FAT DEPOTS WITHIN AND AROUND THE SKELETAL MUSCLE IN RHEUMATOID ARTHRITIS AND THEIR ROLE IN PHYSICAL FUNCTION

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Altered body composition towards greater body fat mass and lower lean mass is a well-known manifestation of Rheumatoid Arthritis (RA). However, there is limited information on fat accumulation in and around the skeletal muscles, and whether they contribute to functional limitations and low physical activity levels that persist in this population despite well-controlled disease. The main objectives of this dissertation were to 1) characterize accumulation of skeletal muscle fat (SMF), intermuscular adipose tissue (IMAT) and subcutaneous adipose tissue (SAT) in individuals with RA and determine the associations of SMF, IMAT and SAT with physical function and physical activity measures; and 2) compare SMF, IMAT and SAT between individuals with RA and healthy individuals.

This cross-sectional ancillary study used data from previously conducted studies in adults with RA and healthy adults. SMF, IMAT and SAT were measured from computed tomography images of the mid-thigh region. Physical function in the RA cohort was measured using self-report and performance-based tests, and physical activity was assessed using an accelerometer-based activity monitor. Associations between each fat depot, and physical function and activity were assessed by multiple linear regression models. To compare SMF, IMAT and SAT in RA and non-RA subjects, those with RA were matched in sex and BMI to healthy adults of same age and to older healthy adults who were 10-20 years older. The differences between groups were assessed using related samples tests.

In subjects with RA, higher SMF significantly contributed to lower physical function and activity ($R^2\Delta$ range=.08-.25, $p<0.05$), whereas IMAT and SAT accumulation were not associated with physical function or activity. Individuals with RA had significantly higher SMF (10% difference, $p < 0.05$) whereas IMAT and SAT accumulation were similar to healthy individuals of same age. There were no differences in SMF, IMAT and SAT between the RA and matched older healthy groups.

Study findings demonstrate that SMF accumulation is an important contributor to low physical function and activity, and accumulates more in RA. These results provide preliminary evidence for future longitudinal studies to investigate the impact of SMF on disability and promote health in individuals with RA.

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PREFACE

The journey towards a PhD is not one that is taken alone. I would like to take this opportunity to express my gratitude towards all those who have provided me with the support, guidance and strength required to complete this arduous yet rewarding process.

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1.0 PROPOSED RESEARCH STUDY

1.1 INTRODUCTION AND SPECIFIC AIMS

About two-thirds of people with Rheumatoid Arthritis (RA) experience a loss of body fat free mass and a concomitant increase in body fat mass which associates with low physical function and disability.[1] Rheumatoid Arthritis is a systemic, inflammatory disease of auto-immune origin and unknown etiology that affects about 1% of the population. Systemic inflammation in RA largely affects the synovial joint causing swelling, stiffness, pain and erosion of joint surfaces. The systemic inflammation also affects body composition by perpetuating the degradation of body cell mass (fat-free mass), and concomitantly increasing fat mass.[1] Evidence from cross-sectional studies shows that overall body fat is higher and body fat free mass is lower in RA compared to healthy populations,[2-4] and that high body fat mass and low body fat free mass are associated with greater disability and lower physical function.[4,5]. While evidence suggests a relationship between overall body composition and physical function, it is uncertain whether specific compartments or depots of fat are responsible for lower physical function and contribute to disability in RA.

In particular, there is limited information about fat content within and in close proximity to skeletal muscle tissue in RA. High fat content within and in close proximity to skeletal muscles can directly affect physical or metabolic properties of the skeletal musculature, and

consequently influence overall physical function. Currently, it is not known whether fat within and around the muscle is higher in RA compared to healthy populations, and if it contributes to low physical function and higher disability in RA. Fat compartments around the muscle are: i) intermuscular adipose tissue, which is present inside the muscle fascia and between the muscle groups, and ii) subcutaneous adipose tissue, which is below the skin and above the muscle.[6] Fat depots present inside the muscle is skeletal muscle fat, and it can be further differentiated into intramyocellular lipid, which lies inside the individual muscle cell, and extramyocellular lipid which lies between individual fibers.[7,8] Both skeletal muscle fat and intermuscular adipose tissue have shown to play a role in muscle function in healthy and non-RA populations, and likely play a similar role in RA, however this role has not been investigated.

Skeletal muscle fat is an important predictor of the physical and metabolic muscle properties in non-RA populations. Higher skeletal muscle fat is associated with lower muscle strength and lower physical function in older adults,[9,10] and also associated with low grade inflammation and higher insulin resistance in elderly[11-14], obese[15-18], and glucose intolerant populations.[17,19] Similarly, intermuscular fat is positively associated with low grade inflammation[13] and mobility restrictions in older adults.[20] Due to the positive association between skeletal muscle fat and intermuscular fat with inflammation in non-RA populations, it is possible that people with RA (who experience high grade systemic inflammation) could have high skeletal muscle fat and intermuscular fat which could affect their physical function in a similar manner observed in older healthy adults. In contrast, the contribution of subcutaneous adipose tissue to physical function is less conclusive. Few studies demonstrated no associations between SAT and physical function,[9,21] while others reported small but statistically significant associations between SAT and risk of mobility limitation in women but not men.[22,23] Studies

have not characterized skeletal muscle fat, intermuscular adipose tissue or subcutaneous adipose tissue in RA nor investigated their role in physical function and disability, hence, the overall aim of this study is to characterize skeletal muscle fat, intermuscular and subcutaneous adipose tissue depots in RA, compare them to a healthy matched group and determine the contribution of each of these fat depots to physical function and disability in individuals with RA.

Specific Aim 1

To characterize skeletal muscle fat content (intramyocellular lipid and extramyocellular lipid), intermuscular adipose tissue (IMAT) and subcutaneous adipose tissue (SAT) in a cohort of people with RA, and to explore their associations with RA disease characteristics (i.e disease duration and disease activity).

For this descriptive aim, skeletal muscle fat (SMF) will be quantified by both direct and indirect techniques. Direct technique includes histo-chemical analysis (light microscopy using Oil Red O staining) of muscle sample obtained from needle biopsy to quantify intramyocellular lipid content. Indirect technique consists of assessing tissue attenuation coefficients derived from Computerized Tomography (CT) imaging of the mid-thigh region. Tissue attenuation coefficients will identify skeletal muscle fat (intra and extramyocellular lipid combined), IMAT and SAT.

Specific Aim 2

To compare SMF, IMAT and SAT content in people with RA to that of age, sex, and BMI matched healthy controls, as well as sex and BMI matched healthy elderly controls. SMF, IMAT and SAT will be quantified from CT imaging and biopsy samples.

Hypothesis 2a

SMF, IMAT and SAT will be higher in people with RA compared to their age, sex and BMI matched healthy controls.

Hypothesis 2b

SMF, IMAT and SAT will be similar in people with RA compared to sex and BMI matched elderly healthy controls 10 to 20 years older than the RA subjects.

Specific Aim 3

To explore the association between SMF, IMAT and SAT with measures of physical function, physical activity, and disability in subjects with RA.

Physical function will be measured by a battery of physical performance tests that includes the chair rise test, the stair climbing, the 4 meter walk, the single leg balance test, and the quadriceps muscles strength test. Physical activity will be measured by an accelerometry-based portable physical activity monitor. Disability will be measured by the self-reported Health Assessment Questionnaire.

Hypothesis 3a

In people with RA higher SMF and higher IMAT will be associated with lower measures of physical function, lower physical activity, and increased disability. SAT will not be associated with measures of physical function, physical activity, and disability in people with RA.

Hypothesis 3b

In people with RA, SMF and IMAT will contribute to physical function, physical activity and disability even after accounting for potential confounders of the relationship such as sex, body size, muscle strength, and muscle area.

1.2 BACKGROUND

1.2.1 Disease Overview

Rheumatoid Arthritis (RA) is a systemic, inflammatory disease that mainly affects the synovial joints. It occurs early in life with the peak age of onset being 50-75 years; and affects approximately 1% of the population of which around two-thirds are women.[24] RA is an auto-immune disorder of unknown cause.[24] The exact cause of RA is not clear, but individuals with genetic predisposition and a family history are more likely to have RA.[24,25] The severity and rate of progression varies, and can lead to significant disability in daily activities,[26] reduced physical function,[27] decreased work productivity,[28-30] and increased morbidity and mortality.[31-33]

The hallmark clinical feature in RA is inflammation in the synovial joint capsules and the formation of a thick tissue within the membrane, known as “pannus”. The inflamed synovium and pannus formation gives rise to symptoms of joint pain, swelling and tenderness. As RA progresses, the thickened synovial membrane leads to destruction of articular cartilage and joint surfaces,[34] which then increases physical disability,[35,36] and in the long term may lead to joint deformities.[37] Usually smaller, distal joints such as the fingers, wrist, elbow or knee are affected, while proximal joints like the hips and shoulders are less commonly affected.[24] The natural course of RA varies with some individuals experiencing cyclic episodes of symptom exacerbation and remission, and others individuals whose symptoms do not remit but gradually progress over time.[38,39]

Rheumatoid arthritis is diagnosed based on history and clinical examination. There is no gold standard for diagnosis of RA, but several classification criteria have been developed by rheumatology task forces to identify persons with RA. The 1987 American College of

Rheumatology Classification criteria for RA was most widely used by researchers and clinicians; and more recently 2010 ACR/EULAR classification criteria was developed to improve the 1987 classification and enable diagnosis of RA during early stages of the disease. Both criteria demonstrate good sensitivity (~0.8-0.9) and specificity (~0.5-0.8) in those with RA.[40,41] Pooled sensitivity was greater by 0.11 in 2010 ACR criteria compared to 1987 ACR criteria, while pooled specificity was lower by 0.04.[40]

1.2.2 Non-articular manifestations in RA – Altered Body Composition

As a systemic disease, RA not only affects articular surfaces but also other organs and tissues;[42] altered body composition is one such non-articular manifestation experienced by about two-thirds of people with RA, and is implicated in increasing disability and morbidity in RA.[1,43] Individuals with RA tend to have body composition which has a lower amount of lean mass and a higher amount of fat mass.[3,4] Alteration in body composition is a result of loss in body cell mass due to increased cell protein degradation, and is perpetrated by systemic inflammation.[43,44] A more severe form of body cell mass loss is referred to as "Rheumatoid Cachexia".[5,43,45,46] Body cell-mass is part of the cell that is fat-free, and excludes extracellular fluid or solids; it is also the location for majority of the cell metabolic activity. Body cell mass is further differentiated into skeletal, visceral and immune system cell masses, with skeletal tissue accounting for around 60% of body cell mass.[1,43]

Loss of body cell mass is associated with inflammation in RA, (higher amount of inflammation indicating lower body cell mass);[5,47] however, the exact mechanisms by which inflammation favors a state of increased protein degradation is not completely understood. Inflammatory factors such as tumor necrosis factor-α and interleukin-1β have been implicated in

increasing whole body protein degradation.[43,48,49] Other cytokines that have also been implicated in increasing protein breakdown are interferon-gamma (IFN-γ), and transforming growth factor-beta (TGF-β1). TNF-α and IFN-γ activate nuclear factor kappa B that suppresses MyoD RNA during post transcriptional phase, which in turn suppresses skeletal muscle differentiation.[43]

The temporal relationship between disease duration and loss of body cell mass is not completely clear due to lack of longitudinal evidence as most studies in this area are cross-sectional, and have been conducted in RA patients with a disease duration of more than 5 years (average duration of RA in the studies ranged from 6-13 years).[4,5,48-50] Furthermore, as individuals with RA are also less physically active,[51-53] a sedentary lifestyle combined with increased body catabolic activity can also contribute to altered body composition by perpetrating further muscle loss, and increase in fat mass. Altered body composition could thus potentially result from the cumulative effects of RA inflammatory activity, disease duration and lifestyle changes.

1.2.3 Evidence Review of Altered Body Composition and Fat distribution in RA

Altered body composition in RA has been mainly studied in cross-sectional studies; some studies included a healthy control group,[2-4,47-49] while some compared body composition findings in RA to available reference values from historical healthy cohorts.[5,50] The majority of the studies measured body composition using Dual X Ray absorptiometry (DXA), which differentiates between bone, muscle and fat mass.[2-5] Few studies have directly quantified body cell mass by measuring total body potassium,[48,49] which does not differentiate between tissue types, and few used bio-electrical impedance which can measure fat and fat free masses.[47,50] Studies have mainly been conducted in people who have had RA for more than 5 years[4,5,48-50] and few in

people with disease duration of less than 3 years.[2,3] Except for study by Elkan et al[50] which included RA subjects with low inflammatory activity, all other studies included those with moderate or high inflammatory activity.

The combined evidence from RA body composition studies demonstrates that body fat tends to be higher, and this finding is predominant in women with RA. Regional body composition measures showed that the trunk was more likely to have greater amount of fat, while higher appendicular fat and lower appendicular lean mass were only present in some RA cohorts. Among the studies that used DXA derived body composition measurements, Giles et al compared a cohort of RA subjects to an age, sex and race (87% Caucasians) matched healthy non-RA cohort and found that differences were more predominant in women with RA. They had about 11% greater total body fat, around 13% lower skeletal muscle mass to fat mass ratio, and 13% higher truncal fat mass compared to women without RA; similar differences were not observed in men with RA. Appendicular lean mass did not differ between RA and healthy women, but was about 5% lower in men with RA compared to healthy men.[4] Book et al conducted a similar cross-sectional comparison of patients with early RA to healthy matched controls and found similar results: women with RA had 12% higher total body fat and 19% greater truncal fat mass but similar appendicular lean mass compared to healthy women; men with RA only demonstrated 11% lower appendicular lean mass and 7% lower total body lean mass compared to the healthy men.[2] Dao et al demonstrated in a cohort of Vietnamese women that those with RA had 12% higher total body and 21% higher truncal fat mass, and 8% lower appendicular lean mass compared to women without RA.[3] Among the studies that quantified body cell mass, two cross-sectional case control studies showed that compared to healthy age, BMI and sex matched controls, body cell mass (measured by total body potassium) was lower in

RA by 14-16%.[48,49] In a cohort of Chinese individuals, Chen et al did not find any difference in body cell mass measured by bio-electrical impedance between RA and non- RA cohort matched on sex and age.[47]

Body composition phenotypes were also described in the studies by Giles et al[4] and Dao et al[3] and a higher prevalence of abnormal body phenotypes was found in women with RA. Body phenotypes are based on previously defined criteria present in the literature, and consist of sarcopenia, which is described as having a skeletal muscle mass index of <5.75 kg/m^2; over-fat, which is described as excess fat on DXA using age-, sex-, and race-stratified cut points of body fat percentage from a large cohort of healthy adults;[54] and sarcopenic obesity, which is present when both criteria for sarcopenia and is satisfied. Both studies demonstrated around 9-13% higher prevalence of sarcopenia, around 10-22% higher prevalence of over-fatness, and 9% higher prevalence of sarcopenic obesity in women with RA. Giles et al additionally demonstrated that the odds of having sarcopenia were 3 times higher, the odds of being over-fat were 2 times higher, and the odds of being both over-fat and sarcopenic were 5 times greater in women with RA. Giles et al did not find a higher prevalence in abnormal phenotypes in men with RA, and Dao et al study only included women. Engvall et al used fat free mass index and fat mass index to define abnormal body phenotypes in a cohort of RA individuals, and compared them to reference values from a healthy cohort. Based on the percentile values of fat mass and fat free mass indexes, they found that 50% of individuals with RA had lower lean mass (fat free mass index below 10th percentile of reference population), 45% were obese (greater than 90th percentile on Fat mass index of reference population), and 38% had cachexia (Fat free mass below 10th percentile and fat mass index above 25th percentile).[5]

Thus, the evidence from studies described above suggests an overall higher body fat mass in RA compared to healthy populations, but there is no information about which specific compartments or depots of adipose tissue are affected. While DXA technology recognizes and differentiates overall body fat from muscle and bone, it cannot separately identify or quantify subcutaneous fat, or fat within the muscle (intermuscular fat and skeletal muscle fat).

1.2.4 Fat within the Muscle and its Importance

The roles of fat within (SMF) and around the muscle (IMAT) have been described in non-RA populations and were shown to have detrimental influences on health that are in contrast to SAT, which has not been associated with adverse physical or metabolic health complications.[55-57] Because different fat depots may have varying associations with health, it is relevant to assess them separately. Higher IMAT and SMF have been established in obese populations who have greater body fatness,[16,18] and were found to be associated with lower insulin sensitivity and higher inflammation in older adults and obese adults.[7,13,19,58,59] One study showed that obese adults and those with diabetes had the highest levels of IMAT and also the lowest insulin sensitivity. The same study also showed that while IMAT was associated with changes in insulin sensitivity, SAT did not seem to play a similar role and was not associated with insulin sensitivity.[16] Beasley et al reported a similar relation of IMAT with insulin sensitivity and inflammation in older adults, where higher levels of IMAT were associated with greater insulin resistance and inflammation.[60] As people with RA experience both altered insulin metabolism and systemic inflammation, they may also have higher amount of SMF and IMAT.

SMF plays a role in muscle strength, physical function and mobility, and was studied on a large scale in an epidemiological study known as the Healthy, Aging and Body Composition

study,[9,10,15,61-63] that consisted of a cohort of healthy older adults between 70-79 years, who were followed over a period of 14 years. Studies from this cohort demonstrated that higher skeletal muscle fat in older adults contributed to lower levels of mobility, decreased strength and reduced physical function.[9,10,61] The contribution of SMF to lower physical function and mobility restrictions was present even after accounting for individual characteristics, muscle strength, muscle cross-sectional area, and body fat.[10,61] Mobility limitations were increased in those with greater SMF (Hazards Ratio for women = 1.98 and for men = 2.16).[10] SMF was an important predictor of lower extremity physical function, after accounting for individual characteristics, body fat and muscle area.[61] Higher muscle fat content in the mid-thigh region was also associated with hip fracture with RR of 1.58 (95% CI: 1.18-2.12) after accounting for muscle cross-sectional area, muscle strength, and lower extremity physical performance measures.[63] On further adjustment of the model with femur bone mass density, higher skeletal muscle fat was still independently associated with risk of hip fracture (RR: 1.42, 95% CI :1.03, 1.97).[63]

SMF can be further divided into: i) intramyocellular lipid,[7] located within each individual muscle fiber and is a metabolically active energy source for the muscle, and, ii) extramyocellular lipid, interspersed between muscle fibers and functions as a long term storage of fat depots.[8] While greater SMF was associated with lower insulin sensitivity in older and obese adults,[64,7,19,65] the opposite was found in endurance trained athletes, who showed higher amounts of intramyocellular lipid and greater insulin sensitivity. This is known as the athletes' paradox, and was a finding that initiated the idea of exercise being a mediator between intramyocellular lipid and insulin resistance.[66] As intramyocellular lipid is a source of energy for the muscle, the presence of adequate lipid utilization and fatty acid oxidation during bouts of physical activity necessitates replenishment of larger depots of intramyocellular lipid for optimum performance.

However in a situation where lipid is not adequately utilized, as in a sedentary person, or a high fat diet, intramyocellular lipid begins to accumulate, and favors the release of lipo-toxic metabolites and impair fat oxidation capacity.[67] We theorize two possibilities with respect to IMCL content in RA. Either that they have high IMCL content as they are largely sedentary and may not be adequately utilizing their stores of intramyocellular lipid, or that they may have lower than normal IMCL content due to the hypermetabolic state in the muscle brought upon by high grade systemic inflammation. [49]

Thus, from the evidence described above SMF and IMAT seem to be an important predictor or indicator of the "health" of muscle, and can act as a predictor of physical as well as metabolic function in people with RA. However, SMF and IMAT have not been studied or characterized in RA. Studies of body composition have reported greater body fat percentage in RA compared to healthy populations, however, these studies could not determine differences in SMF, IMAT and SAT as they utilized DXA which cannot identify or differentiate types of fat.

1.2.5 Fat Infiltration in the RA Muscle

Studies that investigated skeletal muscle involvement in RA largely explored vascular and inflammatory changes in the muscle but did not investigate fat infiltration in RA muscle. Analysis of muscle biopsy samples from case studies and small RA cohorts revealed necrosis of blood vessels, mononuclear and polynuclear cell infiltration around the circumference of the blood vessels, which explained clinical features of rheumatoid vasculitis, [68-71] and infiltration of macrophages and leucocytes and inflammatory cells into the muscle tissue, which explained myositis.[72-74] Current evidence also suggests that increased protein degradation mediated by inflammatory markers results in loss of muscle in RA.[1,48,75] Selective atrophy of Type 2 muscle

was also observed in RA and is considered an RA muscle atrophy pattern.[76] The combined available evidence indicates that muscle is affected in RA, but little is known whether fat content within the muscle is also altered in RA. Although it is a well-accepted notion that skeletal muscle mass in RA is reduced and seems to be replaced by an increased amount of fat, it is not clear whether there is an increased amount of fat within the muscle (IMCL +EMCL depots).

