The association between amount and distribution of protein intake with body composition, resting energy expenditure, substrate oxidation, and muscle function in bariatric surgery candidates: a preliminary study

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Abstract

The association between amount and distribution of protein intake with body composition, resting energy expenditure, substrate oxidation, and muscle function in bariatric surgery candidates: a preliminary study

Niloufar Ghaderian

Background: Obesity is associated with disrupted energy metabolism and skeletal muscle dysfunction. Dietary protein is important for the preservation of skeletal muscle mass and strength. **Objectives:** The objectives of this preliminary study were: 1) to assess the association of dietary protein intake with body composition, and muscle function; 2) to examine weather protein distribution pattern throughout the day are associated with body composition and substrate oxidation; and 3) to explore the association between body composition with substrate oxidation and energy expenditure in males and females with severe obesity. **Methods:** This cross-sectional study included 17 male and female bariatric surgery candidates. Dietary data, including protein intake was collected using a 3-day food journal and 24-hour food recall. Body composition was assessed using dual-energy X-ray absorptiometry. Substrate oxidation and resting energy expenditure were measured by indirect calorimetry. Muscle function was assessed by handgrip strength and 6-minute walk test. **Results:** Mean age and BMI were 43.9 ± 7.8 y and 46.9 ± 4.9 kg/m^2, respectively. Daily protein distribution was uneven and had a skewness toward dinner. Males who ate ≥ 20 g protein at each meal had greater ALM in comparison to those who did not (38.0 ± 0.9 vs 35.2 ± 0.7, $P = 0.03$). ALM was an independent predictor of REE and fat oxidation rate. Females had altered substrate metabolism and decreased rates of fat oxidation. **Conclusion:** This study provided preliminary results that will help improve study design and considerations in the continued recruitment of participants.

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Table of Contents

List of Figures

List of Tables

Introduction

The prevalence of obesity (Body Mass Index (BMI) > 30kg/m^2) and overweight (BMI>25 kg/m^2) has been increasing during the last decades. In 1980, 28.8% and 29.8% of males and females had a BMI of 25 kg/m^2 or greater, respectively; however, in 2013, these proportions reached 36.9% in males and 38.0% in females (Vandevijvere et al., 2015). In other words, about one third of the global population is currently classified as overweight or obese. According to the World Health Organization (WHO) obesity is the most common chronic health condition worldwide (Yumuk et al., 2015). In Canada, two thirds of adults and one third of children are obese or overweight (Shentow-Bewsh and Zuberi, 2018). Obesity increases the risk of several chronic conditions and diseases such as cardiovascular disease, type 2 diabetes mellitus, cancer, and osteoarthritis. Obesity reduces the quality of life and increases mortality risk (Kalyani et al., 2014; Seidell and Halberstadt, 2015) especially in people with BMIs higher than 35 kg/m^2 (Flegal et al 2013).

Obesity, BMI, and Body composition:

WHO defines obesity as excessive fat accumulation to the degree that impairs health (Carbone et al., 2019). Although BMI is commonly used to define overweight and obesity and is a very useful tool in epidemiological studies, BMI is not an accurate marker to assess metabolic status (Ponti et al., 2019). Body mass is composed of different compartments (Figure 1) that are not reflected in BMI but are important factors in health and disease, and also useful to define conditions such as sarcopenia (Kalyani et al., 2014; Carbone et al., 2019)

Fat mass, fat distribution, and lean mass are better related to metabolic status, for instance fat distribution pattern has a major effect on cardiovascular health and metabolism; however, BMI is incapable of quantifying the amount of fat and lean mass or assessing fat distribution (Carbone et al., 2019). Imaging techniques using magnetic resonance imaging (MRI), computed tomography (CT), and dual x-ray absorptiometry (DXA) scans provide a precise assessment of body composition compared to BMI (Ponti et al., 2019). A retrospective study on type 2 diabetes patients used both BMI and whole body DXA to determine obesity; the study demonstrated that while android fat mass was an independent risk factor for cardiovascular events in type 2 diabetes, BMI did not show any association with cardiovascular events (Fukuda et al., 2018).

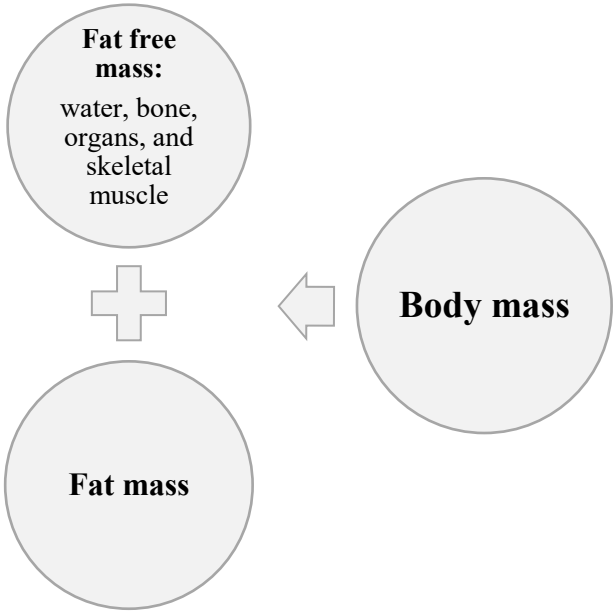

Figure 1. Body mass compartments (Dulloo et al., 2010; Carbone et al., 2019)

Skeletal Muscle:

Skeletal muscle (SM) is the largest organ in the body and consists of more than 500 different muscles (Tieland et al., 2018). SM in lean individuals accounts for 40 to 50% of body mass (Pedersen and Febbraio, 2012; Kalyani et al., 2014; Periasamy et al., 2017). The primary function of SM is to generate force and to produce movement through muscle contraction (Sweeney and Hammers, 2018). SM is made of muscle fibres which are responsible for contraction and relaxation (Tieland et al., 2018). In a study in older adults, ALM (appendicular lean mass) was positively associated with handgrip strength (Bani Hassan et al., 2019). Another study in young and older women reported that handgrip strength was positively correlated to ALM/h^2 (Sui et al., 2020).

In addition to physical performance, SM has an important role in various metabolic pathways. SM is a major player in determining metabolic rate, substrate metabolism, and glucose homeostasis (Periasamy et al., 2017; Tieland et al., 2018; Gutierrez-Monreal et al., 2020). In healthy adults, insulin mediates glucose uptake in myocytes (Gemmink et al., 2017) thus, SM accounts for about 80% of insulin-mediated glucose uptake (Nicholson et al., 2018). SM is also involved in lipid oxidation because fat is the main source of energy production in myocytes (Wolfe, 2006). Overall, SM is responsible for 40 to 50% of free fatty acid uptake from the circulation in the post-absorptive state (Jensen, 2003). The importance of the SM in metabolism is manifested in aging when age-related muscle loss leads to decreased glucose uptake and lower capacity for fat oxidation with both conditions contributing to insulin resistance (Welch et al., 2020).

During recent years, SM has been viewed in a new light as an organ with secretory features. SM secretes myokines, with favorable effects on inflammation, and fat metabolism in

healthy individuals (Pedersen and Febbraio, 2012; Wu and Ballantyne, 2017; Gomarasca et al., 2020; Kirk et al., 2020). Myokines also allow SM to interact with other organs in the body (Tieland et al., 2018).

Skeletal muscle: preservation and loss:

As mentioned above, SM has a key role in human metabolism. Muscle mass is negatively associated with mortality rates in patients with heart disease, cancer, burn injuries, and peritoneal dialysis (Curtis et al., 2015). Skeletal muscle mass is involved in bone mass regulation across life span and is positively correlated with bone mineral density (Deutz et al., 2019). SM preservation needs continuous repair and regeneration (Akhmedov and Berdeaux, 2013), and whether muscle mass is preserved or lost is the result of the equation between muscle protein synthesis and muscle protein breakdown (Hudson et al., 2020; Smeuninx et al., 2020).

SM is a key player in protein metabolism because it acts as a reservoir of amino acids in the body. When the amino acids levels in the blood are not sufficient, for example in the fasting state or due to inadequate dietary intake, SM tissue is broken down to provide amino acids for other vital organs (Wolfe, 2006; Landi et al., 2019). On the other hand, dietary protein as the precursor of the muscle fibre, improves muscle protein synthesis and to some extent decreases muscle protein breakdown (Hudson et al., 2020; Smeuninx et al., 2020). Ingesting enough protein results in the rapid increase of muscle protein synthesis to its peak which then lasts for up to 3 to 4 hours (Jäger et al., 2017; Kerksick et al., 2017). However, if the rate of muscle protein breakdown is greater than the rate of muscle protein synthesis it will eventually result in muscle mass loss (Landi et al., 2019).

Obesity and muscle loss:

Obesity has a detrimental effect on SM mass and strength (Kalinkovich and Livshits, 2017). Valenzuela et al., (2020) investigated the prevalence of poor muscle quality (based on muscle function per unit of muscle mass) in young, middle-age, and older adults with obesity. They reported that in their study 75% of young adults, and 92% of middle-aged and older adults with obesity had poor muscle quality in comparison to reference values obtained from healthy young adults (Valenzuela et al., 2020).

In obesity, excessive fat stores ectopically in SM tissue and causes lipotoxicity by accelerating cell senescence, inducing mitochondrial dysfunction, disrupting fat oxidation, and increasing reactive oxygen species production (Kalinkovich and Livshits, 2017; Tam et al., 2020). By supressing the production of anti-inflammatory myokines and enhancing the production of pro-inflammatory myokines and cytokines, obesity increases inflammation in SM which can affect myocytes insulin sensitivity (Kalyani et al., 2014; Kalinkovich and Livshits, 2017; Wu and Ballantyne, 2017). In an in-vitro study, when adipocytes, obtained from visceral adipose tissue of people with morbid obesity, were added to the human muscle cell culture, adipocytes caused reduced gene expression of contractile proteins in myotubes and induced atrophy (Pellegrinelli et al., 2015).

In obesity, increased levels of free fatty acids in circulation leads to increased fatty acid uptake by myocytes, and this excess flow of fatty acids in SM affects muscle metabolism (Wu and Ballantyne, 2017). Obesity also alters SM fibre composition; obesity is associated with more fast compared to slow muscle fibres. Slow type muscle fibres have higher insulin sensitivity and greater glucose uptake, therefore, a part of metabolic impairments caused by obesity can be attributed to altered SM composition (Tallis et al., 2018). In a study on women with and without obesity, those with obesity had a lower percentage of slow muscle fibres compare to individuals with a healthy weight, 41.5% vs. 54.6, respectively (Tanner et al., 2002).

In contrast with studies mentioned above, some studies reported a positive association between obesity and muscle function. For example, Abdelmoula et al. (2012) reported that male adolescents with severe obesity had higher absolute and relative knee extension strength compare to controls with healthy weight. In another study on adolescent girls with and without obesity, those with obesity had greater muscular strength compare to those with healthy weight (Garcia-Vicencio et al., 2016). This higher muscle strength in people with obesity may be because of the greater force that is needed to move a higher body mass (Tallis et al., 2018). Few studies have studied SM and muscle function in people with severe obesity (Abdelmoula et al., 2012; Coen et al., 2013; Bollinger et al., 2015) and more research is needed to investigate the effects of severe obesity on muscle mass and function.

Obesity and muscle protein turnover:

Individuals with obesity show altered rates of muscle protein synthesis compare to healthy weight controls (Beals et al., 2019). Some studies suggested that obesity supresses muscle protein synthesis which in turn results in muscle mass loss and subsequently, muscle strength decline (Akhmedov and Berdeaux, 2013; Lipina and Hundal, 2017). Insulin resistance and inflammation have been suggested as mechanisms via which obesity may affect muscle protein turnover, but the evidence is not consistent (Beals et al., 2019).

Guillet et al. (2009) reported that non-diabetic young people with obesity had impaired protein metabolism manifested by a lack of stimulation of mitochondrial protein synthesis by insulin and amino acid, and a lower inhibition of protein breakdown by insulin and amino acid in comparison with people with a healthy weight. Another study on young adults demonstrated that individuals with overweight and obesity had diminished myofibrillar protein synthesis in response to protein ingestion compare to participants with healthy weight; while in the group with healthy weight the myofibrillar protein synthesis rates increased by 1.6 fold above the baseline rate, those with obesity demonstrated no increases in postprandial myofibrillar protein synthesis rates (Beals et al., 2016). Also, Smeuninx et al. (2017) compared young and older adults observing that following ingestion of 15 g of milk protein, the rate of muscle protein synthesis increased in older lean subjects but this increase was not seen in older subjects with obesity, indicating an impaired muscle protein synthesis in obesity. Other endocrine conditions can affect SM metabolism, including diabetes, hypogonadism, hyperthyroidism, and vitamin D deficiency (Kalyani et al., 2014).

Assessing muscle function:

Muscle function consists of muscle strength, muscle power and muscle endurance (Beaudart et al., 2019) and assessment of muscle function needs reliable measurement methods (Cebrià I Iranzo et al., 2020). Various tests and methods are used to assess muscle function. One of the reliable methods to assess muscle function is measuring handgrip strength (Zaccagni et al., 2020). Handgrip strength is considered as a major indicator for diagnosing skeletal muscle loss (Lee and Gong, 2020). In a cross-sectional study on female candidates for bariatric surgery, the appendicular skeletal muscle mass had a significant relationship with handgrip strength (Crispim Carvalho et al., 2019). In another study protein intake had a significant positive association with handgrip strength (Fanelli Kuczmarski et al., 2018).

Walk tests are a useful method to evaluate functional status (Solway et al., 2001). The six-minute walk test evaluates the functional exercise capacity (Enright, 2003) and was originally designed to evaluate functional exercise capacity in people with chronic respiratory disease (Singh et al., 2014). The 6-minute walk test is considered as a mirror which can reliably reflect the daily life activities. It is also used to assess treatment effects (Solway et al., 2001). Ekman et al. (2013) used 6-minute walk test to evaluate a weight loss intervention in adults with obesity and found that weight loss in the subjects resulted in a significant increase in the 6-minute walk distance. In another study on women with obesity, 6-minute walk distance was positively correlated with weight (Luchesa et al., 2020).

