FIT OR FAT: THE RELATIONSHIP OF INFLAMMATION, INTRAMUSCULAR FAT AND MUSCLE, AND MOBILITY FUNCTION IN OLDER ADULTS

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Intramuscular adipose tissue (IMAT) is an important negative predictor of muscle and mobility function in older adults. While IMAT has been theorized to increase with aging, more recent evidence suggests that IMAT may be a byproduct of inactivity rather than aging per se. The mechanism of IMAT's influence on muscle and mobility function is unknown but similar to the negative consequences attributed to other ectopic fat depots, may involve an inflammatory pathway. In order to clarify if IMAT is an obligatory consequence of age or if it is related to inactivity, the primary aim of this study was to compare IMAT, muscle and mobility function, and muscular inflammation in age and BMI matched older frail and nonfrail adults. A secondary aim was to examine the relationship of IMAT and visceral adipose tissue (VAT), muscular inflammation, muscle function and mobility function in a subgroup of older adults. Two groups of older nonobese adults were recruited. A sedentary frail group was classified as at least moderately frail with modified physical performance test scores of < 25 and an active nonfrail group had modified physical performance test scores of > 32. Magnetic resonance imaging (MRI) was used to determine mean cross sectional area of thigh IMAT and VAT. Maximal voluntary isometric contractions were used to determine muscle function, and 6 minute walk and stair ascent time were used to determine mobility function. A sub group of participants from both groups who were free from disease and medications known to influence inflammation underwent percutaneous muscle biopsies

of the vastus lateralis. Western blot and real time polymerase chain reaction were used to determined local muscular expression of mRNA and protein for the proinflammatory cytokines interleukin 6 (IL-6) and tumor necrosis alpha (TNF-a). We found significant differences in IMAT, and IL-6 mRNA and protein expression between frail and nonfrail adults. We also found significant correlations between IMAT, VAT, IL-6, and muscle and mobility function. These findings suggest that IMAT is a product of inactivity and disease rather then aging alone and provide a potential mechanistic link between increased IMAT and decreased muscle and mobility function in older adults.

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CHAPTER 1

INTRODUCTION

Muscle's structural composition is an important factor underlying muscle strength and physical function in older adults. There is an increasing amount of research to support the clear disassociation between the loss of muscle lean tissue mass and strength with aging.[1-3] This disassociation between lean tissue and strength implies factors other than lean muscle mass alone are responsible for the decreases in strength and function seen with aging. Intramuscular adipose tissue (IMAT), or the fat found beneath the fascia of a muscle, is a significant predictor of both muscle function and mobility function in older adults.[4-7]

The majority of the work concerning the relationship of IMAT with muscle and physical function has to date been reported from the Health ABC study.[4, 8-10] In this large study of over 2,500 healthy nonmobility limited individuals age 70-79 Goodpaster et al. first reported that increased levels of IMAT were associated with both decreased muscle strength and muscle quality, or the force produced per unit of muscle area.[4] In this same study population of older adults Visser et al. found that individuals with the highest levels of IMAT also had the lowest mobility scores,[7] and in a follow up study 2.5 years later that those with the highest amounts of IMAT and the lowest quartile of strength were most at risk for developing self reported mobility limitations in the future.[9]

In addition to the Health ABC studies, smaller clinical studies have also demonstrated similar relationships between IMAT and mobility function in older adults. Hilton et al. showed that in 9 obese individuals with diabetes IMAT levels are highly correlated (r=-0.92) with mobility function as measured by the physical performance test.[5] More recently, in the only study to date to look at mobility limited individuals, Marcus et al. examined the relationship of IMAT with mobility and strength in older mobility limited adults.[6] In this study of over 100 mobility limited individuals, multiple regression analysis was used to examine the relationships between IMAT, lean muscle tissue, strength, BMI and mobility function (as measured by six minute walk, timed up and go, stair ascent and stair descent). In every analysis IMAT was one of, if not the most, robust predictors of mobility function.[6] Taken together, these studies have demonstrated a clear relationship between IMAT and muscle and mobility function in older adults and IMAT is now recognized as a significant structural component associated with muscle related dysfunctions in this vulnerable population.

The reason for increased IMAT levels in older adults however, is unknown. There is ongoing debate about if IMAT is an inevitable consequence of aging or alternatively a consequence of inactivity that often accompanies aging. Numerous studies theorize that an increase in IMAT is an associated consequence of aging.[1, 11-13] Marcus et al. found a moderate correlation (r=0.47) between aging and IMAT[12] and Gallagher et al. demonstrated an average increase of 9g/year in IMAT in sedentary adults spanning from 20 to 80 years old.[11] In perhaps the most compelling study to date examining aging and IMAT, Delmonico et al. reported, a consistent increase of 16-75% in IMAT over 5 years in healthy adults between the ages of 70 and 79 (n>1500).[1] This increase in IMAT was

consistently found in those who were weight stable, those who gained weight (a 75% increase in IMAT) and surprisingly even in those who lost weight (a 16% increase in IMAT). However this study did not fully account for disease state or activity levels and more recent studies[14-16] have suggested that increased levels of IMAT may be instigated by either or both of these variables.

Increased IMAT is found in individuals with a higher body mass index[4] and in those with numerous chronic disease conditions that are often associated with decreased activity levels such as chronic kidney disease[17] and diabetes.[18-20] Higher levels of IMAT are also noted postspinal cord injury[21] and postcerebrovascular accident[22] and it has been speculated that increased levels of IMAT postinjury are related to decreased muscle activity, as spasticity can blunt the increase in IMAT.[23] Manini et al. also found increased IMAT levels in the thigh and calf muscles of young (19-28 years old) individuals after 30 days of single limb suspension. While muscle volume decreased 7.4% and 7.7% in the thigh and calf respectively, IMAT volume increased 15% in the thigh and 20% in the calf suggesting that even in young healthy individuals increased IMAT is a consequence of decreased activity levels. Additionally, the relative accumulation of IMAT, in this study, exceeded the loss of muscle volume in both the thigh and calf. A regression analysis demonstrated that the loss of muscle mass was only able to explain 26% of the variance in IMAT accumulation suggesting that the accumulation of IMAT is more than just a replacement of lost muscle mass. Further evidence to support the notion that increased IMAT is a consequence of inactivity rather then aging per se can be found in cross sectional studies of older adults examining activity levels and IMAT. Tuttle et al. found a moderate relationship in older obese adults with diabetes between the volume of IMAT in

the calf and the number of steps taken per day ($r=-0.44$, $p<0.05$).[15] Wroblewski et al. conducted a cross sectional study that examined the relationship of IMAT and age in master athletes. This cross sectional study examined 40 master athletes age 40-81 categorized into 10 year age groups 40-49,50-59, 60-69 and 70 plus. They reported no significant differences in the amount of IMAT between the age groups and concluded that increases in IMAT are not inevitable with aging but are likely related to factors such as muscle disuse and disease.

The finding that physical activity can mitigate increased IMAT provides additional evidence linking inactivity and IMAT accumulation. Goodpaster et al. identified that walking two times per week for 12 months resulted in a blunting of increases in IMAT in the midthigh of healthy mobile older adults age 70-89. The control group in this study, who did not participate in any physical activity intervention, had an average increase of 18% in IMAT over 12 months. However, the active intervention group that walked an average of just two times per week for the 12 months had only a 2% increase in IMAT. Furthermore multiple studies have demonstrated that aerobic, resistive, or the combination of the two results in decreases in IMAT levels post training.[12, 24-27] Marcus et al. reported an 11% decrease in IMAT with 12 weeks of resistance training in older individuals with multiple comorbidities[12] and Durheim et al. found that 8-9 months of aerobic training resulted in decreases in IMAT in older men.[24]

IMAT is consistently negatively associated with muscle and mobility function.[4, 5, 7, 9, 14] While it is becoming increasingly clear that IMAT may be more a product of the inactivity that often accompanies aging rather than simply age itself, whether IMAT is merely a marker of muscle dysfunction or if it plays a more active role in muscle and

mobility dysfunction remains unknown. The potential role of IMAT, and adipose tissue in general is evolving.

Adipose tissue is no longer considered to be an inert storage depot for extra calories but is now known to be an active endocrine organ that, among other things plays an important role as the initiator of the chronic inflammatory response.[28, 29] Chronic inflammation, a long term elevation of proinflammatory cytokines, is one of the most important physiological correlates of the frailty syndrome.[30, 31] High levels of proinflammatory cytokines in older individuals are related to an increased risk of mortality,[32] sarcopenia,[33, 34] reduced muscle strength,[10] and decreased mobility.[35, 36] Visceral adipose tissue (VAT) is an active endocrine tissue that secretes inflammatory cytokines and the amount of VAT is strongly related to systemic chronic inflammatory levels.[37] Like VAT, IMAT may also be a metabolically active component of muscle and a potential source of inflammatory regulation in older individuals.[1, 8, 14, 38, 39] While IMAT is a much smaller fat depot then subcutaneous or visceral adipose tissue, it has been associated with systemic levels of the proinflammatory cytokines tumor necrosis alpha (TNF-a), Interleukin-6 (IL-6), and c-reactive protein (CRP).[38] TNF-a, IL-6, and CRP are proinflammatory cytokines released from adipose tissue whose basal levels have been closely associated with muscle and mobility function. While it has been theorized by multiple authors[1, 8, 14, 38, 39] that IMAT's negative relationship with muscle and mobility function may be attributed to IMAT's release of proinflammatory cytokines within the muscle, to date this relationship has not been clarified. Increased levels of inflammation in the subcutaneous fat as well as in the serum have been associated with increased levels of IMAT.[38, 39] The independent parallels between inflammation and IMAT, with strength

and mobility loss in older individuals, may in fact be linked by the release of inflammatory cytokines from IMAT.

In this dissertation I will examine the relationship of IMAT with local (as measured in the muscle) levels of inflammatory cytokines. I will also examine the relationship of IMAT, inflammation, muscle and mobility function in older frail and nonfrail individuals. The first chapter of this dissertation will be focused on a review of the relationships between inflammation, aging, adiposity, and muscle and mobility function in older adults. The second chapter will examine the relationships of IMAT and gene expression of IL-6 and TNF-a (both proinflammatory cytokines) with muscle and mobility function in both frail and nonfrail older individuals. My third chapter will examine location specific fat depots and their relationship with local (as measured in the muscle) proinflammatory mRNA and protein expression in older frail and nonfrail adults. A discussion of each of these studies as well as their implications and future recommendations will be provided within each section.

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CHAPTER 2

INFLAMMATION, AGING, AND ADIPOSITY: IMPLICATIONS FOR PHYSICAL THERAPISTS

Odessa Addison, Paul C. LaStayo, Leland E. Dibble, Robin L. Marcus

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Special Interest Papers

Inflammation, Aging, and Adiposity: Implications for Physical Therapists

Odessa Addison, DPT[1]; Paul C. LaStayo, PT, PhD,[1,2,3]; Leland E. Dibble, PT, PhD,[1,2]; Robin L. Marcus, PT, PhD[1,2]

ABSTRACT

Background: Physical therapists treat older individuals, characterized as both a needy and expanding population. Frailty, a predisability condition with links to chronic inflammatory conditions, is estimated to affect 7% of individuals older than 60 years and 40% of people older than 80 years. Chronic inflammation is one of the most important physiologic correlates of the frailty syndrome and high levels of proinflammatory cytokines, related to both aging and increasing adiposity in older individuals are related to an increased risk of mortality, sarcopenia, reduced muscle strength and decreased mobility.

Purpose: The purpose of this narrative review is to inform the physical therapist of the effects of aging and increasing adiposity on chronic inflammation and the association of inflammation with muscle loss, strength, and mobility impairments in older adults; and to review the current evidence to provide clinical recommendations on physical activity and exercise regimes that may mitigate chronic inflammation in older adults.

Discussion: As physical therapists help manage and treat an increasingly older population, understanding how the inflammatory milieu changes with aging and increasing adiposity and how these changes can be impacted by physical therapists via exercise and physical activity is critical.

Conclusion: Exercise is a potent preventive intervention strategy and countermeasure for chronic inflammation and adiposity. Exercise can also benefit the frail older individual by combating the negative effects of chronic inflammation and optimally balancing the production of pro and anti-inflammatory cytokines. In addition to providing an anti-inflammatory environment within muscle to mitigate the effects of chronic inflammation, exercise has the added benefit of improving muscle mass and function and decreasing adiposity in older adults.

