
T11	14.3	T08	25.0
T12	33.3	T10	42.8
T13	37.5	T15	50.0
T14	16.7	T16	50.0
T17	44.4	T18	14.3
T21	47.0	T20	42.9
T26	33.3	T23	55.6
U03	25.0	T25	50.0
U14	66.7	T27	50.0
U16	33.3	T28	33.3
U26	33.3	U02	50.0

Untrained/Low	kJ/kg	Untrained/High	kJ/kg
U05	50.0	T19	37.5
U06	42.9	T30	50.0
U10	42.6	U01	40.0
U11	16.7	U04	50.0
U13	40.0	U07	50.0
U17	37.5	U08	62.5
U19	33.3	U09	37.5
U20	62.5	U12	50.0
U22	40.0	U15	37.5
U25	40.0	U18	55.6
U27	50.0	U21	40.0
U28	50.0	U23	28.6
U29	60.0	U30	50.0
U38	37.5	U31	50.0
		U33	33.3
		U34	40.0
		U35	42.9
		U37	42.9
		U39	42.9

Total Monocytes

Trained/Low	Count	Trained/High	Count
T01	20077	T03	14943
T02	11409	T04	13455
T06	19716	T05	24994
T09	15646	T07	14124
T11	26679	T08	22567
T12	15555	T10	18132
T13	18716	T15	35656
T14	23951	T16	13071
T17	19844	T18	19154
T21	17936	T20	26384
T26	16994	T23	17804
U03	10701	T25	17155
U14	19067	T27	8573
U16	13641	T28	20903
U26	26510	U02	17352

Untrained/Low	Count	Untrained/High	Count
U05	22648	T19	16802
U06	23529	T30	30044
U10	28509	U01	13447
U11	26005	U04	12386
U13	33222	U07	20364
U17	9015	U08	12277
U19	19257	U09	26673
U20	15831	U12	15868
U22	23802	U15	8668
U25	22501	U18	22690
U27	30749	U21	16487
U28	20307	U23	18077
U29	25788	U30	14437
U38	16693	U31	16409
		U33	18781
		U34	16701
		U35	16559
		U37	20251
		U39	9292

CD14+CD16+

Trained/Low	Count	Trained/High	Count
T01	17961	T03	13098
T02	8345	T04	11169
T06	16668	T05	20459
T09	13571	T07	12570
T11	20715	T08	18718
T12	13442	T10	15880
T13	16854	T15	31866
T14	20855	T16	10041
T17	14527	T18	16232
T21	15679	T20	19445
T26	14288	T23	14526
U03	7657	T25	13895
U14	13154	T27	7063
U16	12332	T28	15342
U26	22926	U02	15740

Untrained/Low	Count	Untrained/High	Count
U05	18438	T19	12311
U06	21234	T30	27096
U10	25141	U01	11383
U11	23980	U04	10382
U13	28389	U07	17430
U17	7658	U08	10336
U19	16013	U09	22943
U20	12205	U12	13915
U22	14623	U15	6514
U25	17076	U18	20072
U27	26913	U21	14829
U28	17272	U23	15185
U29	22402	U30	12093
U38	14600	U31	9206
		U33	15991
		U34	14478
		U35	9483
		U37	18363
		U39	7645

CD14++CD16+

Trained/Low	Count	Trained/High	Count
T01	378	T03	18
T02	529	T04	558
T06	610	T05	1109
T09	478	T07	305
T11	721	T08	853
T12	391	T10	563
T13	516	T15	739
T14	880	T16	359
T17	1114	T18	286
T21	458	T20	4231
T26	509	T23	684
U03	407	T25	261
U14	4644	T27	224
U16	297	T28	463
U26	424	U02	307

Untrained/Low	Count	Untrained/High	Count
U05	621	T19	739
U06	367	T30	600
U10	790	U01	469
U11	352	U04	467
U13	2229	U07	326
U17	229	U08	613
U19	584	U09	1042
U20	1365	U12	507
U22	5704	U15	312
U25	642	U18	347
U27	1431	U21	457
U28	453	U23	836
U29	929	U30	342
U38	326	U31	236
		U33	511
		U34	647
		U35	380
		U37	340
		U39	394

