

**DO OBESITY AND PHYSICAL INACTIVITY UNDERLIE THE INSULIN
RESISTANCE OF AGING?**

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University of Pittsburgh, 2009

Insulin resistance (IR) is the hallmark of type 2 diabetes (T2DM) and can precede its onset for many years. Since the prevalence of T2DM is higher among older adults, it has been suggested that aging is associated with IR. While some studies support the concept of age-related IR, others support the hypothesis that IR may not be associated with aging but rather with lifestyle patterns linked with aging, such as physical inactivity and obesity.

To determine the effects of older age on IR independently of physical inactivity and obesity, we compared 7 older and 7 younger normal weight sedentary volunteers matched by gender, body mass index (BMI) and physical inactivity. In normal weight sedentary subjects, i.e., after accounting for both obesity and level of chronic physical activity, aging per se was not associated with IR.

To determine the effects of obesity on IR independently of age and physical inactivity, we compared 7 older normal weight sedentary subjects to 14 obese sedentary subjects matched by age, gender and physical inactivity. After accounting for both age and physical inactivity, obesity was associated with both peripheral and hepatic IR.

To determine the effects of chronic exercise on IR independently of age and obesity, we compared 14 older endurance trained athletes to 7 normal weight sedentary subjects matched by age and BMI. Within subjects of similar age and body weight, and after adjusting for body fat,

higher physical activity was associated with greater peripheral insulin sensitivity, but not with greater hepatic insulin sensitivity.

Intramyocellular lipids (IMCL) and fatty acid metabolites such as diacylglycerols (DAG) and ceramides (Cer) may play an important role in the pathophysiology of IR. We demonstrated that intramyocellular triglycerides (IMTG) were higher but ceramide content was lower in athletes. Moreover, the distribution of DAG and ceramide species was different in athletes compared to obese sedentary subjects.

In conclusion, these data indicate that IR is not associated with age per se but rather is determined by obesity and physical activity. This study further elucidates the association among intramyocellular lipid content, aging, obesity, physical activity and IR.

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To Silvia and Daniele

An immense Thank you Boss!

1 INTRODUCTION

1.1 RATIONALE

Insulin resistance is a crucial factor for the development of type 2 diabetes (1). Obesity has been linked with insulin resistance (2). The increasing prevalence of obesity and type 2 diabetes in the older population (3) has detrimental health and economic consequences (4).

Since insulin resistance has been shown to precede the development of type 2 diabetes (5), and the prevalence of type 2 diabetes is higher among older adults, it has been suggested that aging is associated with insulin resistance (6). The literature on age-associated insulin resistance, however, is inconsistent. Some studies support the concept of age-related insulin resistance (7, 8) while others, at least in overweight and obese populations, have shown that insulin resistance may not be associated with aging but rather with lifestyle patterns linked with aging, such as a reduced physical activity (9) and obesity (10). Therefore, it is not clear whether insulin resistance associated with older age is secondary to acquired effects of physical inactivity and/or obesity.

No studies have yet examined whether insulin resistance is a feature of older age in both normal weight and obese subjects (Specific Aim 1). Moreover, while insulin resistance is associated with obesity in younger adults, the literature is sparse concerning whether obese older subjects are more insulin resistant than normal weight older subjects. Within a homogenous group of older overweight to obese men and women, our research group has observed that the

amount of body fat was not associated with insulin resistance ($R^2 > 0.01$, $p = 0.99$). This lack of association between body fat and insulin resistance suggests that generalized adiposity may play a relatively minor role in insulin resistance in older subjects. To our knowledge no studies have yet examined whether generalized obesity is associated with insulin resistance in the older population (Specific Aim 2).

Physical inactivity is likely another key factor in determining insulin resistance as people age. Chronic physical activity (i.e. exercise training) enhances skeletal muscle insulin response through many mechanisms, including increases in glucose transporters (GLUT 4) and enhanced insulin signaling. Prior studies have shown that young athletes are more insulin sensitive than young sedentary subjects, but it is not known whether older 'Masters' athletes are more insulin sensitive than sedentary older subjects after accounting for their body fatness (Specific Aim 3).