Key Words: chronic inflammation, frailty, exercise, muscle

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INTRODUCTION

Physical therapists are increasingly treating an older population. By the year 2030 it is expected that 1 in 5 Americans will be older than 65 years and over the next decade there will be a 15% increase in the number of Americans older than 85 years.[1] Frailty, a predisability condition with links to chronic inflammatory conditions, is estimated to affect 7% of individuals older than 60 years and 40% of people older than 80 years.[2] Frailty is marked by dysfunction and decline across multiple physiologic systems including the neuromuscular, neuroendocrine, and immune system,[3] and can result in significant health consequences such as frequent falls,[4,5] fractures,[4,5] and an increased risk of mortality.[6,7] Chronic inflammation is one of the most important physiologic correlates of the frailty syndrome[8,9] and high levels of proinflammatory cytokines in older individuals are related to an increased risk of mortality,[10] sarcopenia,[11,12] reduced muscle strength,[13] and decreased mobility.[14,15] Physical therapists help manage and treat an increasingly older population; therefore, understanding how the inflammatory milieu changes with aging and how it can be impacted by physical therapists via exercise and physical activity is important.

Chronically elevated systemic levels of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and c-reactive protein (CRP) play a central role in the etiology of numerous chronic diseases commonly seen in rehabilitation settings including type 2 diabetes,[16] cardiovascular[17-19] and cerebrovascular disease,[20] dementia,[21] and sarcopenia.[22-24] Cytokines are hormone like proteins that are involved in cell-to-cell communication that regulate the intensity and duration of the immune response. Transient increases in the proinflammatory cytokine cascade associated with injury and illness are part of the normal healing process and assist the immune system in promoting tissue repair and regeneration by activation of the complement system and increased phagocytosis. Long-term elevation of proinflammatory cytokines, however, are detrimental leading ultimately to tissue damage,[23,24] muscle loss,[13] and even death.[10]

When tissue is injured due to trauma or illness macrophages are activated to assist in tissue repair and regeneration (Figure 1). Activated macrophages secrete various cytokines that have autocrine, paracrine, and

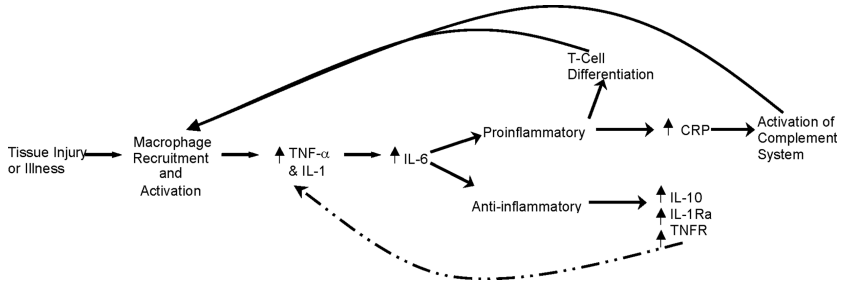

Figure 1. The inflammatory pathway. In the normal inflammation pathway, tissue injury or illness results in macrophage recruitment and activation. Activation of macrophages leads to an increase in multiple proinflammatory cytokines including IL-6 that increases T-cell differentiation and recruitment of further macrophages. Interleukin-6 also serves as an anti-inflammatory cytokine recruiting other anti-inflammatory cytokines to bring an end to the inflammatory process. Chronic inflammation such that may occur with aging and/or increasing fat mass results in excessive macrophage recruitment and activation leading to an abnormal increase in proinflammatory cytokines. Because of currently unknown reasons, in chronic inflammation, the cytokines produced by the anti-inflammatory pathway are unable to bring a halt to the inflammation, creating a positive feedback loop that facilitates on-going inflammation.

endocrine function. The first actions of these cytokines are to influence the cells that produced them and then go on to affect surrounding cells as well. Eventually the production of these cytokines results in a spill over from the tissue in which they are produced to increases in circulating levels in serum which allow these cytokines to act in an endocrine function. Among the first cytokines released by macrophages are TNF-α and interluekin-1 (IL-1). Both are powerful proinflammatory cytokines that result in production of further cytokines such as IL-6 as well as the recruitment of additional macrophages and an induction of acute phase protein synthesis.

Interleukin-6 has been classified as both a "pro" and an "anti" inflammatory cytokine. When produced in response to TNF-α production from macrophages IL-6 results in T-cell differentiation and assists T-cells in resisting apoptosis. Interleukin-6 production also results in the production of CRP by hepatocytes and activation of the complement system resulting in increased phagocytic activity of immune cells. Besides these proinflammatory effects, IL-6 also has several powerful anti-inflammatory properties. The release of IL-6 also results in an increase in the production of IL-10, tumor necrosis factor soluble receptor, and IL-1 receptor agonist, all of which assist in decreasing the production of TNF-α and IL-1 and limit the proinflammatory cascade. More recent work has also demonstrated that IL-6 can be produced by muscle in a TNF-α independent manner. Repetitive muscle contraction results in the production of IL-6 without an increase in TNF-α (Figure 2). In fact, production of IL-6 via the TNF-α independent pathway actually suppresses the production of TNF-α resulting in a powerful anti-inflammatory pathway with exercise. The contradictory effects of IL-6 are most likely due to the environment in which it is produced and the presence or absence of TNF-α.

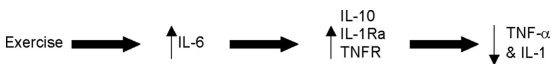

Figure 2. Interleukin-6 production in the TNF-α independent pathway. In the TNF-α independent pathway, contracting myocytes during exercise produce IL-6 in the absence of TNF-α. This leads to an increase in multiple anti-inflammatory cytokines and ultimately a decrease in proinflammatory cytokines such as TNF-α and IL-1.

Systemically elevated inflammation in older adults is particularly troublesome as it is one of the most important physiological correlates of the frailty syndrome.[8,9] Older adults with chronic increases in proinflammatory cytokines are more likely to suffer from a loss of lean tissue[13] and strength,[13] and to experience mobility limitations and disability[22] than those with lower levels of inflammation. Moreover, several studies have demonstrated that older adults with chronically elevated levels of inflammation are at an increased risk of mortality.[6,10,25] In a large study of nondisabled older adults, Harris et al[10] found that those older individuals in the highest quartile for IL-6 serum markers of inflammation were twice as likely to die in the next 4 years than those individuals in the lowest quartile of inflammation. Although increased levels of inflammatory cytokines are associated with increasing age, chronic inflammation may not be an obligatory manifestation of aging per se. Evidence suggests that older individuals with higher activity levels consistently show lower levels of both systemic[26-28] (as measured in the blood) and regional[29] (as measured in the muscle) inflammation. In addition, several studies have demonstrated that aerobic training[30-40] and strength training[41,42] can diminish chronic inflammation in older adults. Because the positive inflammatory impact resulting from increased physical activity and exercise are integral components of physical therapy management in older adults, an awareness of how chronic inflammation is impacted by these variables is important.

The purpose of this narrative review is 3-fold. First, to inform the physical therapist of the effects of aging and increasing adiposity on chronic inflammation and the association of inflammation with muscle loss, strength, and mobility impairments in older adults; and second, to review the current evidence on the impact of physical activity levels and

exercise on chronic inflammation in this population. Finally, we will provide evidence-based clinical recommendations for physical activity and exercise regimes with the aim of mitigating chronic inflammation in older adults. Literature targeted for this narrative review included peer reviewed cross-sectional, longitudinal, epidemiologic, and clinical studies in humans and animals that were related to aging, adiposity, inflammation, mobility, strength, physical activity, and exercise.

AGING AND INFLAMMATION

The relationship of systemic inflammation with increasing age is currently a hotly debated topic. Some have reported consistent positive correlations between aging and inflammation,[43-49] though not all studies have found this to be the case.[50-53] It has been suggested that aging-related increases in proinflammatory cytokines may be due to the presence of multiple comorbidities,[45,50] dysregulation of the immune system,[54-56] or an increase in adipose tissue.[57] Multiple studies have demonstrated an age-related increase in the proinflammatory markers of IL-6, and CRP.[43-49] Wei et al[49], McKane et al[47], and Hager et al[46] have independently reported significant positive correlations between serum IL-6 and age; this relationship also exists in nonhumans.[44] The caveat of most of these studies is that few took into consideration the influence of disease state, comorbidities, and adipose tissue on the relationship between aging and the specific inflammatory cytokines studied. Cartier et al and Ferrucci et al both found an initial positive relationship between aging and inflammation; however, further statistical analysis established that this relationship was greatly blunted after accounting for the amount of visceral adipose tissue[43] or cardiovascular risk factors[45], thus exposing the possibility that it is comorbid disease conditions or increases in adipose tissue rather than age itself that drives these relationships. That is, the increase in proinflammatory cytokines seen with aging may in fact be attributed to underlying comorbidities such as cardiovascular disease or increases in adipose tissue.

Further support for this assertion is found in both clinical[50] and epidemiologic[45] evidence. Beharka et al[50] screened 20 young and 26 older males for chronic disease or illness prior to admission to their study and found no differences in circulating serum IL-6 between the young and old subjects. Because of the detailed screening process that was used in this study and not reported by previous authors, these findings suggest the possibility that previously reported associations between age and chronic inflammation may be at least in part attributed to the presence of underlying disease. Ferrucci et al[45] examined the relationship of proinflammatory cytokines with age in more than 1000 adults aged 20 to 102 years. In both men and women, older age was associated with increased circulating levels of IL-6 and CRP; however, when these results were adjusted for the presence of cardiovascular risk factors and of subclinical cardiovascular disease, the relationships between IL-6 and CRP with age, though still present, were much less robust.[45] Collectively, these results suggest that although the contribution of comorbid disease conditions to chronic inflammation in older adults should not be ignored, there does seem to be a degree of immune system dysregulation that occurs with advancing age.

Dysregulation of the immune system in older adults has been explored in multiple in vitro and in vivo studies. In vitro studies of cytokine production have been equivocal, as increased,[58-63] unaltered,[48,64] and decreased[50,65,66] cytokine production in older adults have been reported. Possible reasons for these differences include the time points at which cytokine production are measured, the types of cells studied, and the sex and health status of the participants. Fewer studies have examined the effects of aging on dysregulation of the immune system in vivo. When both young (20-27 years old) and older adults (61-69 years old) are given an endotoxin injection of *Escherichia coli*, the older adults consistently demonstrate a larger increase in body temperature and serum levels of TNF-α[56] when compared to younger individuals. Furthermore when examining both young and older adults who have been diagnosed with an acute pneumococcal infection older adults demonstrate an increased and prolonged immune response with higher levels of TNF-α and IL-6 than young infected and healthy age matched controls up to 7 days postinfection,[54,55] indicating a prolonged inflammatory response and possible dysregulation of the immune system. It has also been noted that older adults with gastric cancer have higher levels of IL-6 postsurgery and that these levels fell more slowly than younger patients with gastric cancer undergoing similar surgeries. Finally, Marik et al noted that the oldest patients (those older than 85 years) admitted to the intensive care unit for septic shock had the highest levels TNF-α on admission.[67] That older adults, both in the presence of and in the absence of disease, appear to have some level of immune system dysregulation is well supported in the literature, though the mechanism of this dysregulation is currently not well established. Possible reasons theorized for the apparent dysregulation include decreased production of sex steroids,[68] the presences of undiagnosed comorbidities such as atherosclerosis,[45] and higher amounts of adipose tissue.[57]

The increase in adipose tissue leading to immune system dysregulation is an attractive hypothesis as excessive adiposity has also been mentioned as a potential mechanism involved in age-related chronic inflammation.[43,68] Excessive fat mass and obesity are directly linked to increased levels of the circulating proinflammatory cytokines TNF-α, IL-6, and CRP in both the young[69] and old.[57,70] Studies have also demonstrated a decrease in these same cytokines with weight loss.[69,70] Currently, it is thought that as adipocytes increase in size with weight gain some adipocytes become dysfunctional due to the increased presence of lipids in the cell and local hypoxia within the adiopocyte.[71] This combination of dysfunction and hypoxia leads to death of the adipocyte. As adipocytes die macrophages are needed to assist in clearing the dead cells. This increased presence of macrophages leads to an increase of proinflammatory cytokines and in a feed forward manner recruits additional macrophages to the tissue, thus precipitating the increase

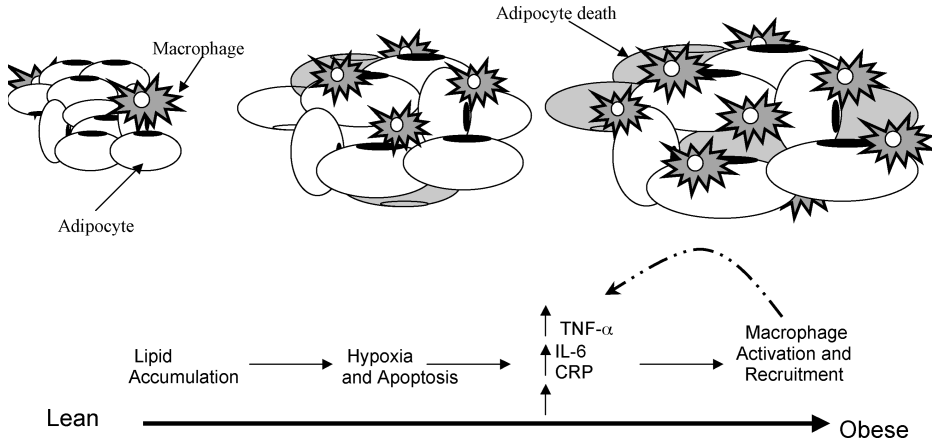

Figure 3. Increased inflammation with increasing adipose tissue. As an individual moves on the continuum between lean and obese, lipids accumulate in adipocytes, causing the adipocytes to enlarge. Some enlarged adipocytes experience local hypoxia from decreased access to the blood supply. The increased presence of lipids as well as local hypoxia leads to death of enlarged adipocytes (apoptosis). As adipocytes die local proinflammatory cytokine levels increase (TNF-α, IL-6, and CRP) to recruit macrophages to clear the dead adipocytes. The increased presence of macrophages leads to a further increase in proinflammatory cytokines and recruits further macrophages resulting in chronic inflammation.

in proinflammatory cytokines, leading to chronic inflammation (Figure 3).[71] Cesari et al have reported that both CRP and IL-6 serum levels are positively associated both with body mass index and with total fat mass in older individuals.[57] Taken together, this literature suggests that age-related immune system dysregulation may stem from increased body fat, a factor that has also been linked to chronic systemic inflammation in older adults.