To date, only one study reported findings on SMF in RA. Kramer et al used CT to measure skeletal muscle fat and total thigh fat in a cohort of RA individuals. The study did not differentiate between IMAT and SAT, and did not use any direct method to quantify or describe intramyocellular lipid in RA. Furthermore they did not have a control group to determine any differences in fat distribution.[77]. Observations in healthy sedentary, older adults, obese, insulin resistant and type 2 diabetic populations suggest an overall increase in elevation of normal intramyocellular lipid content due to inefficient fat oxidation, and under-utilization of fat stores in presence of a high fat diet and low physical activity,[16,18,19] but evidence is limited in the RA population. As people with RA are less physically active, and at a high risk of obesity[4,78] as well as metabolic syndromes,[78-81] they could also experience similar elevations in skeletal muscle fat content due to a combination of decreased or impaired fat utilization and disease inflammation. As skeletal muscle fat has not been adequately investigated in this population, the first aim of this study would focus on gathering information to describe and quantify skeletal muscle fat in a cohort of people with RA.

In the proposed study, SMF, IMAT and SAT will be investigated by CT imaging. Skeletal muscle fat (intramyocellular lipid) will additionally be measured by histo chemical staining of lipid droplets in muscle samples obtained from biopsy of the vastus lateralis muscle.

This study will be the first to explore intramyocellular lipid content in RA using muscle samples and will serve as a preliminary reference for amount of intramyocellular lipid in RA.

1.2.6 SMF, IMAT and SAT in RA compared to a Non-RA

Although people with RA have a higher rate of sarcopenia and increase in body fat percentage compared to their healthy counterparts,[3,4,82] there is limited evidence to suggest that SMF, IMAT and SAT quantity in RA are higher compared to those without RA. Kramer et al quantified SMF using CT attenuation values in individuals with RA, but, they did not compare findings with a healthy matched control group, nor did they differentiate between IMAT and SAT.[77] Two studies by Matschke et al compared muscle architecture in cachetic RA subjects to healthy controls and revealed that cross-sectional muscle area in RA was about 13-16% lower compared to healthy controls, indicating a compromise in muscle mass; however, muscle area was quantified by ultrasonography, which does not permit the quantification of SMF, IMAT or SAT.[83,84] Hence, the second aim of this study is to compare the quantity of SMF, IMAT, and SAT in RA to age, sex and BMI matched healthy individuals. The results of the second aim will provide important information if the RA disease affects skeletal muscle fat content.

Higher mortality in RA has raised the idea of "accelerated aging" in this population, and it was suggested that early aging in RA was the result of senescence in several body systems, including the musculoskeletal system.[85] Muscle in RA has been theorized to undergo premature degeneration similar to what is observed in aging adults, but this theory has not been directly assessed. In healthy aging adults however, longitudinal studies have demonstrated a trend of increased body fatness and decreased lean mass.[86-90] Muscle strength and muscle mass decline have been shown to decline annually by about 3% and 1% respectively, in healthy elderly

adults,[91] and greater loss in muscle strength was attributed to gains in SMF. Changes in body fat distribution with aging favored an increase in abdominal and visceral fat, and a decrease in limbs.[92] Older adults generally also tend to lose SAT and gain IMAT.[88]

Muscle alteration in older adults can be comparable to people with RA because of similar mechanisms that drive muscle loss in both populations. One such common factor associated with muscle loss in both RA and older adults is presence of systemic inflammation. Muscle loss in older adults has also been attributed to a low grade systemic inflammation, due to higher expressions of cytokines such as TNF alpha, IL-6 IL-1 receptor antagonist (IL-1ra), IL-18, C-reactive protein (CRP), and fibrinogen.[60] Evidence suggests that TNF alpha induces age related sarcopenia by altering the expression of genes and signaling proteins that facilitate protein synthesis for muscle cell growth and differentiation.[93,94] A five year longitudinal study in adults older than 60 years demonstrated that the odds of losing appendicular lean mass were about 3 times higher in older adults with greater circulating levels of IL-6 and CRP.[95] Elevated intramyocellular lipid was also observed in older adults, and was attributed to a down-regulation of genes that are associated with lipid oxidation and transport.[96] The combined evidence in aging and RA suggest that high grade systemic inflammation for a relatively short period in RA could result in premature muscle changes with respect to SMF that would normally be present in old age (caused by low grade systemic inflammation for a longer period). However, as no study to date has directly compared SMF in RA to those of healthy elderly adults, part of the second aim will also focus on the comparison of SMF, IMAT, and SAT in RA subjects with sex, BMI-matched healthy adults who are at least 10-20 years older than the RA subjects. The results of this comparison will provide preliminary evidence to support or refute the theory that RA muscle changes resemble the process of muscle changes observed in normal aging.

1.2.7 SMF, IMAT and SAT and their Association with Physical Function in RA

While studies in RA demonstrate that greater body fat and lower lean mass are associated with greater disability, higher RA disease activity and overall reduced physical function,[3,5,82,97] the individuals roles of SMF, IMAT or SAT on disability and physical function in RA have not been investigated. It is important to address the associations of each fat depot on physical function, as they may have differential associations with physical function. In older adults, greater skeletal muscle fat has been associated with lower muscle strength and lower physical function, [9,61] and is also an independent predictor of hip fracture,[63] lower bone density,[98] and mobility restrictions.[10] Moreover, SMF continues to predict physical function and mobility even after accounting for muscle strength and area, total body fat and other biomedical or demographic characteristics.[10,61,63] Increase in IMAT was also associated with lower physical function in older and obese adults, but similar associations with SAT were not reported in the literature. Based on the non-RA evidence, SMF, IMAT and SAT may also play a crucial role in affecting physical function in individuals with RA, who are already at a high risk of impaired muscle strength and increased fracture risk due to their disease, [4,5,27,37,99-102]

One recent cross-sectional study did investigate the relationship of thigh CT derived muscle composition measurements of muscle and fat area, and muscle attenuation with physical function in a cohort of 197 patients with RA.[77] Longer disease duration, higher number of tender joints, use of prednisone and higher IL-6 levels associated significantly with lower thigh muscle density (attenuation), and explained almost 41% of its variability. Using multiple linear regressions, they found that thigh muscle attenuation significantly contributed to reduced physical performance and functional abilities after accounting for thigh muscle and fat area, and RA related disease characteristics. These findings are novel in the RA population as they

emphasize the importance of SMF, and its associations with physical function in people with RA. However, the study did not investigate effects of muscle strength on the relationship between SMF and physical function, which could be a key factor in altering the strength of this relationship. In order to understand how SMF independently influences physical function muscle strength must be taken into account during analysis.

Our research group conducted few preliminary analyses on associations between skeletal muscle fat and one measure of physical function; cross-sectional data of RA subjects who were enrolled in an ongoing clinical trial were used to analyze the association between quadriceps skeletal muscle fat and postural balance. Postural balance was assessed using the single leg balance test, in which the amount of time (up to 30 seconds) a subject can balance on a single leg is measured. To understand the contribution of SMF to postural balance, we controlled for quadriceps isometric strength and cross-sectional area using hierarchical regression analysis. The analysis revealed that quadriceps skeletal muscle fat contributed to balance even after accounting for quadriceps strength and muscle area (R^2 change = 14%, F = 3.32, $p < 0.1$ for right leg; R^2 change = 17%, F = 3.91, $p<0.1$ for left leg). These findings illustrate that factors other than muscle force generating capacity or muscle size may contribute to physical function. In this case it was skeletal muscle fat content that independently contributed to postural balance after accounting for muscle strength and size.

Although the preliminary analysis was limited to the associations between SMF and balance, a similar relationship with other measures of physical function may exist, and need to be further explored. Currently, there is no clear mechanism to explain how SMF affects balance, but the following hypothesis may be considered: lipid or fat infiltration could impede the function of muscle spindles. Three histological studies on muscle spindles in RA conducted about 40 years

ago demonstrated accumulation of fluid and thickening of the muscle spindle capsule, and fibrotic changes in the intrafusal muscle fibers, which could affect sensitivity of the muscle spindle.[103-105] It is possible that fat infiltration might also give rise to inflammatory processes that may perpetrate fluid accumulation in the muscle. Further investigation would be necessary to understand how muscle density affects postural balance, and other measures of physical function. Hence, the third aim of this study will assess the associations of SMF, IMAT and SAT with measures of physical function, disability and physical activity. These associations will also be explored after accounting for potential confounders, such as muscle strength and area, or individual characteristics such as BMI or age.

1.2.8 Measuring SMF, IMAT and SAT in RA

To address the objectives of this study, appropriate techniques that accurately quantify and describe SMF, IMAT and SAT in RA need to be utilized. Currently, various techniques to measure fat exist. While some techniques only estimate overall body fat, others have the capability of differentiating different fat depots based on anatomical location. The current section will summarize the evidence from different techniques to measure fat, as well as describe the techniques selected to address the study aims.

Body Composition Analysis Methods

Majority of the evidence in RA related to body composition (higher body fat mass and lower lean mass) has been assessed using techniques that provide surrogate measures of body composition, or an overall estimate of body fat. Surrogate measures of body composition in studies of RA include body mass index, anthropometry, and skin-fold thickness,[101,106,107] which

do not accurately estimate body fat percentage. Some studies also employed more sophisticated techniques that can differentiate between fat and fat-free mass, such as bioelectrical impedance analysis[47,50,108] and Dual X-Ray Absorptiometry (DXA). [2-5,50,97,109] Bioelectrical impedance analysis measures body water content and with the help of valid formulas and equations that convert impedance signals into fat estimates.[110,111] DXA uses photon energy to differentiate body tissues of different densities. Tissues with the highest density absorb the largest amount of photon and vice versa. DXA can differentiate between bone, muscle mass, and fat mass, and provide quantification and proportion of each tissue in the whole body or a particular region. [112]

Although DXA and bioelectrical impedance analysis are relatively reliable and give an accurate estimation of whole body and regional fat and fat free masses, neither can identify IMAT or SMF. DXA can provide some estimates of subcutaneous and visceral fat, but not of IMAT or skeletal muscle fat. Hence, when assessing different fat depots, both DXA and bioelectrical impedance have limited utility.

Muscle Biopsy and Imaging Methods to Assess SMF, IMAT and SAT

Direct analysis of muscle tissue has been widely used to assess skeletal muscle fat (mostly intramyocellular lipid). Muscle tissue samples are obtained through percutaneous muscle biopsies, which are conducted under local anesthesia, by physicians. Muscle samples allow direct, specific and microscopic analysis of intramyocellular lipid content within each individual muscle fiber.[7,65] Histo-chemical analysis and staining of frozen muscle cross-sectional slices using Oil Red O is a widely used and reliable method to quantify intramyocellular lipid.[16] Oil Red O stains intramyocellular fat (mainly triglycerides), and the stained gray areas indicates lipid content. Darker or higher intensity gray areas indicate greater intramyocellular lipid content.[16]

As muscle biopsies are invasive, alternative methods have been developed to capture SMF. Non-invasive soft tissue imaging has been used to illustrate fat distributions for entire muscle groups or body region. Computed tomography (CT) is one such soft tissue imaging technique that can identify SMF, IMAT and SAT, and was used on a large scale for the first time in the Healthy, Aging, and Body Composition study.[9,10,15,61-63,91,113-115] CT provides a 2-dimensional map of the body in pixels. Within each pixel, the CT identifies tissue based on its the attenuation value. The attenuation value or coefficient is based on the physical density and electron and proton content per unit mass of that tissue, and is measured on a linear scale called Hounsfield Units (HU), which uses water as the reference at 0 HU. Tissue that is less dense than water, such as adipose tissue, would have attenuation values lower than zero, while tissues that are denser than water, like muscle, would have attenuation values greater than zero. The type of body tissue is identified by the degree of grayness, and each tissue lies within a specific range of attenuation values; for example, muscle is a lighter shade of grey, and has positive attenuation values that range from 0-100 HU, while fat is darker gray, and has negative attenuation values that range from -190 to -30 HU. The average of all pixels on a given CT image slice that fall within a given range would provide the average or mean attenuation coefficient over the entire area of that particular tissue. [116,117]

SMF, IMAT and SAT are viewed in axial images of body regions such as upper or lower limbs or trunk. SMF is present within and between the individual muscle fibers, and is distinct and separate from SAT and IMAT.[7] Skeletal muscle fat is recognized as part of muscle tissue but its presence lowers muscle density. Depending on the extent of fat infiltration, muscle with higher fat infiltration can also be classified as low density muscle with attenuation values that range between 0-35 HU, while muscle with low fat infiltration is classified as normal density

muscle with attenuation values between 35-100 HU.[116] IMAT and SAT have negative attenuation values and are distinguished from each other based on their location. SAT is present above the muscle fascia, while IMAT is present below the muscle fascia and interspersed between muscle groups.

The method used to identify SMF from CT muscle attenuation values was shown to moderately correlate with actual intramyocellular lipid content.[15] Goodpaster et al reported high correlations between CT attenuation values and lipid concentration of phantom lipid (r =.99), and modest correlations between CT attenuation values and intramyocellular lipid obtained from percutaneous muscle biopsies (r = -0.43). The modest rather than strong correlation between histo-chemical analysis of intramyocellular lipid and CT derived muscle attenuation coefficients was likely because CT scans cannot discern between intramyocellular lipid and extramyocellular lipid, and also because muscle attenuation represents the average density present within a pixel, which is attributed to intramyocellular lipid, extramyocellular lipid, and water content in the muscle.

Muscle attenuation from CT has also shown to be a stable and reliable measure, and a single cross-sectional area from one particular body region can be generalized to the entire body. The stability and reliability of muscle attenuation coefficients was assessed by comparing CT images of closely adjacent cross-sectional slices from mid-thigh region within the same leg, and from the opposite leg were conducted. Minimal variability was observed between muscle attenuation coefficient values from adjacent slices in the mid-thigh, and on the opposite limb mid-thigh region (coefficient of variation $\leq$ 3.3%). Further, moderate correlations of muscle attenuation values from mid-thigh cross-sectional images with cross-sectional images from other distinct muscle groups, such as mid-calf, psoas major and erector spinae were also demonstrated

(r ≥0.6). Test-retest reliability also showed low variability (coefficient of variation ≤ 1%) in muscle attenuation coefficients, and was conducted by performing two consecutive CT scans of the mid-thigh and mid-calf region in a subset of volunteers. Stranberg et al confirmed test-retest reliability of CT muscle attenuation and also observed small within subject variances between different muscle groups and between different slices in the same muscle group.[118]

Due to its reliability and stability, the main measure of SMF in the current study will be muscle attenuation obtained from CT scans. The non-invasive nature make CT a feasible outcome measure that can be assessed on a large scale. Analysis of muscle biopsy samples will also be conducted in a smaller subset of subjects in whom biopsy samples are available.

1.3 SIGNFICANCE AND INNOVATION OF PROPOSED STUDY

To date, the individual roles of SMF, IMAT and SAT on physical function in RA are not well-established. Hence, the current study would address the gaps in knowledge related to skeletal muscle fat infiltration in RA, and whether specific fat depots are linked to physical function deficits. Further, the study would also assess whether the associations of SMF and IMAT is unique and independent of the associations of other known physical function predictors, such as age, body size, muscle area or strength. The current study was also the one of the first to use a combination of indirect (CT imaging) and direct (muscle tissue histology) techniques to describe SMF, IMAT and SAT in RA.

RA is an inflammatory condition which mainly manifests as a polyarticular disease in which joint synovitis is a widely recognized source of disability and physical inactivity, however, loss of muscle mass and gain in fat mass due to protein catabolism can also aggravate

disability and physical inactivity by affecting muscle function which is depicted in Figure 1. Currently, it is not known whether increase in muscle is a direct result of the RA disease and not merely due to increased body size. Infiltration of fat within the skeletal muscle has been previously observed in neurological and musculoskeletal conditions that directly affect muscles, such as Duchene's muscle dystrophy,[119] nerve injury[120] and post rotator cuff tear, [121] and in aging, obesity and insulin resistant populations.[9,19,58,122] Currently, there are no normative values for a normal SMF index. Therefore the first aim of the study was to characterize each fat depot (SMF, IMAT and SAT) in a cohort of individuals with RA. The second aim of this study was to address whether these fat depots are affected independent of body mass index by comparing persons with RA to those without RA of the same age, sex and BMI (represented by pathways "a" in Figure 1). This study was also the first to investigate the distribution of SMF, IMAT and SAT in RA compared to a matched older cohort to test the notion of accelerated aging.

Moreover, it is not completely understood whether fat infiltration within and around the skeletal muscles in RA is directly linked to low physical function and low physical activity. Increase in muscle fat depots could be postulated to reduce muscle function by affecting the metabolic environment within the muscle tissue, and thereby worsen physical function and perpetuate physical inactivity. Alternatively, metabolic alterations in the muscle (such as reduced oxidative capacity) due to increase in muscle fat depots could directly result in lower physical function and physical inactivity independently of loss in muscle strength. Further, physical inactivity and sedentary lifestyle can likely lead to an increase in muscle fat depots and also reduce muscle function due to disuse. Thus, the relationship between muscle fat depots and physical function and physical activity are cyclic in nature. The third aim of this study was to address whether there are associations between muscle fat depots and physical function and

physical inactivity that are independent of known predictors of physical function and physical activity (age, sex, BMI, muscle area and strength) (represented by pathways “b” in Figure 1). The results of this study would provide preliminary evidence for future longitudinal studies to investigate whether muscle fat depots directly affect physical function and physical activity in the RA population.

The study findings are a pivotal step in understanding the link between physiological changes in fat in the RA muscle and physical function, and the role of skeletal muscle beyond contraction. Muscle function in terms of force generating capacity has always been an important outcome for physical therapists and rehabilitation professionals; however, as rehabilitation and movement experts, we generally do not consider the role of fat within the muscle or whether the metabolic changes in the muscle due to fat infiltration influence physical function. The results of this study will therefore, potentially change the rehabilitationist’s perspective of muscle function to include muscle metabolic health as a contributor to physical function deficits and disability.

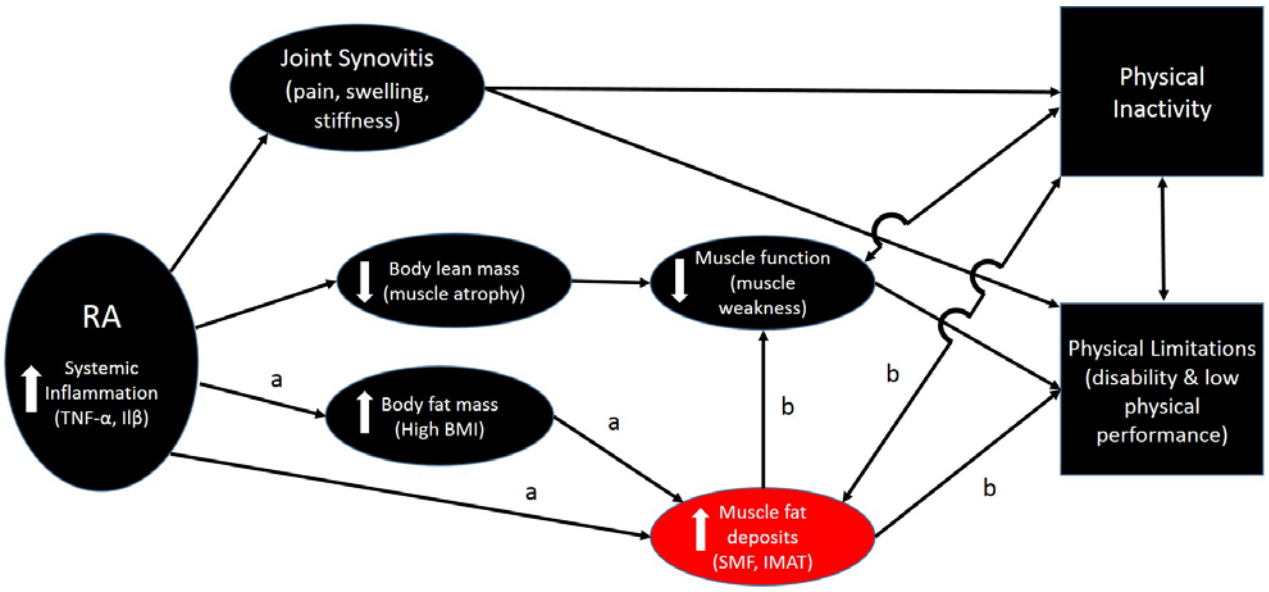


Figure 1. Theoretical Model of Muscle Fat as a Contributor to Physical Function and Physical Activity in RA

Excess production of the inflammatory cytokines (mainly tumor necrosis factor-α and interleukin-1β) lead to joint and bone degradation, which causes joint pain, swelling, and stiffness, which in turn reduce physical activity and physical function. Excess inflammation in RA also shifts protein metabolism toward net catabolism leading to reduced lean body mass predominantly in skeletal muscle (muscle atrophy) which leads to muscle weakness, and a concomitant reduction in physical activity and function. Loss of lean body mass is also accompanied by concomitant gains in fat mass and increased body adiposity (manifests as higher body mass index (BMI)). Increased BMI can lead to increases in fat depots within the skeletal muscle (skeletal muscle fat-SMF) and fat in close proximity to the skeletal muscle (intermuscular adipose tissue-IMAT). However, systemic inflammation in RA may directly lead to increased muscle fat depots, independent of BMI. Increase in these fat depots could lead to metabolic alterations that may affect the functioning of organelles in the muscle at the cellular level (e.g. mitochondria function), which in turn can affect muscle function and reduce strength and endurance and consequently affect physical function and physical activity. These pathways could also be cyclic in nature, as low physical activity levels can also lead to increased muscle fat depots and further muscle weakness.