In addition to defects in insulin signaling and lower GLUT 4 content within skeletal muscle, ectopic lipid deposition, defined as an excess accumulation of triglycerides in non adipose tissue such as within the muscle fibers (intramyocellular triglycerides, IMTG), are also positively associated with obesity and insulin resistance (11, 12). Furthermore, the accumulation of IMTG is often cited as being a key determinant in insulin resistance (13). In addition to playing an important role in obesity (14) and type 2 diabetes (15), ectopic fat deposition is also observed in common conditions such as aging (16) and physical inactivity (17). Paradoxically, regular physical activity (i.e. exercise training) improves insulin sensitivity while increasing IMTG (18). This observation has led several investigators to examine the hypothesis that IMTG do not directly affect insulin action, but, depending on the metabolic context, e.g., high turnover or utilization of this energy pool during physical activity, merely provide a surrogate for the

accumulation of potentially damaging lipid metabolites, namely diacylglycerols (19) and ceramides (20). It is not yet clear whether chronic exercise affects partitioning of fatty acids into and out of these various lipid pools. Moreover, it is not clear whether insulin resistance in aging may be due to excess accumulation of harmful lipid metabolites due to chronic physical inactivity and/or obesity. Based on the above, clinical investigations into the separate effects of aging, obesity and physical inactivity on insulin resistance and IMTG and other intramyocellular lipids (IMCL) are clearly needed.

1.2 PURPOSE

The purpose of this study was to separate the effects of aging, obesity and exercise on insulin sensitivity and intramyocellular lipids (IMCL), by performing three cross-sectional comparisons:

1) To determine whether or not older age is associated with insulin resistance independently of obesity and of chronic exercise, insulin sensitivity was compared in younger normal weight sedentary subjects vs. older normal weight sedentary subjects.

2) To determine whether or not obesity is associated with insulin resistance independently of physical activity specifically within older subjects, insulin sensitivity was compared in older obese sedentary subjects vs. older normal weight sedentary subjects.

3) To determine whether or not chronic exercise training is associated with insulin resistance in older subjects independent of obesity, insulin sensitivity was compared in older normal weight sedentary subjects vs. older endurance-trained athletes.

1.3 SPECIFIC AIMS AND HYPOTHESIS

1.3.1 Specific Aim 1: To examine the effects of older age on insulin resistance in sedentary subjects.

Hypothesis 1 We hypothesized that normal weight younger subjects would be more insulin sensitive than normal weight older subjects, when matched by BMI and by physical activity level (all sedentary subjects).

To test this hypothesis, we measured insulin sensitivity in sedentary normal weight older subjects and compared them to BMI matched sedentary normal weight younger subjects.

This hypothesis would be supported if sedentary normal weight older subjects have lower insulin sensitivity, i.e., they would be more insulin resistant, compared with sedentary normal weight younger subjects.

1.3.2 Specific Aim 2: To examine the effects of obesity on insulin resistance and intramyocellular lipids (IMCL) in sedentary older subjects

Hypothesis 2.1 Sedentary obese subjects would have lower insulin sensitivity compared to age matched sedentary normal weight subjects.

Hypothesis 2.2 Lower skeletal muscle insulin sensitivity in sedentary obese older subjects would be observed in the context of higher intramyocellular triglycerides (IMTG) and with higher

levels of lipid metabolites, including diacylglycerols and ceramides, compared to sedentary normal weight older subjects.

To test these hypotheses, we measured insulin sensitivity, IMTG and lipid metabolites in sedentary obese older subjects and compared them to age-matched sedentary normal weight subjects.

These hypotheses would be supported if sedentary obese subjects have lower insulin sensitivity and higher IMTG with a higher content of diacylglycerols and ceramides as compared with the sedentary normal weight subjects.

1.3.3 Specific Aim 3: To examine the effects of chronic exercise on insulin resistance and IMCL in older subjects.

Hypothesis 3.1 Older endurance-trained ‘Masters’ athletes would have greater skeletal muscle insulin sensitivity compared to age matched sedentary normal weight subjects independently of their total body fat content.