CD14+CD16++

Trained/Low	Count	Trained/High	Count
T01	27	T03	0
T02	1505	T04	1328
T06	1672	T05	2824
T09	1157	T07	844
T11	4550	T08	2248
T12	1040	T10	1338
T13	904	T15	2215
T14	1505	T16	489
T17	3595	T18	1279
T21	1238	T20	2194
T26	1604	T23	1628
U03	1204	T25	2576
U14	906	T27	1027
U16	906	T28	1276
U26	2169	U02	613

Untrained/Low	Count	Untrained/High	Count
U05	3017	T19	1427
U06	1354	T30	1061
U10	1611	U01	988
U11	778	U04	819
U13	2157	U07	2165
U17	615	U08	888
U19	1120	U09	1933
U20	805	U12	16
U22	2640	U15	1282
U25	2079	U18	991
U27	1499	U21	1
U28	81	U23	620
U29	1791	U30	1291
U38	1108	U31	446
		U33	1626
		U34	118
		U35	1124
		U37	1061
		U39	830

CD14+CD16- TLR4+ (MFC)

Trained/Low	MFC	Trained/High	MFC
T01	11.55	T03	11.14
T02	11.97	T04	11.55
T06	11.72	T05	11.97
T09	13.82	T07	10.75
T11	10.75	T08	11.97
T12	10.37	T10	12.41
T13	11.14	T15	12.86
T14	12.86	T16	16.55
T17	10.75	T18	10.00
T21	10.00	T20	11.55
T26	11.97	T23	10.37
U03	14.33	T25	11.97
U14	12.41	T27	11.97
U16	8.66	T28	14.86
U26	10.75	U02	12.41

Untrained/Low	MFC	Untrained/High	MFC
U05	15.40	T19	12.86
U06	10.75	T30	9.65
U10	10.00	U01	11.14
U11	11.97	U04	10.37
U13	11.14	U07	10.75
U17	10.37	U08	9.31
U19	14.33	U09	8.98
U20	10.75	U12	11.14
U22	12.86	U15	11.97
U25	11.55	U18	11.56
U27	13.34	U21	11.14
U28	9.65	U23	12.86
U29	14.33	U30	11.55
U38	9.65	U31	10.37
		U33	10.00
		U34	13.34
		U35	11.55
		U37	11.14
		U39	10.00

CD14+CD16+ TLR4+ (MFC)

Trained/Low	MFC	Trained/High	MFC
T01	13.34	T03	31.62
T02	18.43	T04	13.82
T06	16.03	T05	15.96
T09	19.81	T07	13.82
T11	12.86	T08	13.82
T12	12.86	T10	16.55
T13	13.82	T15	15.40
T14	17.15	T16	22.07
T17	11.14	T18	13.82
T21	11.55	T20	14.33
T26	12.86	T23	12.86
U03	22.07	T25	14.33
U14	16.55	T27	13.10
U16	10.00	T28	17.78
U26	12.86	U02	15.96

Untrained/Low	MFC	Untrained/High	MFC
U05	24.58	T19	17.15
U06	14.33	T30	12.41
U10	11.97	U01	13.34
U11	15.40	U04	13.82
U13	14.33	U07	13.82
U17	16.55	U08	12.41
U19	21.29	U09	10.37
U20	33.98	U12	12.86
U22	15.40	U15	15.96
U25	12.86	U18	15.40
U27	17.78	U21	14.33
U28	11.55	U23	13.82
U29	18.43	U30	13.82
U38	11.55	U31	13.34
		U33	12.86
		U34	15.96
		U35	14.86
		U37	12.86
		U39	10.37

CRP (mg/L)

Trained/Low	CRP	Trained/High	CRP
T01	0.097	T03	0.661
T02	0.149	T04	0.832
T06	0.364	T05	0.670
T09	0.166	T07	0.660
T11	0.474	T08	0.684
T12	0.065	T10	0.126
T13	0.245	T15	2.568
T14	0.281	T16	4.499
T17	0.063	T18	0.174
T21	3.993	T20	5.194
T26	0.175	T23	0.150
U03	5.621	T25	1.360
U14	2.413	T27	0.832
U16	0.756	T28	0.431
U26	3.053	U02	1.784