Although the precise mechanisms behind age-related chronic inflammation are not fully understood, it does appear that the rise in proinflammatory cytokines observed with increasing age may result from of a combination of inherent immune system dysregulation, the presence of comorbid disease conditions, decreased physical activity, and increased fat mass as individuals age. Regardless of the cause, the relationships between chronic inflammation and many of the negative consequences of aging cannot be ignored. These consequences include but are not limited to increased risk for a loss of muscle mass and strength,[13] decreased mobility,[25] and ultimately an increased risk of frailty[22,72] and mortality.[6,10,25]

MUSCLE MASS, STRENGTH, AND INFLAMMATION

Although normal aging is accompanied by a progressive loss of muscle mass and force producing ability,[73] large epidemiological studies have suggested that relative to those with lower TNF-α and IL-6 levels, older adults with higher levels are at increased risk of muscle impairments. In a study of more than 3000 adults aged 70 to 79 years, Visser et al[13] reported that older adults with high levels of TNF-α or IL-6 (defined as levels above the population median) had smaller muscle area, lower appendicular muscle mass, and decreased grip and knee extensor strength. These findings were present even after statistically controlling for potential confounding factors such as age, height, total body fat, physical activity, health status, the use of anti-inflammatory medications, and smoking. The results were even more robust when looking at individuals who had increased levels of both IL-6 and TNF-α. Using this same population of individuals from the Health ABC study Schaap et al[12] used a multiple linear regression model to examine the effects of increased IL-6 and TNF-α on thigh muscle cross-sectional area as well as grip strength. Even after controlling for multiple covariates those individuals with the highest levels of these proinflammatory cytokines experienced a decrease in muscle cross-sectional area as well as grip strength over a 5-year time period.[12] Though the decrease in muscle cross-sectional area was attenuated when accounting for weight changes over the 5 years, a subanalysis of weight stable individuals revealed that higher TNF-α was again associated with decreased thigh muscle area and grip strength. Levinger et al[74] recently reported that 19 older patients with knee osteoarthritis had elevated levels in various inflammatory cytokines including IL-6 within the quadriceps when compared to age and body mass index–matched individuals without osteoarthritis. Though they did not report the association between IL-6 and muscle strength, they did observe a significant negative correlation ($r = -0.37$) between strength and Monocyte chemotactic protein-1 (MCP-1), an inflammatory protein, whose expression is increased by the presence of TNF-α and IL-6.[74]

Chronic inflammation may be mechanistically linked to muscle loss through the direct catabolic effects of TNF-α, originally known as cachectin, due to its direct contribution to catabolic muscle wasting in numerous inflammatory conditions such as chronic obstructive pulmonary disease,[75] chronic heart failure,[76] human immunodeficiency virus,[77] and cancer.[78] Animal models, through injection and infusion of TNF-α have revealed the direct catabolic effects of

increased levels of this proinflammatory cytokine. Mice, rat, and canine models have all demonstrated a loss of muscle mass directly related to the increased presence of TNF-α.[23,79,80] The loss of muscle mass that occurs in the presence of elevated TNF-α is most likely due to the multifactorial effect TNF-α has on muscle tissue. High levels of TNF-α result in cell apopotosis,[81] a decreased rate of protein synthesis, and an inhibition of myoblast differentiation[82] ultimately resulting in an impaired ability to increase muscle mass. In a small study of 8 older frail adults, Greiwe et al[41] demonstrated that the rate of muscle protein synthesis after 3 months of resistance training was inversely related ($r = -0.53$) to TNF-α protein levels in the muscle. Tumor necrosis factor-α may also inhibit muscle repair via apoptosis of muscle satellite cells[83] resulting in a slow but progressive loss of muscle mass over time as the muscle is unable to repair itself from micro-injuries resulting in an overall decline in muscle mass as one ages.

Solely attributing overall declines in muscle strength to loss of muscle mass in older adults is inaccurate as the aging-related loss of muscle mass explains as little as 5% of the accompanying loss of muscle strength.[84] Compounding muscle atrophy in older adults is a decrease in the specific force production of a muscle, defined as force produced per cross-sectional area of the muscle fiber.[85] Various factors may contribute to this decrease in specific force production including the slowing of muscle contractile properties and rate of force development, reduced rate of cross-bridge cycling, alterations in excitation-contraction coupling, and changes in the muscle architectural properties. Although age-related force production decline likely results from multiple factors including diminished physical activity, the impact of chronic inflammation should not be ignored. The presence of elevated levels of proinflammatory cytokines in older adults may help to explain some of the diminished force producing capability observed in this population.

Evidence supporting this assertion is found in animal models that have revealed decreased muscle specific force production only hours after a TNF-α injection.[86] Of note, this strength loss occurs prior to any loss of muscle mass, indicating that an inflammatory milieu contributes to impaired muscle force production in the absence of muscle atrophy. Furthermore, a 12-week resistance training study of frail elders with elevated baseline levels of TNF-α receptors (another measure of TNF-α activity), reported that the number of TNF-α receptors is inversely correlated with muscle strength gains.[87] Those with the highest levels of TNF-α receptors experienced the lowest strength gains with training, indicating that the gain in muscle strength may be negatively influenced by the presence of high TNF-α levels.[87]

Strength decreases in the presence of increased levels of TNF-α may be due to an interruption in the excitation-contraction coupling process.[88-92] Where exactly this disruption takes place is still under debate. The loss of strength may also be due to the removal of actin and myosin proteins resulting in an inhibition of the development of force even prior to a loss of muscle mass.[86,89] High levels of TNF-α also decrease the resting membrane potential of the sarcolemma and the cells ability to regulate calcium release from the sarcoplasmic reticulum, both contributing to decreased force production independent of any protein loss.[88,91]

Smaller clinical studies as well as larger epidemiological studies support the hypothesis that chronic increases in proinflammatory cytokines impair both strength and muscle mass in older individuals. Although the mechanism behind muscle mass and strength loss in the presence of increased proinflammatory cytokines is still not clearly understood, it does appear that increased levels of TNF-α in particular are troublesome for older adults and may contribute to muscle impairments in this population.

MOBILITY AND INFLAMMATION

The loss of muscle mass and strength in the presence of high levels of proinflammatory cytokines may also partially explain why increased levels of inflammation have consistently been tied to decreased function and mobility in older adults. Increased levels of TNF-α, IL-6, and CRP have been linked to lower walking speed,[25] poor physical function,[93] a decreased ability to perform activities of daily living,[22] and ultimately increased levels of disability and frailty.[22,72]

Large epidemiologic studies reveal that high levels of proinflammatory cytokines increase the risk for developing disability over 2 to 5 years.[22,94,95] Ferrucci et al[96] have reported that individuals with the highest baseline levels of IL-6 were also at the highest risk for the progression of disability and this could be at least partially explained by the loss of muscle strength also associated with high levels of IL-6. These study findings were confirmed by Penninx et al[94] in a study of almost 3000 adults aged 70 to 79 years. In this investigation, those with the highest levels of TNF-α, IL-6, and CRP at baseline were at the highest risk of developing mobility limitations and disability over the next 30 months.

This relationship is consistent across comorbidities common in an aging population and across the aging spectrum. Brinkley et al[93] examined several separate populations including those with chronic obstructive pulmonary disease (COPD), congestive heart failure, self-reported disability, and those at high risk for a cardiovascular event. They found that across all disease conditions and comorbidities that increased levels of serum CRP and IL-6 were associated with longer times to complete the 4-m walk and lower short physical performance battery scores.[93] As well, across the aging spectrum multiple studies of individuals aged 50 to more then 90 years have reported that increased levels of proinflammatory cytokines are related to decreased physical performance and ability to perform activities of daily living.[97] Tiainen et al[97] demonstrated that in nearly 200 adults aged 90 years and older increased serum levels of IL-6 and CRP were associated with a worse Barthel Index score, a 10-item measure of ability to perform activities of daily living independently.

Though we cannot assign cause and effect, the literature clearly identifies relationships between inflammation and decreased muscle mass and strength, between inflammation and impaired mobility and between decreased muscle mass and impaired mobility in older individuals[98] (Figure 4).

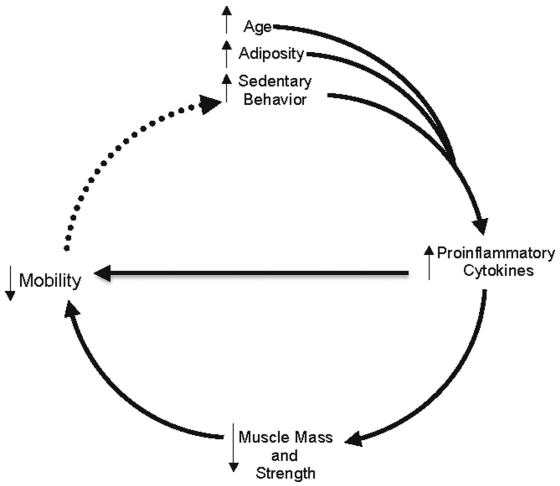

Figure 4. Proinflammatory cycle. Established (solid lines) and theorized (dashed lines) relationships were between chronic inflammation, decreased muscle mass and strength, impaired mobility, age, adiposity, and sedentary behavior. Although speculative, impaired mobility may lead to sedentary behavior and increased adiposity in older adults that in turn perpetuates a cycle of chronic inflammation.

Although speculative, it is intuitive that impaired mobility would lead to sedentary behavior in turn creating the environment for a self-perpetuating cycle of inflammation. In this theoretical cycle, inflammation, decreased muscle size and strength, and impaired mobility combine to create a sedentary lifestyle that further compounds the proinflammatory adverse responses in a feed-forward fashion. In summary, this underscores the important role of the physical therapist in minimizing mobility limitations in older adults with interventions aimed at interrupting this detrimental inflammatory cycle.