1.4 PROPOSED RESEARCH METHODS

The proposed study examined cross-sectional baseline data of individuals from three different cohorts: those with RA, age-matched healthy controls, and elderly healthy controls. These individuals have been consented and enrolled in four clinical trials (one RA, one healthy older adults and two overweight and obese adults) at the University of Pittsburgh. Aim 1 and 3 was a cross-sectional analysis of baseline data from the RA cohort, Aim 2 was a cross-sectional matched design that compared an RA cohort to healthy adults matched on age, BMI and sex, and to compare the RA cohort to healthy older adults (10-20 years older) matched on sex and BMI. Parent trials were approved by the University of Pittsburgh Institutional Review Board (IRB), and all participants gave written consent prior to participation in the parent studies. As the proposed study was an ancillary analysis using de-identified data from prior approved clinical trials, it was approved by the University of Pittsburgh IRB and exempt from needing additional participant consent.

1.4.1 Specific Aim 1: Design, Methods and Analyses

To characterize skeletal muscle fat content (intramyocellular lipid and extramyocellular lipid), intermuscular adipose tissue (IMAT) and subcutaneous adipose tissue (SAT) in a cohort of individuals with RA

Aim 1 was addressed in a cross-sectional ancillary analysis that used data from a randomized clinical trial investigating the effects of two distinct strengthening interventions to help reverse muscle atrophy in a cohort of RA subjects. This randomized clinical trial was conducted at the Department of Physical Therapy in the University of Pittsburgh and is funded by the National Institutes of Health.

1.4.1.1 Study Participants

Participants for the randomized clinical trial were recruited from the Greater Pittsburgh area by way of general public announcements, approved research participant registries, and physician referrals. Those recruited for the trial undergo a telephone interview to screen for eligibility, as per criteria stated below:

Inclusion Criteria for RA participants

Participants were included in this study if they:

- Were greater than 21 years of age
- Have a confirmed diagnosis of RA by their rheumatologist (as per criteria stated by the American College of Rheumatology)
- Can ambulate independently
    - Are native English speakers
    - Are sedentary (< 2 days per week of regular physical activity)

Exclusion Criteria for RA participants

To ensure safety during all study procedures, participants are excluded if they had:

- Any known cardiovascular disease
- Unstable hypertension (>140/90)
- Surgery to the lower extremity within 6 months of enrolling in the study
- Current or anticipated pregnancy
- Allergy to local anesthesia
- Presence of bleeding disorders

To maximize experimental procedures, participants are excluded if they had:

- Less than 70 degrees of passive knee flexion

To avoid confounding effects, participants will be excluded if they had

- History of any neurological disorder that may affect lower extremity function (such as Parkinson's disease, peripheral neuropathy or cerebrovascular accident)
- History of muscle disease or dystrophy
- Currently taking cholesterol lowering medications
- Any change in RA medications within one month of enrolling in the study

1.4.1.2 Experimental Procedures and Data Acquisition

All eligible RA participants signed an informed consent approved by the University of Pittsburgh Institutional Research Board prior to any study procedures. Participants undergo baseline testing at the University of Pittsburgh and University of Pittsburgh Medical Center. CT scans and muscle biopsies were conducted to gather baseline information on muscle and muscle fat measures. Demographics, biomedical information, and disease related information such as disease duration, and disease activity were collected.

CT scan of Mid-Thigh

A 10-mm thick axial image of the mid-thigh region was obtained bilaterally. The axial mid-thigh slice was taken from a location that is at the center of a line joining the lateral most part of the greater trochanter to the lateral femoral epicondyle. Trained technicians/personnel at the Radiology Department perform the CT scan. Subjects were asked to lay supine in the scanner, and to remove any metal objects in their clothing or person. Once the subject was in supine, the technician makes sure both femurs are in neutral position. The technician may tape the subject's feet together, while they are in supine to ensure the femur remains in neutral. The parameters for scanning were 120kVp and 200-250mA.

The axial CT images obtained were processed using the Slice-O-Matic software to quantify areas of SAT, IMAT and SMF in and around the mid-thigh region. The slice-o-matic software was available through Dr. Goodpaster's lab in the Endocrinology Division of the Department of Medicine. This software identifies different body tissues based on their attenuation values. The software uses a color code system which is set manually by the user to identify tissues within a specified range of attenuation values. Areas of SAT and IMAT, were identified by two separate color codes with pixels of attenuation coefficients between -190 to -30 HU. SAT was differentiated from IMAT based on their anatomical location. The image was observed closely by the investigator (Khoja) for a thin membrane that surrounds the muscle, known as the sub-fascial line; a border was then drawn to demarcate this sub-fascial line. Adipose tissue present above the sub-fascial line is SAT and below is IMAT. The investigator (Khoja) utilized a paint brush like tool to color pixels representing areas of SAT and IMAT, and the cross-sectional area of each colored regions was provided by the software in square millimeters. Similarly, to identify skeletal muscle fat, color codes were set to identify pixels with attenuation coefficients from 0 to 100 HU. As skeletal muscle fat was characterized by low muscle attenuation, the mean attenuation value of entire thigh muscle area serves as a proxy value for skeletal muscle fat. In addition, separate color codes to identify areas of low density muscle (from 0 to 35 HU), and normal density muscle (35 to 100 HU) were set. The quadriceps muscle was identified based on anatomical landmarks and the exact same procedure to determine skeletal muscle fat located within the quadriceps was repeated.

This technique of identifying skeletal muscle fat from CT shows high intra-rater and inter-rater reliability with ICCs >0.98, and coefficients of variation less than 2% in the general population.[15,118] CT scans are quick and easy to administer, and lower in costs compared to

magnetic-resonance imaging (MRI); also, the slice-o-matic software used to interpret and quantify fat from CT images is user-friendly and does not require an advanced technical background.

Variables obtained from CT (All continuous):

- Mid-thigh mean muscle attenuation
- Intermuscular adipose tissue area (IMAT)
- Subcutaneous fat area (SAT)
- Mid-thigh total muscle area
- Mid-thigh Low Density Muscle and Normal Density Muscle areas.
- Quadriceps mean muscle attenuation
- Quadriceps muscle area,
- Quadriceps Low Density Muscle and Normal Density Muscle areas.

Muscle Biopsy and Histochemical Analysis of Muscle Tissue

Direct quantification of skeletal muscle fat, in particular intramyocellular lipid was done by histo-chemical analysis of muscle cryosections obtained from biopsy samples of vastus lateralis muscle. Muscle biopsies were outpatient procedures and are scheduled in the mornings after an overnight fast. Participants were instructed to avoid any strenuous activity 48 hours prior to the biopsy and 48 hours post biopsy. All biopsies for this study were conducted by one experienced physician, and performed unilaterally on the left leg. The physician began the procedure by giving the subject local anesthesia to numb the skin around upper lateral surface of the thigh, and then made an incision of less than 0.25 inches on the anesthetized area to assist with needle insertion. Once the biopsy needle (Bergstrom needle) was inserted within the muscle tissue, the physician suctioned a small muscle sample into the needle using a tube attached to a syringe. The

procedure usually takes about 30 minutes. This biopsy technique has been used in several studies and was found to be safe and reliable.[123]

The muscle sample trapped in the needle was transferred onto the petri dish for cleaning and mounting. The muscle samples usually weigh about 100-150 mg, and were dried and trimmed of excessive water or blood clots, and any visible adipose tissue. The specimen was viewed under a microscope to find muscle fibers oriented in the same direction; these fibers were separated, mounted on a block using a mounting medium, (Miles,Incl,Elkhart, IN) and frozen in isopentane (methyl butane) cooled by liquid nitrogen at -160 degrees Celsius.

Transverse muscle cryosections of 8μm were mounted on a glass slide and prepared for histo-chemical analysis. Muscle lipid content was identified using a procedure described by Goodpaster et al.[124] The mounted cryosections were air dried for 15 minutes, and then stained in Oil Red O solution. To identify fiber types, the slides were stained with 2 primary antibodies that stain specifically for each fiber type (I and IIA). A light microscope was used to examine the muscle section, and digital images of the muscle sections were captured with a camera. Several images were captured in order to find sections that were free of artifact and had at least 100 muscle fibers for reliable quantitative analysis of lipid content. Using the Northern Eclipse Image software a threshold of the Oil Red O staining intensity was set, and the software then computes the average intensity of gray, which is a measure of lipid content in the muscle. Darker areas represent higher lipid content. The Lipid Accumulation Index was an additional method of quantifying intramyocellular lipid content. It is defined as the average gray per unit of muscle fiber area.

Histo-chemical analysis of muscle cryosections described above has been used in several other studies in non-RA populations, and has shown good reliability and reproducibility.[16] As no

study has yet examined or described intramyocellular lipid in a cohort of RA subjects, results obtained from this study would generate a reference value for lipid content in individuals with RA.

Variables from Histo-chemical Analysis of Biopsy Samples (All continuous):

- Muscle Fiber Area
- Total average gray
- Average gray for Type I and Type II fibers
- Total Lipid Accumulation Index
- Lipid Accumulation Index for Type I and Type II muscle fiber type.

The number of subjects that undergo CT scan was larger in this study compared to those who undergo biopsy. As we had a smaller subset of subjects with muscle biopsies, all variables from histo-chemical analyses were exploratory in nature.

Demographics and Biomedical Variables

These consisted of age, sex, race, body mass index (BMI), education level, number of co-morbidities, disease duration and disease activity score (DAS-28). The disease activity score (DAS-28) is a reliable and valid tool used to indicate how active the RA is in an individual.[125] It involves examination of 28 joints for tenderness and swelling, erythrocyte sedimentation rate from a blood test, and patient reported global health on a scale of 0 to 100. Scores for the DAS-28 range from 0 to 9.4 and are calculated using a validated formula that includes the total number of swollen and tender joints, the erythrocyte sedimentation rate and patient global health score. Scores ≤ 3.2 indicate low disease activity, scores $\geq 3.2 \leq 5.1$ indicate moderate disease activity and scores > 5.1 indicate high disease activity.[126]

1.4.1.3 Analysis Plan and Power Calculations

Aim 1: To characterize skeletal muscle fat content (intramyocellular lipid and extramyocellular lipid), intermuscular adipose tissue (IMAT) and subcutaneous adipose tissue (SAT) in a cohort of individuals with RA, and to explore their associations with RA disease characteristics (i.e disease duration and disease activity) and individual characteristics (age, and BMI).

Analysis for Aim 1 was descriptive and exploratory in nature. Normality of variables was tested using the Shapiro-Wilk test. Depending on data distribution, continuous variables are described as mean ± SD, or median and 25th-75th percentiles. Categorical variables were described in frequencies or percentage. Correlations between the CT variables, histo-chemical variables, and disease characteristics were also explored. Depending on distribution of data either Pearson or Spearman's correlation coefficient were used. Where appropriate CT variables were averaged for both legs. When exploring associations between CT and histo-chemical variables data from the left leg was used because muscle biopsies are performed unilaterally. The variables from CT and Histo-chemical analysis would be stratified by individual (age, sex, BMI) and disease related characteristics (RA duration and severity) to observe trends or differences in skeletal muscle fat among the stratified groups. All statistical analyses were performed using the IBM SPSS software, version 21. (IBM Corporation)

<u>Power calculations</u>

As the first aim is to characterize muscle fat in RA population, the precision with which we are able to detect a true population mean is the main concern. We assumed that the variability of our primary measure (muscle attenuation) from population of older adults is similar in RA. With a sample of 60 subjects from the parent RA trial, the width of the 95% confidence interval for the

sample muscle attenuation is ± 2 HU. Thus, we would be 95% confident that this interval captures the true population mean for muscle attenuation.

1.4.2 SPECIFIC AIM 2: Design, methods and Analyses

To compare skeletal muscle fat, IMAT and SAT content in people with RA to that of age, sex, and BMI matched healthy controls, as well as sex and BMI matched healthy elderly controls.

A matched design was utilized to address aim 2. The first part of this aim compared skeletal muscle fat, IMAT and SAT content in people with RA to that of age (+/- 5 years), sex, and BMI (+/- 2.0 kg/m^2) matched healthy controls. Because the amount of fat varies by age, sex and body size, these were included as matching factors. The second part compared skeletal muscle fat, IMAT and SAT content in people with RA to an older (>10 years) but sex and BMI (+/- 2.0 kg/m^2) matched healthy controls. All cases were manually paired by the investigator (Khoja). For the matching process a data spreadsheet containing only the study identification number, age, sex and BMI of the RA cases, and non-RA controls was provided by the respective PIs to investigator (Khoja), the PI of the current dissertation study. Ms. Khoja was blinded to CT and biopsy data during the match process, and attempted to match one healthy age matched control and one elderly control for each RA case. Variables from CT and biopsy were added to the data spreadsheet for analyses once the matching process is complete.

1.4.2.1 Study Participants

The RA cases for the second aim were obtained from the same cohort as Aim 1(Sub-section 4.1.1). The matched healthy controls are individuals who have already participated in other studies at the University of Pittsburgh. One cohort of healthy individuals was obtained from a trial investigating the effect of weight loss on regional adiposity and insulin sensitivity. The other cohort of individuals was obtained from a study that investigated the effect of a strengthening

program to induce muscle hypertrophy in older, healthy adults. Both studies had similar inclusion criteria as that of the RA parent trial, comprised of relatively healthy and sedentary adults, and utilized similar measures for fat content as the RA parent trial. De-identified data on baseline measures of fat content from these matched controls were provided by the respective studies' Principal Investigators. As muscle attenuation from CT were the primary outcome measures in the clinical trials, we expected to find a larger subset of subjects who agreed to undergo CT scan data than those who underwent both CT scan and muscle biopsy.

Inclusion Criteria for non-RA participants

- Adults between 30 - 80 years
- Sedentary (<2 days/week of regular physical activity)
- Non-smoker
- Able to ambulate independently

Exclusion Criteria for non-RA participants

- History of cardiovascular disorders
- Unstable hypertension or on antihypertensive medications
- Hyperlipidemia (plasma triglycerides $\geq$ 350 mg/dl or cholesterol $\geq$ 300 mg)

1.4.2.2 Experimental Procedures and Data Acquisition

Experimental procedures for the RA subjects were exactly the same as outlined in Aim 1 (Sub-section 4.1.2). All non-RA participants used in matching were also tested at the University of Pittsburgh and have undergone the same procedures for mid-thigh CT scan and muscle biopsy as the RA subjects. We obtained the same variables from CT and Muscle biopsy as outlined in Aim 1.

1.4.2.3 Analysis Plan and Power Calculation

The primary outcome variable for Aim 2 was muscle attenuation for skeletal muscle fat, and secondary outcome variables are IMAT and SAT areas, all obtained from CT. All variables measuring intramyocellular lipid from histo-chemical analyses were exploratory in nature.

Hypothesis 2a: People with RA will have higher skeletal muscle fat content, higher IMAT content and higher SAT content compared to their age, sex and BMI matched healthy controls.

The hypothesis was tested using primary and secondary outcome variables from CT. Normality of data is assessed using the Shapiro-Wilk test, and appropriate descriptive statistics for each outcome variable were reported. Depending data distribution, either parametric test such as paired student t-test or a non-parametric equivalent of the paired t-test, such as Wilcoxon-signed rank test for hypothesis testing was used. We chose paired t-tests as both groups are matched on individual characteristics, and hence, not independent of each other. The alpha level of significance was set at 0.05. In addition, the same paired analysis of the exploratory variables from histo-chemical analysis in a subset of matched pairs was conducted. All statistical analyses were performed using the IBM SPSS software, version 21. (IBM Corporation)

<u>Power Calculations</u>

Power analysis was based on our primary outcome variable mid-thigh muscle attenuation (MA). We shall attempt to detect a 10% difference in muscle attenuation between the RA cohort and healthy cohort, assuming 40 HU to be the average for healthy adults. The power analysis for the paired t-test was calculated assuming a correlation of 0.5 between the standard deviation (SD) of both groups. For the study to have 80% power to detect a significant difference at alpha level of

0.05 in our primary outcome measure, we needed at least 19 matched pairs to detect a difference of 4.0 HU with SD of difference of 5.8 HU. SD of difference of 5.8 HU was calculated from pre and post SDs of 5.4 HU and 6.1 HU, respectively, assuming a correlation of 0.5. These SDs were obtained from a previous study that reported changes in mid-thigh muscle attenuation.[127] All statistical analyses were performed using the IBM SPSS software, version 21. (IBM Corporation)

Hypothesis 2b: People with RA will have similar skeletal muscle fat content, similar IMAT content, and similar SAT content compared to sex and BMI matched elderly healthy controls 10 to 20 years older than the RA subjects.

The hypothesis was tested using primary and secondary outcome variables from CT. Normality of data was assessed using the Shapiro-Wilk test, and appropriate descriptive statistics for each outcome variable were reported. Depending data distribution, either parametric test such as paired student t-test or a non-parametric equivalent of the paired t-test, such as Wilcoxon-signed rank test for hypothesis testing was used. We chose paired t-tests as both groups are matched on individual characteristics, and hence, not independent of each other. The alpha level of significance is set at 0.05. In addition, the same paired analysis of the exploratory variables from histo-chemical analysis in a subset of matched pairs was conducted. All statistical analyses are performed using the IBM SPSS software, version 21. (IBM Corporation). (IBM Corporation)

<u>Power Calculations</u>

Similarly for hypothesis 2b we attempted to detect a 10% difference in muscle attenuation between the RA cohort and the healthy older cohort assuming 40 HU to be the average for healthy adults. The sample size for paired t-test was calculated assuming a correlation of 0.5 between the SD of both groups. For the study to have 80% power to detect a significant

difference at alpha level of 0.05 in our primary outcome measure, we needed 19 matched pairs to detect a difference of 3.5 HU with SD of difference of 5.8 HU. SD of difference of 5.8 HU was calculated from pre and post SDs of 5.4 HU and 6.1 HU, respectively, assuming a correlation of 0.5. These SDs were obtained from a previous study that reported changes in mid-thigh muscle attenuation.[127]

1.4.3 SPECIFIC AIM 3: Design, methods and Analyses

To assess the associations of skeletal muscle fat, IMAT and SAT to measures of physical function, physical activity, and disability in subjects with RA.

Specific Aim 3 was addressed using a cross-sectional, ancillary analysis of baseline data from the parent randomized clinical trial that investigated the effects of two distinct strengthening interventions to help reverse muscle atrophy in individuals with RA. The RA parent trial was conducted at the Department of Physical therapy in the University of Pittsburgh and is funded by the National Institutes of Health (NIH).

1.4.3.1 Study Participants

The study participants' inclusion and exclusion criteria were outlined in Aim 1. (Sub-section 4.1.1)

1.4.3.2 Experimental Procedures and Data Acquisition

Experimental procedures for demographics, biomedical characteristics, CT, and muscle biopsy, were exactly as described in Aim 1 (Sub-section 4.1.2). Experimental procedures for physical function, physical activity and disability is described in this section.

Physical Function

Measures of physical function involved a battery of physical performance tests. These measures included quadriceps strength test, timed chair rise, 4 meter walk, stair climb, and single leg

balance. These measures are relatively quick and easy to perform in a clinical setting, reflect functional activities, and do not require special equipment. The stair climb and chair rise tests reflect lower limb muscle power and strength[128,129] and have been validated to capture lower limb performance in high functioning adults.[128-130] The single leg balance test is a reliable and valid tool to measure balance in high functioning adults (r = 0.69). We selected these functional tasks as we expected to have subjects who were younger in age, and had higher function than older adults, as RA is an early onset disease. Each of the performance tests was timed; a shorter time to complete each task indicated better performance, except for single leg stance test, whereas a longer time indicated better performance. All performance tests were conducted by a trained physical therapist who is blinded to the subject's intervention allocation in the parent trial.

Timed Chair Stand Test: Subjects were instructed to rise from a chair and sit back down, as fast, and safely as they can, five times in a row and the time (in seconds) to perform the test was recorded.

Stair Climb Test: Subjects were instructed to climb up and down one flight of stairs, and the time to climb up the stairs and total time in seconds (up + down) was recorded.

Four meter walk Test: Subjects were instructed to walk at their normal pace between two cones four meters apart and the gait speed was calculated in meters/second.

Single Leg Stance Test: Subjects were instructed to stand on one leg for as long as 30 seconds without losing balance. Subjects were asked to repeat the single leg stance thrice on each side, and average time (in seconds) during the three trials was calculated.