Dietary protein and skeletal muscle mass:

Dietary protein is an essential macronutrient for human health. Protein has many functions including in the immune system, as enzymes, and as hormones (van der Zanden et al., 2014). Protein also affects weight regulation by increasing satiety (Schollenberger et al., 2016). Moreover, dietary protein is a major factor in muscle protein synthesis and inadequate protein intake can result in lean tissue loss (van der Zanden et al., 2014; Hudson et al., 2020). Ingesting a meal that contains a moderate amount of protein results in 30 to 100% increase in muscle protein synthesis. However, this strong response is temporary and lasts for 1 to 4 hours after consuming a meal (Jäger et al., 2017).

Cohort studies have shown higher dietary protein intake is protective against loss of muscle mass and muscle strength in middle-aged and older adults. Beasley et al. (2013) reported that middle-aged and older women with higher protein intake at baseline had a slower decline in handgrip strength. In Framingham Offspring cohort, after 6 years of follow up the handgrip strength of participants in the lowest dietary protein decreased while in individuals in the highest quartiles of dietary protein increased (McLean et al., 2016). In another study on middle-aged and older adults, higher dairy protein intake was associated with a lower risk of SM loss during a 12-year follow-up (So and Joung, 2020). However, obesity may impair the protective effect of dietary protein intake on muscle preservation, as a cohort study on middle aged adults reported that normal weight participants with higher protein intake at baseline had higher lean mass after a 12 years follow-up but this protective effect was not found in participants with obesity (So et al., 2019).

The current recommended daily allowance (RDA) for dietary protein for adults of 18 years or older is 0.8 g protein/kg body weight/day of good quality. This level of protein intake is considered enough to prevent protein deficiency in healthy adults (Institute of Medicine, 2005). However, it is suggested that the guidance for 0.8 g protein/kg body weight/day is based on data from young male athletes and may not represent the protein needs of older adults or people with special conditions (McLean et al., 2016).

Dietary Protein: importance of distribution pattern:

In recent years, in addition to total daily protein intake, protein content of each meal and protein distribution pattern throughout the day are becoming increasingly important considerations as potential factors that can affect muscle protein synthesis and breakdown (Cardon-Thomas et al., 2017; Hudson et al., 2020; Yasuda et al., 2020). It is suggested that the distribution of protein intake within the day can affect muscle protein synthesis, with an even protein intake distribution over the day improving muscle synthesis and an uneven distribution of protein intake increasing muscle breakdown (Hudson et al., 2020; Smeuninx et al., 2020). The current literature on the effect of the daily distribution of dietary protein on SM is limited and inconsistent (Hudson et al., 2020). A randomized 7-d crossover feeding study on healthy young adults demonstrated that an even distribution of high-quality protein 3 times a day resulted in 25% higher muscle protein synthesis in 24-h compared to if protein intake was skewed toward the evening meal (Mamerow et al., 2014). In contrast, in another study on healthy older adults the protein distribution pattern did not have any effect on muscle protein synthesis (Kim et al., 2015). Overall, the number of studies investigating the potential effects of protein distribution pattern on muscle protein turnover is limited and does not include diverse populations.

It is suggested that a dose-response relationship exists between dietary protein and muscle protein synthesis (Morton et al., 2015) and some studies indicated that 20 g of protein per meal was the best effective dose of protein per meal in healthy young adult males to stimulate muscle protein synthesis both at rest and after exercise (Moore et al., 2009; Witard et al., 2014). The participants of the current study were bariatric surgery candidates in the preoperative stage. The protein recommendation for bariatric surgery patients is at least 60 g/day (Busetto et al., 2017; Steenackers et al., 2018) and post-surgery patients are recommended to take 4 to 6 meals per day with an emphasis on high-protein foods to meet their protein needs (Sherf Dagan et al., 2017). An intake of 20 g protein per meal is a pattern that would ensure meeting the minimum recommended protein after the surgery, though some studies reported that preoperative eating behaviors did not have any association with post-surgery success (Konttinen et al., 2015; Figura et al., 2017), however, these studies did not investigate protein distribution pattern pre and post surgery and its relationship with loss of lean and fat mass. Future studies will need to address if the protein distribution pattern is as important as total daily protein intake to maximize lean mass preservation with successful weight loss in bariatric surgery patients.

Metabolic rate and substrate oxidation:

Reduced total energy expenditure can be a factor involved in obesity etiology (Siervo et al., 2017; Hollstein and Piaggi, 2020). Total energy expenditure has three parts: resting energy expenditure (REE), the thermic effect of food, and energy needed for daily activities. In normal conditions REE constitutes the major proportion of the total energy expenditure (Wolfe, 2006). REE is the amount of energy that an individual needs to maintain the body at rest (Noreik et al., 2014). An increase in lean tissue mass results in an increase in the whole-body REE because lean mass has a higher metabolic rate compare to fat mass (Dulloo et al., 2010). Brain, heart, kidneys, liver, and skeletal muscle constitute important components of lean mass. While the mass of brain, heart, kidneys, and liver is relatively constant in different individuals, skeletal muscle mass is the only component of lean mass that can significantly change in a person's life time and differ among individuals (Wolfe, 2006; Dulloo et al., 2010; Periasamy et al., 2017). For example, age-related SM loss can cause a 30% decrease in basal metabolic rate in older adults compare to young adults (Kalyani et al., 2014). The findings on the effect of obesity on metabolic rate are conflicting. While some studies reported a positive association between REE and body fat in people with overweight and obesity (Kunz et al., 2000; Hirsch et al., 2017), Faria et al. (2012) found a negative association between body fat and REE in people with severe obesity. In another study on female participants, no difference was found in REE between participants with obesity and participants with healthy weight (Di Renzo et al., 2006). More studies are needed to investigate the association between obesity and REE in different degrees of obesity.

Metabolic flexibility:

Metabolic flexibility is defined as the ability to adapt fuel oxidation in response to fuel availability, i.e. to switch from mainly fat oxidation to carbohydrate oxidation in the fed state, and from carbohydrate oxidation to mostly fat oxidation in the fasting state (Galgani et al., 2008). After eating, SM shifts to more glucose oxidation and glucose storage from a predominantly fat oxidative state. At the same time, adipose tissue lipolysis is suppressed, and fat storage is enhanced. During a fasting state, lipolysis in adipose tissue and fat oxidation in SM increases. Exercise causes a substantial increase in lipolysis in adipose tissue. In SM, exercise increases both fat and glucose oxidation to meet increased energy demand during exercise (Goodpaster and Sparks, 2017). The capacity to switch substrate oxidation in response to fuel availability and energy demands is a key component of a healthy metabolism (Boyle et al., 2017).

Impaired fuel transition or metabolic inflexibility, especially impaired fat oxidation, has been associated with obesity (Venables et al., 2005; Goodpaster and Sparks, 2017; Piaggi, 2019). In a study on healthy young and middle-aged adults, participants with obesity demonstrated a diminished postprandial fat oxidation compare to participants with healthy weight (Blaak et al., 2006). Obesity causes impaired fat oxidation not only at a whole-body level, but also in SM (Berggren et al., 2008). In vitro experiments conducted on human SM cell cultures from individuals with healthy weight or with obesity demonstrated significantly lower rates of fat oxidation in SM cells from people with obesity compare to lean individuals (Berggren et al., 2008; Boyle et al.,2017). Insulin resistance and inflammation have been considered major factors

that contribute to metabolic inflexibility in obesity by causing mitochondrial dysfunction and impaired fat oxidation (Goodpaster and Sparks, 2017; Hong and Choi, 2020).

Assessment of energy expenditure in obesity:

The common equations predicting resting energy expenditure are reliable in healthy subjects but not accurate enough to predict resting energy needs in people who are over BMI of 35 kg/m^2. For these individuals, indirect calorimetry is considered the gold standard in measuring resting energy expenditure (Delsoglio et al., 2019). Indirect calorimetry assesses energy expenditure by measuring oxygen consumption and carbon dioxide production (Lam and Ravussin, 2017).

Sex differences in body composition and fat metabolism:

Though males and females are both susceptible to obesity, they do not have similar body composition. With the same BMI, males have more lean mass than females, while females have higher amounts of fat mass (Bredella, 2017). Males and females also demonstrate different fat distribution. Females usually have higher subcutaneous fat compare to males and are more likely to deposit fat around the hip and thigh areas (Santosa and Jensen, 2015; Ofenheimer et al., 2020). In males, fat accumulation is more likely to be in abdominal region (Power and Schulkin, 2008). Compare to females, males have higher absolute and relative SM mass (relative to body mass). Moreover, age-related SM loss is greater in males compare to females (Bredella, 2017). Sex hormones have a central role in sex-related body composition differences. Testosterone increases lipolysis, inhibits lipoprotein lipase activity, and decreases fat accumulation in adipose tissue (Santosa and Jensen, 2014). Estrogen promotes subcutaneous fat deposition, and increases proliferation of preadipocytes (Power and Schulkin, 2008).

Sex differences also exist in fat metabolism; with higher amounts of fat uptake, fat storage, and fat utilization in females compare to males. This difference can be attributed to the higher levels of lipoprotein lipase in females (Gheller et al., 2016). Lipoprotein lipase is a rate-limiting enzyme which plays a central role in plasma triglyceride hydrolysis (Xie et al., 2010). In vitro studies indicate that lipoprotein lipase can also mediate fat uptake into cells independent of its catalytic function (Merkel et al., 1998). In a study on healthy adults, the level of lipoprotein lipase in females was 35% higher than males (Magkos et al., 2009). In addition to lipoprotein lipase, females have higher levels of other proteins that are involved in fat mobilization and oxidation (Gheller et al., 2016). For example, a study on healthy adults reported that females had 1.5 to 2 times greater levels of muscle perilipin5 protein compare to males; a protein that appears to be associated with fat utilization and oxidation in SM (Peters et al., 2012). However, in contrast with these findings some studies reported lower rate of fat oxidation in females compare to males (Nagy et al., 1996; Kien and Bunn, 2008).

Rationale:

Obesity is associated with metabolic alterations and impaired substrate oxidation, SM metabolism and regeneration (Berggren et al., 2008; Kalyani et al., 2014; Kalinkovich and Livshits, 2017; Beals et al., 2019). Obesity also affects muscle function (Bales et al., 2017). Dietary protein is an essential macronutrient in muscle protein synthesis and although studies have shown that higher protein intake is protective against SM loss (Beasley et al., 2013; McLean et al., 2016; So and Joung, 2020), few studies have investigated the association between protein distribution pattern within the day and SM, especially in people with severe obesity (Mamerow et al., 2014; Kim et al., 2015).

In-vitro studies demonstrated reduced capacity of fat oxidation in obese SM (Berggren et al., 2008; Boyle et al.,2017). Also, males and females have different fat oxidation capacities. However, studies on sex differences in fat oxidation between males and females had conflicting results, with some studies reporting higher fat oxidation rates in males compare to females(Nagy et al., 1996; Kien and Bunn, 2008) and others finding the contrary (Venables et al., 2005; Numao et al., 2009; Siervo et al., 2016).

Thus, further investigation is needed that examines protein intake and distribution and its association with body composition in those with severe obesity. Since there are conflicting results regarding differences in fat oxidation between males and females with severe obesity, further studies are needed to clarify these differences.

Objectives:

The objectives of this preliminary study were: 1) to assess the association of dietary protein intake with body composition, and muscle function; 2) to examine weather protein distribution pattern throughout the day are associated with body composition and substrate oxidation; and 3) to explore the association between body composition with substrate oxidation and energy expenditure in males and females with severe obesity.

Methods

Study design and participants:

Participants were recruited from the bariatric surgery clinic of the Montreal General Hospital. Participants were included if they were 18 years or older and had no mobility issues. Subjects were excluded if they had conditions affecting protein metabolism in the body (e.g. diabetes and/or kidney disease), had undergone bariatric surgery previously, or consume cannabis more than twice per month (Sharma et al., 2012). Those who weighed more than 204.11Kg (the weight limit of the DXA Table) were also excluded. The study was approved by Le comité central d'éthique de la recherche.

Study visits:

All study visits were conducted at the PERFORM Centre. On the morning of the study visit, participants arrived fasted for at least 8 h. They were also asked to avoid alcohol and caffeine for at least 12 hours and avoid exercise or strenuous physical activity for at least 24 hours prior to the visit. At each visit, anthropometric, body composition, energy expenditure and substrate oxidation, and muscle function assessments were conducted.

Anthropometric measures and Body composition:

Height was measured without shoes with a stadiometer to the nearest 0.1 cm. Participants were weighed without shoes with empty pockets using an electronic scale ((Seca 216, Seca Corp., Chino, CA). Body Mass Index (BMI) was calculated as weight (kg)/square of height (m^2).

Whole body and regional measures of lean and fat mass were obtained by DXA (GE Lunar iDXA, GE Healthcare, Madison, WI, USA). Quality assurance was done before each scan by investigators. Participants were asked to wear a gown and to remove all metallic objects such as jewelry. To perform the test the subject laid down in a supine position on the scanning bed, aligned with the vertical lines on the surface. The feet were fastened together with a Velcro strap. Though all participants were within the weight limits of the machine, none were able to fit entirely within the scanning field. For all the participants the right side of the body was scanned thoroughly and for the missing parts in the left side, the right-side measures were used to produce whole body measures (Rothney et al., 2009). The ROIs (Region of Interest) were adjusted following the GE Healthcare Lunar encore-based X-ray Bone Densitometer User Manual (http://medicaloutfitter.net/wp-content/uploads/2014/09/enCORE_V13.5_EN_English .pdf). The appendicular lean mass (ALM) was calculated as the sum of arms and lean leg mass and was adjusted for height in meter (ALM kg/ht^2) to calculate relative ALM (Mangano et al., 2017).