PHYSICAL ACTIVITY, EXERCISE, AND INFLAMMATION

Physical activity may be a powerful countermeasure to combat chronic inflammation and its deleterious effects in older adults. Physical activity includes any body movement produced by skeletal muscle that results in energy expenditure above resting basal levels and includes leisure time, recreational, occupational, and transport activities.[99] In contrast, exercise is a subset of physical activity that is planned repetitive movement with the object of improving or maintaining fitness.[99] Multiple epidemiological and cross-sectional studies have demonstrated a strong inverse relationship between levels of physical activity and markers of chronic inflammation across ages, body mass indexes, and comorbidities.[26-28,100,101] These relationships are observed whether considering levels of proinflammatory cytokines in the serum[26-28,100,101] or in the muscle.[29]

Though these studies have consistently revealed an inverse association between physical activity and chronic inflammation, exercise intervention studies aimed at reducing proinflammatory cytokine levels in older adults are more variable. Although a majority of studies that employ aerobic training,[30-36,38-40] resistance training,[41,42] or some combination of the two[37,102] suggest that exercise decreases proinflammatory cytokines, some studies have not found this to be the case.[86,103-107] Of the resistance training studies[86,103] that reported no statistical difference in proinflammatory cytokines both reveal a trend for decreased IL-6 postintervention, thus, suggesting a lack of statistical power. Alternatively, the inconsistent findings between studies may be explained by the populations studied, and the time between the final exercise bout and the postintervention measures. Most studies that found no effect postexercise intervention do not report how long postexercise intervention blood draws for serum inflammatory markers were conducted[37,87,104,106] and in at least one study blood draws were conducted as long as 2 weeks after the completion of the intervention.[107] Thompson et al[39] demonstrated that while it takes 12 weeks to see significant changes in serum markers of inflammation with exercise, it only takes 2 weeks of detraining to see returns on almost baseline levels.[39] A prolonged time period between exercise intervention and blood draws contributes to additional variability in these investigations. Finally, study outcomes may differ based on pretraining cytokine levels as individuals with the highest levels of preexercise inflammatory markers usually benefit the most from exercise.[39]

Consistent with the cross-sectional and epidemiologic studies,[26-28,100,101] those intervention studies reporting no change in proinflammatory cytokines with exercise[86,103-107] have employed serum markers. Two studies have demonstrated that even in the presence of minimal change in serum markers of inflammation with exercise, there may be a very large change in the proinflammatory cytokines within the muscle postexercise intervention.[30,32] Gielen et al[32] examined serum and muscle markers of inflammation in 20 males with congestive heart failure pre- and post-6 months of an aerobic exercise intervention. Patients in this study exercised 20 minutes a day every day and additionally participated in 1 hour a week of supervised group aerobic training. Despite a significant decrease of more than 30% in muscle-specific IL-6 and TNF-α, these authors reported no change in serum inflammatory markers postexercise training. Lambert et al[108] reported similar findings in a group of obese frail older adults. After 12 weeks of exercise (90 minutes, 3 times per week of combined aerobic and resistance exercise) the authors noted no decrease in serum IL-6 or TNF-α. However, muscle biopsies showed a 50% decrease in levels of IL-6 and TNF-α within the muscle tissue.[108] Bruun et al[30] also examined the combined effects of 15 weeks of aerobic activity with a hypocaloric diet in 27 severely obese individuals. The aerobic intervention consisted of 2 to 3 hours of moderate activity 5 days/week. At the end of 15 weeks, the authors noted a significant decrease in serum measures of IL-6 and CRP but not in TNF-α. However, when examining the muscle specific markers for IL-6 and TNF-α, large decreases that exceeded those seen in serum were noted to be more than 50% for IL-6 and 25% for TNF-α.

Decreased muscular expression of IL-6 in the absence of serum level changes with exercise may be explained by the influence of muscle contraction on the release of IL-6. As noted previously, contracting muscle is able to produce IL-6 in a TNF-α independent pathway. The IL-6 produced by contracting muscle is a powerful anti-inflammatory and, in fact, can suppress the release of TNF-α. Three hours of cycling can produce enough IL-6 in the muscle to suppress the release of TNF-α even when an individual is injected with an endotoxin.[109] Repeated bouts of exercise such as those employed by Gielen et al and Lambert et al would lead to repeated release of IL-6 from the contracting muscle. This "myokine" would then provide a powerful anti-inflammatory environment within the muscle leading to overall decreases in the release of proinflammatory cytokines within the muscle tissue itself. (For a comprehensive review of this concept the reader is encouraged to see reviews on this topic by Pedersen et al[110,111] for further details.) Overall exercise-induced fat mass reduction provides another potential hypothesis to explain proinflammatory cytokine decreases observed with exercise. Several studies have suggested that exercise, diet, or a combination of the 2 reduces adipose tissue, an effect that may contribute to an overall reduction in the proinflammatory cytokines that are released from inflamed adipocytes.[70,105,112] Although this may be an attractive hypothesis, the observation that at least 1 study has failed to report decreased proinflammatory cytokines when only weight loss is used independent of exercise,[108] raises further questions about fat mass as a potential mechanistic link between exercise and chronic inflammation.

Regardless of the precise cause, there appears to be strong evidence that both formal exercise[30-42,102,105] and simply increased levels of physical activity[100] can have a positive influence on chronic inflammation in older adults. Furthermore, it is likely that the anti-inflammatory effects of exercise are present in muscle even if no change is seen in serum markers of inflammation.[41,108] These findings provide yet another clear indication of the beneficial effects of exercise and increased daily physical activity in this vulnerable population.

CLINICAL RECOMMENDATIONS

Chronic increases in proinflammatory cytokines are associated with a myriad of impairments and dysfunction in older adults. Exercise is a potent preventive and intervention strategy for chronic inflammation and appears to be beneficial even in frail older adults.[41] Exercise dosage that has proven effective in decreasing proinflammatory cytokines have ranged from a frequency of 2 to 5 days a week and in daily duration from 20 minutes to 3 hours. Exercise modes have included home walking programs, treadmill walking, cycling, and resistance training using machines and free weights. Aerobic exercise intensity has varied between moderate walking and vigorous aerobic activity at 80% of age–predicted maximum heart rate. Resistance training has been reported effective utilizing 8 to 10 exercises with 8 to 10 repetitions to target all major muscle groups done 2 to 3 times per week at an intensity of 65% to 80% of a one repetition maximum.[41,42,102]

Though exercise of any mode and duration may positively impact chronic inflammation, there is currently limited literature to provide specific guidelines as to the most effective mode and dose of exercise to decrease chronic inflammation. Although further research is needed for the most effective exercise prescription to target chronic inflammation, the current American College of Sports Medicine (ACSM) guidelines provide a reasonable initial approach with the aim of reducing chronic inflammation and improving physical function in older adults. Exercise to combat not only the proinflammatory cytokines but also the deleterious effects that stem from them should include a combination of aerobic and whole body resistance training a minimum of 3 days a week for 30 to 60 minutes and the initial training program should last at least 12 weeks.[37,39,102,113] Thompson et al[39] demonstrated that it takes at least 12 weeks to see changes in the serum levels of IL-6 but only 2 weeks of detraining to return to baseline. In addition, one long-term study examining the effects of 12 months of aerobic exercise on serum levels of IL-6 found that there was an additional decrease of serum IL-6 at 12 months compared with 6 months.[37] These studies underscore the importance of regular physical activity in this population as positive changes in proinflammatory cytokines may continue to take place weeks and months after the initiation of exercise but may disappear in as little as 2 weeks after cessation of exercise.

Activation of large amounts of muscle mass are critical to the muscular release of IL-6 from the TNF-α independent pathway, activities that engage large amounts of muscle are most effective at producing IL-6 in this manner.[111] Activities such as walking, running, biking, or using an elliptical trainer would be advised. The addition of resistance exercises 2 to 3 days a week targeting all major muscle groups would also be beneficial to both decreasing the proinflammatory cytokines[41,42] as well as increasing muscle protein synthesis.[41]

As physical therapists see a larger proportion of older adults who suffer the functional consequences of frailty, there is an increasing interest in how best to address the treatment and prevention of this debilitating syndrome. Chronic inflammation is one of the strongest correlates of the frailty syndrome and can lead to a myriad of problems including muscle and strength loss and mobility limitations. Physical activity is a powerful anti-inflammatory tool that physical therapists can use not only to combat the effects of chronic inflammation but also to combat the production of proinflammatory cytokines. Exercise and increased physical activity can provide an anti-inflammatory environment within the muscle that may mitigate many of the effects of chronic inflammation on muscle. Future research should focus on the causes of chronic inflammation as well as the most effective treatments and exercise regimes to decrease inflammation.

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CHAPTER 3

INFLAMMATION, INTRAMUSCULAR FAT AND MUSCLE AND MOBILITY FUNCTION DIFFERENCES BETWEEN HEALTHY AND FRAIL OLDER ADULTS

Introduction

Sarcopenia, a loss of lean muscle mass, has traditionally been identified as a major reason for a loss of muscle and mobility function in older adults,[1-4] however, recent research has documented a clear disassociation between the loss of lean muscle mass and function in older adults.[5-8] Intramuscular adipose tissue (IMAT), an ectopic fat depot found beneath the deep fascia of the thigh, is increasingly recognized as a negative predictor of both muscle and mobility function in older adults and presents a potential link between the two.

Increased levels of IMAT have been associated with decreased strength and mobility and correlated with an increased risk of mobility limitations in older adults.[9-11] Because of this, increased levels of IMAT may be an unwanted but inevitable consequence of aging, even in individuals who lose weight over years.[5] Increased activity levels, however, may actually blunt or eliminate this age-related increase in IMAT.[12,13]

Master athletes demonstrate no differences in the levels of IMAT across 10 year age groups 40-49,50-59, 60-69 and 70 and older (mean age 60.1 years) reinforcing the notion that increases in IMAT are not inevitable with aging but are likely related to factors such as muscle disuse and disease. Whether a high level of IMAT is merely a marker of muscle dysfunction in older inactive individuals, or if it plays a more active role in muscle and mobility dysfunction, remains unknown.

The mechanism underlying IMAT's negative influence is currently unknown but has been speculated that like other ectopic fat depots it may involve an inflammatory pathway.[5, 14-17] Excessive fat mass has been directly implicated in increased circulating levels of numerous proinflammatory cytokines such as tumor necrosis factor alpha (TNF-a), interleukin-6 (IL-6), and C-reactive protein (CRP) in both young and old individuals.[14, 18-21] Increased levels of visceral adipose tissue, also an ectopic fat depot, directly contribute to the increase in circulating proinflammatory cytokines.[19]

TNF-a, IL-6 and CRP basal circulating levels have all been shown to be closely associated with muscle and mobility function in older adults.[22-26] However few studies have examined the effect of increased levels of inflammation in locomotor muscle of older adults. Higher systemic levels of proinflammatory cytokines in serum are associated with decreased strength and mobility in older adults[22-26] and increased circulating levels of proinflammatory cytokines result in cell apoptosis,[27] a decreased rate of protein synthesis,[28] and decreased muscle quality[29], all of which may contribute to decreased strength and mobility in older adults.[30] Several studies on the effects of increased systemic levels of circulating proinflammatory cytokines show a relationship

between inflammation and muscle and mobility function,[22, 23, 26, 31, 32] however, no study to date has looked at relationship of inflammation, muscle and mobility function in the muscle of older adults.

In order to clarify if IMAT is a by-product of age per se or if it is related to inactivity the primary aim of this study was to compare muscle and mobility function, IMAT and muscular inflammation in age and BMI matched older frail and nonfrail adults. A secondary aim was to examine the relationship of IMAT, muscular inflammation, muscle function, and mobility in older frail and nonfrail adults. We hypothesized that IMAT would be related to inactivity rather then aging and that older frail adults would have decreased muscle and mobility function and higher levels of IMAT and muscular inflammation when compared to nonfrail adults. We further hypothesized that a relationship between muscle and mobility function, IMAT and muscular inflammation exists in this group of older adults.

Methods

Participants

An accessible population of older adults in our local community was recruited using local health care clinics, newspaper ads, target mailings, church groups, senior centers, and health fairs. To maximize potential differences in IMAT, two groups of older non-obese adults (a frail sedentary and a nonfrail active group) were recruited. Due to the difficulty in finding and recruiting frail individuals we planned on recruiting twice as many healthy individuals and sought to power this study based on our primary aim of

determining differences in IMAT, muscle and mobility function and inflammation between frail and nonfrail individuals. Based on prior studies effects sizes of 1.36 to 2.39 for differences between active and sedentary adults we estimated that we would need between 5 and 12 nonfrail individuals and 3 to 6 frail individuals.[33, 34] We conservatively aimed to enroll at a minimum 12 active individuals and 5 frail individuals. Inclusion criteria for both groups included individuals over the age of 65 years and a BMI of less then 30. Additionally the sedentary frail group was required to be classified as at least moderately frail by a modified physical performance test (MPPT) score of < 25 and reported little to no planned physical activity over the last 12 months.[35-37] The active nonfrail group was made up of individuals who had MPPT scores of > 32 and reported at least 90 minutes of moderate activity a week over the last year.[35-37] Exclusion criteria for both groups included diabetes, COPD, active cancer, chronic heart failure, or chronic infection. Additionally a subgroup of participants were asked to undergo a percutaneous a muscle biopsy if they were free from heart disease or any other condition known to increase systemic inflammatory levels. Participants in this subgroup were also excluded if they were using any medication known to influence inflammation such as tobacco products, corticosteroids, nonsteroidal anti-inflammatories, hormone replacements or anticoagulants. Any individuals in this subgroup who were on statin medications were asked to hold all statin medication for at least 7 days prior to the muscle biopsy. Figure 3.1 demonstrates the recruitment flow from initial contact to recruitment for a muscle biopsy.