Untrained/Low	CRP	Untrained/High	CRP
U05	0.114	T19	2.905
U06	1.408	T30	0.172
U10	0.224	U01	0.881
U11	0.081	U04	0.010
U13	1.640	U07	4.850
U17	0.255	U08	0.366
U19	1.392	U09	0.247
U20	0.221	U12	1.062
U22	2.203	U15	0.563
U25	1.990	U18	0.119
U27	4.349	U21	6.433
U28	2.223	U23	2.439
U29	1.238	U30	0.372
U38	1.118	U31	0.118
		U33	0.134
		U34	5.017
		U35	4.581
		U37	2.284
		U39	0.522

IL-6 Control (pg/mL)

Trained/Low	CTRL	Trained/High	CTRL
T01	35.855	T03	37.416
T02	46.560	T04	89.062
T06	36.251	T05	65.943
T09	96.498	T07	64.273
T11	63.432	T08	15.226
T12	17.277	T10	23.699
T13	22.164	T15	75.653
T14	68.643	T16	10.617
T17	26.092	T18	52.418
T21	44.197	T20	34.545
T26	15.797	T23	37.926
U03	2.542	T25	49.249
U14	2.569	T27	22.652
U16	1.104	T28	21.414
U26	22.346	U02	8.651

Untrained/Low	CTRL	Untrained/High	CTRL
U05	31.482	T19	30.170
U06	3.914	T30	17.689
U10	50.879	U01	79.768
U11	78.045	U04	98.831
U13	31.480	U07	9.594
U17	3.665	U08	17.279
U19	18.299	U09	40.137
U20	65.881	U12	17.020
U22	314.551	U15	3.665
U25	112.590	U18	54.901
U27	14.445	U21	388.792
U28	43.744	U23	23.226
U29	157.262	U30	54.956
U38	52.205	U31	56.837
		U33	50.268
		U34	10.368
		U35	15.022
		U37	76.668
		U39	16.231

IL-6 Stimulated (pg/mL)

Trained/Low	STIM	Trained/High	STIM
T01	2635.3	T03	4638.4
T02	3761.0	T04	1014.8
T06	4698.4	T05	1767.9
T09	6802.4	T07	2905.0
T11	5959.6	T08	4022.2
T12	1420.4	T10	6626.1
T13	4091.7	T15	4745.4
T14	1420.4	T16	3779.0
T17	7857.2	T18	5377.9
T21	6873.7	T20	6873.3
T26	1519.4	T23	2826.1
U03	3847.6	T25	5483.9
U14	1701.6	T27	3793.1
U16	3830.1	T28	4967.2
U26	5039.7	U02	4074.8

Untrained/Low	STIM	Untrained/High	STIM
U05	2885.6	T19	2133.2
U06	3691.6	T30	9013.5
U10	9552.4	U01	2736.3
U11	5195.1	U04	14859.6
U13	5579.4	U07	4925.3
U17	2433.8	U08	2905.0
U19	5015.4	U09	4461.0
U20	5450.2	U12	4478.7
U22	10354.9	U15	7088.3
U25	6758.5	U18	7870.0
U27	4131.0	U21	12976.3
U28	4208.5	U23	4564.4
U29	4137.0	U30	7553.8
U38	7686.6	U31	2887.5
		U33	3367.0
		U34	6781.8
		U35	3730.9
		U37	7551.6
		U39	2466.0

IL-6 Production (pg/mL)

Trained/Low	IL-6	Trained/High	IL-6
T01	2599.4	T03	4601.0
T02	3714.4	T04	925.7
T06	4662.1	T05	1702.0
T09	6705.9	T07	2840.7
T11	5896.2	T08	4007.0
T12	1403.1	T10	6602.4
T13	4069.5	T15	4669.7
T14	1351.8	T16	3768.4
T17	7831.1	T18	5325.5
T21	6829.5	T20	6838.8
T26	1503.6	T23	2788.2
U03	3845.1	T25	5434.7
U14	1699.0	T27	3770.4
U16	3829.0	T28	4945.8
U26	5017.4	U02	4066.1