Quadriceps Strength Test: Maximum voluntary isometric contraction of the quadriceps for each leg was tested using an isokinetic dynamometer (Biodex,Inc). The subject performed five trials

of isometric knee extension for each leg, and the best of three trials were selected for data analysis. Subjects were seated on the dynamometer with the knee at 60 degrees of flexion. The seat was adjusted and straps are used to ensure proper positioning and stabilization of the subject during testing. After positioning, the subject was instructed to kick as "hard" and as "strongly" as possible for 5 trials. Each trial lasted for about 5 seconds, with a 60 second rest period between each trial. While performing the isometric test, the subjects were able to view the torque they are producing on a computer screen in front of the dynamometer. The tester used this visual feedback to encourage the participants to give their best effort for each trial. The intra-rater and inter-rater reliability of this procedure in our laboratory is high (ICC = 0.97 and 0.82, respectively) (unpublished pilot data). This technique to assess quadriceps strength with an isokinetic dynamometer has been used in previous studies with RA individuals and was tolerated well.[131]

Physical Function Variables:

Total time in seconds for chair rise test, 4 meter walk test, and stair climb test, and single-leg balance test were recorded into the parent study database during the testing visit. Quadriceps strength was measured in Newton meters (N-m), and in strength per unit area of quadriceps muscle (specific force).

Physical Activity

Physical activity levels were measured using a multi-sensor activity monitor called Sense Wear Armband (Bodymedia, Inc), that calculates energy expenditure by combining information from sensors that detect heat flux (heat dissipated by the body), galvanic signals from sweat rates, skin temperature, and movement from a biaxial accelerometer. Physical activity measured by Sense Wear Armband showed moderate reliability in healthy and older adults and correlates well to

reference standards such as doubly labeled water and (correlation coefficients ranging from 0.48 to 0.81)[132,133] and indirect calorimetry (correlation coefficients ranging from 0.4 to 0.92).[134-137]

Subjects wore the activity monitor on their right arm, midway between their shoulder and elbow, and were instructed to wear the activity monitor throughout the day (including sleep), except during any water related activities (e.g. baths, showers or swimming). Subjects wore the monitor for a total of 10 days including the days that the monitor was handed over and returned. This gave us 8 full days of data, which was appropriate as a minimum of 4 days is required to procure reliable estimations of physical activity energy expenditure (ICC = 0.82).[138]

Data gathered on the activity monitor were processed using SenseWear Professional software v6.1 (BodyMedia Inc., Pittsburgh, PA). The software utilizes proprietary algorithms developed by Bodymedia Inc that combine the information from its sensors with participant biomedical characteristics (age, BMI, sex) to calculate time spent or energy expenditure during physical activity. The software enables the researcher to classify physical activity energy expenditure further into light, moderate, and vigorous physically activity.

Physical Activity Variables:

Time spent in moderate or higher intensities of physical activity.

Disability

Health Assessment Questionnaire (HAQ)

The scores from the self-reported Health Assessment Questionnaire (HAQ) were used to assess level of disability in this cohort of RA subjects. It is a widely used validated tool which assesses functional disability during activities of daily living. It includes 20 activities of daily living, and score ranges from 0 (no disability) to 3 (severe disability).[139]

1.4.3.3 Analysis Plan and Power Calculations

For Aim 3 we tested associations of skeletal muscle fat, IMAT and SAT with measures of physical function, physical activity and disability.

Hypothesis 3a: Higher SMF and higher IMAT will be associated with lower physical function, lower physical activity. SAT will not be associated with physical function, physical activity and disability.

Normality of data from the primary (muscle attenuation) and secondary outcome variables (IMAT and SAT) was assessed using the Shapiro Wilk test, and appropriate descriptives are reported. Associations between the variables were tested using Pearson's correlation coefficient (r) for variables that meet the assumption of normality, and Spearman's correlation coefficient (ρ) for variables that did not meet the assumption of normality. Strength of associations were classified as small for coefficient values around 0.2, moderate for coefficient values around 0.5, and large for coefficient values around 0.7. Associations of exploratory variables from histo-chemical analysis with physical function, physical activity and disability in a subset of subjects who underwent biopsy were conducted. All statistical analyses were performed using the IBM SPSS software, version 21. (IBM Corporation)

Power Calculations:

Study power was calculated based on the primary outcome variables from CT: mid-thigh mean muscle attenuation and quadriceps mean muscle attenuation. With a sample size of at least 60, we had 80% power to detect significance at alpha = 0.05 for an association of low-moderate strength (r = 0.35)

Hypothesis 3b: SMF and IMAT will contribute to physical function, physical activity and disability after accounting for muscle strength and muscle area.

After testing the bivariate correlations, the contribution of SMF and IMAT to physical function and physical activity independent of muscle strength and muscle area, and confounders such as age, sex and BMI was also assessed. Quadriceps muscle attenuation was selected as the primary predictor or independent variable for this hypothesis, and IMAT as the secondary predictor or independent variable. The dependent variables consisted of chair rise time, stair climb time, gait-speed, single-leg balance time, time spent during moderate physical activity, and HAQ scores. Separate models were created for each dependent variable (physical function and physical activity outcomes) with each predictor variable (skeletal muscle fat and IMAT).

To test the independent contribution of skeletal muscle fat or IMAT, the first step of the regression analysis was to identify potential confounders or modifiers of the relationship between independent and dependent variable. Potential confounders selected were age, sex, BMI, quadriceps strength and quadriceps area and were each added separately into the model with the independent variable. If the potential confounder produced a change of 10% in the beta coefficient of the independent variable or increased its significance, (decreased p-value) it was added in the final model. In the final model all identified confounders were added in the first step, followed by the predictor variable. The independent contribution of the skeletal muscle fat and IMAT in the final model was assessed by observing the change in R^2 when skeletal muscle fat or IMAT was entered into the model. Regression diagnostics were run and data transformations were used when needed to ensure that the assumptions for linear regression (i.e., normality of the error distribution, linearity, homoscedasticity) were not violated. All statistical analyses were performed using the IBM SPSS software, version 21. (IBM Corporation)

2.0 STUDY 1- SKELETAL MUSCLE FAT AND ITS ASSOCIATION WITH PHYSICAL FUNCTION IN RHEUMATOID ARTHRITIS

The first study includes the results and discussion for Specific Aims 1 and 3 from the original research proposal. This study only addresses the outcomes of skeletal muscle fat, intermuscular adipose tissue and subcutaneous adipose tissue that were assessed using computed tomography imaging techniques. The results and discussion for the exploratory analysis on intramyocellular lipid using muscle biopsy samples is addressed in Chapter 4.0.

2.1 SUMMARY

Objective: To characterize skeletal muscle fat (SMF), intermuscular adipose tissue (IMAT) and subcutaneous adipose tissue (SAT) in individuals with rheumatoid arthritis (RA), and assess the associations between these fat depots and physical function and physical activity. Methods: Cross-sectional analysis from an RA cohort. SMF, IMAT and SAT were measured using computed tomography imaging of the mid-thigh cross-sectional region. Physical function was measured with the Health Assessment Questionnaire (HAQ) and a battery of performance-based tests that included quadriceps muscle strength, gait speed, repeated chair-stands, stair ascend, and single leg-stance. Physical activity was assessed by accelerometry. Associations between SMF, IMAT and SAT, and physical function and activity were assessed by multiple linear regression

models adjusted for age, sex, body mass index, muscle area, and muscle strength. Results: Sixty subjects with RA (82% female, age 59 ± 10 years, BMI: 31.79 ± 7.16) were included. In the adjusted models, lower SMF showed moderate to large associations with faster gait speed, longer single leg stance time, greater quadriceps strength, lower HAQ scores, and greater physical activity ($R^2\Delta$ range .08-.25, $p < 0.05$); whereas IMAT did not associate with physical function or activity; and SAT was negatively associated with HAQ scores ($R^2\Delta$= .13 $p<0.05$) and weakly but positively associated with muscle strength. ($R^2\Delta$= .023, $p<0.05$). Conclusions: Fat infiltration within the muscle seems to independently contribute to low physical function and activity in contrast to IMAT or SAT accumulation. Longitudinal studies are necessary to confirm the impact of SMF on disability and promoting health in persons with RA.

2.2 INTRODUCTION

Rheumatoid Arthritis (RA) is characterized by systemic inflammation that promotes protein degradation, leading to loss of body cell mass (mostly lean mass) and concomitant increase in fat mass.[49,140] This loss of lean mass and gain in fat mass could be accompanied by increase in fat content within or around the skeletal muscles. Increase in fat depots in and around the skeletal muscle may affect physical function and physical activity participation in those with RA. Investigating these fat depots may provide insight on alternate sources of disability and low physical activity levels [141] that persist in this population, despite relatively well-controlled disease and advanced medical management.[142,143]

The mechanisms by which fat accumulation inside and around the muscle influence physical function have not been established, but, some studies have indicated that excessive fat

infiltration may be responsible for perpetrating chronic inflammatory pathways and producing toxic lipid by-products that could interfere with the normal muscle metabolic and contractile functions.[144-146] These alterations in muscle physiological functions due to fat may consequently influence physical function. The fat depots inside and around the skeletal muscle are generally classified as skeletal muscle fat (SMF), intermuscular adipose tissue (IMAT), and subcutaneous adipose tissue (SAT) (Figure 1). SMF is located within individual muscles and includes the fat inside the myocytes and around the muscle fibers. IMAT lies within the muscle fascia and is interspersed between groups of muscles. SAT lies outside the muscle fascia and directly underneath the skin.[147,148]

The role of SMF, IMAT and SAT on physical function has mostly been studied in healthy elderly adults in large longitudinal studies. However, the findings of these studies have not been consistent despite robust study designs and attempts to account for confounders. Higher SMF accumulation was associated with lower physical function in one longitudinal and two cross-sectional studies, [10,21,61] whereas no associations were reported in another longitudinal study.[149] Higher IMAT accumulation was significantly associated with decline in physical function in two longitudinal studies[22,23], while no associations were found in another longitudinal study.[149] Higher SAT accumulation was associated with decline in physical function over time among women, but not in men,[22,23] whereas one cross-sectional study reported no associations between SAT and physical function.[21] The inconsistent findings from these studies may be driven by variation in the samples in terms of lifestyle, presence of co-morbidities, and level of disability and obesity. Only three of the above studies examined the associations between fat depots and physical function independently of muscle area and muscle strength; and also reported conflicting results. [10,61,149] As muscle area and strength are known to contribute to

physical function, and also shown to associate with SMF[9], adjusting for these muscle characteristics would inform whether these fat depots influence physical function beyond what is already explained by muscle size and force generating capacity.

Findings from healthy populations may not directly translate to populations with chronic conditions such as RA, who experience a greater degree of functional limitations, are less active, and have more loss of muscle mass and gain in fat mass compared to healthy populations. Currently, there is limited insight on how SMF, IMAT and SAT contribute to physical function and physical activity in RA. While previous RA studies have investigated the role of overall body adiposity [2,3,47,97] in physical function, these studies used methods [2,3,97] [47] that do not quantify SMF or IMAT. To date, we are aware of only study in RA that used methods to distinguish these different fat depots and explored their associations with physical function.[77] In cross-sectional analyses, SMF and total fat outside the muscle (IMAT and SAT combined) were associated with lower physical function in RA,[77] but muscle strength was not taken into account when assessing the associations.

The current study sought to characterize SMF, IMAT and SAT separately, assess the associations of each fat depot with measures of physical function and physical activity, and determine whether the contributions of SMF, IMAT and SAT are unique and independent of commonly known factors that directly influence physical function such as age, sex, BMI, muscle area, and muscle strength. We hypothesized that after accounting for confounding SMF, IMAT and SAT would associate with physical function and physical activity.

2.3 METHODS

2.3.1 Study Design and Participants

The current study was an ancillary, cross-sectional analysis of baseline data from 60 individuals with RA who participated in a randomized clinical trial that compared two strengthening exercise programs to reverse muscle atrophy (Clinical Trials Registration NCT00924625). The study was conducted in the Department of Physical therapy at the University of Pittsburgh between December 2009 and September 2013. Computed tomography (CT) imaging was obtained at the University of Pittsburgh Radiology Department, and physical function measures were obtained in the Department of Physical Therapy.

Participants were included if they were above 21 years of age, diagnosed with RA by a rheumatologist as per the 1987 American College of Rheumatology criteria, and able to ambulate independently. Participants were excluded if they had contra-indications that precluded safe participation in strength training of the major lower extremity muscles, such as cardiovascular disease, uncontrolled hypertension, neurological or muscular conditions affecting the lower extremities, or recent surgery of the lower extremity. All eligible participants signed an informed consent approved by the University of Pittsburgh Institutional Review Board prior to study enrollment.

2.3.2 Measurement of SMF, IMAT and SAT

SMF, IMAT and SAT were obtained from mid-thigh CT imaging using previously described methods.[15,116] Briefly, a 10-mm thick axial image of the mid-thigh region was obtained at the

femoral mid-point, which is the center of a line joining the lateral most part of the greater trochanter and the lateral femoral epicondyle. Scanning parameters were 120kVp and 200-250mA, and subjects were positioned in supine with both femurs in neutral position in the scanner. CT images were processed using the Slice-O-Matic software to quantify SMF, IMAT and SAT. The software differentiates between muscle, fat, and bone tissue based their physical density properties which is measured on an interval scale in Hounsfeld Units (HU), with water as the reference at 0 HU. Tissues denser than water such as muscle and bone have positive values (above 0 HU) while less dense tissues such as adipose tissue have negative values (below 0 HU). Muscle density ranges between 0 to 100 HU, while adipose tissue density range between -190 to -30 HU. SMF accumulation is assessed using the average muscle density in HU, with lower average muscle density corresponding to higher amounts of SMF accumulation, and higher muscle density corresponding to low SMF accumulation. The average muscle density (HU) and muscle cross-sectional area (square centimeters) were obtained for both the quadriceps muscle and total mid-thigh muscle area. SAT and IMAT were separated by tracing the muscle fascia, and their cross-sectional areas measured in square centimeters. (Figure 1) For analyses, all CT variables were averaged for both legs. The method used to assess muscle area and fat content using CT imaging is reliable and valid with ICCs >0.98, and coefficients of variation less than 2%. [118]

2.3.3 Physical Function and Physical Activity

Physical function was measured using the self-reported Health Assessment Questionnaire (HAQ) questionnaire and a battery of physical performance tests shown to be reliable, valid, and well-tolerated in the RA population.[128-131] The HAQ assesses limitations during 20 activities of daily

living, and is scored between 0 (no disability) to 3 (severe disability).[139] Maximum voluntary isometric strength of the quadriceps was measured using an isokinetic dynamometer (Biodex,Inc). Subjects were seated on the dynamometer with 70 degrees of knee flexion and performed five trials of isometric knee extension per leg. The highest three trials for each leg were averaged, and then further averaged for both limbs. Repeated chair-stand test consisted of the time taken to stand up from a chair five times. Stair climbing test recorded the time taken to ascend one flight of 12 stairs. Self-selected gait speed was calculated from the time taken to walk four meters. The single-leg stance test measured the time (up to 30 seconds) a subject could stand on one leg without losing balance. Each leg underwent three trials for single-leg stance, and the values from both limbs were averaged.

Physical activity was measured by a reliable and valid multi-sensor activity monitor, the Sense Wear Armband (Bodymedia, Inc).[150,151] worn on the right upper arm for 8 days, up to 24 hours per day, except during water related activities. We calculated the daily time spent in activities of moderate or higher intensities. A minimum of 4 days with at least 10 hours of data was required to yield reliable estimates of physical activity. [138]

Demographics and biomedical characteristics included age, sex, race, education, body mass index (BMI), and disease duration and activity. BMI was calculated based on weight and height measured on-site. Disease activity was measured by the disease activity score (DAS-28), a widely used validated tool that involves the examination of 28 joints for tenderness and swelling, erythrocyte sedimentation rate from a blood test, and patient reported global health on a scale of 0 to 100. Validated algorithms provide scores that range from 0 to 9.4, with scores ≤ 3.2 indicating low disease activity, $\geq 3.2 \leq 5.1$ indicating moderate disease activity and scores > 5.1 indicating high disease activity.[125 126]

2.3.4 Power Calculation and Data Analysis:

Sixty subjects were recruited into the parent trial and were included in the current secondary, ancillary analysis. With 60 subjects, the current study had 80% power to detect small to moderate associations ($\rho = 0.35$, $\alpha=0.05$). The sample also achieved 80% power to detect an R-square of 0.16 with up to 4 covariates in a regression model, and an R-square increment of 0.11 with one predictor in the main set.

All subject characteristics, and study outcome measures were described using means and standard deviations or medians and 25-75 interquartile ranges for continuous variables, and frequencies and percentages for categorical variables. We also calculated the 95% CI around the measures of fat depots, and physical function and activity measures to provide estimates of precision. Univariate associations of average muscle density (proxy measure of SMF), and IMAT and SAT areas with physical function and physical activity were assessed using Pearson's (r) or Spearman's (r_s) correlation coefficients, depending on data distribution. The strength of the correlations were interpreted based on the values provided by Cohen.[152]

We used multiple linear regression to assess the independent contribution of muscle density, IMAT and SAT (independent variables) towards physical function and physical activity (dependent variables) after accounting for potential confounders. We selected age, sex, BMI, muscle area and strength as potential confounders because of their known associations with physical function. Separate models were created for each independent variable and its ability to explain the variance for each dependent variable. Regression modeling entailed two steps. First, the confounding effect of age, BMI, sex and muscle area was assessed by adding each potential confounder separately into the model with the independent variable. The potential confounder was included in the final adjusted model if it produced a change of 10% or higher in the effect

size estimates of the independent variable (unstandardized beta coefficients).[153] Quadriceps strength was a dependent variable, but, was also selected as a potential confounder for the hierarchical models built to predict the other physical function and physical activity variables. This was done to assess whether the role of muscle fat in physical function and PA was beyond what could be explained by muscle force generating capacity. After the confounding variables were entered in the regression models, the final step was to enter the independent variable to determine the magnitude of effect by observing the R^2 change (R^2 Δ) and the beta coefficients. Regression diagnostics were run and data transformations were used when needed to ensure that the assumptions for linear regression (i.e., normality of the error distribution, linearity, homoscedasticity) were not violated. All statistical analyses were performed using the IBM SPSS software, version 21. (IBM Corporation)

For clarity of presentation, the results only contain data on muscle density and muscle cross-sectional area derived specifically from the quadriceps muscle. This was done for two reasons. First, the average density for the total mid-thigh muscle and quadriceps only were highly correlated ($r > 0.8$). Second, because muscle strength was assessed only for the quadriceps muscles.

2.4 RESULTS

Subjects were mostly white (83%),female (82%), and obese (mean BMI: 31.79 ± 7.16) The average age was 59 ± 10 years, median RA duration was 13.5 years, and subjects had moderate disease activity (Table 1). Quadriceps density (SMF accumulation) was normally distributed with relatively lower variability and narrower confidence intervals compared to IMAT and SAT

areas that were positively skewed with larger variability and wider confidence intervals. (Table 2)

Univariate associations indicated that higher muscle density (lower SMF) associated moderately with lesser time taken to ascend 12 steps, faster self-selected gait speed, greater single leg stance time, higher quadriceps strength, lower HAQ scores, and higher physical activity ($p < 0.05$). (Table 2) Higher IMAT showed weak to moderate associations with slower self-selected gait speed, greater repeated chair stand time, and greater time to ascend 12 steps, higher HAQ scores and lower physical activity levels ($p < 0.05$). Higher SAT area was weakly associated with greater time for to ascend 12 steps and lower physical activity levels ($p < 0.05$). (Table 2)

After identifying and controlling for confounders, higher muscle density (lower SMF) demonstrated moderate to large positive associations with gait speed, single leg stance time and physical activity, and negative associations with HAQ scores ($R^2\Delta$ range .08-.25, $p < 0.05$). Higher muscle density (lower SMF) also had a weak but significant positive association with quadriceps strength ($R^2\Delta = .023$, $p < 0.05$), and there were no associations with stair ascent and repeated chair stand time, ($p > 0.05$). (Table 3) IMAT did not associate with any measures of physical function or physical activity after controlling for confounding, (Table 4) while SAT showed moderate negative associations with HAQ scores ($R^2\Delta = .13$ $p < 0.05$), and weak positive associations with quadriceps strength ($R^2\Delta = .023$, $p < 0.05$). (Table 5)

2.5 DISCUSSIONS AND CONCLUSIONS

To our knowledge, this is the first study to investigate the individual roles of SMF, IMAT, and SAT on objectively measured physical function and physical activity outcomes in RA. Study findings demonstrate that muscle density (SMF) significantly contributed to most of the physical function measures (four out of six) and to physical activity in individuals with RA, even after accounting for confounders such as muscle area and strength, BMI, age, and sex. In contrast, the contribution of IMAT and SAT to physical function and physical activity was significantly attenuated after accounting for muscle area, strength, BMI, age or sex. These findings are unique and suggest that physical function and physical activity are not solely influenced by the amount of muscle or its ability to generate force, but, also by the amount of fat within the muscle.