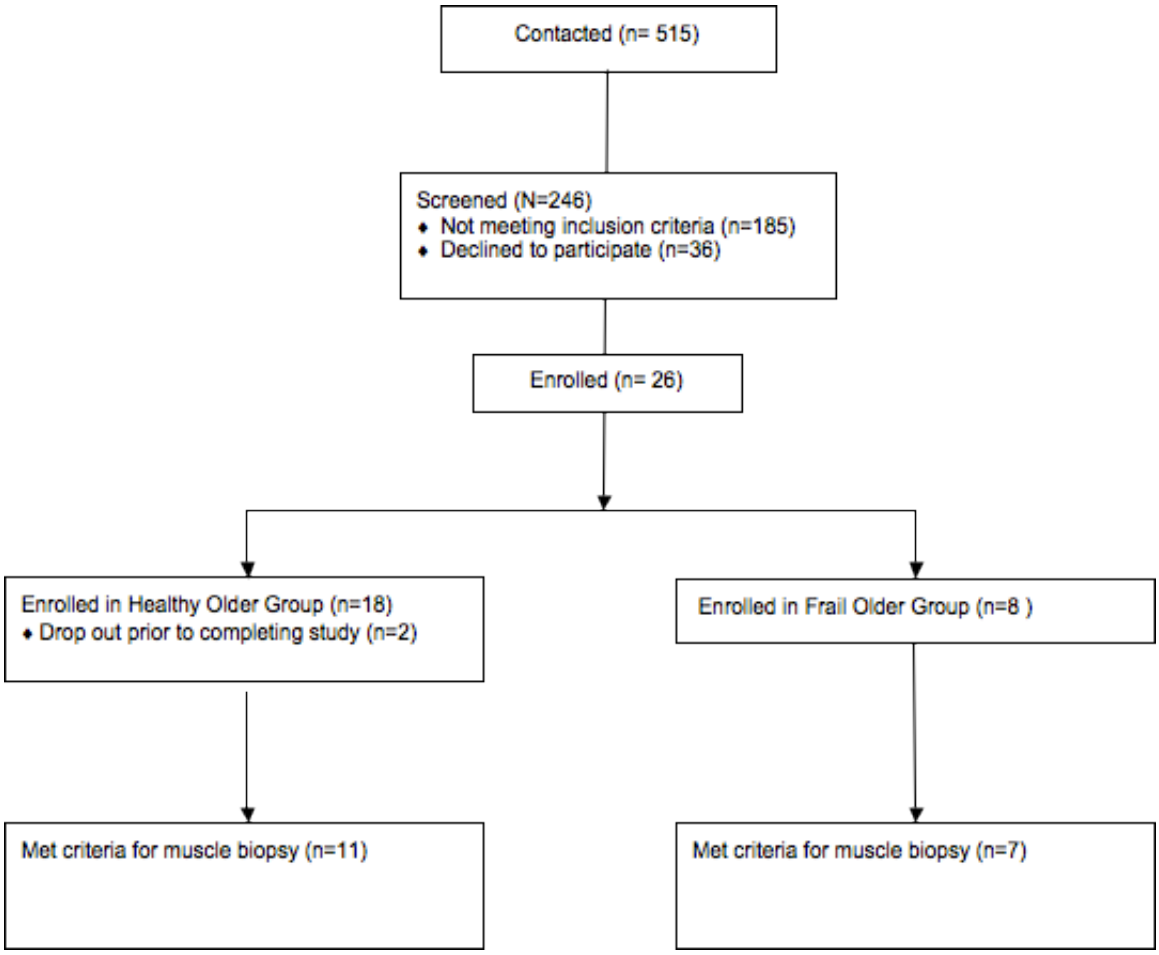

Figure 3.1 Recruitment and Consort Diagram

Study Design

Potential participants completed a telephone screen followed by an in person screen to determine eligibility. During the in person screen the modified physical performance test was performed and scored by a physical therapist to ensure the participants met the requisite scores.[37] Prior to all screening tests, all eligible volunteers signed an institution approved consent form. Immediately after the in-person screening, demographic data were gathered from the participants and their first clinic testing visit was scheduled within 2 weeks. During the first clinic testing session, individuals underwent functional testing to determine mobility, strength testing to determine muscle function, and an MRI of their legs to determine the cross sectional area of lean muscle mass and IMAT. Within 1 week of functional testing individuals who met the inclusion criteria for a muscle biopsy underwent a muscle biopsy to determine levels of local inflammation within the muscle.

Muscle Function Testing

Muscle function was operationally defined in this study as maximum knee extension voluntary strength. A tester blinded to group assignment collected muscle force production via knee extension strength in both legs. Knee extension strength was determined via a maximum voluntary isometric contraction (MVIC) on a KinCom dynamometer (Chattanooga Inc., Hixon, TN) as follows: participants were stabilized by chest and thigh straps and seated with their knees fixed at 60 degrees of flexion with their arms folded across their chest. Prior to testing, participants practiced submaximal contractions at 50 and 75% of their perceived maximal effort prior to one practice

maximal contraction trial. After a 2 minute rest period, three separate maximal contractions were performed. Each maximal contraction was held for 5 seconds with a 3 minute rest between trials. The outcome variable muscle force was calculated as the average force of three trials. The order of testing (right versus left) was randomized among subjects.[38] This test has been shown to have a high degree of reliability (ICC=. 99).[39]

Mobility Function Testing

A tester blinded to group collected all mobility testing measures. Mobility was determined using three tests: (1) a six-minute walk (6MW), (2) stair ascent (StairA) and (3) self-selected gait speed (GS). These performance tests were chosen to represent mobility function and have been shown to be both valid and reliable in this population. [40-42] The 6MW test, a measure of the distance a subject walks in 6 minutes, was used to assess overall mobility. Participants were asked to cover as much distance as possible in six minutes without running. Distance was recorded in meters. The stair A test required participants to ascend one flight of stairs under close or contact supervision as quickly and safely as possible. Time was recorded to the nearest 0.01 second from a verbal go signal to final foot placement on a standard flight of 10 stairs, and the average of three trials was recorded. Self-selected gait speed was measured over a 50 foot course. Individuals were instructed to walk at a comfortable pace starting at the word "go." They were asked to walk out 25 feet and back. Timing took place from the command "go" until the starting line was crossed on the way back.[37, 43] Participants were allowed to use any walking aid they used on a daily basis. All instructions, encouragement and

measurements for all functional tests were carried out in a consistent manner according to standardized instructions.

Lean Muscle Mass and IMAT Determination

MRI methods for determination of lean muscle mass and IMAT have been described in detail elsewhere.[38] Briefly bilateral magnetic resonance imaging (MRI) scans of the thighs were obtained. The respective IMAT and lean tissue cross sectional areas were calculated from the MRI scans. Subjects were placed supine in a 3.0 Tesla whole body MR imager (Siemens Trio, Siemens Medical, Erlangen, Germany). The legs were scanned in a coronal plane and the midpoint of the thigh was determined and defined as half way between the superior margin of the femoral head and the inferior margin of the femoral condyles. Axial imaging (5mm thick slices at 1 cm intervals) of the legs was then performed over 1/2 the length of the femur, centered at the midpoint of the thigh. Separate fat and water images were created with custom software using the three-point Dixon method. A tissue model was then used to calculate estimates of total fat and nonfat volume fractions on a per pixel basis, which were displayed in image form. All sequences were performed with a phase array torso/abdomen coil. Eleven images from the middle 1/3 of each thigh were used to determine average cross-sectional area (cm^2) of IMAT and lean tissue. Manual tracing eliminated subcutaneous fat and bone and isolated the fascial border of the thigh to create a subfascial region of interest (ROI). Total IMAT and lean tissue were calculated by summing the value of percent fat fraction and percent lean tissue fraction over all pixels within the ROI using custom written image analysis software (MATLAB; The MathWorks, Natick, Massachusetts). This sum was multiplied

by the area of each pixel to give total fat and lean tissue CSAs within the ROI. This method accurately measures fat and lean tissue in pixels that contain both by allowing fractional contributions to the fat and lean tissue CSA calculations.[44] This allows microscopic fat within muscle tissue as well as thin planes of fat adjacent to fascial planes to be accurately taken into account, even when image resolution is inadequate to delineate these visually. The same investigator, blinded to time point of the scan and slice location, performed measurements of individual participants. This technique has demonstrated high levels of intrarater reliability,[45] test-retest reliability,[46, 47] and concurrent validity when compared to imaging of a cadaveric phantom limb.[45] To normalize IMAT for thigh size the percent of IMAT was calculated for each individual. This was calculated by dividing the cm^2 of IMAT by the cm^2 of the thigh excluding subcutaneous adipose tissue and bone.

Muscle Biopsy

All muscle biopsies in eligible participants were performed within one week of functional testing. Participants were asked to refrain from any strenuous activity within 48 hours of the muscle biopsy. All individuals presented in the morning after a 12 hour fast for a percutaneous needle biopsy.[48, 49] The skin and fascia 12-15 cm above the lateral joint space was anesthetized with 5cc of 1% lidocaine and a small incision was made. The biopsy needle was inserted 3-5 cm beyond the fascia into muscle and 3 passes (bites) made to yield approximately 200 mg of muscle tissue. Following biopsy, deep pressure was applied for 20 minutes, followed by a steri-strip bandage and pressure dressing. The

sample obtained was dissected free from blood and visible fat, snap frozen in liquid nitrogen and stored at -80 C until analysis.

Proinflammatory Cytokines Measures in the Muscle

IL-6 and TNF-a were the dependent variables chosen to represent proinflammatory cytokines levels within the muscle. In order to measure the expression of these proinflammatory cytokines within the muscle both mRNA and protein expression of IL-6 and TNF-a were measured. To determine the mRNA expression of IL-6 and TNF-a in the muscle tissue of the participants total RNA, cDNA synthesis and real time qPCR were conducted as previously reported.[50] Total RNA was extracted by homogenizing 15-20 mg muscle tissue with a handheld homogenizing dispenser (PowerGen 125; Fisher Scientific) in a solution containing 0.75 ml Tri reagent (LS; Molecular Research Center, Cincinnati, OH) and 0.25 ml nuclease free water. The RNA was separated into an aqueous phase using 0.2 ml of chloroform and precipitated using 0.5 ml of isopropanol. Isolated RNA was washed with 1 ml of 75% ethanol, dried, and then suspended in a known amount of nuclease-free water (1.5 ml/mg tissue). RNA was DNase-treated using a commercially available kit (TURBO DNase-free, Life Technologies, Carlsbad, CA). RNA concentration was determined with a NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA). Afterwards, 0.5 mg of total RNA was reverse transcribed into cDNA according to the manufacturers' directions (iScript, BioRad, Hercules, CA). All isolated RNA and cDNA samples were stored at -80°C until analyzed. Real-time qPCR was carried out with an Applied Biosystems 7900HT fast sequence detection system. Taqman predesigned primers (IL-6 and TNF-a) were purchased from

Life Technologies. Values were normalized to beta 2-microglobulin then fold change values were calculated using the $2^{-\Delta\Delta Ct}$ method.[51]

To determine protein expression of IL-6 and TNF-a in the muscle western blots were used. Frozen muscle tissue was homogenized in a buffer cocktail with protease inhibitors in a prechilled glass tube under ice. Muscle homogenates were centrifuged at 6000 rpm for 10 minutes at 4°C and the supernatant was subsequently collected and transferred to a new microcentrifuge tube. Total protein concentration for each sample was determined on a spectrophotometer using a colorimetric protein assay (Bio-Rad; Bradford) and an albumin standard curve. Whole muscle homogenates were diluted 1:1 in a 2X sample buffer. Homogenates were loaded at equal protein concentration on a Criterion Tris-HCL precast polyacrylamide gel (Bio-Rad) and subjected to SDS-PAGE (150V) for 1h in running buffer. Each gel contained alternating Healthy and Frail samples loaded in duplicate, and a molecular weight ladder. An internal control was loaded in duplicate on each gel for band normalization and comparisons across blots. Protein was transferred (50V; 1h) to a polyvinylidene diflouride membrane in ice cold transfer buffer then blocked for 1h at room temperature with 2% nonfat dry milk (NFDM) in Tris-buffered saline in 0.1% Tween-20 (TBST). Membranes were incubated overnight in primary antibody diluted in 2% NFDM in TBS. The next morning, blots were rinsed in TBST for 5 minutes, rocked in secondary antibody for 1h at room temperature in 2% NFDM in TBS then serially washed (15 minutes, 4 x 5 minutes) in TBST. Chemiluminescence reagent (ECL Plus, GE Healthcare) was applied to each blot for 5 minutes. Optical density measurements were obtained with a digital imager (Bio-Rad).

Membranes were stripped (Restore PLUS, Thermo Scientific) of primary and secondary antibodies then reprobed for a-tubulin (1:50,000; Sigma Aldrich, St. Louis, MO). Densitometric analysis was performed using Quantity One software (Bio-Rad). After subtracting out background, all western blot data were normalized to the internal control and replicate samples were averaged. The following antibodies were used in this experiment: IL-6 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), TNF-a (1:500; Cell Signaling, Boston, MA), and Toll-like receptor 4 (1:500; Santa Cruz Biotechnology). Donkey anti-rabbit and goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (1:6000) were purchased from Santa Cruz Biotechnology.

Data Analysis.