Resting Energy Expenditure and Substrate Oxidation:

Prior to resting measurements, participants were asked to lay supine for 90 minutes. They were permitted to sleep, read, or listen to calming music during this rest period. The lights were dimmed, and the environment was kept calm. After this rest period, a clear plastic canopy was placed over the participant's head and respiratory gas exchange was measured with the participant lying awake using a calibrated indirect calorimetry system (Sable Systems International). To calculate substrate oxidation the following equations were used (Livesey and Elia, 1988):

CHOox (g/min) = 4.59 VCO_2 (L/min) – 3.23 VO_2 (L/min)

Fatox (g/min) = 1.70 VO_2 (L/min) – 1.70 VCO_2 (L/min)

Handgrip strength:

The handgrip strength was measured by an adjustable Jamar hand-held dynamometer to the nearest kilogram (Guler et al., 2019). After adjusting the dynamometer grip size, participants were asked to squeeze the dynamometer with maximum effort in a standing position with feet hip width apart and hands not touching their body. On each hand two trials were done. The best result for each hand was summed up to calculate the combined handgrip strength in kilograms (Perna et al., 2016).

6-minute walk test:

The six-minute walk test was used to evaluate the participants' functional exercise capacity (Enright, 2003). The 6-minute walk test was conducted according to the American Thoracic Society guidelines (2002) in a designated walk-test hallway. The participants were asked to walk fast for 6 minutes, back and forth over a 30-meter course. The participants walked as much as possible for 6 minutes, back and forth in the walking course. Each completed lap was recorded. The total distance was calculated as follow:

(Number of completed laps*30) + Distance covered in the partial lap

The total distance was measured to the nearest meter (American Thoracic Society, 2002).

Dietary data:

The dietary intake was assessed with the use of three-day food journal and 24-hour food recall. The 24-hour food recall was done on the day of the visit at the PERFORM Centre. The investigator interviewed the participants to get detailed information about all food and beverages they had consumed during the past 24 hours. Three-dimensional food models (Nasco International, Modesto, CA, USA) were used to help participants remember and report their intake with higher precision. Food Processor® software (Food Processor, ESHA, Salem, OR,

USA) was used to calculate average daily total energy intake (kcal) and protein intake. The protein intake was expressed in gram per day (g/d) and in gram per kilogram of body weight per day (g/kg bw/d).

Statistical analysis:

The statistical analysis was performed with SAS software (v 9, SAS Institute Inc., Cary, NC, USA). The normality of the data was tested using the Shapiro-Wilk test. All variables were normally distributed except for lean arm mass and BMI. The differences between the means were tested by repeated measures ANOVA with Tukey post hoc test (meals protein content), and Student's t test for independent samples (between males and females). For the variables not distributed normally, the Wilcoxon test was used. To adjust for fat free mass analysis of covariance was used. Multiple linear regression was performed to examine the association between protein intake, sex, age, body composition, resting energy expenditure, substrate oxidation, and muscle function. Since the lean arm mass was not normally distributed, the log transformed data for lean arm mass was used in multiple regression. All values are expressed as Mean ± SD. P-values <0.05 were considered significant.

Results

Characteristics of participants:

Seventeen male and female participants were recruited. The characteristics of the participants are presented in Table 1. The mean age of subjects was 43.9 ± 7.8 years and 29.4% were males. The average BMI was 46.9 ± 4.9 kg/m^2 with no significant difference between males and females (45.7 ± 3.4 kg/m^2 vs. 47.4 ± 5.5 kg/m^2).

In comparison to females, males had higher total fat free mass (76.7 ± 3.7 kg vs. 56.8 ± 6.0 kg, *P* <0.0001), absolute and relative ALM (*P* <0.0001 and 0.02, respectively), higher amount of lean mass in arms (9.8 ± 1.3 kg vs. 5.8 ± 0.8 kg) and legs (27.5 ± 1.3 kg vs. 20.6 ± 2.9 kg, *P*= 0.002 and 0.0002, respectively) (Table 1). Total fat mass was not significantly different between males and females (63.3 ± 10.2 kg vs. 66.0 ± 9.2 kg, *P* = 0.61), while females had greater percentage of body fat compare to males (53.7 ± 2.0% vs. 45.0 ± 3.5%, *P* <0.0001).

Table 1. Characteristics of the participants in the study

Characteristics	**Total** (n = 17)	**Males** (n = 5, 29.41%)	**Females** (n = 12, 70.59%)	*P*
Age, y	43.9 ± 7.8	47.0 ± 6.9	42.6 ± 8.1	0.31
Height, cm	165.7 ± 9.7	175.3 ± 5.8	161.8 ± 8.1	0.004
Weight, kg	128.7 ± 16.1	140.2 ± 11.7	123.9 ± 15.5	0.052
BMI, kg/m^2	46.9 ± 4.9	45.7 ± 3.4	47.4 ± 5.5	0.75
Relative ALM, kg/m^2	10.7 ± 1.6	12.1 ± 1.2	10.1 ± 1.5	0.02
ALM, kg	29.1 ± 5.8	36.9 ± 1.7	26.4 ± 3.5	<0.0001
Lean arm mass, kg	6.9 ± 1.9	9.8 ± 1.3	5.8 ± 0.8	0.002
Lean leg mass, kg	22.6 ± 4.1	27.5 ± 1.3	20.6 ± 2.9	0.0002
Fat free mass, kg	62.6 ± 10.8	76.7 ± 3.7	56.8 ± 6.0	<0.0001
Total fat mass, kg	65.4 ± 9.0	63.3 ± 10.2	66.0± 9.2	0.61
Total body fat, %	50.7 ± 4.9	45.0 ± 3.5	53.7 ± 2.0	<0.0001

Values are Mean ± SD. *P*-values for comparison between males and females

Protein and energy intake:

The average total protein and energy intake was 98.1 ± 25.6 g/d and 2443 ± 529 kcal/d, respectively (Table 2). The average protein intake per kilogram of body weight was 0.76 ± .17 g/kg bw/d. Compared with females had a higher total daily protein intake (121.1 ± 14.6 g/d vs. 88.5 ± 23.1 g/d, $P = 0.01$).

Table 2. Daily energy and protein intake

	Total	**Males**	**Females**	*P*
Number	17	5	12	
Total Energy intake, kcal/d	2443 ± 529	2823 ± 435	2285 ± 496	0.054
Total Protein, g/day	98.1 ± 25.6	121.1 ± 14.6	88.5 ± 23.1	0.01
Protein intake, g/kg bw/d	0.76 ± 0.17	0.87 ± 0.12	0.71 ± 0.17	0.09

Values are Mean ± SD. *P*-values for comparison between males and females

Dietary protein distribution:

Protein intake at each meal is presented in Table 3. Participants' daily protein intake was distributed unevenly across meals, with dinner containing the highest amount of protein in the pooled data (42.78 ± 19.02 g, $P < 0.0001$). In males and females, the average protein content of lunch and dinner was not significantly different (males: lunch = 35.29 ± 13.92g, dinner = 55.76 ± 29.54 g; females: lunch = 28.98 ± 13.84 g, dinner = 37.37 ± 10 g). In females both protein intake at lunch and dinner were significantly higher than at breakfast (14.90 ± 8.50 g, $P = 0.008$) whereas in males only the protein content of dinner was significantly higher than breakfast (20.91 ± 11.33g, $P < 0.0001$)(Figure 2).

No significant difference was observed between males and females regarding the protein distribution across meals (Figure 3).

Males who ate at least 20 g protein at each meal 3 times a day had significantly higher ALM compare to those who did not (38.0 ± 0.9 kg vs. 35.2 ± 0.7 kg, $P = 0.03$) (Table 4). In females, those who ingested 20 g or more of protein per meal were younger and had a higher fat oxidation rate than females who did not fulfill the 20 g protein per meal (102.7 ± 16.0 vs. 76.1 ± 20.7, $P = 0.04$).

Table 3. Protein distribution pattern

	Break fast (Protein g)	**Snacks** (Protein g)	**Lunch** (Protein g)	**Dinner** (Protein g)	***P***
Total (n = 17)	16.66 ± 9.47	10.23 ± 7.14	30.83 ± 13.75	42.78 ± 19.02	<0.0001
Males (n = 5)	20.91 ± 11.33	11.77 ± 10.80	35.29 ± 13.92	55.76 ± 29.54	0.008
Females (n=12)	14.90 ± 8.50	9.59 ± 5.49	28.98 ± 13.84	37.37 ± 10	<0.0001

Values are Mean ± SD. *P*-value for the model

a. b. c.

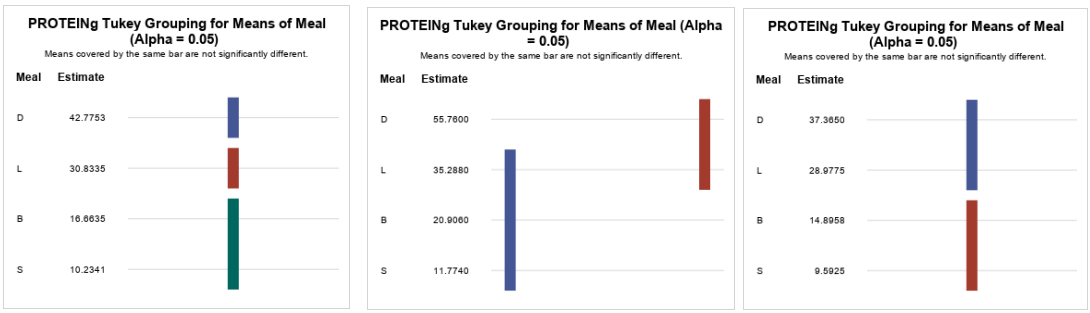

Figure 2. Dietary protein intake at breakfast (B), lunch (L), dinner (D), and snacks (S) in all participants (a), males (b), and females(c)

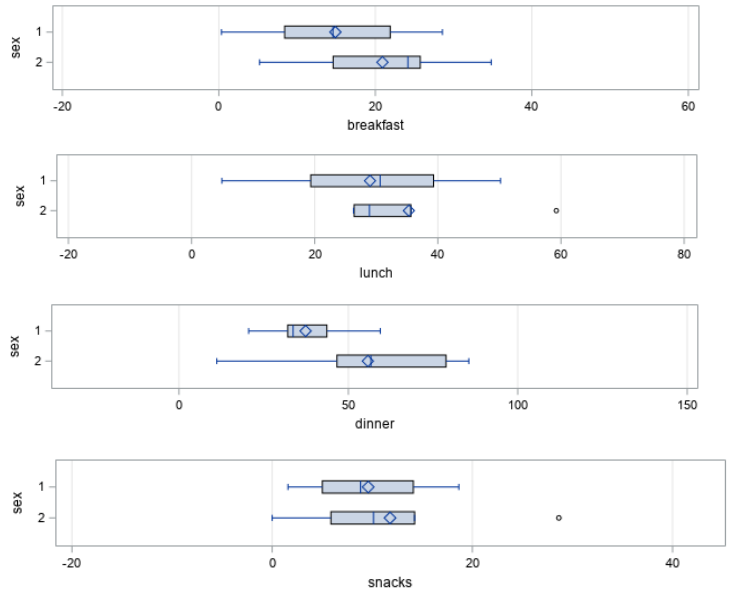

Figure 3. Protein distribution across meals between females (1) and males (2)

Table 4. Body composition, energy expenditure and substrate oxidation according to a daily protein intake under or over 20 g/meal

	Males (n=5)			**Females** (n = 12)		
	≥20g protein per meal (n=3)	<20g protein per meal (n=2)	*P*	≥20g protein per meal (n=5)	<20g protein per meal (n=7)	*P*
Age, y	47.3 ± 4.7	46.5 ± 12.0	0.92	36.4 ± 8.0	47.0 ± 4.8	0.02
REE, kcal/d	2576 ± 242	2241 ± 233	0.22	2104 ± 140	1917 ± 171	0.07
CHOox, mg/min	151.7 ± 87.8	125.9 ± 3.66	0.72	135.4 ± 43.7	177.7 ± 59.0	0.21
Fatox, mg/min	131.3 ± 28.5	116.6 ± 18.8	0.58	102.7 ± 16.0	76.1 ± 20.7	0.04
ALM, kg	38.0 ± 0.9	35.2 ± 0.7	0.03	28.3 ± 2.1	25.1 ± 3.8	0.12
ALM/h^2, kg/m^2	12.8 ± 1.3	11.3 ± 0.5	0.28	10.2 ± 1.1	10.1 ± 1.8	0.92
Lean arm mass, kg	9.9 ± 0.4	8.7 ± 1.34	0.25	6.1 ± 0.8	5.6 ± 0.7	0.37
Lean leg mass, kg	28.1 ± 1.2	26.5 ± 0.7	0.22	22.2 ± 1.7	19.5 ± 3.2	0.12

Functional tests:

Males had higher handgrip strength compare to females (84.4 ± 9.9 kg vs. 63.3 ± 12.5 kg, *P* = 0.004) (Table 5).

Table 5. Handgrip strength and 6-minute walk test

	Total (n = 17)	**Males** (n = 5)	**Females** (n = 12)	*P*
Handgrip strength, kg	69.5 ± 15.2	84.4 ± 9.9	63.3 ± 12.5	0.004
6-minute walk test, m	478.2 ± 58.7	501.2 ± 50.7	468.7 ± 61.1	0.31

Values are Mean ± SD. *P*-values for comparison between males and females

Resting energy expenditure and substrate oxidation:

Females had a significantly lower REE (1995 ± 180 kcal/d vs. 2442 ± 277 kcal/d) and absolute resting fat oxidation rate (87.2 ± 22.7 mg/min vs. 125.4 ± 23.6 mg/min) compared to males (*P* 0.001 and 0.007, respectively) (Table 6). When fat oxidation rate was statistically

adjusted by fat free mass the difference between males and females was no longer significant ($P = 0.55$)

Table 6. Resting energy expenditure and substrate oxidation

	Total (n = 17)	**Males** (n = 5)	**Females** (n = 12)	*P*
RER	0.81 ± 0.04	0.79 ± 0.04	0.82 ± 0.04	0.23
REE, kcal/d	2126 ± 293	2442 ± 277	1995 ± 180	0.001
CHOox, mg/min	154.6 ± 56.5	141.4 ± 63.7	160.1 ± 55.4	0.55
Fatox, mg/min	98.4± 28.6	125.4 ± 23.6	87.2 ± 22.7	0.007

Values are Mean ± SD. *P*-values for comparison between males and females

Multiple regression analysis

We performed multiple regression analysis to examine the relationship between variables, with sex, age, and protein intake, either total protein intake or protein intake per kilogram of body weight, as independent variables, and body composition (Table 7), or functional tests (Table 8) as dependent variables. In these models sex (female vs male) was the only independent predictor for ALM (estimate = -9.1, $P = 0.001$), total body fat percentage (estimate = 9.0, $P = 0.0004$), lean arm mass (estimate = -0.4 , $P = 0.0005$), lean leg mass(estimate = -6.0, $P = 0.006$) and handgrip strength (estimate = -16.6, $P = 0.04$).