We are only aware of one previous study in RA that investigated SMF with respect to physical function.[77] Kramer et al reported that SMF significantly contributed to lower physical function in regression models simultaneously adjusted for subject demographics, RA disease characteristics, muscle area, and thigh fat area (SAT +IMAT combined). However, the unique variability explained by SMF, SAT and IMAT on physical function was not ascertained, and the role of muscle strength was not assessed in their study. To our knowledge, our study is the first to demonstrate that SMF independently explains an important amount of variability ($R^2\delta$ range .08-.25) in physical function and physical activity independent of muscle strength. These findings suggest that there might be underlying mechanisms not related to muscle force generating capacity that can also affect an individual's physical functioning or activity participation. It has been postulated that fat encroachment within the skeletal muscle can affect the contractile, neuromuscular, and metabolic functions by altering the muscle's extracellular matrix and connective tissue properties,[146] and through the release of toxic lipid by-products.[145]

However, these pathways have not been directly studied. Few small RA studies, conducted over 40 years ago, explored the histological properties of muscle spindles in RA and found accumulation of fluid, thickening of the muscle spindle capsule, and fibrotic changes in the intrafusal muscle fibers.[103-105] There is a possibility that fat encroachment around the muscle spindles may affect the intrafusal muscle fibers in a similar fashion, however, this investigation would require studies that directly assess muscle ultrastructure and intramyocellular lipid content in RA.

The only two physical function measures that did not associate with SMF were stair ascend time and repeated chair stand time. One possible explanation for this could be the type of energy source predominantly used during these two activities. SMF largely represents the intramyocellular lipid stores [15] which are utilized mainly during continued low intensity activities such as muscle endurance activities, and not during sudden bursts of strong muscle contraction, where glycogen is the main source of energy. Since time taken to ascend stairs and repeated chair stands represent quick and powerful quadriceps muscle contractions, the amount of SMF in the quadriceps may not affect the ability of the quadriceps muscles to produce a sudden burst of activity.

In contrast to SMF, IMAT did not explain any additional variability in physical function or physical activity after controlling for confounders. BMI was the most consistent confounder and attenuated the associations between IMAT and physical function outcomes. The only outcome that IMAT may have had a small but important contribution was physical activity, ($R^2\delta$ =.039), but, due to small sample size (N = 51), this model may have been underpowered to detect significance. The role of IMAT on physical function has not been previously reported in RA, but, there seems to be conflicting results between non-RA studies that also adjusted for the effect of

BMI and other confounders.[23,149] Murphy et al reported that IMAT was associated with a small but significant risk of developing mobility limitations over time (Hazard Ratio range: 1.00-1.47, (95% CI 1.00-2.02)), [23] whereas Reinders et al observed no associations between IMAT and worsening of physical function measures over time (Odds ratio range: 1.00-1.14, (95% CI: 0.82-1.54)).[149] Based on the current RA and previous non-RA findings, it is likely that the magnitude of the independent associations between IMAT and physical function or physical activity is small after accounting for a measure of overall body adiposity such as BMI. Although IMAT was not a significant predictor of physical function or activity, its role should not be dismissed as it has been associated with metabolic complications, which were not assessed in the current study. IMAT accumulation has been attributed to poor glucose metabolism and perpetrating chronic inflammatory processes in the elderly, obese and those with diabetes.[13,122,147,154-156] Also, recently, a cross-sectional study in individuals with RA found IMAT accumulation to be associated with greater insulin resistance.[157]

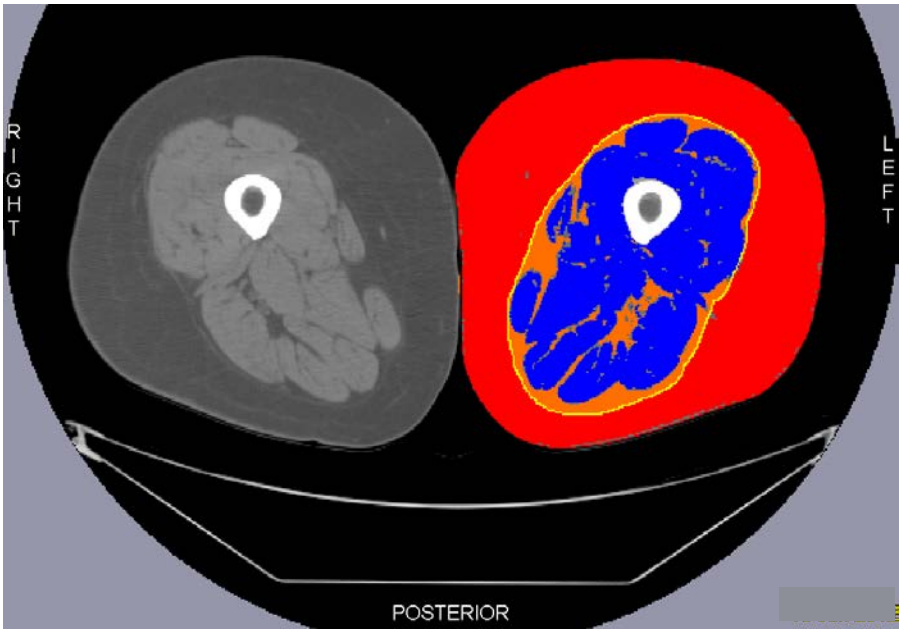
SAT accumulation did not associate with most physical function variables or physical activity, but, was positively associated with strength and negatively with HAQ scores ($R^2\delta$ = .023 and .127, respectively). Similar to IMAT, most associations between SAT and physical function or activity were significantly attenuated after accounting for BMI, as well as age, sex, and muscle area. Although SAT has not been specifically studied previously in RA, prior RA studies have reported on the associations between regional adiposity and disability, and have reported results that contradict the current study.[77,97] Giles et al and Kramer et al observed a detrimental effect of overall body and appendicular fat mass, and total thigh fat area (SAT and IMAT combined), respectively, on HAQ scores, in adjusted models.[77,97] In healthy elderly populations, the associations between SAT and physical function is inconsistent among studies,

but, none reported a protective effect as observed in the current study. Among studies that accounted for BMI and demographics, Therkelson et al reported no associations,[21] whereas Murphy et al, and Beavers et al reported significant associations between SAT and worsening of physical function over time in women, but not men. [22,23] There does exist some literature in healthy elderly and obese populations suggesting that subcutaneous fat mass, particularly in the lower body region, is not associated with adverse metabolic complications, and may be attributed to favorable metabolic profile [55,56,156]. In RA, being overweight and obese was associated with lower mortality, [158,159] but, the RA studies only used BMI as a proxy measure of body fat, and did not assess SAT specifically. Therefore, SAT may not be a fat depot that adversely affects physical or metabolic health, however, further investigation in RA will be necessary to confirm these findings.

The current study is not without limitations. The study sample consisted mainly of Caucasian women. However, this sample is also fairly representative of the RA populations examined in most western developed societies in terms of sex distribution, BMI, and disease characteristics. Also, the cross-sectional design precludes any temporal or causal inferences. To that end, future longitudinal studies are necessary to confirm how different fat depots affect physical function and activity behavior.

In summary, this study demonstrates that fat infiltration within the muscle (SMF) has a unique contribution to physical function and physical activity in individuals with RA, that is independent of body size, muscle area or muscle strength. These findings suggest that fat encroachment may drive some metabolic and physical changes within the muscle that may consequently contribute to worse physical function and low physical activity levels in RA. In contrast, fat infiltration outside the muscle (IMAT and SAT), does not seem to explain much

variability in physical function or physical activity beyond what is already explained by overall body size (BMI), demographics, or muscle area. These findings are clinically relevant because they suggest that fat within the muscle may be an alternate source of disability in this population. Future longitudinal studies assessing the effect of fat infiltration within muscle on physical function and activity will be necessary to confirm these findings, and to examine whether targeting these fat depots may be beneficial in promoting the health of those with RA.



The right mid-thigh cross-section shows the original image obtained by Computed Tomography, and the left mid-thigh cross-section represents the processed image with the fat depots.

The blue region represents the mid-thigh muscle area. Skeletal muscle fat is represented by the average attenuation values of the blue colored region in Hounsfield Units (HU). The yellow outline represents the borders of the muscle fascia. The orange region underneath the yellow line represents the intermuscular adipose tissue cross-sectional area. The red region outside the yellow line represents the subcutaneous adipose tissue cross-sectional area

Figure 2.Cross-sectional Bilateral Images of the Mid-thigh Region Illustrating Skeletal Muscle Fat, Intermuscular Adipose Tissue and Subcutaneous Adipose Tissue.

Table 1. Characteristics of the RA Study Sample

Variables	Total Sample (N=60)
Age in years, mean ± SD	59.0 ± 9.8
Number of Females, (%)	49 (82)
Number of Caucasians, (%)	50 (83)
Education Level, N (%)	
- High School	16 (27)
- College Education	44 (73)
Marital status, N (%)	
- Married	32 (53)
- Single/never married	12 (20)
- Other (divorced, widowed, etc.)	16 (27)
Employment Status, N (%)	
- Regular Full Time or Part time	20 (33)
- Retired (not due to health)	20 (33)
- Retired or unable to work due to health	11 (18)
- Other (e.g. modified light job, home-maker)	9 (14)
Height in meters, median (25th-75th percentile)	1.63 (1.59-1.69)
Weight in kg, mean ± SD	84.9 ± 21.1
BMI in kg/m^2 mean ± SD	31.2 ± 7.2
RA duration in years, median (25^{th}-75^{th} percentile)	13.5 (6-22)
DAS-28 score, mean ± SD	4.0 ± 1.3
HAQ score, median (25^{th}-75^{th} percentile)	0.88 (0.38-1.25)
Charleston Co-morbidity index, median (25^{th}-75^{th} percentile)	
Raw Score	0 (0.0 – 1.0)
Age Adjusted Score	2.0 (1.0 – 3.0)

BMI: Body Mass Index, DAS: Disease Activity Score, HAQ: Health Status Questionnaire

Table 2. Descriptive Statistics of Physical Function and Physical Activity Measures, and Fat Depots along with their Univariate Associations

	Mean ± SD or Median (25th-75th Percentile) 95% Confidence Interval	Univariate Correlation Coefficients (Pearson's r or Spearman's rho)		
		Skeletal Muscle Fat (Quadriceps Density), HU	Intermuscular Adipose Tissue, sq.cm	Subcutaneous Adipose Tissue, sq.cm
Dependent Variables				
Quadriceps Strength, Newton-meters‡	141.6 (114.8 – 165.9) 134.3 - 158.7	.454*	.072	.075
Single leg stance, seconds‡	12.7 (3.2-23.5) 11.0 -16.3	.508*	-.211	-.071
Gait speed, meters/second	1.06 ± 0.25 0.99 – 1.12	.397*	-.389*	-.219
5-Chair-stand time, seconds	12.5 (10.4-15.1) 12.0 -14.9	-.244	.270*	.128
Stair ascend time, seconds	6.3 (5.1 – 7.7) 6.1 -7.6	-.576*	.372*	.289*
HAQ	0.88 (0.38-1.25) 0.72 – 1.04	-.418*	.424*	.190
Physical Activity, minutes/day	34.0 (16.0 -47.0) 31.1 -52.4	.578**	-.388*	-.321*
Independent Variables‡				
Skeletal muscle fat (quadriceps density, HU)	46.4 ± 4.7 45.1 – 47.6			
Intermuscular adipose tissue area (sq. cm)	11.8 (7.9 – 17.3) 11.4 – 15.3			
Subcutaneous adipose tissue area (sq.cm)	106.3 (69.4 – 153.4) 101.3 – 134.9			

N = 60, except for HAQ (N = 59), Gait speed (N = 59), and Chair-stand (N=58) due to missing data, and Physical Activity (N=51) due to insufficient accelerometry data (<10 hours per day on 4 days).

*significant at alpha level 0.05

‡ Values represent the averages of the right and left leg.

HAQ: Health Assessment Questionnaire, HU: Hounsfeld Units, sq.cm: square centimeter

Table 3. Adjusted Regression Models of Associations between Skeletal Muscle Fat (Quadriceps Density, HU) and Physical Function and Physical Activity

Dependent Variable	Step †	Confounder variable	Independent variable	Beta-coefficient	R^2	$R^2\Delta$	R^2 Adjusted	F-change	p-value
Quad Strength	1	Age BMI Quad Area		.104 -.143 .896*	.771		.758	62.727	<.001
	2		SMF	.212*	.794	.023	.779	6.118	.017
Stair Climb (Inverse)	1	Age BMI Strength		-.444* -.541* .410*	.627		.607	31.426	<.001
	2		SMF	.006	.627	.000	.600	.002	.961
Gait Speed	1	BMI		-.276*	.142		.127	9.438	.003
	2		SMF	.306*	.226	.084	.198	6.051	.017
Single Leg Stance	1	Age		-.213	.172		.157	12.027	.001
	2		SMF	.416*	.304	.132	.279	10.801	.002
Chair Rise Time (Inverse)	1 2	Age BMI		-.298 -.383*	.195		.166	6.660	.003
			SMF	-.004	.195	.000	.150	.001	.979
HAQ	1	Age BMI Strength		-.200 .240 -.211	.230		.188	5.471	.002
	2		SMF	-.345*	.286	.056	.233	4.237	.044
Physical Activity (Sqrt)	1	BMI Strength Quad Area		-.296 -.547* .395	.167		.133	4.818	.012
	2		SMF	.530*	.415	.248	.377	19.881	<.001

*significant at alpha level 0.05

†Step 2 in controlling for variables in Step 1

SMF: Skeletal Muscle Fat, BMI: Body Mass Index, Quad: Quadriceps muscle, HAQ: Health Assessment Questionnaire, Sqrt: Square root transformed

Table 4. Adjusted Regression Models of the Associations between Intermuscular Adipose Tissue (area in square cm), and Measures of Physical Function, and Physical Activity

Dependent Variable	Step†	Confounder Variable	Independent Variable	Beta Coefficient	R^2	$R^2 \Delta$	R^2 Adjusted	F-change	p-value
Quad Strength	1	Age		.099	.788		.772	51.025	<.001
		BMI		.176					
		Sex		-.294*					
		Quad Area		1.111*					
	2		IMAT	-.037	.788	.001	.769	.150	.700
Stair Climb (Inverse)	1	BMI		-.701*	.438		.419	22.242	<.001
		Quad Area		.593*					
	2		IMAT	.046	.439	.001	.409	.100	.753
Gait Speed	1	Age		-.262*	.212		.184	7.539	.001
		BMI		-.353*					
	2		IMAT	-.093	.216	.004	.174	.294	.590
Single Leg Stance (log)	1	BMI		-.215	.164		.135	5.602	.006
		Quad Area		.362*					
	2		IMAT	-.216	.186	.022	.143	1.516	.223
Chair Rise Time (Inverse)	1	BMI		-.275	.179		.149	6.008	.004
		Sex		.255*					
	2		IMAT	-.101	.184	.005	.139	.323	.572
HAQ	1	BMI		.168	.125		.110	8.133	.006
	2		IMAT	.258	.157	.032	.127	2.141	.149
Physical Activity (Sqrt)	1	BMI		-.198	.162		.145	9.495	.003
	2		IMAT	-.285	.202	.039	.168	2.369	.130

*significant at alpha level 0.05

†Step 2 in controlling for variables in Step 1

IMAT: Intermuscular Adipose Tissue, BMI: Body Mass Index, Quad: Quadriceps muscle, HAQ: Health Assessment Questionnaire, Sqrt: Square root transformed

Table 5. Adjusted Regression Models of the Associations between Subcutaneous Adipose Tissue (area in square cm), and Measures of Physical Function, and Physical Activity

Dependent Variable	Step	Confounder Variable†	Independent Variable	Beta Coefficient	R^2	$R^2\,\Delta$	R^2 Adjusted	F-change	p-value
Quad Strength (sqrt)	1	Age		.112	.788		.772	51.025	<.001
		BMI		-.567*					
		Sex		.084					
		Area		1.143*					
	2		SAT	.305*	.811	.023	.793	6.497	.014
Stair Climb (Inverse)	1	Age		-.303*	.619		.591	22.349	.000
		BMI		-.809*					
		Sex		.221					
		Area		.615*					
	2		SAT	.086	.621	.002	.586	.256	.615
Gait Speed	1	Age		-.262*	.213		.170	4.973	.004
		BMI		-.605*					
	2	Sex		-.054					
			SAT	.239	.227	.014	.170	.985	.325
Single Leg Stance	1	Age		-.333*	.288		.236	5.557	.001
		BMI		-.595*					
		Sex		.029					
		Area		.303					
	2		SAT	.237	.302	.014	.237	1.060	.308
Chair Rise Time (Inverse)	1	Age		-.254*	.240		.198	5.685	.002
		BMI		-.356					
	2	Sex		.232					
			SAT	-.041	.240	.000	.183	.030	.864
HAQ	1	BMI		.894*	.133		.102	4.311	.018
	2	Sex		.361*					
			SAT	-.718*	.260	.127	.220	9.433	.003
Physical Activity (Sqrt)	1	Age		-.333*	.324		.280	7.497	.000
		BMI		-.657*					
	2	Sex		-.408*					
			SAT	.286	.344	.020	.287	1.408	.241

*significant at alpha level 0.05

†Step 2 in controlling for variables in Step 1

SAT: Subcutaneous Adipose Tissue, BMI: Body Mass Index, Quad: Quadriceps muscle, HAQ: Health Assessment Questionnaire, Sqrt: Square root transformed

3.0 STUDY 2 – ACCUMULATION OF FAT DEPOTS IN RHEUMATOID ARTHRITIS COMPARED TO HEALTHY INDIVIDUALS

This chapter focuses on the comparisons between skeletal muscle fat, intermuscular adipose tissue and subcutaneous adipose in RA versus healthy cohorts. Two cross-sectional studies are reported in this chapter. The first study compares the three fat depots in RA and a healthy cohort matched on age, sex and body size, and the second study compares the three fat depots in RA and a healthy cohort matched on sex and body size but who were 15 +/- 5 years older than their RA counterparts.

3.1 SUMMARY

Objective: To compare skeletal muscle fat (SMF), intermuscular adipose tissue (IMAT) and subcutaneous adipose tissue (SAT) between individuals with rheumatoid arthritis (RA), and healthy individuals of the same age, and with healthy individuals who were 15 +/- 5 years older than those with RA. Methods: Two cross-sectional studies were conducted. In the first study RA 21 subjects were matched with healthy adults who were +/- 5 years of age, same sex, and BMI +/- 2.0 kg/m^2. In the second, 23 RA subjects were matched by sex and BMI +/- 2.0 kg/m^2 to adults who were 15 +/- 5 years older. Bilateral computed tomography images of the mid-thigh

region from the RA and non-RA individuals were used to assess SMF, IMAT and SAT. SMF, IMAT and SAT were compared between the RA and non-RA cohorts by either parametric or non-parametric related samples tests. Results: In the first study SMF was significantly higher in the RA cohort compared to their age-matched healthy counterparts (mean difference= -3.5 HU (95% -6.2, -0.9), p = 0.011), but IMAT and SAT were similar between cohorts. In the second study, SMF, IMAT and SAT were not significantly different between the RA and matched healthy cohorts who were 15 +/- 5 years older. Conclusion: Accumulation of fat within the skeletal muscle in RA is higher than in healthy adults of similar age, and those with RA seem to have similar pattern of SMF, IMAT and SAT accumulation as adults who are 10-20 years older. This study provides preliminary information for future studies to assess whether targeting the muscle in an attempt to reduce SMF may be beneficial to improve health in RA.