Data were analyzed with SPSS Statistics 20.0 (SPSS, Chicago, IL). Descriptive statistics were calculated for demographic variables and dependent measures.

Aim One: Comparing Muscle and Mobility Function, IMAT and Muscular Inflammation. For our first aim of comparing muscle and mobility function, IMAT and muscular inflammation in age and BMI matched older frail and nonfrail adults independent t-tests were used. The independent variable was group (frail or nonfrail) and the dependent variables were percent IMAT, mRNA and protein expression for IL-6 and TNF-a, and all mobility and strength measures.

Aim Two: The Relationship of IMAT, Muscular Inflammation and Muscle and Mobility Function. For our second aim of examining the relationship of relationship of IMAT, muscular inflammation, muscle function, and mobility in older frail and nonfrail adults pearson product correlations were used. Using these correlations we examined the

relationship of percent IMAT, mRNA expression of IL-6 and TNF-a with all mobility (6MW, SA, GS) and MVIC in both groups. The level of significance was set at P<.05 and a bonforonni correction was used for multiple comparison.

Results

Participant Characteristics

A total of 26 individuals were enrolled in the study. In accordance with our a priori power calculation 18 nonfrail and 8 frail individuals were enrolled. A subgroup of 7 frail and 12 nonfrail individuals met the inclusion criteria for a muscle biopsy. During the MRI, suspected cancer was found in one nonfrail individual and they were eliminated from the study. Additionally one non-frail individual refused the MRI and was thus eliminated from the study. All remaining individuals completed all testing procedures for the study resulting in data for 16 nonfrail and 8 frail completed the study. The demographics for both groups of participants are summarized in Table 3.1.

Table 3.1 Participant characteristics

	Frail	Healthy
N	8	16
Age (years)	83.3 (4.5)	78.1 (5.7)
BMI (Kg/m^2)	25.0 (3.3)	23.9 (2.6)
MPPT test	16.3 (7.7)	35.2 (1.2)
% Lean	82.0 (4.9)	88.3 (2.0)
% IMAT	18.0 (4.9)	11.7 (2.0)

Mean [± SD]; BMI = Body Mass Index; MPPT = modified physical performance test score; % lean= percentage of thigh lean tissue average cross sectional area as measured with MRI excluding subcutaneous fat and bone in the biopsied leg; %IMAT= percentage thigh intramuscular adipose tissue average cross sectional area as measured with MRI excluding subcutaneous fat and bone in the biopsied leg.

Aim One: Comparing Muscle and Mobility Function, IMAT and Muscular Inflammation. Significant differences were found between the frail and nonfrail groups for all mobility and muscle function measures. The frail group was found to cover less distance on the 6MW ($p<.001$), took more time to complete SA ($p=.03$), and had a slower self-selected GS ($p=.002$). The frail group was also significantly weaker MVIC ($p=.03$). Despite having similar ages and BMI's, significant differences between the frail and nonfrail group was also found for the % lean and the % IMAT in the thigh. The frail group was noted have less lean tissue mass ($p=.004$) and more IMAT ($p=.004$) than the nonfrail group. Table 3.2 demonstrates the means for all muscle and mobility measures collected for both the frail and healthy groups.

Table 3.2 Differences between frail and healthy older adults for muscle and mobility data

	Frail	Healthy
6MW (meters)	278.50 (147.6)	540.81 (77.9)*
SA (sec)	29.0(25.9)	4.26 (.9)*
GS (m/sec)	.67(.21)	1.23 (.3)*
MVIC (N)	161.9 (49.6)	323.1(121.4)*
% IMAT	18.02 (4.9)	11.6 (2.0)*
% Lean	82.0 (4.9)	88.3 (2.0)*

6MW=distance in meters covered in 6 minute walk test test; SA=time in seconds to ascend one flight of stairs; GS=self selected gait speed in meters/second; MVIC= maximal voluntary isometric contraction of knee extensor muscles in Newtons; % IMAT= percentage of IMAT in thigh; % lean = percentage of lean tissue in the thigh. *significant difference between groups. ($P<.05$)

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In the subgroup analysis the mRNA(p=.015) and protein expression (p= .016) of IL-6 was significantly higher in the frail group then the nonfrail group (p=.015) as seen in Figure 3.2 A and B. There was no difference found in the mRNA (p= .132) or protein expression (p= .265) for TNF-a between the groups as seen in Figure 3.2 C and D.

Aim Two: The Relationship of IMAT, Muscular Inflammation and Muscle and Mobility Function. Intramuscular adipose tissue was strongly correlated with mobility and muscle function. Percent IMAT in the thigh was significantly correlated with distance covered in the 6MW (r=-0.726, p<.001), SA (r=0.699, p=.001), self-selected GS (r=-0.676, p=.001), and MVIC (r=-.752, p<.001). A significant positive correlation (r=0.565 p=.009) was found between the % IMAT in the thigh skeletal muscle expression of IL-6 mRNA. IL-6 mRNA expression was also significantly correlated with 6MW (r=-0.685, p=.001) and self-selected GS (r=-0.617, p=.004). While SA was significantly correlated with (r=0.535 p=.013) IL-6 mRNA expression after the bonforoni correction this was no longer statistically significant. IL-6 mRNA expression was also moderately correlated with MVIC (r=-0.370) but it did not reach the level of significance (p=.072). IL-6 protein expression was significantly correlated with self-selected gait speed (r=-.70, p=.001) and MVIC (r=-.549, p=.009) but was not significantly correlated with 6MW (r=-.540, p=.01) after the bonforoni correction. There was also no significant correlation between IL-6 protein expression and % IMAT (r=.252, p=.157) No significant correlations (r ranged .023 to .163, p values ranged from .153 to .465) were found for TNF-a mRNA or protein expression with any mobility, MVIC, or % IMAT.

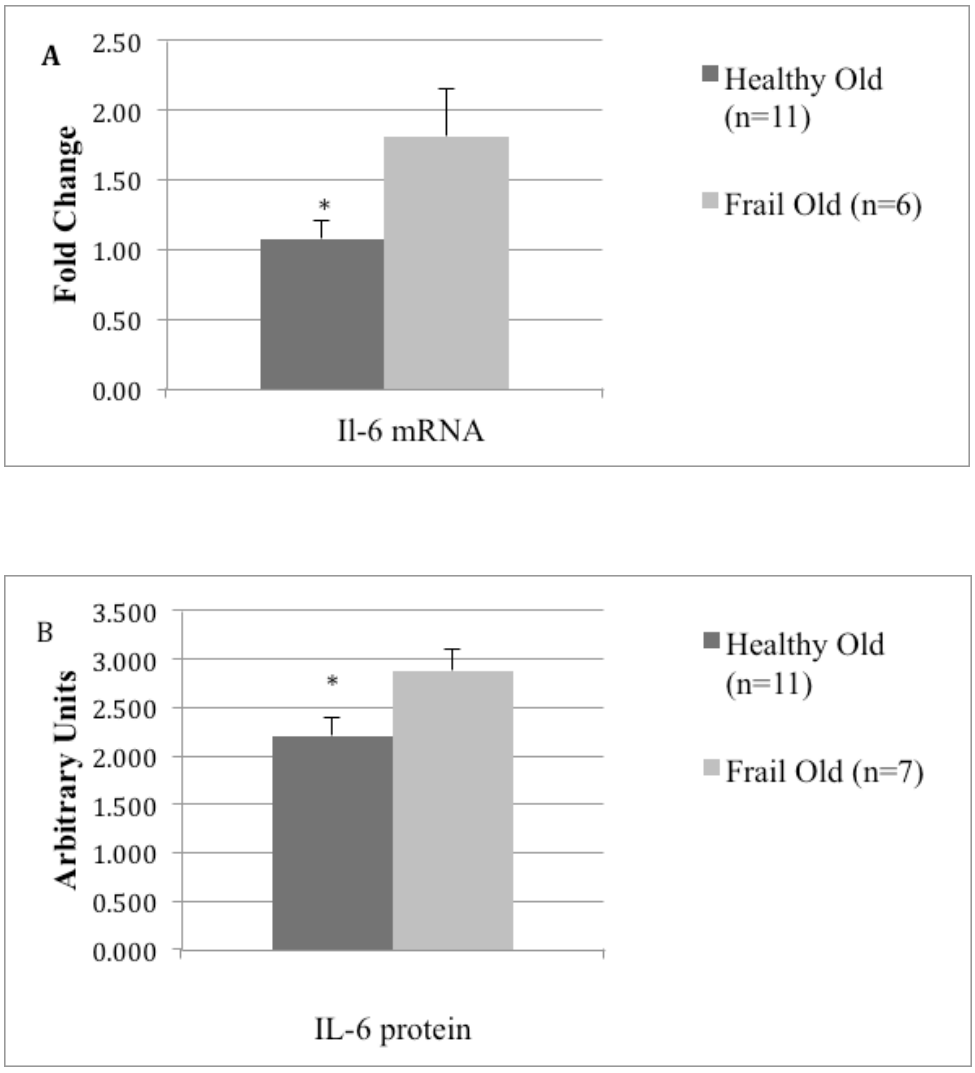
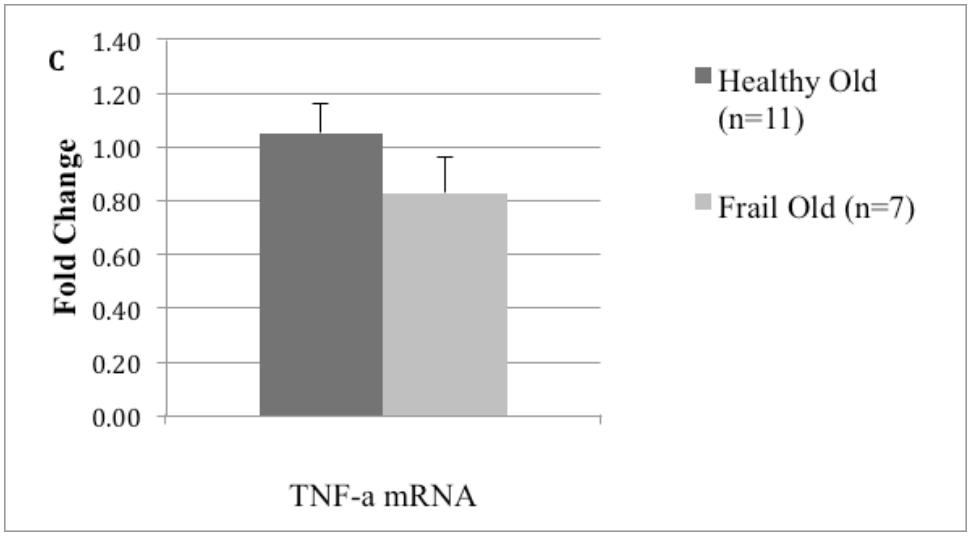


Figure 3.2 IL-6 mRNA and Protein Expression in Muscle of Healthy vs Frail Adults. Mean expression of (A) IL-6 mRNA, (B) IL-6 protein and (C) TNF-a mRNA and (D) TNF-a protein in the vastus lateralis of healthy and frail older adults. * Statistically significant differences between healthy and frail adults ($P<0.05$).



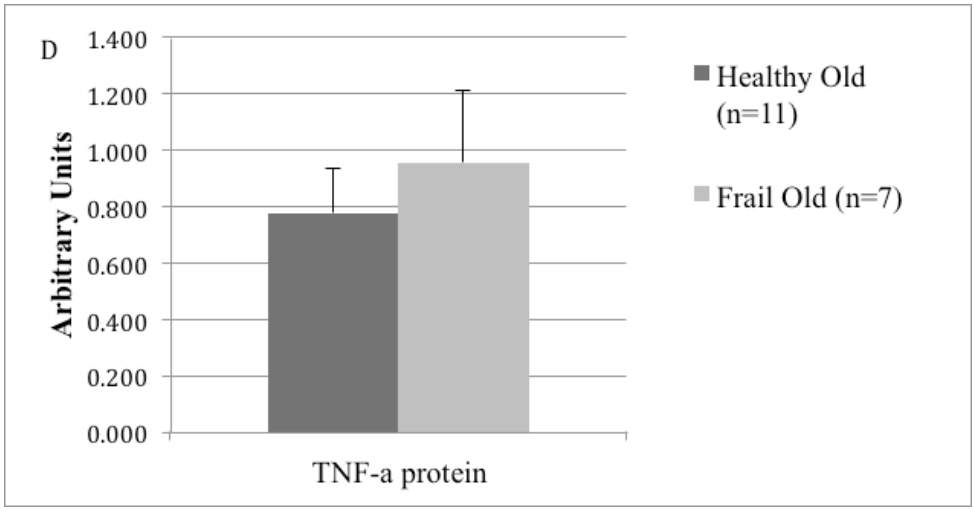


Figure 3.2 Continued

Discussion

This study is the first study that we are aware of that examines the relationships of IMAT and inflammation in aging muscle tissue. Our primary aim was to clarify if IMAT was a by-product of age or related to inactivity by comparing muscle and mobility function, IMAT and muscular inflammation in age and BMI matched older frail and non-frail individuals. To better understand the potential harmful nature of IMAT our secondary aim was to examine the relationships of IMAT, inflammation, and muscle and mobility function in older adults. As hypothesized, we found that IMAT was related to inactivity rather then aging and that older frail adults had both increased levels of IMAT and inflammation when compared to nonfrail adults. We also found significant relationships between IMAT, inflammation, and muscle and mobility function in this group of older adults.