In models with REE, RER, or substrate oxidation rates as the dependent variable and sex, age, and body composition compartments as independent variables (Table 9) ALM and lean leg mass were independent predictors of REE (estimate = 43.5, $P = 0.02$; estimate = 36.5, $P = 0.02$, respectively). ALM was also the independent predictor of fat oxidation rate (estimate = 0.004, $P = 0.04$). In one model with REE as the dependent variable and sex, age and ALM/h^2 as independent variables, predictors of REE were sex (estimate = -417.5, $P = 0.004$) and age (estimate = -13.4, $P = 0.04$).

In three models we found a significant interaction between the independent variable and sex (Table 10 and 11) for which stratified analysis was performed on males and females (Table 12).

Table 7. Multiple regression on body composition as the dependent variable and protein intake, sex, and age as independent variables

Total protein intake, g/d n =17	**ALM, kg**		**ALM/h^2, kg/m^2**		**Total body fat, %**		**Total fat mass, kg**		**Lean arm mass, kg**		**Lean leg mass, kg**	
	Estimate	$^{\dagger}P$	Estimate	$^{\dagger}P$	Estimate	$^{\dagger}P$	Estimate	$^{\dagger}P$	Estimate	$^{\dagger}P$	Estimate	$^{\dagger}P$
Intercept	34.6	0.0009	12.5	0.009	45.4	<0.0001	62.3	0.02	2.1	<0.0001	26.5	0.002
Protein, g/d	0.05	0.2	0.002	0.9	0.02	0.6	0.14	0.2	0.002	0.3	0.04	0.3
Sex	-9.1	0.001	-1.9	0.1	9.0	0.0004	5.8	0.4	-0.4	0.0005	-6.0	0.006
Age	0.09	0.4	-0.01	0.8	-0.05	0.6	-0.3	0.3	-0.001	0.8	-0.08	0.4
		R^2=0.79 $^{\ddagger}P$=0.0001		R^2= 0.31 $^{\ddagger}P$= 0.7		R^2=0.76 $^{\ddagger}P$= 0.0003		R^2= 0.27 $^{\ddagger}P$= 0.2		R^2= 0.80 $^{\ddagger}P$= <0.0001		R^2= 0.70 $^{\ddagger}P$= 0.001
Protein intake, g/kg bw/d												
Intercept	43.0	<0.0001	14.0	0.001	49.4	<0.0001	90.8	0.0009	2.3	<0.0001	33.5	<0.0001
Protein, g/kg bw/d	0.6	0.9	-1.2	0.6	-1.1	0.8	-4.3	0.8	0.1	0.6	-0.4	0.9
Sex	-11.0	<0.0001	-2.2	0.03	8.1	0.0002	-0.3	0.9	-0.5	<0.0001	-7.5	0.0005
Age	-0.1	0.2	-0.02	0.7	-0.07	0.4	-0.5	0.1	-0.003	0.6	-0.1	0.2
		R^2= 0.76 $^{\ddagger}P$= 0.0003		R^2= 0.33 $^{\ddagger}P$= 0.2		R^2= 0.75 $^{\ddagger}P$= 0.0003		R^2= 0.19 $^{\ddagger}P$= 0.4		R^2= 0.79 $^{\ddagger}P$= 0.0001		R^2= 0.67 $^{\ddagger}P$= 0.002

$^{\dagger}P$-value for the estimate, $^{\ddagger}P$-value for the model

Table 8. Multiple regression on functional tests as the dependent variable and protein intake, sex, and age as independent variables

Total protein intake, g/d				
n =17	**Handgrip Strength** (n=17)		**6-minute walk test** (n=17)	
	Estimate	†*P*	Estimate	†*P*
Intercept	45.3	0.2	780.5	0.0001
Protein, g/d	0.3	0.1	-0.6	0.4
Sex	-12.0	0.2	-71.5	0.09
Age	0.2	0.7	-4.4	0.03
		R^2= 0.53 ‡*P*= 0.02		R^2= 0.34 ‡*P*= 0.1
Protein intake, g/kg bw/d				
Intercept	59.4	0.045	741.1	<0.0001
Protein, g/kg bw/d	29.5	0.1	-62.7	0.5
Sex	-16.6	0.04	-59.7	0.1
Age	-0.01	0.9	-3.9	0.04
		R^2= 0.52 ‡*P*= 0.02		R^2= 0.33 ‡*P*= 0.1

† *P*-value for the estimate, ‡ *P*-value for the model

Table 9. Multiple regression on REE and substrate oxidation as the dependent variable and body composition, sex, and age as independent variables

n =17	REE		RER		CHO Oxidation		Fat Oxidation	
Lean arm mass, kg								
	Estimate	†*P*	Estimate	†*P*	Estimate	†*P*	Estimate	†*P*
Intercept	1850	0.07	1.1	0.0002	0.5	0.1	-0.03	0.8
Lean arm mass	528.8	0.2	-0.1	0.2	-0.2	0.2	0.9	0.06
Sex	-246.6	0.3	-0.03	0.5	-0.06	0.4	-0.0001	1
Age	-12.6	0.06	0.0003	0.8	-0.0006	0.7	-0.001	0.3
		R^2= 0.70		R^2= 0.24		R^2= 0.14		R^2= 0.60
		‡*P*= 0.001		‡*P*= 0.3		‡*P*= 0.6		‡*P*= 0.006
Lean leg mass, kg								
Intercept	1670.2	0.02	0.9	<0.0001	0.3	0.2	0.03	0.7
Lean leg mass	43.5	0.02	-0.005	0.3	-0.005	0.4	0.004	0.07
Sex	-188.1	0.2	-0.006	0.9	-0.02	0.7	-0.01	0.6
Age	-9.0	0.1	0.0001	0.9	-0.001	0.7	-0.001	0.5
		R^2= 0.77		R^2= 0.19		R^2= 0.07		R^2= 0.59
		‡*P*= 0.0002		‡*P*= 0.4		‡*P*= 0.8		‡*P*= 0.007
ALM, kg								
Intercept	1518.9	0.03	1.0	<0.0001	0.4	0.2	0.003	0.9
ALM	36.5	0.02	-0.01	0.2	-0.01	0.3	0.004	0.04
Sex	-104.4	0.6	-0.02	0.6	-0.04	0.6	0.001	0.9
Age	-9.0	0.1	0.0001	0.9	-0.001	0.7	-0.001	0.5
		R^2= 0.78		R^2= 0.22		R^2= 0.09		R^2= 0.62
		‡*P*= 0.0002		‡*P*= 0.4		‡*P*= 0.7		‡*P*= 0.005
ALM/h^2, kg/m^2								
Intercept	2513.1	0.0003	0.8	<0.0001	0.1	0.6	0.2	0.04
ALM/h2	46.5	0.2	-0.0004	0.9	0.01	0.7	0.002	0.6
Sex	-417.5	0.004	0.03	0.3	0.03	0.5	-0.04	0.02
Age	-13.4	0.04	0.001	0.6	-0.0001	0.9	-0.001	0.2
		R^2= 0.70		R^2= 0.11		R^2= 0.04		R^2= 0.48
		‡*P*= 0.001		‡*P*= 0.7		‡*P*= 0.9		‡*P*= 0.03
Total body fat, %								
Intercept	2397.6	0.03	1.0	0.0005	0.5	0.1	0.01	0.9
Total body fat	14.7	0.5	-0.01	0.3	-0.01	0.3	0.004	0.1
Sex	-632.1	0.01	0.07	0.1	0.1	0.2	-0.1	0.006
Age	-13.1	0.06	0.0004	0.8	-0.001	0.7	-0.001	0.3
		R^2= 0.66		R^2= 0.19		R^2= 0.11		R^2= 0.55
		‡*P*= 0.002		‡*P*= 0.4		‡*P*= 0.7		‡*P*= 0.01

† *P*-value for the estimate, ‡ *P*-value for the model

Table 10. Interaction between protein intake and sex on total body fat percentage, lean leg mass, and ALM

Protein intake, g/kg bw/d		
	Total body fat, % (n=17)	
	Estimate	†*P*
Intercept	67.5	<0.0001
Protein, g/kg bw/d	-23.2	0.02
Sex	-13.9	0.1
Age	-0.05	0.5
Protein*Sex	26.3	0.02
		R^2= 0.85
		‡*P*= <0.0001
Lean leg mass, kg		
	REE (n=17)	
	Estimate	†*P*
Intercept	-1584.6	0.3
Lean leg mass	164.5	0.008
Sex	3332.5	0.04
Age	-10.5	0.047
Lean leg mass*Sex	-130.9	0.03
		R^2= 0.85
		‡*P*= <0.0001
ALM		
	REE (n=17)	
	Estimate	†*P*
Intercept	-2204.6	0.1
ALM	137.5	0.003
Sex	3851.5	0.02
Age	-9.1	0.06
ALM*Sex	-109.6	0.01
		R^2= 0.87
		‡*P*= <0.0001

† *P*-value for the estimate, ‡ *P*-value for the model

Table 11. Multiple regression on total fat mass percentage as the dependent variable and protein and age as independent variables in males and females

Protein intake, g/kg bw/d	**Total body fat, %** Males (n=5)		**Total body fat, %** Females (n=12)	
	Estimate	†*P*	Estimate	†*P*
Intercept	69.1	0.03	53.1	<0.0001
Protein, g/kg bw/d	-22.9	0.2	3.2	0.4
Age	-0.1	0.7	1.1	0.6
		R^2= 0.68 ‡*P*= 0.3		R^2= 0.11 ‡*P*= 0.6

† *P*-value for the estimate, ‡ *P*-value for the model

Table 12. Multiple regression on resting energy expenditure as the dependent variable and lean leg mass and ALM and age as independent variables in males and females

Lean leg mass, kg	**REE** Males (n=5)		**REE** Females (n=12)	
	Estimate	†*P*	Estimate	†*P*
Intercept	-1022.1	0.5	1438.9	0.008
Lean leg mass	168.9	0.5	39.0	0.02
Age	-25.0	0.07	-5.8	0.3
		R^2= 0.94 ‡*P*= 0.06		R^2= 0.62 ‡*P*= 0.01
ALM, kg				
Intercept	-1608.1	0.3	1495.8	0.009
ALM	131.3	0.04	29.9	0.04
Age	-16.9	0.1	-6.9	0.2
		R^2= 0.95 ‡*P*= 0.046		R^2= 0.57 ‡*P*= 0.02

† *P*-value for the estimate, ‡ *P*-value for the model

Discussion

This study examined the association between protein intake and protein distribution with body composition, energy expenditure, substrate oxidation and muscle function in a group of participants with class 3 obesity. We found that males who ate at least 20 g protein at each meal 3 times a day had higher ALM compared to those who did not. We also found that sex but not the protein intake was the factor that independently predicted body composition in our participants.

Protein distribution:

In our study, daily dietary protein intake was distributed unevenly across the meals throughout the day. For the entire sample, the protein distribution was skewed toward the dinner which is consistent with similar studies on Western populations (Hone et al., 2020; Smeuninx et al., 2020). A randomised clinical trial on healthy young males, reported that compared to when the protein content of the breakfast was low and unevenly distributed, optimal and even distribution of dietary protein across three meals resulted in better muscle protein synthesis (Yasuda et al., 2020).

The effect of protein distribution pattern on body composition are unclear. One reason for the lack of clarity in the existing studies is because of differing definitions of protein distribution from varying amounts per meal to different frequencies. In the current study, we compare people who ate at least 20 g protein per meal throughout the day to those who did not meet this criterion. In our study, males with an even daily protein intake of at least 20 g protein per meal had significantly higher ALM compare to males with an uneven daily protein intake. Similarly, another study showed that adults who ate at least 30 protein per meal, twice per day had greater lean leg mass compare to those who did not (Loenneke et al., 2016). In contrast, another study in older adults did not find any association between protein distribution and muscle mass (Gingrich et al., 2017). This contradictory finding may be due to impaired muscle protein synthesis in older adults as Wall et al. (2015) reported that post-prandial muscle protein synthesis rates in older adults were 16% lower compare to their younger counterparts. Nevertheless, considering the small number of males in our study (n=5) these results should be tested in larger studies.

In our study, females who ate at least 20 g protein per meal had a significantly higher fat oxidation rate than others. However, one caveat was that females in this higher protein intake group were about 10 years younger than those who consumed less protein per meal. Thus, this higher rate of fat oxidation may not be due to an even daily protein intake but because of the higher levels of estrogen in younger females. Estrogen has been shown to be involved in fat oxidation, as higher levels of circulating estrogen in younger premenopausal females have been associated with higher rates of fat oxidation (Purdom et al., 2018). Estrogen also plays a role in muscle mass preservation in females (Kirk et al., 2020) and age-related loss of lean mass in older female participants can result in reduced rates of fat oxidation (Kalyani et al., 2014).