Untrained/Low	IL-6	Untrained/High	IL-6
U05	2854.1	T19	2103.0
U06	3687.7	T30	8995.8
U10	9501.5	U01	2656.5
U11	5117.1	U04	14760.8
U13	5547.9	U07	4915.7
U17	2430.1	U08	2887.7
U19	4997.1	U09	4420.9
U20	5384.3	U12	4461.7
U22	10040.3	U15	7084.6
U25	6645.9	U18	7815.1
U27	4116.6	U21	12587.5
U28	4164.8	U23	4541.2
U29	3979.7	U30	7498.8
U38	7634.4	U31	2830.7
		U33	3316.7
		U34	6771.4
		U35	3715.9
		U37	7474.9
		U39	2449.8

APPENDIX 3 – PROTOCOLS

3.1 FACS Analysis

Materials

- Blood collection tube, EDTA treated (Becton Dickinson, Franklin Lakes, NJ)
- 12x75 mm Falcon tubes (Becton Dickinson, Franklin Lakes, NJ)
- Human Fc Receptor Blocking Inhibitor (eBioscience, San Diego, CA)
- CD14-FITC antibody (eBioscience, San Diego, CA)
- CD16-PE antibody (eBioscience, San Diego, CA)
- CD284 (TLR4)-APC antibody (eBioscience, San Diego, CA)
- Mouse IgG1 FITC isotype control antibody (eBioscience, San Diego, CA)
- Mouse IgG1 PE isotype control antibody (eBioscience, San Diego, CA)
- Mouse IgG2a APC isotype control antibody (eBioscience, San Diego, CA)
- Red blood cell lysis buffer (Sigma Aldrich, St. Louis, MO)
- 1x PBS
- 1-2% formaldehyde (37% diluted with PBS; Sigma Aldrich, St. Louis, MO)

Methods

1. Collect blood sample following 10 hour fast in EDTA treated tubes
2. Label tubes for isotype controls and test samples, as well as tubes for a single autofluorescent control and single-color controls. Only one set of autofluorescent and single-color controls need to be provided for each set of samples.
3. Aliquot 100 μL of blood from each sample into both control and test tubes. Blood from any sample may be used for autofluorescent and single-color controls.

4. Pipette 20 µL of blocking inhibitor to each sample and incubate at room temperature for 20 minutes. Vortex to mix.
5. Pipette 5 µL of each antibody into test sample tubes and 5 µL of control antibody into isotype control tubes. Use 5 µL of PBS for the autofluorescent control, and 5 µL of the respective test antibody for each single-color control. Incubate at room temperature in the dark for 30 minutes. Vortex to mix.
6. Pipette 2 mL of RBC lysis buffer into each tube and invert to mix. Incubate at room temperature for 10 minutes in the dark.
7. Centrifuge at 1000 xg for 8 minutes at room temperature. Decant the supernatant into a beaker containing bleach. Do not pipette supernatant.
8. Pipette 2 mL of 1x PBS to each tube. Vortex to mix to dislodge cell pellet.
9. Centrifuge at 1000 xg for 8 minutes at room temperature. Decant the supernatant into a beaker containing bleach. Do not pipette supernatant.
10. Vortex cell pellet in remaining liquid. Pipette 200 µL of 1-2% formaldehyde in a dropwise fashion to fix cells.
11. Samples are ready for analysis. Store at 2-8°C until analysis.