3.2 INTRODUCTION

Altered body composition as a result of loss in lean mass and concomitant gain in fat mass is a well-known extra-articular manifestation of rheumatoid arthritis (RA).[160] Cross-sectional studies that compared individuals with RA to age and sex matched healthy individuals reported that those with RA had 13-16% higher total body fat mass and 5-11% lower lean mass compared to their healthy counterparts.[2-4,47,48] However, there is limited information on whether increase in total body fat reported in RA extends to abnormal accumulation of fat within and around the skeletal muscles. The limited information is mostly because the body composition methods used in previous studies consisted of bioelectrical impedance, that does not provide location of fat,[47] or dual X-ray absorptiometry, that mostly estimates subcutaneous adipose tissue (SAT) that is

superficial to the skeletal muscles.[2-4] An example of an appropriate method to measure fat within and around the skeletal muscles is Computed Tomography (CT).[147,148] CT enables the assessment of skeletal muscle fat (SMF), which represents the fat present within the individual muscle cells or around individual muscle fibers, and intermuscular adipose tissue (IMAT), that refers to fat depots underneath the muscle fascia that lie outside the muscle and is interspersed between muscle groups.[148]

Understanding whether accumulation of SMF and IMAT is affected in RA is clinically relevant because it may help explain the persistent disability and higher cardiometabolic risk in this population. Both SMF and IMAT have detrimental influences on health that are in contrast to SAT, which has not been associated with adverse physical or metabolic health complications. [55-57] In non-RA populations,[9,10,61,63,113] as well as one RA study[77], SMF accumulation was shown to contribute to disability and mobility limitations. IMAT has been implicated in metabolic complications, such as greater insulin resistance, dyslipidemia, and chronic inflammation in non-RA populations [13,58,154,161] and recently one study in RA also reported a moderate association between IMAT and insulin sensitivity.[157]

Studies that compared SMF, IMAT and SAT in RA to healthy cohorts are sparse. One study that investigated SMF accumulation in RA versus non-RA individuals [162] reported that SMF accumulation was significantly higher only in RA individuals with low BMI. However, the results of this study are limited because BMI was lower in non-RA cohort compared to RA cohorts.[162] Because BMI has shown to associate with SMF and IMAT,[9] it needs to be adjusted in such investigations. We are aware of one study that matched subjects with RA to healthy individuals by BMI, along with age and sex. This study reported no differences between the groups on SMF, IMAT, and SAT. However, the no differences between RA and non-RA cohorts

could be because their RA sample had low disease activity and RA was in remission in more than 50% of the participants.[157] The conflicting reports on fat accumulation in RA warrant further investigation using study designs that account for potential confounders (such as body size), and also include a representative sample of individuals with more active RA disease.

The RA disease process has also been postulated to simulate changes in muscle and fat mass that are somewhat similar to those observed in normal aging.[44,48,75,163] Systemic inflammation appears to be a common culprit for loss in muscle mass and gain in fat mass in both aging and RA. Exact mechanisms might be different since RA is associated with high-grade inflammation that may occur in acute bouts, while in aging inflammation is low-grade and chronic in nature,[164] but, both processes may have similar consequences on muscle and fat mass. With respect to fat accumulation in healthy aging, there is generally a loss of SAT, while SMF and IMAT tend to increase even without significant changes in body weight.[92,165,166] Studies in healthy aging suggested that loss of SAT could be due to decreased capacity of the adipocytes to store lipid,[92,166,167] and that increase in IMAT may be due to the faulty transformation of precursor muscle satellite cells into mesenchymal adipocyte-like default cells instead.[167] Although the notion of accelerated aging in RA has been speculated, it has not been directly tested with respect to changes in accumulation of specific fat depots.

To address the gaps in RA literature regarding fat accumulation we conducted two studies. In the first study we compared individuals with RA to healthy controls who were matched by age, sex, and BMI; and we hypothesized that individuals with RA would have higher SMF, IMAT, and SAT accumulation. The second study was to compare SMF, IMAT, and SAT in individuals with RA to healthy individuals who were matched on sex and BMI, but 15 +/- 5 years older than the RA subjects. We hypothesized that individuals with RA would have similar

SMF, IMAT and SAT and similar muscle area compared to their sex and BMI matched older healthy counterparts.

3.3 METHODS

3.3.1 Study Design and Participants

A cross-sectional design was used for the two ancillary studies. Data on individuals with RA were obtained from a previous randomized clinical trial (RCT) conducted at the University of Pittsburgh. (Clinical Trials Registration NCT00924625) Data for the age matched and elderly controls were obtained from three RCTs on exercise and/or weight loss interventions that were also conducted at the University of Pittsburgh.

Participants with RA were recruited in the parent RCT between December 2009 and September 2013 if they were above 21 years of age, had a confirmed diagnosis of RA as per the 1987 American College of Rheumatology criteria, able to ambulate independently, and sedentary (<2 days/week of regular physical activity). Participants were excluded if they had been diagnosed with medical conditions that precluded safe participation in a high intensity exercise program, such as cardiovascular disease, uncontrolled hypertension, neurological or muscular conditions affecting the lower extremities, or recent surgery of the lower extremities. Participants were also excluded if they were on cholesterol lowering medications (Statins). All eligible participants signed an informed consent approved by the University of Pittsburgh Institutional Research Board prior to study enrollment.

Non-RA controls were healthy individuals who participated in RCTs on exercise and lifestyle interventions conducted at the Department of Physical Therapy and the Endocrinology and Metabolism Research Center at the University of Pittsburgh. The non-RA studies enrolled adults between 21-80 years of age, who were able to ambulate independently and were sedentary (<2 days/week of regular physical activity). Individuals were excluded if they had a history of cardiovascular disorders or unstable hypertension. All eligible participants signed an informed consent approved by the University of Pittsburgh Institutional Research Board prior to enrollment in the respective studies.

3.3.2 Matching Process

All cases were manually paired by the primary author (SSK). De-identified data spreadsheets containing the study identification number for the RA and healthy subjects, along with their age, sex and BMI was provided by the respective parent study PIs to the primary author. During the matching process the primary author was blinded to imaging data on SMF, IMAT and SAT. For the first study, individuals with RA were matched to healthy adults who were within +/- 5 years of age, same sex, and BMI within +/- 2.0 kg/m^2. For the second study, individuals with RA were matched to adults who were 15 +/-5 years older than them, but, of the same sex, and BMI within +/- 2.0 kg/m^2. Fat and muscle assessment from Computed Tomography (CT) imaging were added to the final data spreadsheets for analyses only after the matching process was complete.

3.3.3 Assessment of Fat Depots and Subject Characteristics

Data on SMF, IMAT, SAT and muscle area were obtained from bilateral CT imaging of the mid-thigh area using similar methods across all parent studies.[15,116] Briefly, a 10-mm thick axial image of the mid-thigh region was obtained at the femoral mid-point, which is the center of a line joining the lateral most part of the greater trochanter and the lateral femoral epicondyle. Subjects were positioned in supine in the scanner with both femurs in neutral position and scanning parameters used were 120kVp and 200-250mA. CT images obtained were processed using the Slice-O-Matic specialized software to quantify fat and muscle outcomes. The software differentiates between muscle, fat, and bone tissue based on their physical density properties, which is measured on an interval scale in Hounsfeld Units (HU), with water as the reference at 0 HU. Tissues denser than water such as muscle and bone have positive values (above 0 HU) while less dense tissues such as adipose tissue have negative values (below 0 HU). Muscle density ranges between 0 to 100 HU, while adipose tissue density range between -190 to -30 HU. SMF was the primary outcome of both studies and was measured by the average density (HU) of the mid-thigh muscle region, with lower HU values corresponding to higher amount of SMF. SAT and IMAT were separated by tracing the muscle fascia, and their cross-sectional areas measured in square centimeters. We also assessed muscle area of the mid-thigh region. All variables were averaged for the right and left leg. This method used to measure muscle area and fat content using CT imaging is reliable and valid (ICCs >0.98, and coefficients of variation < 2%). [118]

For the RA subjects, disease duration, disease activity, and functional limitations were also recorded. Disease activity was assessed by the disease activity score (DAS-28). It involves the examination of 28 joints for tenderness and swelling, erythrocyte sedimentation rate from a blood test, and patient reported global health on a scale of 0 to 100. Scores are calculated using a

validated algorithm and range from 0 to 9.4. Scores ≤ 3.2 indicate low disease activity, scores ≥3.2 ≤ 5.1 indicate moderate disease activity and scores > 5.1 indicate high disease activity. Self-reported functional limitations was assessed using the Health Assessment Questionnaire (HAQ), which is a widely used tool that assesses limitations during 20 activities of daily living, and is scored between 0 (no disability) to 3 (severe disability).[139]

3.3.4 Power Calculation and Data Analysis

Both studies on comparison between RA and age-matched pairs, and between RA and older-matched pairs were powered to detect a 10% difference in the primary outcome of muscle density. In order for each study to have 80% power at an alpha level of significance of 0.05, at least 19 matched pairs were required to detect a difference of 4.0 HU (assuming mean muscle density for healthy adults is 40 HU) using a SD of difference of 5.8 HU derived from a previous study.[127]

Subject characteristics, and CT imaging data were described in terms of mean (standard deviation) for continuous variables and frequencies for categorical variables. Normality of data distribution were assessed using the Shapiro-Wilk test. Depending on data distribution either parametric tests such as the paired student t-test or non-parametric Wilcoxon-signed rank test was used to test for differences in the outcome variables between the RA cases and controls. All statistical analyses were performed using the IBM SPSS software, version 22. (IBM Corporation)

3.4 RESULTS

3.4.1 Comparison of RA subjects with age matched controls

The matching process resulted in 21 pairs of RA cases and healthy controls, among which 67% of the pairs were female. The RA cohort had a mean age of 61.5 years, while the healthy controls were 61.9 years in age, and both cohorts were obese (mean BMI of RA cohort = 31.7 kg/m^2 and Healthy cohort = 32.4 kg/m^2) (Table 6). Those with RA had an average disease duration of 15 years, moderate disease activity and mild functional limitations. Compared to their matched healthy controls, subjects with RA had significantly lower mid-thigh muscle density (-3.5 HU, 95% CI -6.2, -0.9) which represents higher SMF (Table 7). There were no significant differences in the amount of IMAT, SAT, and muscle area between the individuals with RA and age, sex and BMI matched healthy controls (Table 7).

3.4.2 Comparison of RA subjects with older matched controls

The matching process resulted in 23 pairs of RA cases elderly healthy controls, among which 77% of the pairs were female. The RA cohort had a mean age of 57.6 years, and the older controls had a mean age of 71.7 years, and both cohorts were obese (mean BMI of RA cohort = 30 kg/m^2 and elderly cohort = 30.5 kg/m^2) (Table 8). Those with RA had an average disease duration of 17 years, moderate disease activity and mild functional limitations. Compared to their older matched controls, subjects with RA had similar amount of SMF, IMAT and SAT, and muscle area ($p > 0.05$) (Table 9).

3.5 DISCUSSION

3.5.1 Comparison of RA subjects with age matched controls

Study findings partially confirmed our hypothesis. SMF accumulation was higher in RA compared to healthy individuals of the same sex, similar age, and BMI; but IMAT and SAT accumulation were not different between the cohorts. Of note, our results also demonstrated that higher SMF accumulation in the RA was present even though there were no differences in muscle area between RA and healthy controls, which means that SMF accumulation can occur even in absence of muscle atrophy. SMF represents the intramyocellular lipid within the myocyte and also extramyocellular lipid that surrounds the individual muscle. Excess accumulation of these lipids are postulated to release toxic lipid by-products that can give rise to inflammation and affect muscle function.[7,64] Greater SMF accumulation in the muscle has been associated with poor muscle strength, higher disability, and greater risk of mobility limitations and bone fractures.[9,10,61,63,77] SMF infiltration has also been associated with insulin resistance in some non-RA populations such as obese and sedentary individuals.[64,66] Since insulin resistance is a cardiovascular risk factor, high SMF may be directly linked to high risk of cardiovascular disease in RA. Future studies need to investigate whether interventions that directly target the muscle may be beneficial to reduce cardiometabolic risk in RA.

We are aware of two studies in RA that assessed SMF accumulation compared to healthy cohorts, but reported conflicting results.[157,162] Baker et al reported that individuals with RA with low BMI and muscle area had significantly higher SMF compared to the healthy individuals. [162] In the current study, SMF was higher in RA in regardless of BMI, but, we also observed that in the six pairs of RA and non-RA controls who were ≤ 25 kg/m^2, the differences in muscle density

were accentuated (-7.00 HU, 95% CI: -12.8, -1.2) compared to the mean difference in the entire cohort (-3.5 HU, 95% CI:-6.2, -0.9). Thus, our findings concur with those of Baker et al, and also suggest that SMF accumulation in RA is likely influenced by factors other than BMI, such as systemic inflammation. Chronic systemic inflammation in RA may set up a cascade of events that might alter fat metabolism within the myocyte and encourage excess fat encroachment around the individual muscle fibers. Studies in RA have reported significant positive associations of disease activity with higher SMF which support the link between systemic inflammation and muscle fat.[77,162] On the contrary, AbouAssi et al reported no significant differences between SMF in RA and the healthy cohorts, who were matched on age, sex and BMI.[157] The conflicting findings between AbouAssi and the current study could be attributed to the younger age (average age 55 years versus 61 years) and low disease activity (average DAS-28 score of 3.1 versus 4.1) of the RA cohort in their study compared to the current study. Both younger age and lower disease activity may have contributed to the healthier muscle in AbouAssi et al study as compared to our RA sample (average muscle density 50 HU versus 41 HU, and average muscle area 116 sq.cm versus 106 sq.cm).

The findings that IMAT and SAT are not different in RA compared to healthy individuals with the same BMI suggest that these fat depots are largely dependent on obesity, and conflict with previous reports of higher body fat mass in RA compared to healthy matched controls. [2-4] However, these previous studies did not match the RA and non-RA cohorts by BMI, and therefore, the greater prevalence of obesity in their RA cohorts compared to the healthy cohorts likely explains the conflicting findings. On the other hand, our findings are aligned with those of AbouAssi et al, in which no significant differences in SAT or IMAT were reported between RA and healthy cohorts (matched on age, sex and BMI).[157] There were also no differences in muscle

area between the RA and the age matched controls. This may be explained by the controversial obesity paradox in RA, which suggests that being overweight or obese is protective against the severe disease progression of RA and associated with less muscle wasting.[168-170] On the contrary, being underweight in RA is associated with worse disease outcomes, and greater muscle wasting with low adiposity.[169] Baker et al demonstrated this protective effect of high BMI in RA in their study; their findings showed that individuals with high BMI did not show deficits in muscle area and density compared to healthy adults.[162] Since our cohort was on average obese, they may not have had any significant alterations in muscle area due to the supposedly protective effect of the obesity paradox.

3.5.2 Comparisons of RA subjects with older matched controls

This is the first study to compare fat depots and muscle in individuals with RA with healthy individuals of the same sex and BMI who were at least 10 years older. Findings demonstrated that those with RA had similar SMF, IMAT and SAT accumulation, and muscle area, as the healthy elderly individuals, which implies that the accumulation of these fat depots seem to be similar to what is seen in aging. Although systemic inflammation is typically high in RA and fluctuates in short bouts when there is a flare-up of the disease, in aging, there is low-grade chronic systemic inflammation.[164] Systemic inflammation leads to release of cytokines such as TNF- α, interleukin-1β, C-reactive protein and interleukin-6, which are common factors responsible for loss in muscle mass in both populations.[95,171] Evidence suggests that TNF alpha induces sarcopenia by altering the expression of genes and signaling proteins that facilitate protein synthesis for muscle cell growth and differentiation.[94,164] Other suggested factors that are common in aging and RA related to accumulation of muscle fat include hormonal changes (i.e.,

low bioavailability of insulin-like growth factor and reduced production of growth hormones), physical inactivity, and nutritional status.[172,173] Other mechanisms that have been suggested in aging but not studied in RA include the reduced capacity of adipocytes to store SAT, and faulty transformation of non- adipocyte precursor cells, such as muscle satellite cells, into mesenchymal adipocyte-like default cells that store triglycerides.[92,166,167]

Increase in adiposity in aging and loss of muscle mass has been studied extensively. Delmonico et al reported changes in IMAT and SAT over a period of 5 years in individuals between 70-79 years of age, but did not report on SMF.[174] The study reported that IMAT increased in the elderly individuals regardless of changes in weight. In contrast, changes in SAT went along with weight gain or loss, and did not increase in those who were weight stable.[174] Because of the current study's cross-sectional design, it was not possible to determine whether individuals with RA lose SAT over time, as observed in adults who are aging,[92] or whether they experience similar gains in IMAT and SMF as do older adults. Muscle loss or sarcopenia has been reported to affect between 10-52% of adult above 70 years of age, [172] and loss of muscle mass in adults between 70-79 years occurs at the rate of approximately 1% per year.[113] Due to lack of longitudinal studies, it is not known how changes in fat accumulation within and around the muscle occur over time as the RA disease progresses. Because SMF and IMAT are both related to adverse metabolic complications such as dyslipidemia, insulin resistance and risk of developing type 2 diabetes, [92] that are known cardiovascular risk factors, it may be important for future longitudinal studies to investigate whether muscle fat accumulation may be an independent risk factor for developing cardiovascular disease in RA.

3.5.3 Overall Limitations and Conclusions

The two RA studies reported in this paper are not without limitations. The cross-sectional design precludes any temporal inferences related to changes in SMF, IMAT or SAT accumulation in RA over time. Due to relatively small sample sizes of the studies, there might also be a potential for a type II error in the results related to IMAT and SAT. However, the magnitude of differences in IMAT, SAT and muscle area between the cohorts were minimal (<5% difference), and not likely relevant. Strengths of these studies comprise the inclusion of a RA population representative of most RA samples reported in the literature in terms of body size (i.e., majority overweight and obese) and moderate disease activity. Moreover, in our studies the RA and non-RA cohorts were matched on BMI, which enabled a more accurate assessment of whether fat accumulation in and around the muscles are truly altered in RA, and not merely a consequence of obesity.

The results of our studies suggest that SMF accumulation is higher in RA compared to healthy individuals, independent of age, sex, and BMI; while SAT or IMAT accumulation does not appear to be significantly different compared to healthy individuals. The results also demonstrate that SMF, IMAT and SAT accumulation are similar compared to healthy individuals who are 10-20 years older than the individuals with RA, suggesting that fat accumulation in and around the muscles in RA may mimic a premature aging process. The current study provides preliminary information for future longitudinal studies to investigate the rate and extent of accumulation within specific fat depots in RA, as well as the role of SMF accumulation as a potential independent risk factor for cardiovascular disease in RA.

Table 6. Demographics of the RA cohort and the non-RA matched cohort matched by age, sex and BMI

Variables	RA cohort (n=21)	Non-RA cohort (n=21)
Age, years	61.5 ± 10.9	61.9 ± 11.5
Height, cm	164.7 ± 8.4	168.2 ± 6.2
Weight, kg	85.9 ± 21.3	89.5 ± 21.8
BMI, Kg/m^2	31.7 ± 7.9	32.4 ± 7.6
Number of Females (%)	14 (67)	14 (67)
RA duration, years	15.2 ± 12.1	NA
Disability, HAQ scores	0.91 ± 0.67	NA
Disease Activity, DAS-28 scores	4.1 ± 1.4	NA
Variables described as Mean ± SD, unless specified otherwise BMI: Body Mass Index, HAQ: Health Status Questionnaire, DAS: Disease Activity Score		

Table 7. Comparison of mid-thigh skeletal muscle fat, intermuscular and subcutaneous adipose tissue, and muscle area in the RA cohort and the non-RA cohort matched by age, sex and BMI

Mid-thigh CT measures	RA cohort (n=21)	Non-RA cohort (n=21)	Mean Differences between groups (95% CI)	P-value†
SMF, average muscle density in Hounsfeld units	41.2± 4.7	44.7 ± 2.7	-3.5 (-6.2, -0.9)	.011*
Total muscle area (sq. cm)	106.0 ± 25.3	109.0 ± 16.5	-3.0 (-12.6, 6.7)	.527
IMAT area (sq. cm)	13.6 ± 6.3 12.0 (7.8, 19.5)	15.6 ± 13.9 12.4 (6.4, 19.6)	-2.0 (-6.9, 2.8)	.391
SAT area (sq. cm)	113.2 ± 70.2	108.8 ± 61.4	4.4 (-10.6, 19.4)	.547

Values in red represent medians (25th, 75th percentile) for those outcomes that were not normally distributed.

* Significant findings at alpha level of 0.05

† P-values were obtained from parametric (paired t-test) or non-parametric (Wilcoxon-signed rank test) depending on data distribution.

SMF: Skeletal muscle fat, IMAT: intermuscular adipose tissue, SAT: subcutaneous adipose tissue

Table 8. Demographics of RA cohort and the older cohort matched by sex and BMI

Variables	RA cohort (n=23)	Older non-RA cohort (n=23)
Age, years	57.6 ± 6.3	71.7 ± 5.1
Height, cm	164.7 ± 5.3	164.2 ± 9.2
Weight, kg	81.7 ± 21.6	82.4 ± 21.4
BMI, Kg/m2	30.0 ± 7.4	30.5 ± 7.3
Number of Females (%)	21 (78)	21 (78)
RA duration, years	17.0 ± 12.1	NA
Disability, HAQ scores	0.97 ± 0.64	NA
Disease Activity, DAS-28 scores	4.2 ± 1.3	NA
Variables described as Mean ± SD, unless specified otherwise BMI: Body Mass Index, HAQ: Health Status Questionnaire, DAS: Disease Activity Score		

Table 9. Comparison of Mid-thigh CT measures of SMF, IMAT, SAT and Muscle Area in RA cohort and older cohort matched by sex and BMI

Mid-thigh CT outcome measures	RA cohort (n=23)	Older non-RA cohort (n=23)	Mean Differences between groups (95% CI)	P value†
SMF, average muscle density in Hounsfeld units	42.7 ± 3.8	43.5 ± 5.3	-0.8 (-2.3, 0.8)	.335
Total muscle area (sq. cm)	102.7 ± 24.5 98.3 (88.3, 117.1)	98.5 ± 23.0 97.3 (80.9, 106.4)	4.2 (-6.1, 14.4)	.411
IMAT area (sq. cm)	11.1 ± 6.2 8.8 (6.9, 14.5)	15.4 ± 14.2 10.7 (6.2, 18.5)	-4.3 (-9.1, 0.4)	.071
SAT area (sq. cm)	110.9 ± 64.0 97.7 (65.2, 152.7)	117.6 ± 60.6 102.5 (66.8, 193.3)	-6.7 (-25.1, 11.7)	.458

Values in red represent medians (25^{th}, 75^{th} percentile) for those outcomes that were not normally distributed.