IMAT: Aging or Inactivity

The novel findings in this study were that significant differences in both IMAT and the mRNA and protein expression of IL-6 exist within the muscle of frail and non-frail older adults with vast difference in muscle and mobility function. We found these differences in levels of IMAT despite similar ages and BMI's in the frail and non-frail groups of older adults. While it has been previously suggested that increased levels of IMAT were an inevitable consequence of aging our findings would suggest that increased IMAT may be more an effect of inactivity and illness then aging.[5] There are differing results in the literature regarding this questions, Delmonico et al. found in a 5-year

longitudinal study that IMAT levels increased even in individuals who lost weight.[5] However, more recently Wroblewski et al. reported in a cross-sectional study of master athletes ages 40 to 70 plus years that IMAT levels did not increase significantly with age.[13] Further support of the inactivity hypothesis was found by Manini et al. who demonstrated increased IMAT levels in the thigh and calf of young (19-28 years old) individuals after 30 days of single limb suspension. IMAT volume increased up to 20% in the lower extremity suggesting that even in young healthy individuals increased IMAT is a consequence of decreased activity levels.

Taken together, along with our findings, these studies would suggest that increased IMAT levels may be more of a product of illness and disuse then age per se. While we did not have a younger group of individuals to compare with, our data suggests that IMAT differences exist between frail and nonfrail older adults of similar age ranges. If increases in IMAT were solely an age related phenomenon we would expect to see similar levels of IMAT in our frail and nonfrail individuals. The suggestion that IMAT may be related to activity rather then aging per se is important as physical activity may be a treatment to ameliorate or even decrease the increased levels of IMAT found in older frail adults.

Inflammation: Aging or Inactivity

Surprisingly little research has examined the inflammatory state of skeletal muscle in older adults.[52] While previous studies have compared muscular IL-6 mRNA and protein expression in older healthy adults and young healthy individuals,[53-56] or changes in muscular inflammation pre- and post-chronic exercise,[33, 57, 58] this is the first

study that we are aware of that compares mRNA and protein expression of IL-6 and TNF-a in the muscle of frail and nonfrail older adults of similar ages and BMI's. Our results suggest that differences in IL-6 mRNA and protein expression exist in the muscle between older frail and nonfrail individuals. Previous research has found little to no difference in IL-6 mRNA expression in the muscle of young healthy versus older healthy adults.[53-56] Findings have also been equivocal on the effect of aging on TNF-mRNA expression in muscle with some studies finding increases compared to younger individuals[59] and some studies finding no differences compared to younger individuals.[53, 56, 60] In the current study we found no differences in the muscular expression of TNF-a mRNA between frail and nonfrail older adults. It is known that TNF-a release is more variable then IL-6 and this may be one reason why we did not find any differences between frail and nonfrail individuals.[61] It is also possible that with our small sample size we were underpowered to detect differences between frail and nonfrail individuals with regards to TNF-a.

IMAT, Inflammation and Muscle and Mobility Function

There is some speculation that the negative consequences of IMAT on muscle and mobility function may be from the release of pro-inflammatory cytokines that act in a paracrine like manner on nearby muscle tissue.[5, 14-17] Our results suggest that levels of muscular inflammation may relate to levels of IMAT within the muscle as we found a moderate significant correlation between levels of IMAT and levels of IL-6 mRNA within the muscle although we did not find a relationship between IMAT and protein expression of IL-6. It is possible with our small data set that we were underpowered to see any

relationship between IL-6 protein and IMAT. We also found moderate significant correlations between most measures of muscle and mobility function with IMAT and IL-6 mRNA and protein expression. It has been well documented that increased serum levels of IL-6 are closely associated with decreased muscle[24, 26] and mobility[23, 62, 63] function in older adults and multiple studies have demonstrated that even after controlling for total body fat, physical activity and health status, individuals with the highest circulating levels of pro-inflammatory cytokines have the lowest levels of strength and mobility.[22-26] We did not, however, find any relationship between gene or protein expression of TNF-a and IMAT. Nor did we find any relationship between TNF-a mRNA or protein expression with any measures of muscle of mobility function.

Limitations and Directions for Future Research

This study included a small number of individuals and thus our results should be interpreted with caution. Because individuals with disease processes that are known to increase inflammatory levels were purposely excluded from our muscle biopsy analysis, our findings are likely conservative. Gene expression of local inflammatory cytokines in the muscle of older adults may be even more robust if including individuals with diseases typically associated with increases in both IMAT and inflammation such as diabetes. While we did attempt to control for multiple co-morbidities and used a careful screening process to do this, it is also possible that individuals with higher inflammatory levels had undiagnosed co-morbidities that we were not aware of. We also recognize the slight age discrepancy between our groups of frail and non-frail individuals. While the age difference of 5.2 years is small it is possible that the reason for our findings was the age

difference. However given that multiple previous research studies demonstrate no significant differences between IL-6 mRNA expression in young and old healthy individuals this is unlikely.[53-56]

Clinical Relevance

This study is the first to examine the relationship of IMAT and inflammation in aging muscle tissue and may expose new ways in which physical activity can stave off the negative consequences of aging on skeletal muscle. Increased levels of IMAT and IL-6 mRNA and protein expression are both related to muscle and mobility function in older adults. It also appears that a significant relationship exists between IMAT and IL-6 mRNA expression. This IMAT-inflammatory pathway provides a potential mechanism behind IMATs negative influence on muscle and mobility function and is a potential reason for the harmful effects of IMAT on skeletal muscle. Additional studies are needed to more clearly delineate the relationship between IMAT and inflammation in older adults and to explore the relationship between systemic inflammation, muscular inflammation and IMAT.

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CHAPTER 4

RELATIONSHIP OF ECTOPIC FAT DEPOTS AND INFLAMMATION IN THE MUSCLE OF OLDER FEMALES

Introduction

Over the last 20 years there has been a rapid expansion in our understanding of adipose tissue. Once thought to be a passive storage depot for excess calories, important only to energy homeostasis, we now know that adipose tissue is capable of expressing and secreting a multitude of hormones and chemicals that travel throughout the body to act in an autocrine, paracrine, and endocrine manner signaling the heart, musculoskeletal, central nervous and metabolic systems.[1-3] Specifically, the release of pro-inflammatory cytokines from adipose tissue, such as tumor necrosis factor-a (TNF-a), interleukin-6 (IL-6), and c-reactive protein (CRP) are important initiators of chronic inflammation,[1, 4] a process that has been implicated in the pathogenesis of a multitude of diseases including type 2 diabetes, cardiovascular and cerebrovascular disease, and dementia.[5-9] High levels of circulating pro-inflammatory cytokines in older individuals are also associated with increased mortality risk,[10] sarcopenia,[11, 12] reduced muscle strength,[13] and decreased mobility.[14, 15]

Recent studies have suggested that the location of adipose tissue rather than simply total body adiposity, may be important in the systemic increase of circulating pro-inflammatory cytokines.[16-19] In particular visceral adipose tissue (VAT) has a positive

relationship with levels of TNF-a, IL-6 and CRP,[16, 18-20] and the extraction of VAT leads to a decrease in circulating pro-inflammatory cytokines.[21, 22] Multiple studies have also demonstrated a decrease in pro-inflammatory cytokines with diet and exercise- induced weight loss[23, 24] and with physical activity.[25-28]

Advancing age results in a redistribution of fat depots, despite stable or decreasing overall fat amounts, with adipose storage sites changing from subcutaneous locations to the more harmful VAT or ectopic fat depots in the liver and muscle.[3, 29-31] Specifically, intramuscular adipose tissue (IMAT) may also influence the pro-inflammatory milieu, similar to VAT, as positive associations between circulating levels of IL-6 and IMAT in locomotor muscle of older adults have been noted.[16] In addition to its potential inflammatory contribution, IMAT has been associated with muscle weakness and mobility impairment[32-35] and, importantly to risk of future mobility limitation in older adults.[34, 35] It has been theorized that increased IMAT is a normal consequence of aging [36]; however, more recent evidence suggests that increased IMAT may be more a product of inactivity then age per se and therefore may be modifiable.[37-39]

Multiple authors have suggested[16, 36, 37, 40, 41] that IMAT's negative relationship with muscle and mobility function may be attributed to the release of pro-inflammatory cytokines; to date this relationship has not been clarified. The independent parallels between inflammation and IMAT, with strength and mobility loss in older individuals, may in fact be linked to the release of inflammatory cytokines from IMAT. IMAT has been associated with systemic circulating levels of IL-6, though no study to our knowledge has examined the relationship of IMAT with local muscle concentrations of

pro-inflammatory cytokines within the locomotor muscle. The role of IMAT and it's inflammatory contribution to muscle is important as it may provide new therapeutic targets for combating muscle and mobility impairments often seen in older adults. Despite our knowledge of the influence of VAT and IMAT on circulating cytokines much less is known about the influence of these ectopic fat depots on local levels of cytokines. Therefore, the purpose of this study was to examine the relationships of physical activity, location specific fat depots (VAT, SAT, and IMAT), and local (as measured in the muscle) pro-inflammatory cytokine expression in older adults. We hypothesized the active individuals would have both lower VAT and IMAT as well as decreased local inflammation. We further hypothesized that significant relationships would exist between VAT, IMAT and local inflammation.

Methods

Participants

All participants in this study were part of a larger study examining the differences in IMAT, inflammation and mobility in older frail and nonfrail elders. Individuals were recruited from local community groups, health care clinics, mailing and word of mouth referrals. To minimize the contribution of sex or obesity to VAT, IMAT and inflammation inclusion criteria required subjects to be female, over the age of 65, have a BMI of less then 30, and willing to undergo a percutaneous needle muscle biopsy. Exclusion criteria were any history or diagnosis of diabetes, COPD, active cancer, chronic heart failure, chronic infection, heart disease or any other condition known to increase systemic inflammatory levels. Participants were also excluded if they were using any medication

known to influence inflammation such as tobacco products, corticosteroids, non-steroidal anti-inflammatories, hormone replacements or anti-coagulants. Any individuals on statin medications were asked to hold all statin medication for at least 7 days prior to the muscle biopsy.

Study Design

Potential participants completed a telephone screen followed by an in-person screen to determine eligibility. Prior to all in person screening tests, all eligible volunteers signed an institution-approved consent form. Immediately after the in-person screening demographic data were gathered from the participants and participants were asked to fill out the Physical Activity Scale for the Elderly (PASE).[42-44] The PASE is a self-report measure that has been shown to be reliable and valid method for estimating levels of physical activity in older adults and includes not only planned physical activity but also considers the contribution of leisure time, volunteer, and housework activities.[42-44] After completing the PASE participants were scheduled for an MRI within 2 weeks. Individuals underwent a MRI scan of their legs and abdomen to determine the cross sectional area of abdominal VAT and SAT and IMAT found within the legs. Within one week of the MRI a muscle biopsy was performed to determine local muscular levels of pro-inflammatory cytokines.

SAT, VAT and IMAT Determination

MRI methods for determination of lean muscle mass and IMAT have been described in detail elsewhere.[45] Briefly magnetic resonance imaging (MRI) was used to

determine the SAT and VAT at a single abdominal axial cross section of L4-L5 and bilateral imaging of the thighs was performed to determine the average cross sectional area of IMAT.

SAT and VAT determination. Subjects were placed supine in a 3.0 Tesla whole body MR imager (Siemens Trio, Siemens Medical, Erlangen, Germany). Imaging of the abdomen was performed with a single axial slice at the L4-5 level using a 2D gradient recalled echo (GRE) three-point Dixon sequence (TR=20ms, TE=2.75/3.95/5.15ms, matrix size=192x156). Localization of the slice to the L4-5 level was performed with a sagittal T_2 weighted turbo-spin echo (TSE) sequence prior to the axial abdomen imaging. Two acquisitions were performed in a single breath hold and all imaging was performed with a phase array torso/abdomen coil. Separate fat and water images were created with custom software using the three-point Dixon method.[45] A tissue model was then used to calculate estimates of total fat and nonfat volume fractions on a per-pixel basis, which were displayed in image form. SAT was determined by manually defining a region of interest (ROI) between the skin and the fasical boarder external to the abdominal wall. VAT was determined by manually defining a region of interest within the fascial plane of the internal abdominal wall. Total SAT and VAT tissue was calculated by summing the value of percent fat fraction over all pixels within the ROI using custom-written image analysis software (MATLAB; The MathWorks, Natick, Massachusetts). This sum was multiplied by the area of each pixel to give total SAT and VAT cross-sectional area within the ROI.