Body composition:

It is well-established that dietary protein is essential in muscle protein synthesis and maintaining skeletal muscle mass (Jäger et al., 2017). Various studies have reported positive associations between dietary protein and lean and fat free mass (Correa-Rodríguez et al., 2017; Mangano et al., 2017; Celis-Morales et al., 2018; Bi et al., 2019). However, we did not find any association between protein intake and body composition compartments. While this finding is contrary to many other studies that reported positive associations between protein intake and lean mass (Sahni et al., 2015; Loenneke et al., 2016; Mangano et al., 2017; Celis-Morales et al., 2018), our results are consistent with studies on older adults that found no association between protein intake and muscle mass (Mitchell et al., 2003; Gingrich et al., 2017). Thus, although the lack of a significant relationship between protein intake and lean mass in our study may be due to the small sample, the lack of a relationship may also indicate impaired muscle metabolism in people with severe obesity (Beals et al., 2019).

The association between protein intake and fat mass is not consistent. Lemieux and colleagues (2014) reported an inverse association between protein intake and fat mass in postmenopausal females. In contrast, in another study (Genaro et al., 2015) on elderly females, higher levels of protein intake were positively associated with fat mass. Popp et al. (2019) also reported a positive association between protein intake and fat mass. Similarly, another study on middle-aged adult males showed that those who had a higher protein intake had higher lean and fat mass (So et al., 2019).

In our regression models that examined the relationship between protein intake and body composition, sex was the only factor determining body composition; being female was a predictor of lower ALM, lean arm mass, and lean leg mass, and greater total body fat percentage. These results are consistent with other studies that report sex as an effector of body composition (Power and Schulkin 2008; Bredella, 2017). A cross-sectional study on young, middle-aged, and older adults found that males at all age groups had greater absolute and relative ALM compare to females (Suetta et al., 2019). In another study, males had greater lean mass index (lean mass adjusted by square of height) compared to females at all age groups (Imboden et al., 2017). Also, a large study on young, middle-aged, and older adults, females had greater total body fat percentage compare to males in all age groups (Ofenheimer et al. 2020).

Muscle function:

Handgrip strength is a reliable method to assess muscle function. In our study, sex was the only factor that predicted handgrip strength, and males had significantly higher handgrip strength compared to females. Our results are consistent with findings in a study on healthy older adults that did not observe any associations between protein intake and handgrip strength (Gingrich et al., 2017). Our findings are also consistent with previous studies demonstrating that males have greater handgrip strength compare to females (Fanelli Kuczmarski et al., 2018; Tak et al., 2018; Suetta et al., 2019; Zaccagni et al., 2020). While we did not find any relationship between protein intake and handgrip strength, Mishra and colleagues (2018) reported that in middle-aged adults a higher total daily protein intake was positively associated with handgrip

strength. Similarly, another study showed that participants with higher protein intake per kilogram of body weight had higher handgrip strength (Celis-Morales et al., 2018).

In our study, there were no differences in the 6-minute walk test between males and females. Another study in adults with obesity also reported no significant difference between males and females who performed a 6-minute walk test at baseline (Ekman et al., 2013). Previous studies have shown favorable effects of protein intake on walk test result (Bales et al., 2017; Nabuco et al., 2018; Nahas et al., 2019). However, we did not find any association between protein intake and the 6-minute walk test. The 6-minute walk test is often used to measure the response to treatment (Singh et al., 2014). It is difficult to compare our findings with these studies because of the different study designs as most studies had a longitudinal design compared to our study which was cross-sectional. The lack of association between protein intake and the 6-minute walk test in our study could also be due to the fact that one of the inclusion criteria in our study was being able to walk and perform the functional tests. Therefore, all our participants were able to walk and perform their daily activities. Furthermore, protein intake is not the only factor affecting functional capacity and aerobic fitness; other factors involved in aerobic fitness, such as cardiovascular health, may also contribute (Nilsson et al., 2018).

Body composition, resting energy expenditure, and substrate oxidation:

Consistent with another study on bariatric surgery candidates, we also found that males had a significantly higher REE compare to females (Wilms et al., 2018). Two major determinants of REE are body weight and lean mass (Müller et al., 2018). In our sample males tended to be heavier and had significantly higher lean mass in comparison to females.

In our findings, lean leg mass and ALM were independent predictors of REE. Hirsch et al. (2017) also reported a positive association between REE and lean mass in arms and legs in adults with overweight and obesity. Muscle metabolism is a notable part of REE, and the quantity of SM varies substantially in people resulting in differences in energy expenditure among individuals (Dulloo et al., 2010). While at rest, muscle protein turnover, which includes muscle protein synthesis and breakdown, is the major activity consuming energy in muscle tissue (Wolfe, 2006).

In the multiple regression model, we used REE as the dependent variable and sex, age, and total body fat percentage as independent variables. While sex was the only variable that predicted REE in our study, Faria et al. (2012) reported a negative association between REE and body fat percentage in a group of bariatric surgery candidates. However, Bosy-Westphal et al. (2009) observed that the effect of fat mass on REE is dependent on the degree of adiposity. That is to say, when fat mass comprises 40% body mass, the association between REE and fat mass percent is positive (Bosy-Westphal et al., 2009). When % fat mass is over 40%, a negative relationship is observed between %fat mass and REE. This negative relationship may be attributed to the mitochondrial dysfunction observed with greater degrees of obesity (Bosy-Westphal et al., 2009). Despite our participants having an average of 51% fat, we did not find any association between total body fat percentage and REE.

In another multiple regression model with REE as the dependent variable and sex, age, and relative ALM as independent variables, we found that sex (female vs male) and age were

predictors of REE. This is consistent with another study that reported a sex-specific and age-related decrease in REE (Geisler et al., 2016).

In our study, the rate of absolute fat oxidation at rest was lower in females compare to males, which is contrary to some studies that reported higher rates of resting fat oxidation in females (Venables et al., 2005; Numao et al., 2009; Siervo et al., 2016), and consistent with other studies showing males to have higher rates of absolute resting fat oxidation compare to females (Nagy et al., 1996; Kien and Bunn, 2008). It is worthy to note that in our study, males had significantly higher fat free mass compared to females and that fat free mass is positively associated with resting fat oxidation rate (Wolfe, 2006; Robinson et al., 2016). When fat oxidation rate was adjusted for total fat free mass, the difference in fat oxidation rates between males and females was no longer significant. Skeletal muscle tissue in females has higher rates of fat uptake and oxidation compare to males due to higher enzyme capacity for fat metabolism in females (Gheller et al., 2016). Thus, the reduced rate of fat oxidation observed in females may be because obesity has been shown to disrupt fat oxidation, especially by skeletal muscle (Rosenkilde et al., 2010). Despite the increased influx of fatty acids into skeletal muscle in obesity, the oxidative enzyme capacity in skeletal muscle cells has been shown to decrease resulting in lower rates of fat oxidation (Kelley et al., 1999; Wu and Ballantyne, 2017). We also found that ALM was an independent predictor of fat oxidation rate. Since skeletal muscle constitutes the largest component of fat free mass and fat is the primary source of fuel in skeletal muscle, more muscle mass might result in higher fat oxidation (Jensen, 2003; Wolfe, 2006).

Another finding of the present study that indicates perturbed fat metabolism in female participants is that we did not find any significant differences in RER between males and females. In adulthood, females usually have lower RER compared to males because of higher rates of fat oxidation (Carter et al., 2001; Tarnopolsky, 2008; Isacco et al., 2012; Siervo et al., 2016; Purdom et al., 2018). The metabolic inflexibility in our female participants that is represented by lower rates of fat oxidation may be the result of damaging effects of obesity on metabolism (Kelley et al., 1999; Galgani et al., 2008). Comparably, a study in adults with overweight and obesity reported no differences in RER between males and females (Hirsch et al., 2016).

Strengths and Limitations

There were several strengths of the study including the use of gold standard techniques of indirect calorimetry to measure REE and substrate oxidation, and DXA to assess total and regional body composition in participants. Indirect calorimetry measures energy expenditure and substrate oxidation reliably in the research settings (Lam and Ravussin, 2017). In people with obesity, indirect calorimetry provides a more reliable measurement for energy expenditure compared to prediction equations (Madden et al., 2016). The use of DXA for body composition measurement allows for the accurate delineation of several regions of interest that would not be possible with other techniques (Shepherd et al. 2017).

A major limitation of the present study was the study's cross-sectional design, which did not allow us to explore causality in the observed associations. Another limitation of the current study was the small sample size, and the uneven number of male and female participants. The

small sample size also affects the power of the multiple regression analyses to detect relationships where they may exist. We only had 5 male participants in the study and as a general rule, at least 10 observations in each group is recommended per independent variable (Peduzzi et al., 1996). Also, we did not assess and adjust participants' physical activity levels, which could affect muscle function and body composition (Beasley et al., 2013). Recruitment of further participants should assess physical activity of participants.

Conclusion

In conclusion, our findings demonstrated that in adults with severe obesity, ALM was an independent predictor of REE and fat oxidation rate. We also found indicators showing that females may be less metabolically flexible, as demonstrated by altered substrate metabolism and decreased rates of fat oxidation. Additionally, our findings suggested that an even and adequate protein intake throughout the day may be associated with higher lean mass in males in particular. Assessment of physical activity should be conducted for future participants. The results of this preliminary study will help to improve study design for future participants.

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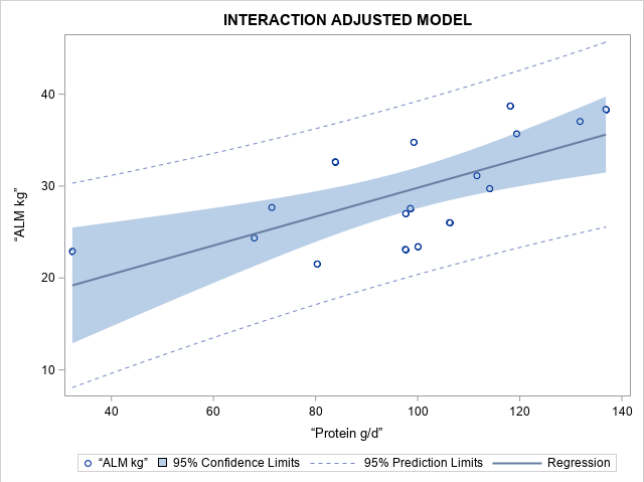
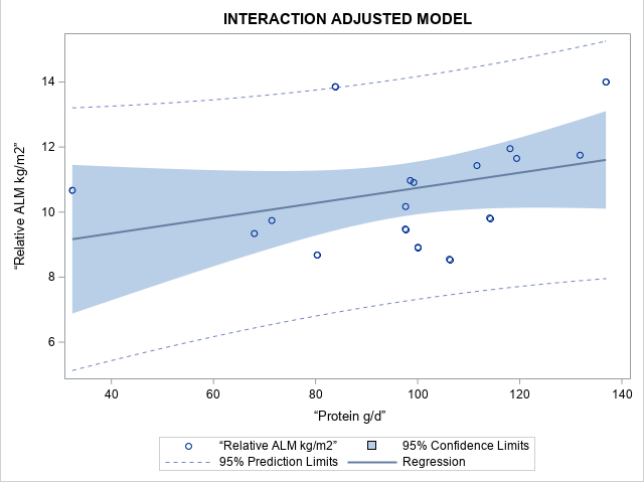
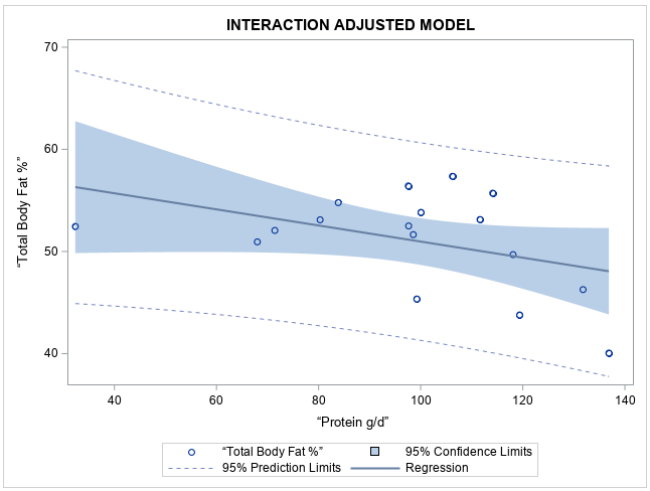
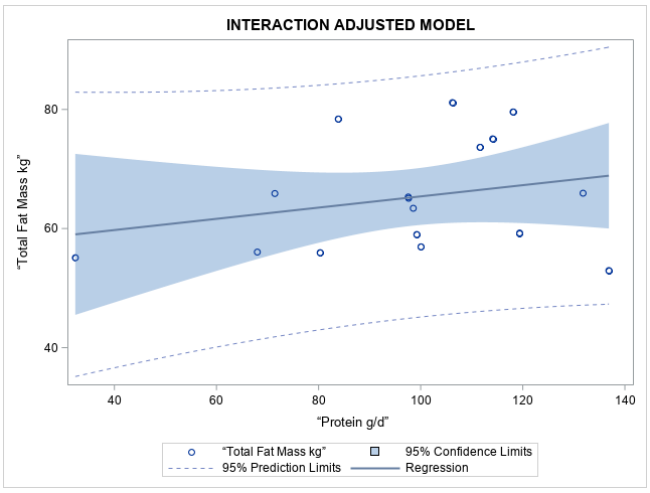
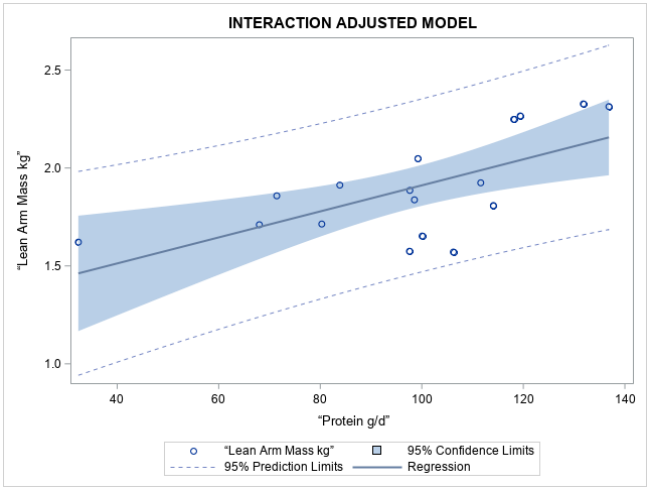
Appendix A. Multiple regression scatter plots