† P-values were obtained from parametric (paired t-test) or non-parametric (Wilcoxon-signed rank test) depending on data distribution.

SMF: Skeletal muscle fat, IMAT: intermuscular adipose tissue, SAT: subcutaneous adipose tissue

4.0 EXPLORATORY STUDY ON INTRAMYOCELLULAR LIPID IN RA

This chapter focuses on the exploratory outcome of intramyocellular lipid (IMCL) content in subjects with Rheumatoid Arthritis (RA). It investigates the associations between IMCL content and disease characteristics, and the outcomes of physical function and physical activity. This chapter also compares IMCL in RA to healthy cohorts, and examines the methodological challenges of measuring IMCL in these dissertation studies.

4.1 INTRODUCTION

IMCL is located within the muscle cell (myocyte) and is mainly made up of neutral triglycerides. It is a metabolically active lipid depot and a direct source of energy for the contracting muscle.[7,145] However, excess deposition of IMCL not utilized for energy may result in the formation of lipotoxic intermediaries that facilitate the release of pro-inflammatory cytokines such as reactive oxygen species, which can impair the metabolic functions of the muscle.[7] In non-RA populations, including the elderly, obese, and those with type 2 diabetes, excess deposition of IMCL was observed due to inefficient fat oxidation, and under-utilization of fat stores in the presence of a high fat diet and low physical activity.[16,18,19] As people with RA are less physically active, and at a high risk of obesity[4,78] and metabolic syndromes,[78-81] they could also experience similar elevations in IMCL content due to a combination of decreased or

impaired fat utilization. Conversely, the accelerated protein degradation at the cellular level due to systemic inflammation in RA may induce the muscles to remain in a catabolic state resulting in lower IMCL stores compared to healthy non-RA individuals. Evidence related to IMCL content in RA is limited, but it is a well-accepted finding that loss of lean muscle mass due to protein breakdown is accompanied by an increase in fat mass. Currently, it is not known whether fat stores within the myocyte are also affected in RA, or if fatty infiltration is restricted to encroachment of surrounding adipose tissue into the muscle fascia (intermuscular adipose tissue).

Excess IMCL deposition in RA is of interest because these individuals are also at a higher risk for cardiovascular disease,[175] and some of the heightened cardiovascular risks, such as insulin resistance,[176] may possibly stem from abnormal muscle metabolism and excess deposition of IMCL.[145] Because of the limited evidence surrounding IMCL content in RA, the current study proposed to investigate IMCL stores in RA, explore their associations with physical function, and compare IMCL in RA to healthy controls. This study will be the first to explore IMCL content in RA using muscle samples and will serve as a preliminary reference for the amount of IMCL in RA.

4.2 METHODS

4.2.1 Study Design and Participants

This was a cross-sectional study. Data were obtained from subjects with RA who participated in the parent trial on strengthening interventions to reverse muscle atrophy. (Clinical Trials

Registration NCT00924625). Participants were included if they were above 21 years of age, diagnosed with RA by a rheumatologist as per the 1987 American College of Rheumatology criteria, able to ambulate independently, and were sedentary (< 2 days/week of physical activity). Participants were excluded if they had contra-indications that precluded safe participation in strength training of the major lower extremity muscles, such as cardiovascular disease, uncontrolled hypertension, neurological or muscular conditions affecting the lower extremities, or recent surgery of the lower extremity. To ensure safety during study muscle biopsy procedures, participants with a history of bleeding disorders or allergy to local anesthesia were excluded. Among the 60 subjects recruited for the randomized trial, 48 consented to undergo unilateral percutaneous needle biopsies of the vastus lateralis muscle, and were included in this study. All eligible participants signed an informed consent approved by the University of Pittsburgh Institutional Review Board prior to study enrollment.

4.2.2 Muscle Biopsy and Immunohistochemistry

The biopsies were carried out by one experienced physician under local anesthesia and with a Bergstrom needle.[65] Subjects were instructed to fast overnight and abstain from strenuous physical activity on the day before the biopsy. Local anesthesia was applied to numb the skin of the upper and lateral part of the thigh, and a small incision (approx. 0.25 inches) was made to assist with insertion of the biopsy needle.[65] A small amount of muscle (~100-150 mg) is suctioned into the needle using a syringe. The specimen is dried, trimmed of any visible adipose tissue and viewed under a microscope to find muscle fibers oriented in the same direction; these fibers are separated, mounted on a block using a mounting medium, (Miles,Incl,Elkhart, IN) and

frozen in isopentane (methyl butane) cooled by liquid nitrogen at -160 degrees Celsius, and stored at -80 degrees Celsius.

Immunohistochemistry was performed using 8μm thick transverse slices of the frozen muscle cryosections using previously described methods.[16,177] These frozen cryosections are stained with Oil Red O solution (ORO), which specifically stains neutral lipids (mostly triglycerides). Briefly, the frozen muscle cryosections are air dried at room temperature for 10 minutes, fixed in 3.7% formaldehyde for 1 hour, and then stained in Oil Red O (ORO) solution for 30 minutes. The slides are then washed in running lukewarm water for 15 minutes to remove excess ORO, and rinsed with Phosphate-buffered saline solution. To identify fiber types, the slides are additionally stained with 2 primary antibodies that stain for each fiber type (I and IIA). (Complete protocol in Appendix A) After staining, the muscle fibers are viewed under a Leica microscope and several magnified (20x objective) digital images of the muscle sections are captured (Retiga 2000R camera; Q Imaging), and are converted into gray scale images that is viewed on the computer. A specialized software (Northern Eclipse, v6.0; Empix Imaging) is used to calculate the average intensity of the lipid that is stained by ORO. This measure is called average gray intensity and it serves as a proxy measure of IMCL content, (higher average gray intensities indicate higher IMCL content). The average gray intensity is obtained from the difference of the intensity of the background slide image (clean empty portion of the slide with no muscle section) and the average intensity obtained from the stained lipid droplets in the muscle section images. We obtained the average gray intensity for each fiber type (I and II), and then also the total average gray with both fiber types combined. All outcome measures from histochemical analysis were exploratory in nature and used to characterize muscle and IMCL in

individuals with RA. Histology procedures were completed at the Endocrinology and Metabolism Research Center, University of Pittsburgh.

4.2.3 Physical Function, Physical Activity and RA Disease Characteristics

Physical function was measured using the self-reported Health Assessment Questionnaire (HAQ) questionnaire and a battery of physical performance tests shown to be reliable, valid, and well-tolerated in the RA population.[128-131] The HAQ assesses limitations during 20 activities of daily living, and is scored between 0 (no disability) to 3 (severe disability).[139] Maximum voluntary isometric strength of the quadriceps was measured using an isokinetic dynamometer (Biodex, Inc). Subjects were seated on the dynamometer with 70 degrees of knee flexion and performed five trials of isometric knee extension per leg. The highest three trials for each leg were averaged, and then further averaged for both limbs. Repeated chair-stand test consisted of the time taken to stand up from a chair five times. Stair climbing test recorded the time taken to ascend one flight of 12 stairs. Self-selected gait speed was calculated from the time taken to walk four meters. The single-leg stance test measured the time (up to 30 seconds) a subject could stand on one leg without losing balance. Each leg underwent three trials for single-leg stance, and the values from both limbs were averaged.

Physical activity was measured by a reliable and valid multi-sensor activity monitor, the Sense Wear Armband (Bodymedia, Inc).[150,151] worn on the right upper arm for 8 days, up to 24 hours per day, except during water related activities. A minimum of 4 days with at least 10 hours of data was required to yield reliable estimates of physical activity. [138] The daily time spent in activities of moderate or higher intensities were obtained.

Demographics and biomedical characteristics included age, gender, race, education, body mass index (BMI), and disease duration and activity. BMI was calculated based on weight and height measured on-site. Disease activity was measured by the disease activity score (DAS-28), a widely used validated tool that involves the examination of 28 joints for tenderness and swelling, erythrocyte sedimentation rate from a blood test, and patient reported global health on a scale of 0 to 100. Validated algorithms provide scores that range from 0 to 9.4, with scores ≤ 3.2 indicating low disease activity, between 3.2 and 5.1 indicating moderate disease activity and scores > 5.1 indicating high disease activity.[125 126]

4.2.4 Matching Process of RA and non-RA cohorts

Non-RA cohorts for matched analyses were obtained from previous studies conducted at the Department of Physical Therapy and the Endocrinology and Metabolism Research Center (EMRC), University of Pittsburgh. Details of the eligibility criteria for these studies are discussed in Chapter 3.0, Section (3.3.2). All cases were manually paired by the primary author (Khoja). For the first comparison, individuals with RA were matched to healthy adults who were within +/- 5 years of age, same sex, and BMI within +/- 2.0 kg/m^2. For the second comparison, individuals with RA were matched to adults who were 15 +/- 5 years older, but, of the same sex, and BMI within +/- 2.0 kg/m^2. De-identified data spreadsheets containing the study identification number for the RA and healthy subjects, along with their age, sex and BMI was provided by the respective parent study PIs to the primary author. During the matching process, the primary author was blinded to the histology data on IMCL.

4.2.5 Data Analysis

All data analyses were exploratory in nature due to small sample size. IMCL content was characterized and displayed using whisker plots. Then, we determined the associations between IMCL, demographics and RA disease characteristics (i.e., disease activity and duration), and also between IMCL and measures of physical function and physical activity. Pearson's or Spearman's correlation coefficients were used for the associations, depending on data distribution. Last, comparisons between IMCL content in RA and healthy controls matched on age, sex, and BMI, and an older cohort (10-20 years older) matched by sex and BMI were conducted using either parametric or non-parametric paired samples tests depending on data distribution.

4.3 RESULTS

IMCL Content and RA

Muscle tissue was available for histological analysis in 46 out of 48 subjects that consented to undergo percutaneous needle biopsy. In two subjects, we were unable to extract muscle tissue during the needle biopsy procedure (i.e., only fat). There were no notable meaningful differences between the subset of individuals in whom muscle histology was available (n=46) and those who participate in the parent study but did not have muscle histology data (n=14). (Table 10). IMCL assessed as average gray intensity is characterized in Figure 2. Higher IMCL content was associated with younger age, but was not associated with BMI, disease activity, or disease duration. (Table 11)

Associations between IMCL content and Physical Function and Physical Activity in RA

Higher IMCL showed a small but significant association with greater single leg stance, meaning that those with more IMCL content were able to balance on one leg for longer time. IMCL content was not associated with any other measures of physical function or physical activity. (Table 12)

Comparison of IMCL content in RA versus Healthy Cohorts

We had 10 pairs of individuals with RA matched to healthy controls on age, sex, and BMI who had muscle histology data available. The RA individuals in these pairs were on average 57 years, obese, had moderate disease activity, and moderate functional limitations. (Table 13) There were no significant differences in IMCL content between the RA and non-RA controls of similar age. (Table 14) For the comparison between RA cohort and an older cohort matched on sex and BMI, we had 6 pairs. The RA individuals in these pairs were 57 years old, obese, had moderate disease activity, and moderate functional limitations. (Table 15). We found that those with RA had significantly higher IMCL content compared to the older cohort. (Table 16)

4.4 DISCUSSION AND CONCLUSIONS

This is the first study to assess IMCL directly from muscle tissue samples in individuals with RA. We included a histological assessment of IMCL because skeletal muscle fat (SMF) obtained using CT imaging does not have the capability of differentiating IMCL from extramyocellular lipid (EMCL). This study provides preliminary information on IMCL content in RA. IMCL

content has been measured in other non-RA populations such as athletes, older adults, obese adults and those with type 2 diabetes.[16,18,19] Due to different methods of reporting IMCL content (i.e. different Arbitrary Unit scales, or area of lipid droplets) in previous studies on IMCL, we were not able to compare our study findings with several of these studies. However, Amati et al used a similar assessment of IMCL (average gray intensity) in their study in normal weight and obese healthy adults over 60 years of age. The IMCL content in our sample (Mean average gray: 5005 AU) was similar to normal weight sedentary adults (Mean average gray: 6000 AU), but lower than obese sedentary adults (Mean average gray: 8000 AU) reported by Amati et al. Minor differences in the sample in Amati et al may explain the discrepant findings in IMCL content, such as higher proportion of males in their study (50%) compared to the current study (18%) and slightly older sample (average age: 66 years) compared to the current study (average age: 59 years). But, the lower IMCL in the current study may also represent a hypermetabolic state in RA that has been described previously by Roubenoff,[160] and we could theorize that this hypermetabolic state could be accentuated with worse disease severity.

In this study IMCL was not associated with BMI, and higher IMCL content was associated with younger age. These findings were unexpected and conflict with previous research that has shown that greater muscle fat infiltration is associated with higher BMI and older age ($r = -0.44$, and 0.58, respectively).[9,178] Our results also indicated that IMCL content in RA was not influenced by length of RA disease or disease activity. Although the non-association between IMCL and disease activity can be explained because disease activity tends to fluctuate throughout the course of the disease, the non-associations between IMCL and length of RA disease was unexpected. We had hypothesized that IMCL would associate with length of disease, given that RA is known to affect muscle metabolism.[5,49] We were not able to determine any

plausible explanations as to why age associated negatively with IMCL, and BMI and length of RA disease did not associate with IMCL. Data distribution does not seem to have affected these findings because the variability in IMCL values was sufficiently large to test the associations. Further, in Chapter 2.0 we found that skeletal muscle fat (SMF) measured by computer tomography (CT) imaging was associated with older age ($r = -.49$), and higher BMI ($r = -.33$). Based on that, we checked the associations of SMF, measured by CT, with length of RA and disease activity, and found that SMF was significantly correlated with length of RA ($\rho = -.33$) but not with disease activity ($r = -.14$). We expected that our findings from IMCL would corroborate with those from SMF as SMF is a valid measurement of muscle lipid. Goodpaster et al reported that SMF (CT muscle density values) had high correlations with phantom lipid droplets ($r = -0.9$) and modest correlations with IMCL assessed using ORO staining ($r = -.43$).[15] Since we were unable to understand why associations of IMCL did not corroborate with SMF, given that they should measure similar constructs of muscle lipid, we suspected that perhaps the method of measuring IMCL may contribute to these unexpected findings.

In this study IMCL content was not associated with most measures of physical function and physical activity, except that greater IMCL was associated with longer single leg stance time. These findings conflict with the results reported in Chapter 2.0 in which higher SMF significantly contributed to shorter single leg stance time. It could be argued that because IMCL is a direct energy source for the contracting muscle, it would be beneficial for muscle function, as opposed to SMF that also includes EMCL, which is not a direct energy source for the muscle. However, if the protective notion of IMCL on muscle function is true, then we would expect to observe an association between IMCL and muscle strength, which directly represents the contractile integrity of the muscle. Additionally, IMCL was not associated with any other

measure of physical function or physical activity to support this protective role compared to our findings in chapter 2.0 in which SMF demonstrated a detrimental role on disability, gait speed, muscle strength, balance and physical activity levels, even after accounting for covariates such as age, BMI, muscle area and strength. (Results from Study 1, Chapter 2.0) Findings from Chapter 2.0 also corroborate with epidemiological studies in elderly adults that measured SMF using CT muscle density values and attributed higher SMF to lower physical function.[10,61,91] Although studies that directly assessed IMCL and investigated its associations with physical function are sparse, one study reported that high IMCL content was associated with lower muscle force production.[179] There is a possibility that the non-associations of IMCL with most measures of physical function and physical activity may be due to inadequate statistical power. However, the strength of the associations were too small (ranging from .020 to .170) to suggest the presence of a Type 2 error. Given that we were not able to consolidate our findings between IMCL and SMF, there is the possibility that the negative association between IMCL and single leg stance is spurious.

The current study is also the first attempt to compare IMCL content in RA individuals compared to a healthy matched control group. The results that the RA cohort had significantly higher IMCL compared to healthy older cohort but had similar IMCL compared to the healthy cohort matched on age were also surprising and do not corroborate with our findings from Chapter 3.0 in which we found that SMF was significantly higher in RA compared to healthy cohorts of the same age, and similar to healthy older cohort. Due to small sample size (N = 10 pairs), there is a possibility of Type II error for the comparison between the RA and the healthy cohort, because there seemed to be a trend of higher IMCL in those with RA. (Table 14) Since we had a much smaller number of pairs with IMCL data for the comparison between RA and

older cohort (N = 6 versus N = 23 with CT data in Chapter 3.0), we also checked to see if there was any characteristics of the RA subjects in the current study that would contribute to the contradictory findings. One probable explanation could be that the 6 RA subjects matched to older adults would have worse disease compared to the 10 RA subjects matched to the healthy cohort of similar age. However, that does not seem to be the case because both RA cohorts had similar RA length, disease activity, disability, age and BMI.

To date, studies have not assessed IMCL content in RA. Since RA disease is associated with altered cellular metabolism, such as accelerated protein metabolism which leads to muscle loss, lower IMCL content could be a marker of worse disease and higher IMCL content may indicate a healthy muscle that is not in a catabolic state. This notion is theoretically sound, and similar to the athletes' paradox, which demonstrated higher IMCL content in athletes compared to sedentary adults, and that high IMCL in athletes was associated with better insulin sensitivity. However, conflicting nature of our findings do not build a solid case to support the notion of a possible RA paradox to indicate higher IMCL content as a marker of good muscle health because: 1) there were no associations between IMCL and subject demographics or disease characteristics; 2) IMCL was only associated with better balance, but did not associate with the other measures of physical function; 3) matched comparisons with healthy adults have shown no differences; and 4) IMCL findings conflict with findings from SMF obtained by CT, and with previous research. These perplexing findings prompted us to investigate our measurement of IMCL in the current study.

In the current study we used the average gray intensity as a proxy measure for IMCL content. The average gray intensity does not directly quantify amount of IMCL (in terms of area occupied by lipid droplets or the number of lipid droplets) in the muscle section, rather, it is only

provides how dark the lipid in the muscle sections stain. Darker staining should represent higher amount of lipid, however, the degree of dark staining could be highly variable, and may not accurately represent the amount of lipid in the muscle section. Staining intensity of the slide can be affected by the amount of ORO that remains on the slides after washing. Although ORO staining is conducted in a controlled environment with a strict protocol, the amount of ORO that gets washed off the slide cannot be completely controlled. Slight differences in the temperature of the water used during the washes, and the age of the ORO stock may affect how much of the dye remains on the slides. Staining intensity is also dependent on the intensity of the background slide (which does not have ORO stain). While analyzing the slides using the Northern Eclipse software utmost care is taken to find a clean background. However, despite the amount of diligence, it is not completely possible to control the variability in ORO staining intensity. Figure 3 illustrates the variability of the Average Gray Intensity measure. This figure shows muscle biopsy block from the same patient (RA13310) that was sectioned and stained by two independent assessors. The same reagents, stock of ORO and antibodies were used for both procedures. From Figure 3 it is clear that the muscle obtained from the same block and same patient stained much darker (panel a) on one occasion compared to the other (panel b).

Thus, the measure of IMCL content in this present study may explain our conflicting findings with previous studies. As SMF from CT imaging has been previously validated against IMCL content,[15] both methods should provide similar findings; however, this was not the case in our current study. We checked whether the associations between SMF and IMCL content reported by Goodpaster et al also hold true in our data. We observed that there was not a significant association ($r = .255$), and the direction of the association was also opposite compared to that reported by Goodpaster et al ($r = -0.43$).[15] One explanation for these opposing findings

could be by how IMCL was measured. Goodpaster et al quantified the area occupied by lipid droplets, while in our study we used a measure of staining intensity of lipid droplets. Previous studies that report on IMCL have also suggested and utilized methods to either quantify the area occupied by the lipid droplets or a measure of lipid density (i.e number of pixels occupied by IMCL per unit area of the muscle).[18,180-182] Quantification of area occupied by the lipid droplets would demand greater magnification (40X objective versus 20X objective for intensity), and a more powerful microscope (confocal versus light microscope). It is also possible that the high variability in the average gray intensity may be responsible for the inaccurate assessments of lipid content. To that end, we conducted a reliability analysis on 9 subjects who had muscle samples that were independently sectioned and stained by two independent testers using the exact same protocol, and the same stock of reagents and ORO dye. We found that the reliability coefficient for average gray intensity was low, compared to the reliability of fiber area (Table 5). Generally, reliability coefficients <0.5 are considered poor while those >0.7 are considered good.[183] The low reliability suggests that staining intensity may not be appropriate to assess IMCL content and is likely the reason for conflicting results reported in this study compared to prior research in IMCL in non-RA populations, and in comparison to our own findings in Chapters 2.0 and 3.0 that used SMF measured by CT imaging.