IMAT determination. The legs were scanned in a coronal plane and the midpoint of the thigh was determined and defined as half way between the superior margin of the femoral head and the inferior margin of the femoral condyles. Axial imaging (5mm thick slices at 1 cm intervals) of the legs was then performed over 1/2 the length of the femur, centered at the midpoint of the thigh. Eleven images from the middle 1/3 of each thigh were used to determine average cross-sectional area (cm^2) of IMAT. Manual tracing eliminated subcutaneous fat and bone and isolated the fascial border of the thigh to create a subfascial ROI. Total IMAT was calculated by summing the value of percent fat fraction over all pixels within the ROI using custom-written image analysis software (MATLAB; The MathWorks, Natick, Massachusetts). This sum was multiplied by the area of each pixel to give total IMAT within the ROI. This method accurately measures fat and lean tissue in pixels that contain both by allowing fractional contributions to the fat and lean tissue CSA calculations.[46] This allows microscopic fat within muscle tissue as well as thin planes of fat adjacent to fascial planes to be accurately taken into account, even when image resolution is inadequate to delineate these visually. The same investigator, blinded to slice location, performed measurements of individual participants SAT, VAT and IMAT. This technique has demonstrated high levels of intrarater reliability, [47] test-retest reliability,[48, 49] and concurrent validity when compared to imaging of a cadaveric phantom limb.[47]

Muscle Biopsy

All muscle biopsies were performed within one week of the MRI scans. Participants were asked to refrain from any strenuous activity in the 48 hours prior to the muscle biopsy. All individuals presented in the morning after a 12 hour fast for a percutaneous needle biopsy.[50, 51] The skin and fascia 12-15 cm above the lateral tibio-femoral joint space was anesthetized with 5cc of 1% lidocaine and a small incision was made. The biopsy needle was inserted 3-5 cm beyond the fascia into muscle and three passes (bites) made to yield approximately 200 mg of muscle tissue. Following biopsy, deep pressure was applied for 20 minutes, followed by a steri-strip bandage and pressure dressing. The sample obtained was dissected free from blood and visible fat, snap frozen in liquid nitrogen and stored at -80 C until analysis.

Pro-inflammatory Cytokine Measures in the Muscle.

IL-6 and TNF-a were the dependent variables chosen to represent pro-inflammatory cytokines levels within the muscle. In order to measures the expression of these pro-inflammatory cytokines within the muscle both mRNA and protein expression of IL-6 and TNF-a were determined. To determine the mRNA expression of IL-6 and TNF-a in the muscle tissue of the participants total RNA, cDNA synthesis and real-time qPCR were conducted as previously reported. [52] Total RNA was extracted by homogenizing 15-20 mg muscle tissue with a hand-held homogenizing dispenser (PowerGen 125; Fisher Scientific) in a solution containing 0.75 ml Tri reagent (LS; Molecular Research Center, Cincinnati, OH) and 0.25 ml nuclease free water. The RNA was separated into an aqueous phase using 0.2 ml of chloroform and precipitated using

0.5 ml of isopropanol. Isolated RNA was washed with 1 ml of 75% ethanol, dried, and then suspended in a known amount of nuclease-free water (1.5 ml/mg tissue). RNA was DNase-treated using a commercially available kit (TURBO DNase-free, Life Technologies, Carlsbad, CA). RNA concentration was determined with a NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA). Afterwards, 0.5 mg of total RNA was reverse transcribed into cDNA according to the manufacturers' directions (iScript, BioRad, Hercules, CA). All isolated RNA and cDNA samples were stored at -80°C until analyzed. Real-time qPCR was carried out with an Applied Biosystems 7900HT fast sequence detection system. Taqman pre-designed primers (IL-6 and TNF-a) were purchased from Life Technologies. Values were normalized to beta 2-microglobulin then fold change values were calculated using the $2^{-\Delta\Delta Ct}$ method.[53]

To determine protein expression of IL-6 and TNF-a in the muscle western blots were used. Frozen muscle tissue was homogenized in a buffer cocktail with protease inhibitors in a pre-chilled glass tube under ice. Muscle homogenates were centrifuged at 6000 rpm for 10 minutes at 4°C and the supernatant was subsequently collected and transferred to a new microcentrifuge tube. Total protein concentration for each sample was determined on a spectrophotometer using a colorimetric protein assay (Bio-Rad; Bradford) and an albumin standard curve. Whole muscle homogenates were diluted 1:1 in a 2X sample buffer. Homogenates were loaded at equal protein concentration on a Criterion Tris-HCL pre-cast polyacrylamide gel (Bio-Rad) and subjected to SDS-PAGE (150V) for 1h in running buffer. Each gel contained alternating samples loaded in duplicate, and a molecular weight ladder. An internal control was loaded in duplicate on

each gel for band normalization and comparisons across blots. Protein was transferred (50V; 1h) to a polyvinylidene diflouride membrane in ice cold transfer buffer then blocked for 1h at room temperature with 2% nonfat dry milk (NFDM) in Tris-buffered saline in 0.1% Tween-20 (TBST). Membranes were incubated overnight in primary antibody diluted in 2% NFDM in TBS. The next morning, blots were rinsed in TBST for 5 minutes, rocked in secondary antibody for 1 hour at room temperature in 2% NFDM in TBS then serially washed (15 minutes, 4 x 5 minutes) in TBST. Chemiluminescence reagent (ECL Plus, GE Healthcare) was applied to each blot for 5 minutes. Optical density measurements were obtained with a digital imager (Bio-Rad). Membranes were stripped (Restore PLUS, Thermo Scientific) of primary and secondary antibodies then re-probed for a-tubulin (1:50,000; Sigma Aldrich, St. Louis, MO). Densitometric analysis was performed using Quantity One software (Bio-Rad). After subtracting out background, all western blot data were normalized to the internal control and replicate samples were averaged. The following antibodies were used in this experiment: IL-6 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), TNF-a (1:500; Cell Signaling, Boston, MA), and Toll-like receptor 4 (1:500; Santa Cruz Biotechnology). Donkey anti-rabbit and goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (1:6000) were purchased from Santa Cruz Biotechnology.

Data Analysis

Data were analyzed with SPSS Statistics 20.0 (SPSS, Chicago, IL). Descriptive statistics were calculated for demographic variables. To examine our hypothesis of the relationships between activity, fat depots, and inflammation pearson product correlations

were used. The correlations between activity levels, BMI (a measure of total body fat), VAT, SAT, IMAT, mRNA and protein expression of TNF-a and IL-6 within the muscle of all participants were calculated. The level of significance was set at $p<.05$.

Results

Participant demographics are presented in Table 4.1. Fourteen women completed the MRI's and biopsies and were thus included in this study. Three women had previous abdominal surgery creating artifact in the MRI that precluded us from obtaining VAT or SAT measurements. IL-6 mRNA was undetectable (i.e., not amplified within 40 PCR cycles) for one individual. Therefore the results presented are for 11 persons for VAT and 13 for IL-6.

There were significant ($p<.05$) correlations between BMI and all body fat depots. (VAT ($r=0.80$), SAT($r=0.57$) and IMAT ($r=0.83$).) BMI was also significantly negatively associated with activity levels as measured by the PASE ($r= -0.52$). IL-6 mRNA expression was significantly associated with IMAT ($r=0.64$), VAT ($r=0.80$), BMI ($r=0.62$) and activity levels ($r = -0.50$). IL-6 protein expression revealed a trend toward significance with activity levels ($r = -0.43$ $p=0.06$). TNF-a mRNA expression was significantly associated with SAT ($r=0.54$) and BMI ($r=0.48$) but was not associated with activity levels. TNF-a protein expression was not significantly associated with any measures.

Table 4.1 Participant Characteristics

N	14
Age (years)	80.1 (5.3)
BMI (Kg/m^2)	23.8 (2.9)
IMAT (cm^2)	17.1 (5.2)
VAT (cm^2)	108.5 (69.6)
SAT (cm^2)	185.1 (55.7)
PASE (minutes of activity/week)	140 (96)

BMI = Body Mass Index; MPPT = modified physical performance test score; IMAT= thigh intramuscular adipose tissue average cross sectional area; VAT = cross sectional area of visceral adipose tissue; SAT = subcutaneous adipose tissue; PASE = Physical Activity Scale self reported minutes of activity/week

Discussion

In this study we sought to determine the relationships of physical activity, location specific fat depots (VAT, SAT, and IMAT), and local (as measured in the muscle) pro-inflammatory cytokine expression in older adults. We hypothesized that active individuals would have both lower VAT and IMAT as well as decreased local inflammation. We further hypothesized that significant relationships would exist between VAT, IMAT and local inflammation.

The Relationship of VAT, IMAT and Inflammation

The most novel finding of this study was that IL-6 mRNA expression within the muscle was correlated with BMI, VAT and IMAT depots and with self-reported physical activity levels in older nonobese females. This suggests that pro-inflammatory cytokines within the muscle are associated both with total body fat as well as local fat depots and activity levels. While BMI, VAT, and IMAT have been shown to be highly correlated in

obese individuals this has not been reported in those with BMI level in the normal range. Our findings suggest that fat deposition in the muscle even in nonobese individuals is associated with a surrogate measure of total body adiposity (BMI) and with VAT. While previous work has demonstrated that up to 30% of circulating levels of IL-6 may be released from adipose tissue in obese subjects,[18] our data suggest that even in the absence of obesity, IL-6 mRNA expression in the muscle is associated with adipose deposition. Our findings are in agreement with Beasly et al., who reported the relationships of circulating IL-6 and TNF-a with VAT, SAT and IMAT in older adults. [16] In this study of over 1,000 older adults they found that all body fat measures (VAT, SAT, and IMAT) were positively and significantly correlated with circulating levels of IL-6. They also identified a trend towards increasing IL-6 with increasing levels of IMAT.[16] While our study is much smaller than Beasly et al. it is the first that we are aware of that examines the relationships of Il-6 and TNF-a mRNA and protein expression in locomotor muscle and multiple fat depots.

Inflammation, IMAT, and Activity

Increased levels of IMAT are associated with decreased strength and mobility in both younger and older adults.[32, 34, 35, 37, 45, 54] Multiple authors have theorized that these negative associations may be due to increased release of TNF-a or IL-6 from IMAT.[16, 36, 37, 40, 41] While we did not find any relationship between any fat depots and IL-6 protein expression within the muscle we did find significant correlations between IL-6 mRNA expression within the muscle and both IMAT and VAT. Increased basal levels of pro-inflammatory cytokines such as IL-6 is one of the most important physiologic correlates

of frailty[55, 56] and increases in circulating levels of IL-6 are known to be associated with decreased strength,[13, 57] mobility,[58, 59] and activity levels in older adults.[60] We also found that both IL-6 mRNA and protein expression within the muscle were significantly correlated with self-reported activity levels. Several studies have reported significant correlations between increased activity levels and decreased levels of circulating pro-inflammatory cytokines.[15, 61, 62] Our findings along with previous work suggests that increasing activity levels may be one treatment for combating chronic inflammation in older adults. Exercise interventions may mitigate increased IMAT in locomotor muscle of this population. Modest walking has been shown to prevent increased IMAT in older adults[63] and combining aerobic and resistance training can decrease IMAT accumulation[64]

While previous research suggests little to no change in circulating cytokines with exercise interventions, large decreases in pro-inflammatory cytokines within the muscle have been reported.[25, 65] Although changes in VAT and IMAT were not examined in these studies, decreases in VAT or IMAT provide a potential explanation for the muscle specific decreases in pro-inflammatory cytokines that accompany exercise training. Further studies are needed to elucidate the relationships of increased activity levels, IMAT and inflammation within the muscle.

Limitations and Directions for Future Research

Due to the limited number of participants in the present study, our results should be interpreted cautiously. However, all participants were carefully screened to minimize the effect of medications or illnesses known to influence inflammatory markers, and

therefore these results may in fact be conservative. The inclusion of individuals with known systemic disease may uncover more robust findings. While it is possible that some participants had an undiagnosed illness that influenced our results, our agreement with previous research support our findings, that both VAT and IMAT as well as physical activity levels influence pro-inflammatory cytokines found within the muscle of older women, even in the absence of obesity lend credence to our findings. Future research should corroborate our findings with larger sample sizes, and importantly determine whether therapeutic interventions aimed at decreasing inflammation affect ectopic fat deposition or vice-versa. Such information will help to inform the mechanisms that link excessive adipose tissue to muscle and mobility decline in older adults and provide targets for therapeutic interventions.

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