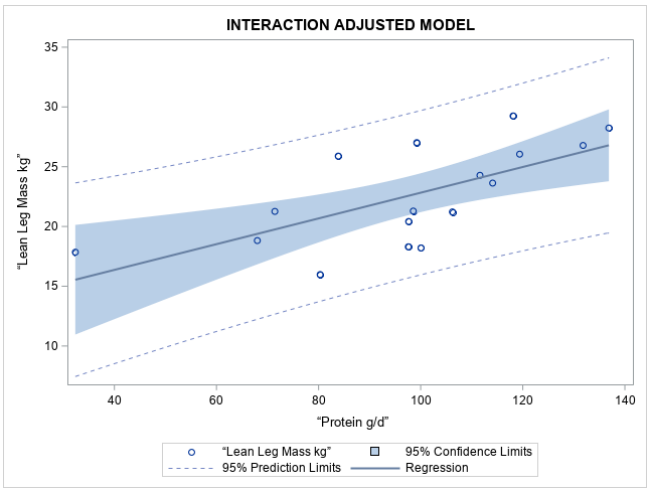


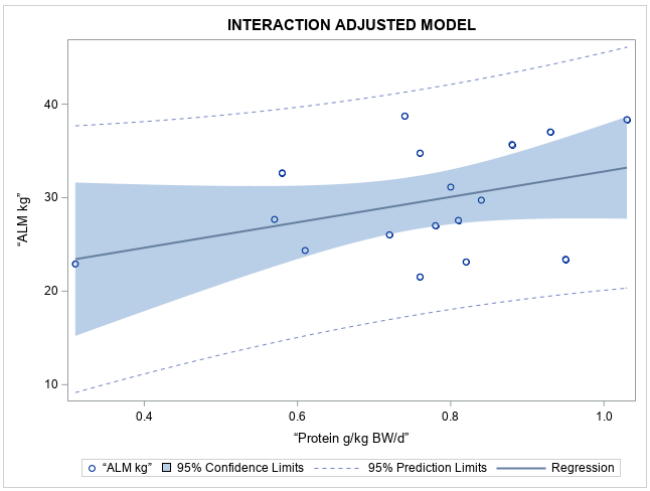


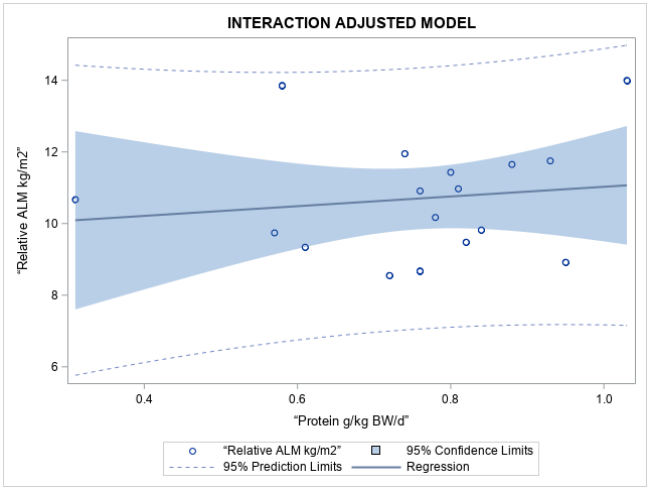

INTERACTION ADJUSTED MODEL
"Total Body Fat %"
70
60
50
40
40
60
80
100
120
140
"Protein g/d"
"Total Body Fat %"
95% Confidence Limits
95% Prediction Limits
Regression

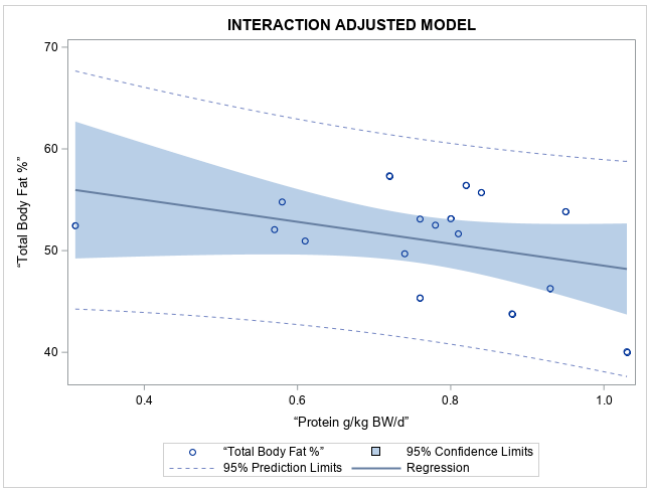

INTERACTION ADJUSTED MODEL
"Total Fat Mass kg"
80
60
40
40
60
80
100
120
140
"Protein g/d"
"Total Fat Mass kg"
95% Confidence Limits
95% Prediction Limits
Regression

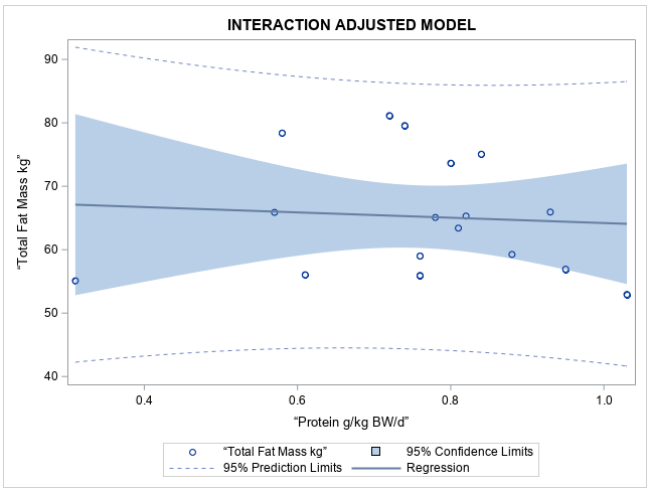

INTERACTION ADJUSTED MODEL
"Lean Arm Mass kg"
2.5
2.0
1.5
1.0
40
60
80
100
120
140
"Protein g/d"
"Lean Arm Mass kg"
95% Confidence Limits
95% Prediction Limits
Regression

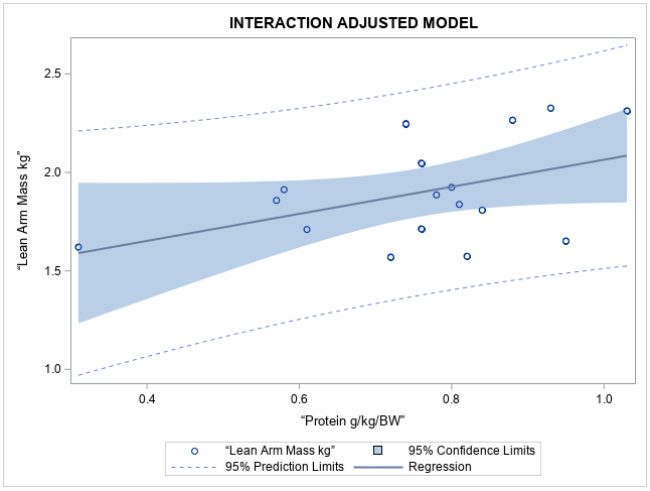

INTERACTION ADJUSTED MODEL
"Lean Leg Mass kg"
35
30
25
20
15
10
40
60
80
100
120
140
"Protein g/d"
"Lean Leg Mass kg"
95% Confidence Limits
95% Prediction Limits
Regression

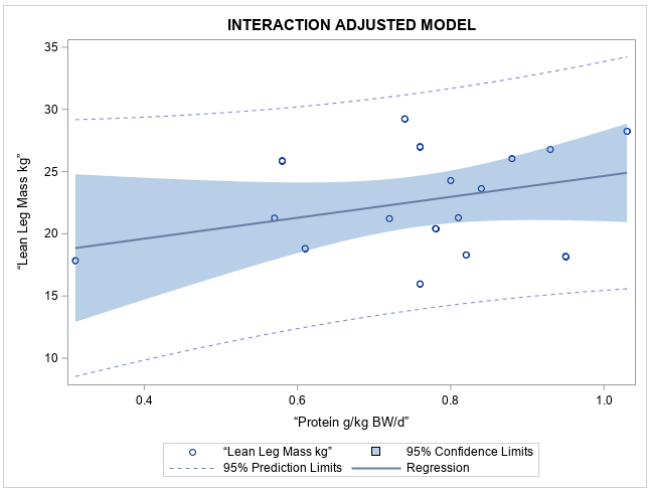

INTERACTION ADJUSTED MODEL
"ALM kg"
40
30
20
10
0.4
0.6
0.8
1.0
"Protein g/kg BW/d"
"ALM kg"
95% Confidence Limits
95% Prediction Limits
Regression

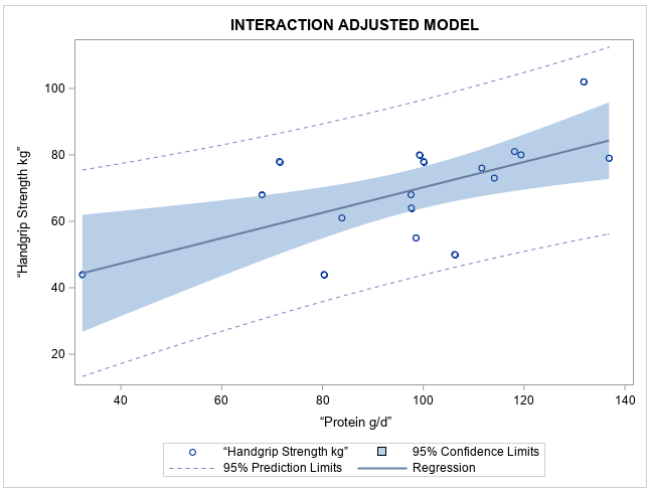

INTERACTION ADJUSTED MODEL
"Relative ALM kg/m2"
14
12
10
8
6
0.4
0.6
0.8
1.0
"Protein g/kg BW/d"
"Relative ALM kg/m2"
95% Confidence Limits
95% Prediction Limits
Regression

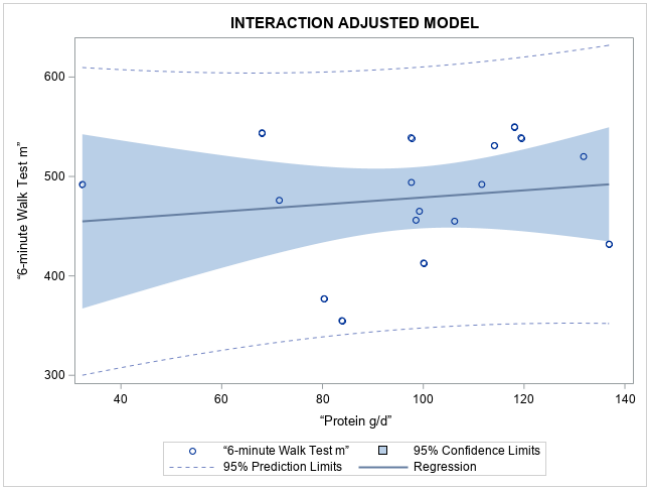

INTERACTION ADJUSTED MODEL
"Total Body Fat %"
70
60
50
40
0.4
0.6
0.8
1.0
"Protein g/kg BW/d"
"Total Body Fat %"
95% Confidence Limits
95% Prediction Limits
Regression

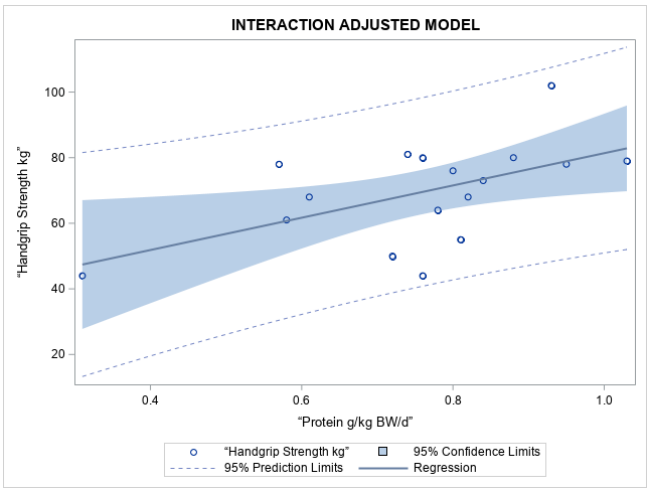

INTERACTION ADJUSTED MODEL
"Total Fat Mass kg"
90
80
70
60
50
40
0.4
0.6
0.8
1.0
"Protein g/kg BW/d"
"Total Fat Mass kg"
95% Confidence Limits
95% Prediction Limits
Regression

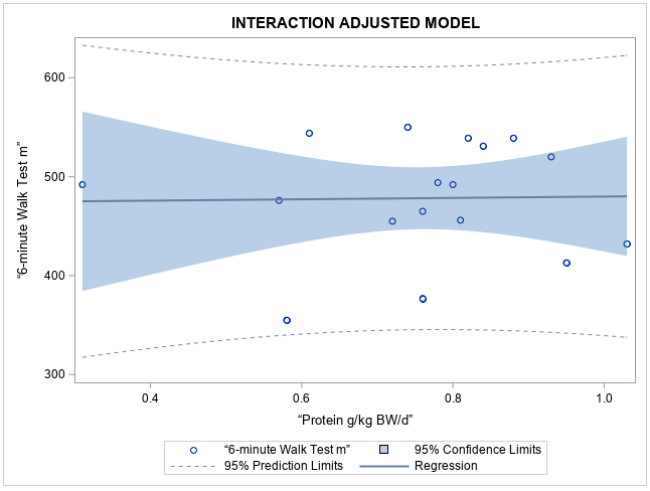

INTERACTION ADJUSTED MODEL
"Lean Arm Mass kg"
2.5
2.0
1.5
1.0
0.4
0.6
0.8
1.0
"Protein g/kg/BW"
"Lean Arm Mass kg"
95% Confidence Limits
95% Prediction Limits
Regression