This exploratory study is the first to attempt to quantify IMCL content in individuals with RA. In this study IMCL content in those with RA was negatively associated with age, and was not associated with BMI, RA disease duration and disease activity. IMCL was not associated with majority of the physical function or physical activity measures. When compared to healthy cohorts, IMCL seemed to be higher in individuals with RA compared to older adults (10-20 years older), but, there was no difference in IMCL content between RA subjects and non-RA

controls who were matched on age. The findings of this study were unexpected and conflicting with previous studies on IMCL as well as our previous studies using SMF from CT imaging. Because there was no plausible explanation of our findings we explored the methodological challenges of measuring IMCL, and found that the measure of average gray intensity was not reliable. Based on the results of this exploratory analysis, we recommend that using a quantifiable measure of IMCL (such as lipid droplet area) may be more appropriate to provide more accurate assessment of IMCL content.

Table 10. Characteristics of the RA Sample with and without muscle histology data

	Total Sample (N=60)	With Muscle Histology Data (N=46)	Without Muscle Histology Data (N = 14)
Variables			
Age in years, mean ± SD	59.0 ± 9.8	58.7 ± 9.8	59.9 ± 1.0
Number of Females, (%)	49 (82)	37 (82)	12 (80)
Number of Caucasians, (%)	50 (83)	38 (84)	12 (80)
Height in meters	1.65 ± 0.08	1.65 ± 0.08	1.65 ± 0.10
Weight in kg, mean ± SD	84.9 ± 21.1	85.2 ± 21.2	83.7 ± 21.8
BMI in kg/m^2 mean ± SD	31.2 ± 7.2	31.4 ± 7.4	30.6 ± 6.7
Education Level, N (%)			
- High School	16 (27)	13 (29)	3 (20)
- College Education	44 (73)	32 (71)	12 (80)
Marital status, N (%)			
- Married	32 (53)	23 (51)	9 (60)
- Single/never married	12 (20)	10 (22)	2 (13)
- Other (divorced, widowed, etc.)	16 (27)	12 (27)	4 (27)
Employment Status, N (%)			
- Regular Full Time or Part time	20 (33)	13 (29)	7 (47)
- Retired (not due to health)	20 (33)	15 (33)	5 (33)
- Retired or unable to work due to health	11 (18)	9 (20)	2 (13)
	9 (14)	8 (18)	1 (7)
RA duration in years,	13.5 (6-22)	14 (7-24)	11 (5-17)
DAS-28 score, mean ± SD	4.0 ± 1.3	4.0 ± 1.3	3.8 ± 1.1
HAQ score	0.88 (0.38-1.25)	.88 (.38-1.25)	1.06 (.43-1.66)
Charleston Co-morbidity index			
- Raw Score	0 (0.00 – 1.00)	0 (0-1)	0 (0-1)
- Age Adjusted Score	2.0 (1.00 – 3.00)	1.0 (1-3)	2 (1-4)

BMI : Body mass index, DAS: Disease activity score, HAQ: Health assessment questionnaire
Values are Median (25-75 IQR) unless indicated otherwise.

Table 11. Associations between RA sample characteristics and Intramyocellular Lipid (N = 46)

	Univariate Correlation Coefficients (Pearson's r or Spearman's rho)			
	Age	BMI	RA Duration	Disease Severity (DAS-28)
IMCL Content in Type 1 Fibers, AU†	-.307*	.026	-.103	.170
IMCL Content in Type 2 Fibers, AU†	-.282	.109	-.218	.247
IMCL Content in Type 1 and Type 2 fibers combined AU†	-.320*¶	.100	-.151	.163

BMI: Body Mass Index, DAS: Disease Activity Score, HAQ: Health Assessment Questionnaire, AU: Arbitrary Units

† Measured as average gray intensity AU (higher average gray values indicate higher IMCL)

Pearson's correlation coefficient r was calculated for normally distributed data and Spearman's rho for non-normally distributed data

*significant at alpha level 0.05

Table 12. Associations between Intramyocellular Lipid and Physical Function and Physical Activity

	Univariate Correlation Coefficients (Pearson's r or Spearman's rho)		
	IMCL Content in Type 1 Fibers, AU†	IMCL Content in Type 2 Fibers, AU†	IMCL Content in Type 1 and Type 2 fibers combined AU†
Quad Strength (N = 46)	.067	.208	.170
Gait Speed (N = 45)	.099	.001	.049
Single Leg Stance (N = 46)	.294	.148	.324*
Stair Climb (N = 46)	-.166	-.051	-.133
Chair-stand (N = 44)	-.118	-.072	-.040
Physical Activity (N = 42)	.040	.079	.020
Self-Reported Disability (HAQ scores	.039	.200	.015

† Measured as average gray intensity in Arbitrary Units (AU), higher average gray values indicate higher IMCL

Pearson's correlation coefficient r was calculated for normally distributed data and Spearman's rho for non-normally distributed data
*significant at alpha level 0.05

Table 13. Demographics of RA and non-RA cohorts matched by age, sex and BMI

Variables	**RA cohort (n=10)**	**Non-RA cohort (n=10),**
Age, years	56.5 ± 11	56.8 ± 11.4
BMI, Kg/m^2	35.0 ± 7.1	35.6 ± 6.4
Number of Females (%)	8 (73)	8 (73)
RA duration, years	11.7 ± 10.5	NA
Disability, HAQ scores	1.02 ± 0.60	NA
Disease Activity, DAS-28 scores	4.5 ± 1.6	NA

Variables described as Mean ± SD, unless specified otherwise

BMI: Body Mass Index, HAQ: Health Status Questionnaire, DAS: Disease Activity Score

Table 14. Comparison of IMCL content in the RA cohort and the non-RA cohort matched by age, sex and BMI

	RA cohort (n=10)	Non-RA cohort (n=10)	Mean Differences between groups (95% CI)	P-value¶
IMCL Content, AU†	6281.79 ± 2152.49	5196.18 ± 2299.18	1085.61 (-995.54, 3166.76)	.268

N = 10 for average gray values, N = 9 for muscle fiber area

† Measured as average gray intensity in arbitrary units (AU), higher average gray values indicate higher IMCL

¶P-values were obtained from parametric (paired t-test) or non-parametric (Wilcoxon-signed rank test) depending on data distribution

Table 15. Demographics of RA and non-RA older cohorts matched by sex and BMI

Variables	RA cohort (n=6)	Non-RA Older cohort (n=6)
Age, years	54.7 ± 6.9	69.5 ± 7.1
BMI, Kg/m^2	33.7 ± 8.8	34.4 ± 8.8
Number of Females (%)	5 (83)	5 (83)
RA duration, years	16.8 ± 13.7	NA
Disability, HAQ scores	1.18 ± 0.62	NA
Disease Activity, DAS-28 scores	4.3 ± 1.1	NA
Variables described as Mean ± SD, unless specified otherwise BMI: Body Mass Index, HAQ: Health Status Questionnaire, DAS: Disease Activity Score		

Table 16. Comparison of IMCL content (Average Gray Intensity) and Muscle Fiber Area in the RA cohort and the non-RA older cohort matched by sex and BMI

	RA cohort (n=6)	Non-RA Older cohort (n=6)	Mean Differences between groups (95% CI)	P-value¶
IMCL Content, AU†	7078.78 ± 1036.02	5043.85 ± 1694.81	2034.93 (414.09, 3655.78)	.023*
† Measured as average gray intensity in arbitrary units (AU), higher average gray values indicate higher IMCL ¶P-values were obtained from parametric (paired t-test) or non-parametric (Wilcoxon-signed rank test) depending on data distribution *Significant at alpha level of 0.05				

Table 17. Reliability of Histology variables between Khoja and Despines

	Intra-class correlation coefficients
IMCL content, Average Gray Intensity *(type 1 and 2 combined)*	0.479
IMCL content in Type 1 fibers, Average Gray Intensity	0.404
IMCL content in Type 2 fibers, Average Gray Intensity	0.385
Type 1 Fiber Area	0.952
Type 2A Fiber Area	0.966
N = 9 for each comparison Sectioning and staining was done by both Samannaaz and Alex in a similar time frame, using the same batch of reagents and antibodies. Circling of fibers and calculation of average gray for all sections (including Despines) was done by Khoja	

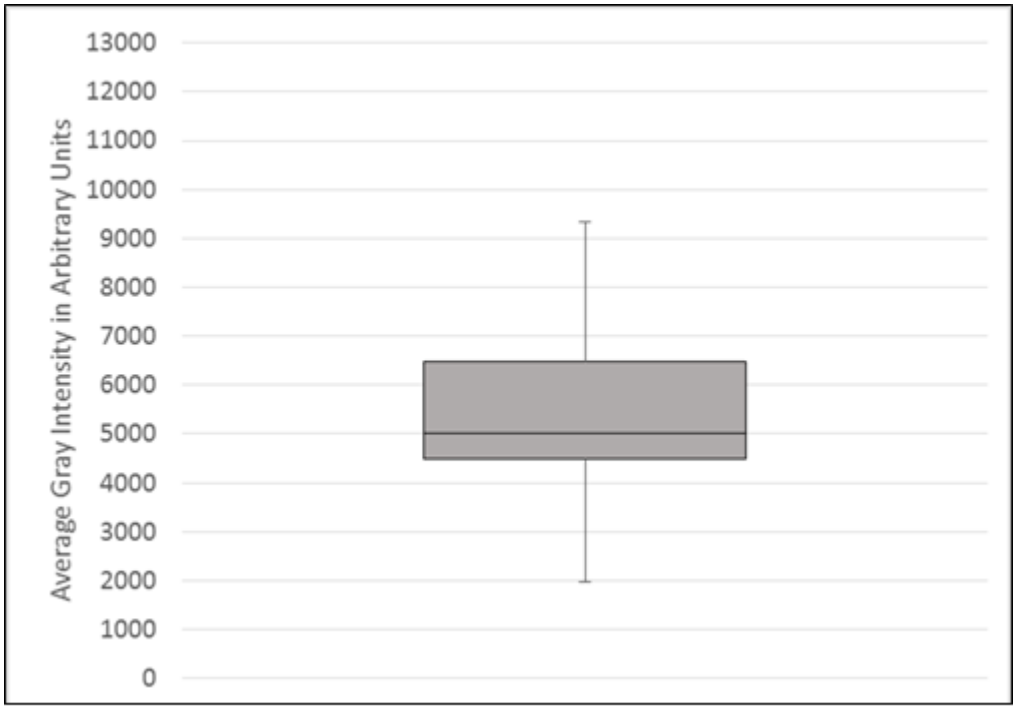


Figure 3. Whisker Plot Characterizing Intramyocellular Content (Measured as Average Gray Intensity) in the RA Sample

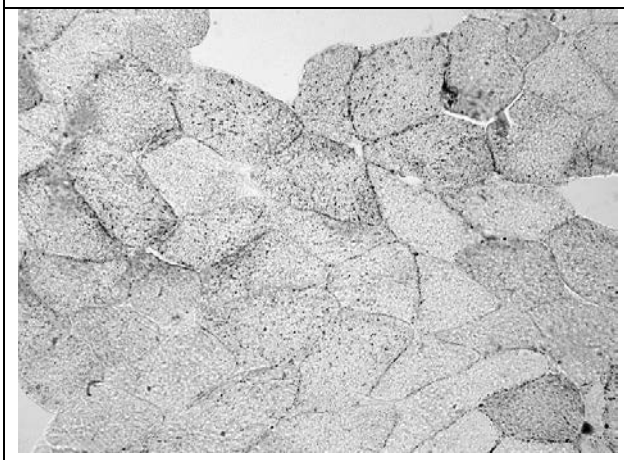
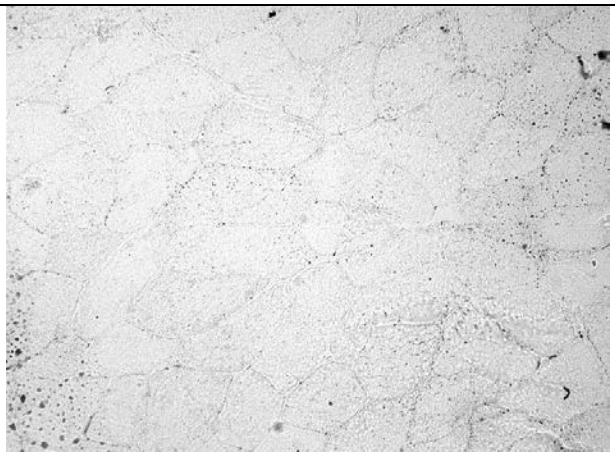
(a) Despines ORO Image	(b) Khoja ORO Image
	
Average Gray Intensity = 10174 AU	Average Gray Intensity = 3016 AU

Figure 4. Difference in ORO staining intensity between raters for the same muscle block

5.0 LESSONS LEARNED AND FUTURE DIRECTIONS

The purpose of this dissertation study was to investigate fat accumulation within and around the skeletal muscles in individuals with Rheumatoid Arthritis (RA). Although altered body composition towards low lean mass and high fat mass is well-known extra-articular manifestation of RA, there is very limited information on the extent of fat accumulation within specific depots. We chose to study fat within and around the muscle because abnormal accumulation in these depots have been attributed to both physical and metabolic complications in non-RA healthy populations, but, have not been adequately investigated in those with RA. Because of the high inflammatory milieu due to the RA disease these individuals are already at a higher risk of altered cellular metabolic activity which can result in abnormal accumulation of fat within the muscles and around it. The overall results of this dissertation project demonstrate that skeletal muscle fat (SMF) is an important contributor to physical function and physical activity in individuals with RA, and is indeed higher in those with RA compared to matched healthy individuals. High SMF accumulation in RA is a clinically relevant finding, as it may potentially explain the persistent disability and high cardiometabolic risk in this population despite well-controlled disease.

The first chapter in this dissertation provides an overview of the evidence and methods to assess skeletal muscle fat, intermuscular adipose tissue and subcutaneous adipose tissue, and the gaps in the literature with respect to these fat depots in RA. The second chapter addresses

associations between the fat depots and measures of physical function and physical activity in a cohort of individuals with RA. In that study we found that SMF had a significant contribution to most measures of physical function, even after accounting for important covariates such as age, BMI, muscle strength, and muscle area. This means that in addition to muscle size and force generating capacity there are other mechanisms related to the metabolic environment in the muscle that could impact physical function in this population. This was not the case with intermuscular (IMAT) or subcutaneous adipose tissue (SAT) accumulation.

In the third chapter we conducted two matched comparisons of the fat depots between RA and non-RA healthy populations. The rationale behind conducting these analyses was to see if SMF, IMAT and SAT accumulation was indeed altered in those with RA compared to matched healthy counterparts, and if individuals with RA undergo similar changes in fat accumulation that have been observed in healthy aging. In these studies we demonstrated that SMF accumulation was indeed higher in those with RA compared to their age, sex and BMI-matched counterparts. This means that SMF accumulation is likely the result of altered fat metabolism in the muscle cells, and our results also showed that the higher SMF in RA was present in absence of muscle atrophy. In contrast, IMAT and SAT were not different between those with RA and healthy individuals, suggesting that body size (i.e., BMI) is likely the most influential contributor to IMAT and SAT accumulation. We also showed that there were no differences between SMF, IMAT and SAT accumulation between individuals with RA and healthy counterparts who were 10-20 years older. These findings suggest that the accelerated aging process that is speculated in RA could extend to fat accumulation.

In this dissertation project, the fat depots were primarily measured using variables from Computed Tomography Imaging. However, we also had muscle biopsy samples available in a

sub-group of our cohort, which enabled us to measure intramyocellular lipid (IMCL) content. IMCL has not been measured in individuals with RA. Having a direct measure of IMCL would confirm whether fat within the muscle is increased in RA, and if it affects physical function. Unexpectedly, we did not find any associations in our exploratory analysis of IMCL with RA disease characteristics and physical function. One reason for non-associations, could be that we were not adequately powered for the analyses. However, the low strength of the associations (i.e., all <0.3) questions whether these associations would be relevant even in larger samples. Further, in our sample, CT muscle density values did not associate with the IMCL variable of average gray intensity. Average gray intensity assesses the average darkness of an image stained for neutral lipids (darker stain is supposed to indicate higher IMCL content). The measure of average gray intensity is possibly limited because it does not directly quantify the area occupied by the lipid droplets. We also observed that this measure was highly variable (in the same muscle block) and not reliable between independent assessors (reliability coefficients <0.5). These limitations of average gray intensity could explain our conflicting results with previous studies that have validated CT muscle density scores against histologically assessed IMCL content (measured using area of lipid droplets). Based on this study, we recommend that future studies assess IMCL using a quantifiable measure such as area occupied by lipid droplets.

We acknowledge some limitations of this dissertation project. For one, the cross-sectional design precludes any temporal inferences of changes in fat accumulation in RA. Second, the study may have been under-powered for the analyses related to the histology variables, and the methods used to measure IMCL may not have been accurate. However, this study provides important preliminary information for future longitudinal studies to investigate changes in fat

accumulation within and around the muscles in RA and their contribution to disability as well as high cardiometabolic risk.

During the course of my PhD training, I have gained significant experience in the process of scientific inquiry as well as the administrative and regulatory processes that go into managing studies in the field of rehabilitation science. Working on this dissertation study also gave me the opportunity to train in bench-work science procedures such as preparing muscle biopsy blocks, sectioning the muscle blocks and conducting immunohistochemistry analyses to measure IMCL and muscle fiber area. I believe that this process was invaluable, as it provided me with some skills and knowledge necessary to conduct clinical and translational research. This project gave me a new appreciation of the methodological issues that may arise with wet-lab procedures, and how to troubleshoot them. One big hurdle to complete this project was equipment related issues. Several steps went into identifying the source of the issue, including checking the microscope settings, the cable, light source, filters and the also the software. To make sure that the issue was not from poorly stained slides, we conducted several iterations of the staining process with new reagents, fresh ORO stock and antibodies. Another important experience I gained through the process of this project is participating in developing and writing grants. We applied for three grants from private foundations (Arthritis Foundation and Rheumatology Research Foundation) to secure funding to recruit additional healthy controls to expand the aims of this dissertation study and add other measures of skeletal muscle fat and mitochondria function. Although our proposals were well received by the reviewers and we obtained good scores, it did not make it to the funding cut-off.

This project provides insight into non-articular manifestations of the RA disease that could contribute to disability in this population. It is well known that the sources of disability and

morbidity and mortality in RA is multifactorial and solely treating joint disease may not be sufficient to alleviate disability or improve quality of life. Individuals with RA also have to deal with fatigue, disturbed sleep, and cardiovascular risks. I would use the knowledge gained from this dissertation project to pursue a related but slightly different research direction. As rehabilitation experts, we have an in-depth understanding of the importance of the muscle as a versatile organ. Therefore, I believe that future longitudinal research in RA also needs to address how muscle function and metabolism may contribute to the health of those with RA. The benefits of both aerobic and resistance exercises are also well-known and safe for those with RA,[184] however, in clinical practice, exercise and physical activity interventions are not widely implemented, and do not even hold a presence in the clinical practice guidelines to treat patients with RA.[185] I would like to leverage my current research to advocate and also investigate practical approaches to improve physical activity and muscle function in those with RA.

APPENDIX

OIL RED O AND FIBER TYPE COSTAINING LAB PROTOCOL

- Air-dry frozen sections 10 min.
- Fix the sections in 3.7% formaldehyde/Phosphate-buffered saline (PBS) for 1 hour in fridge
- Rinse 3x in DH_2O each 30 sec.
- Stain in Oil Red O working solution for 30 min.
- Rinse 3x in DH_2O each 30 sec.
- Rinse in warm running tap water for 15 min.
- Place in PBS for 2 x 5 minutes
- Add primary antibodies (1:25 of A4.840 (mouse IgM anti-slow fiber) and 1:25 of A4.74 (mouse IgM anti-fast fiber 2A)) in 2% BSA/PBS and leave either at RT for 90 minutes or at 4C overnight
- If slides are refrigerated overnight, the next day, keep slides at RT for 30 minutes before proceeding
- Wash 3 x 5 minutes in 1x PBS
- Place secondary Ab in dark (1:200 of anti IgM-R sc-2088, 1:500 of anti IgG-FITC sc-2010) in 2% BSA/PBS and incubate for 1 hour. (briefly spin down Ab mixture before adding to slide). Keep slide in dark from now on.
- Wash 5 minutes x 5 in 0.5% BSA/PBS

- Mount with UltraCruz DAPI Mounting media. Seal with nail polish after 24hours. Store slide in fridge.
- Solutions:

Oil Red O stock: 500 mg oil red o (Sigma, Fluka - 75087) to 100 ml 60% triethyl phosphate (Fluka, 90530) in DH_2O.

Oil Red O working solution: Just prior to staining, mix DH_2O and oil red o stock in a ratio 2:3 (16ml DH_2O and 24ml stock), and filter it with Whatman #42, to remove crystal of oil red o.

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