3.2 LPS Cell Stimulation

Materials

- Blood collection tubes, sodium heparin treated (Becton Dickinson, Franklin Lakes, NJ)
- Lipopolysaccharide, 1 mg/mL (*S. enteriditis*; Sigma Aldrich, St. Louis, MO)
- RPMI-1640 cell culture media (Sigma Aldrich, St. Louis, MO)

- L-glutamine (200 mM)/penicillin (10,000 IU)/streptomycin (10 mg/mL) solution (Sigma Aldrich, St. Louis, MO)
- 24 well plates, 2 mL each (VWR, Radnor, PA)
- Incubator at 37°C with 5% CO_2
- 1.5 mL tubes

Methods

1. Collect blood sample following 10 hour fast in sodium heparin treated tubes.
2. Note: all steps following collection should be carried out in a fume hood. Prepare cell culture media in a 1:100 dilution with glutamine/penicillin/streptomycin mixture. Blood is diluted 1:10 with cell culture media, so a minimum of 3.6 mL RPMI is needed per sample.
3. Prepare a 1:10 dilution of blood samples with RPMI treated with glutamine/penicillin/streptomycin. Samples should be plated in duplicate, so a minimum of 4 mL of prepared blood is necessary.
4. Plate 2 mL of blood in duplicate on plate.
5. Treat stimulated wells with 50 µL of LPS, for a final concentration of 25 µg/mL. Mix well.
6. Treat control wells with 50 µL of RPMI media. Mix well.
7. Incubate at 37°C for 24 hours.
8. Following incubation, centrifuge plate at 800 xg at 4°C for 10 minutes.
9. Harvest supernatant in 1.5 mL tubes.
10. Freeze at -80°C until analysis.

11. Prior to analysis for inflammatory cytokine production, samples should be centrifuged at 800 xg at 4°C to eliminate unavoidable cellular debris.

APPENDIX 4 – ABBREVIATIONS

1,25OH2D	1,25-dihydroxyvitamin D
25OHD	25-hydroxyvitamin D
7DHC	7-dehydrocholesterol
ACSM	American College of Sports Medicine
AI	Adequate Intake
BMI	Body mass index
BMD	Bone mineral density
CRF	Cardiorespiratory fitness
CRP	C-reactive protein
CVD	Cardiovascular disease
DBP	D-binding protein
DRI	Daily Recommended Intake
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
HD	High vitamin D group
IFN-y	Interferon-gamma
IL-10	Interleukin-10
IL-1b	Interleukin-1 beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IOM	Institute of Medicine
IU	International Units
LD	Low vitamin D group
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
NHANES	National Health and Nutrition Examination Survey
NPA	Not physically active groups
NPA-H	Not physically active & high vitamin D group
NPA-L	Not physically active & low vitamin D group
PA	Physically active group
PA-H	Physically active & high vitamin D group
PA-L	Physically active & low vitamin D group
PTH	Parathyroid hormone
RDA	Recommended Dietary Allowance
RPE	Rate of Perceived Exertion

TLR4	Toll-like receptor 4
TNF-a	Tumor necrosis factor-alpha
UV	Ultraviolet
VDR	Vitamin D receptor
W:H	Waist-to-hip ratio

VITA

Laura was born in 1987 in Bloomington, Indiana. She was raised by her parents, Chuck and Marla, and has one younger brother, Will. From an early age, Laura enjoyed physical activity of all sorts, including participating in a variety of organized sports and playing outside with friends. She also established a love for learning, enjoying her time at school and cultivating her love of reading.

Laura graduated with honors from high school in 2006, when she began her undergraduate degree in Biological Sciences at Purdue University. During her time at Purdue, she discovered her interest in research, while also spending many extracurricular hours doing clinical work and becoming a long-distance runner. Through her undergraduate research mentor Dr. Howie Zelaznik, she was introduced to the field of Exercise Physiology, combining her interests in biology, physical activity, and patient care.

Upon graduation from Purdue in 2010, Laura began the doctoral program in the School of Kinesiology at LSU with the mentorship of Dr. Laura Stewart. Throughout her time at graduate school, she completed many hours of bench work with Dr. Tara Henagan at Pennington Biomedical Research Center. This time gave her experience to examine physiological phenomena at the cellular level. Laura finished her Masters in Kinesiology in 2012, en route to finishing her Ph.D.

After finishing her Doctoral degree, Laura plans on pursuing a career in laboratory research. Laura was married to her college boyfriend, Matt, in 2012. They have a dog, Scout, who knows how to get into a lot of trouble but also loves snuggling.