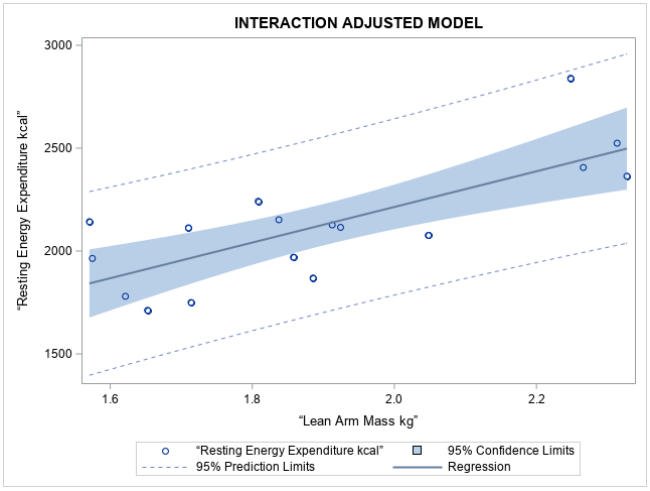

INTERACTION ADJUSTED MODEL
"Lean Leg Mass kg"
"Protein g/kg BW/d"
"Lean Leg Mass kg"
95% Confidence Limits
95% Prediction Limits
Regression

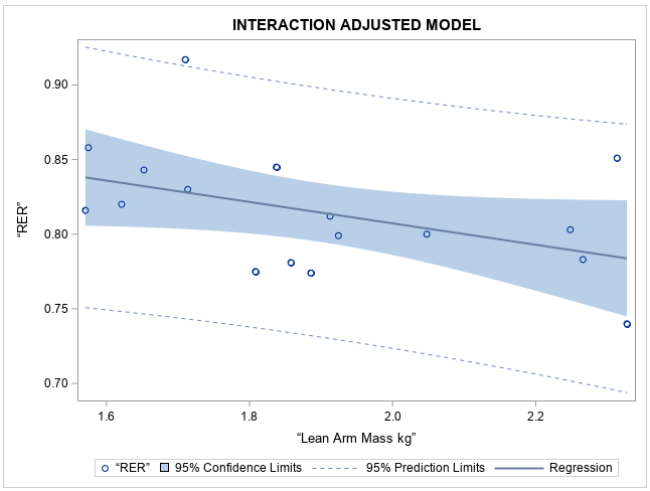

INTERACTION ADJUSTED MODEL
"Handgrip Strength kg"
"Protein g/d"
"Handgrip Strength kg"
95% Confidence Limits
95% Prediction Limits
Regression

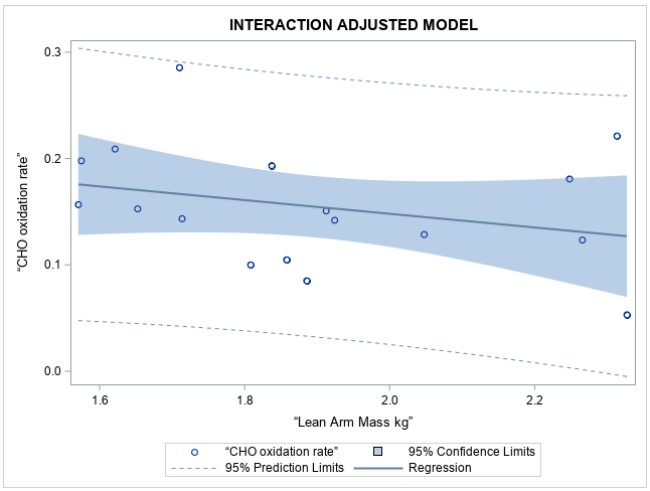

INTERACTION ADJUSTED MODEL
"6-minute Walk Test m"
"Protein g/d"
"6-minute Walk Test m"
95% Confidence Limits
95% Prediction Limits
Regression

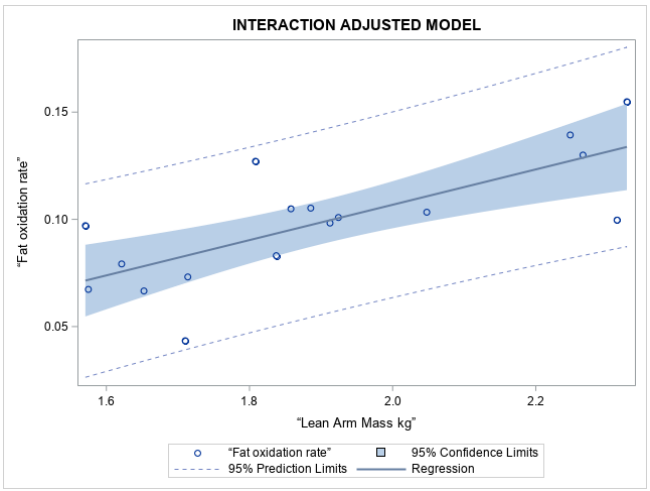

INTERACTION ADJUSTED MODEL
"Handgrip Strength kg"
100
80
60
40
20
0.4
0.6
0.8
1.0
"Protein g/kg BW/d"
"Handgrip Strength kg"
95% Confidence Limits
95% Prediction Limits
Regression

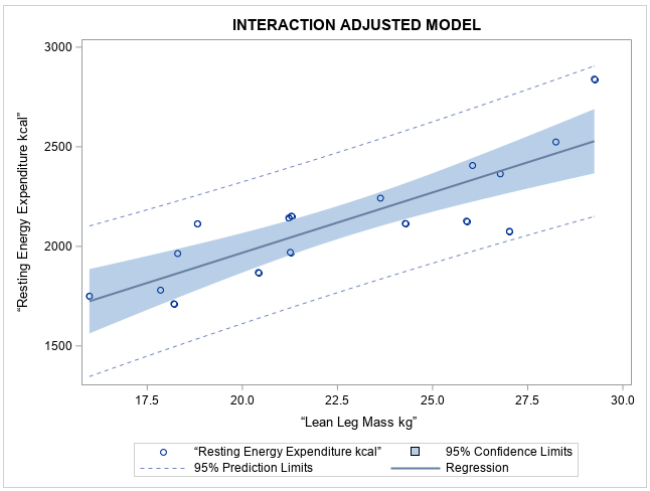

INTERACTION ADJUSTED MODEL
"6-minute Walk Test m"
600
500
400
300
0.4
0.6
0.8
1.0
"Protein g/kg BW/d"
"6-minute Walk Test m"
95% Confidence Limits
95% Prediction Limits
Regression

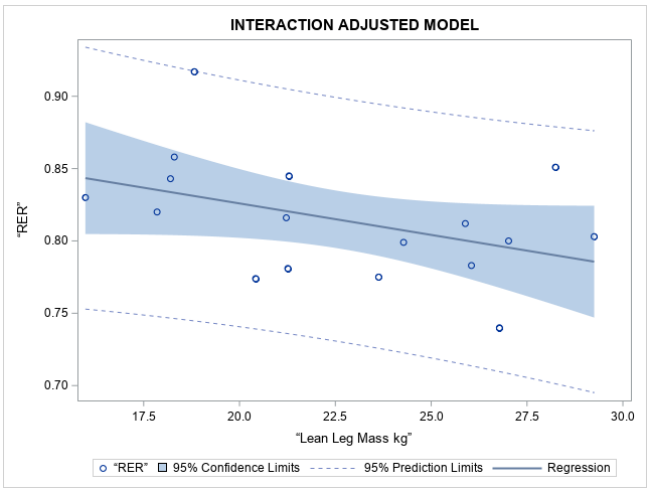

INTERACTION ADJUSTED MODEL
"Resting Energy Expenditure kcal"
3000
2500
2000
1500
1.6
1.8
2.0
2.2
"Lean Arm Mass kg"
"Resting Energy Expenditure kcal"
95% Confidence Limits
95% Prediction Limits
Regression

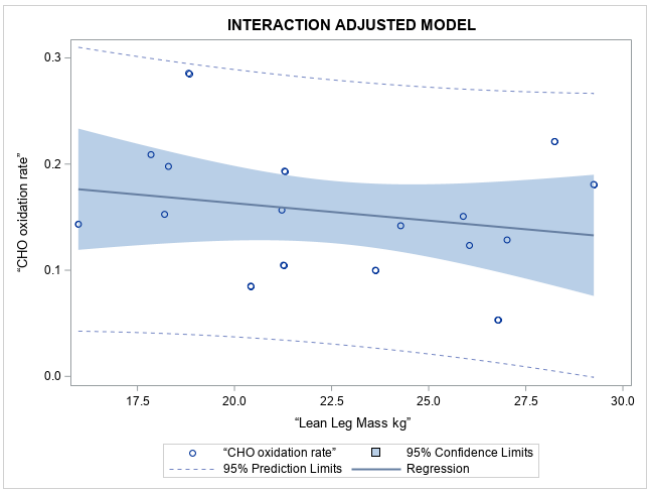

INTERACTION ADJUSTED MODEL
"RER"
0.90
0.85
0.80
0.75
0.70
1.6
1.8
2.0
2.2
"Lean Arm Mass kg"
"RER"
95% Confidence Limits
95% Prediction Limits
Regression

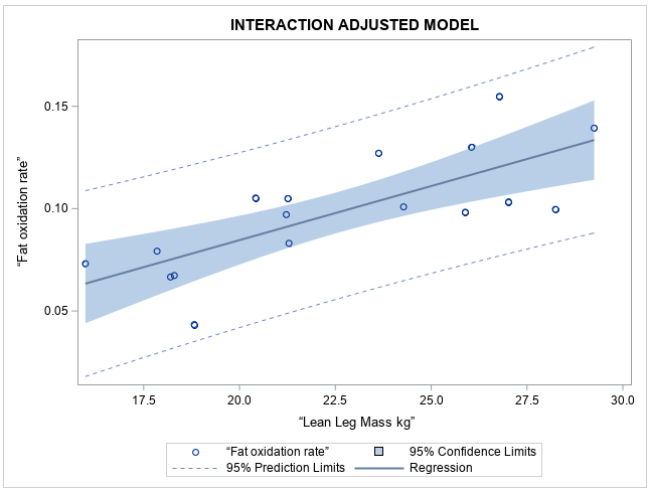

INTERACTION ADJUSTED MODEL
"CHO oxidation rate"
0.3
0.2
0.1
0.0
1.6
1.8
2.0
2.2
"Lean Arm Mass kg"
"CHO oxidation rate"
95% Confidence Limits
95% Prediction Limits
Regression

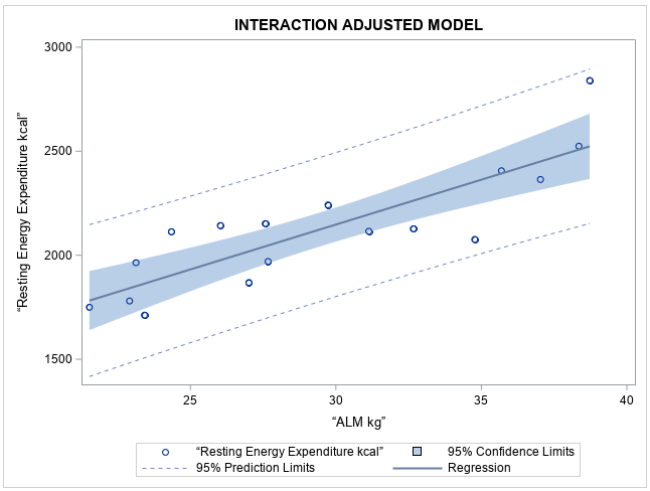

INTERACTION ADJUSTED MODEL
"Fat oxidation rate"
0.15
0.10
0.05
1.6
1.8
2.0
2.2
"Lean Arm Mass kg"
"Fat oxidation rate"
95% Confidence Limits
95% Prediction Limits
Regression

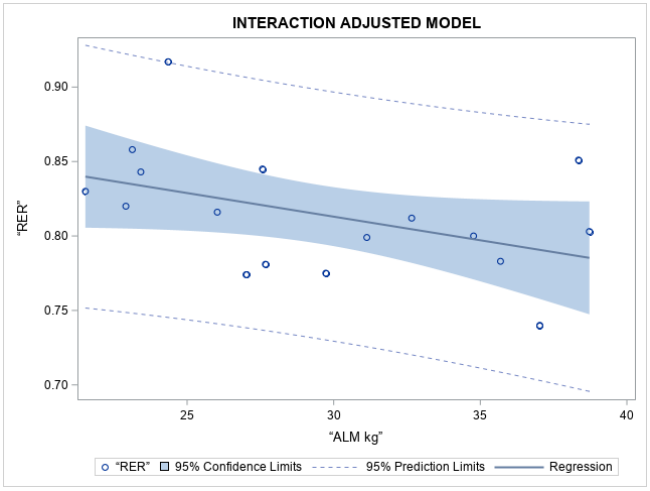

INTERACTION ADJUSTED MODEL
"Resting Energy Expenditure kcal"
3000
2500
2000
1500
17.5
20.0
22.5
25.0
27.5
30.0
"Lean Leg Mass kg"
"Resting Energy Expenditure kcal"
95% Confidence Limits
95% Prediction Limits
Regression

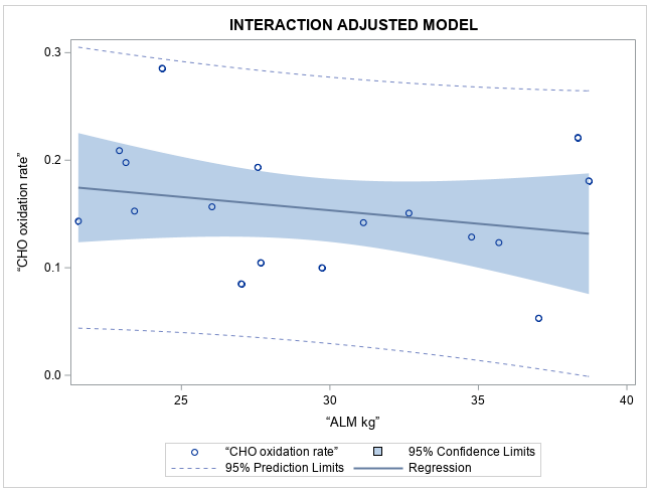

INTERACTION ADJUSTED MODEL
"RER"
0.90
0.85
0.80
0.75
0.70
17.5
20.0
22.5
25.0
27.5
30.0
"Lean Leg Mass kg"
"RER"
95% Confidence Limits
95% Prediction Limits
Regression

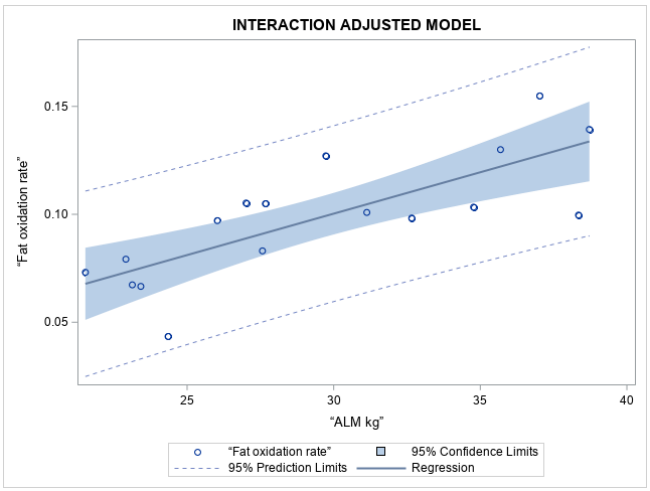

INTERACTION ADJUSTED MODEL
"CHO oxidation rate"
0.3
0.2
0.1
0.0
17.5
20.0
22.5
25.0
27.5
30.0
"Lean Leg Mass kg"
"CHO oxidation rate"
95% Confidence Limits
95% Prediction Limits
Regression

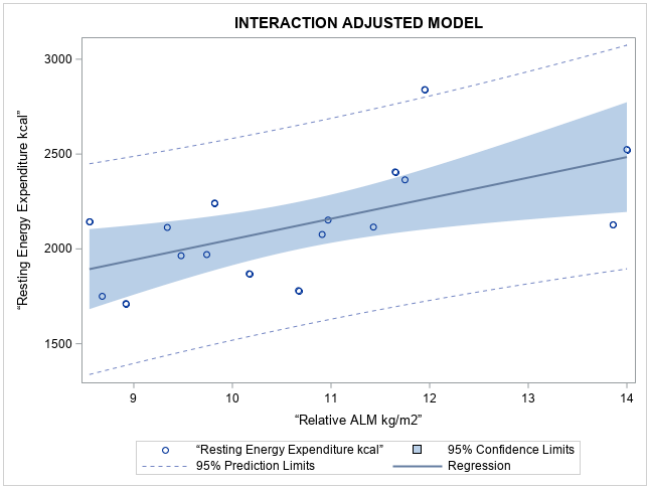

INTERACTION ADJUSTED MODEL
"Fat oxidation rate"
0.15
0.10
0.05
17.5
20.0
22.5
25.0
27.5
30.0
"Lean Leg Mass kg"
"Fat oxidation rate"
95% Confidence Limits
95% Prediction Limits
Regression

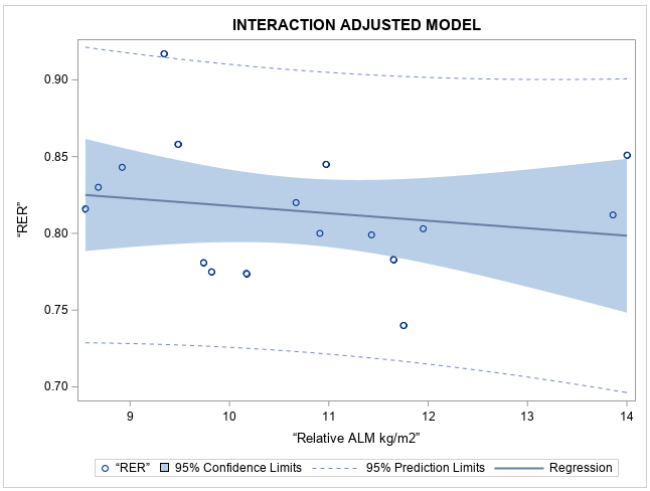

INTERACTION ADJUSTED MODEL
"Resting Energy Expenditure kcal"
3000
2500
2000
1500
25
30
35
40
"ALM kg"
"Resting Energy Expenditure kcal"
95% Confidence Limits
95% Prediction Limits
Regression

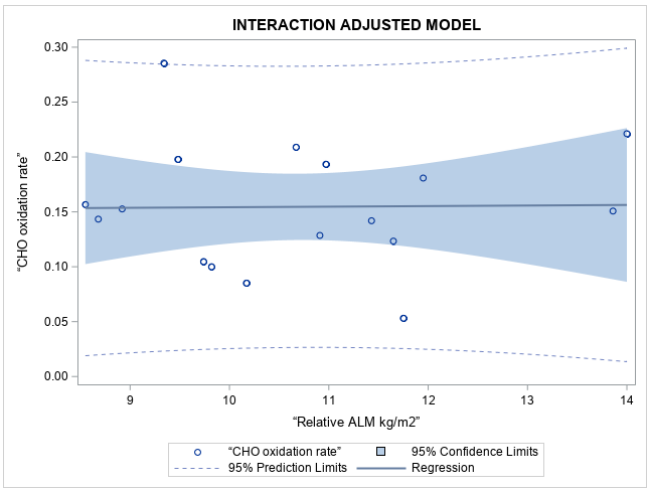

INTERACTION ADJUSTED MODEL
"RER"
0.90
0.85
0.80
0.75
0.70
25
30
35
40
"ALM kg"
"RER"
95% Confidence Limits
95% Prediction Limits
Regression

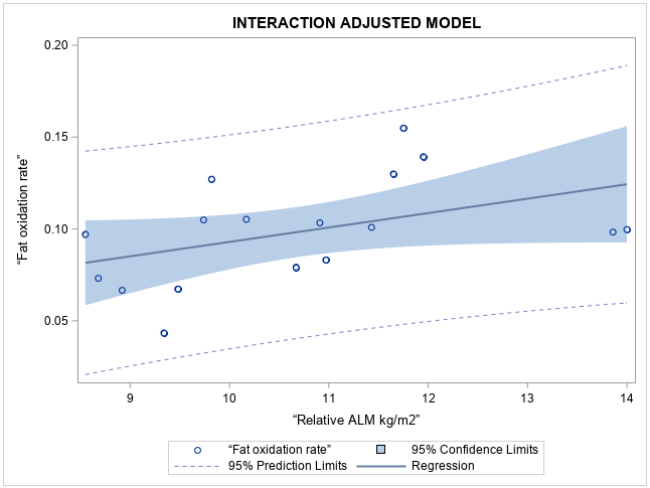

INTERACTION ADJUSTED MODEL
"CHO oxidation rate"
0.3
0.2
0.1
0.0
25
30
35
40
"ALM kg"
"CHO oxidation rate"
95% Confidence Limits
95% Prediction Limits
Regression

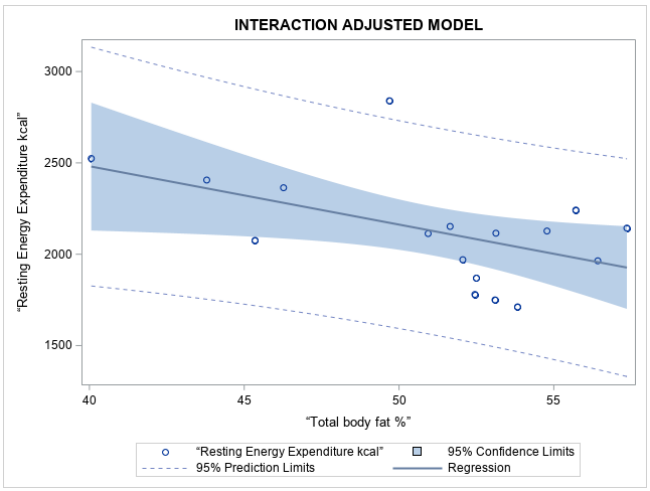

INTERACTION ADJUSTED MODEL
"Fat oxidation rate"
0.15
0.10
0.05
25
30
35
40
"ALM kg"
"Fat oxidation rate"
95% Confidence Limits
95% Prediction Limits
Regression

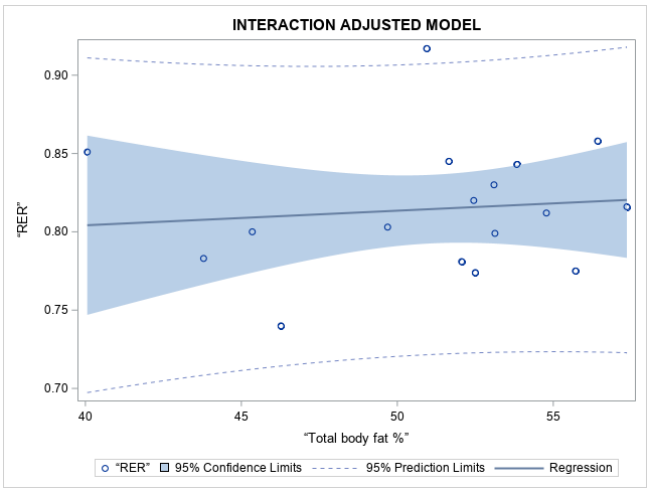

INTERACTION ADJUSTED MODEL
"Resting Energy Expenditure kcal"
3000
2500
2000
1500
9
10
11
12
13
14
"Relative ALM kg/m2"
"Resting Energy Expenditure kcal"
95% Confidence Limits
95% Prediction Limits
Regression

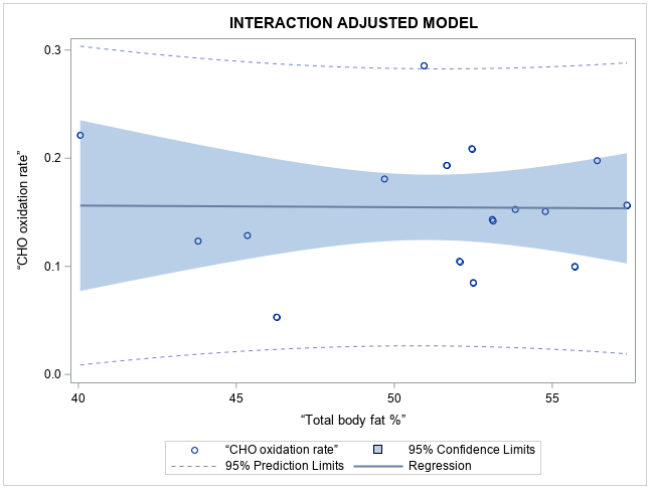

INTERACTION ADJUSTED MODEL
"RER"
0.90
0.85
0.80
0.75
0.70
9
10
11
12
13
14
"Relative ALM kg/m2"
"RER"
95% Confidence Limits
95% Prediction Limits
Regression

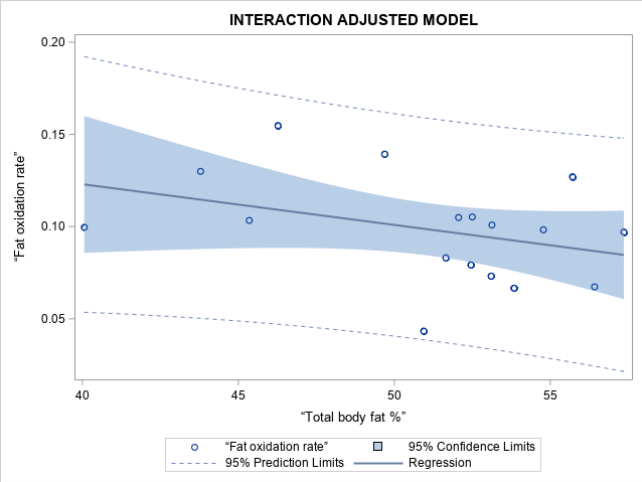

INTERACTION ADJUSTED MODEL
"CHO oxidation rate"
0.30
0.25
0.20
0.15
0.10
0.05
0.00
9
10
11
12
13
14
"Relative ALM kg/m2"
"CHO oxidation rate"
95% Confidence Limits
95% Prediction Limits
Regression


INTERACTION ADJUSTED MODEL
"Fat oxidation rate"
0.20
0.15
0.10
0.05
9
10
11
12
13
14
"Relative ALM kg/m2"
"Fat oxidation rate"
95% Confidence Limits
95% Prediction Limits
Regression


INTERACTION ADJUSTED MODEL
"Resting Energy Expenditure kcal"
3000
2500
2000
1500
40
45
50
55
"Total body fat %"
"Resting Energy Expenditure kcal"
95% Confidence Limits
95% Prediction Limits
Regression


INTERACTION ADJUSTED MODEL
"RER"
0.90
0.85
0.80
0.75
0.70
40
45
50
55
"Total body fat %"
"RER"
95% Confidence Limits
95% Prediction Limits
Regression


INTERACTION ADJUSTED MODEL
"CHO oxidation rate"
0.3
0.2
0.1
0.0
40
45
50
55
"Total body fat %"
"CHO oxidation rate"
95% Confidence Limits
95% Prediction Limits
Regression


INTERACTION ADJUSTED MODEL
"Fat oxidation rate"
0.20
0.15
0.10
0.05
40
45
50
55
"Total body fat %"
"Fat oxidation rate"
95% Confidence Limits
95% Prediction Limits
Regression