Hypothesis 3.2 Higher skeletal muscle insulin sensitivity in Masters athletes would be observed in the context of higher intramyocellular lipids (IMCL) and triglycerides (IMTG) but with lower levels of lipid metabolites, including diacylglycerols and ceramides, compared to sedentary normal weight older subjects.

To test these hypotheses we measured insulin sensitivity, IMCL, IMTG and lipid metabolites in sedentary normal weight older subjects and in Masters athletes.

These hypotheses would be supported if sedentary normal weight subjects have lower insulin sensitivity with a higher content of diacylglycerols and ceramides but lower IMCL and lower IMTG compared to the Masters athletes.

1.4 SIGNIFICANCE

Older adults are at increased risk for the development of type 2 diabetes. In 2006, the prevalence of diagnosed diabetes among people aged 65 to 74 was 12 times that of people younger than 45 years old (18.4% and 1.6%, respectively) (21). Insulin resistance is generally accepted to be a key factor for the development of type 2 diabetes. Age-related insulin resistance has been related to higher levels of intramyocellular lipids and lower oxidative capacity in skeletal muscle. These same phenotypes have also been commonly described as characteristics of obesity and physical inactivity.

The 2003-2006 National Health and Nutrition Examination Survey estimated that about 66% of US adults are overweight or obese. Among men and women of 65 or more years old, the prevalence was 78% and 70% respectively (22). The same survey estimated that 57% of the population over 65 years old was sedentary in terms of their leisure time physical activities (i.e. never engaged in any vigorous, moderate or light physical activities for at least 20 minutes per week). Given that diabetes, obesity and sedentary behavior follow the same epidemiological trend in the aging population, it is important to determine the separate effects of age, obesity and

physical inactivity on insulin resistance. This will help elucidate the etiology of insulin resistance and its underlying mechanisms.

This study will provide novel information concerning the separate effects of chronic exercise, aging and obesity on insulin sensitivity and on IMCL. These hypotheses if confirmed would support the primary role of overweight, obesity and physical activity on insulin resistance in aging. This will be one more step towards the understanding of the separate influences of aging, lifestyle (or decline of physical activity) linked with age. This will provide valuable evidence concerning the importance of physical activity and maintaining a healthy body weight in the prevention of metabolic diseases and with maintaining optimal health in older age.

2 REVIEW OF LITERATURE

2.1 EPIDEMIOLOGY OF AGING, OBESITY, TYPE 2 DIABETES AND PHYSICAL ACTIVITY

Obesity has become a major health care problem in developed countries. In the United States, about 32% of the adult population are obese (body mass index BMI>30kg/m²) and another 33% are overweight (BMI 25-30) (23). This prevalence is proportionately higher in older age groups (24). Even more disturbing is the observation that the incidence of obesity has increased from 14% to 23% to 30% in each of the past decades (25).

Diabetes and the metabolic syndrome (a cluster of metabolic disorders including obesity, type 2 diabetes, hypertension and dyslipidemia) have followed the same pattern (26, 27). In 2005, about 20.8 million persons had diabetes in the U.S., which corresponded to an estimated 7% of the population (3). About 40% of all those diagnosed with diabetes were 65 years of age or older. That same year, the prevalence of diagnosed diabetes among people aged 65-74 (18.5%) was about 12 times that in persons younger than 45 years of age (3). Worldwide, the prevalence of diabetes for all age-groups was estimated to be 2.8% in 2000 with the most important prevalence in the proportion of people >65 years of age (28). Type 2 diabetes accounts for about 90 to 95% of all diagnosed diabetes (3). The impact of the recent increase in incidence allowed the Centers for Disease Control and Prevention to project the total prevalence to more

then double from 2005 to 2050, with an increase of 220% among those aged 65-74 years and 449% among those 75 years or older (29).

In 1997, the National Health Interview Survey conducted by the Centers for Disease Control and Prevention estimated that about 40% of U.S. adults were sedentary, defined as never engaging in any exercises, sports, or physically active hobbies in their leisure time. This survey is conducted yearly in the non-institutionalized civilian population (30). This proportion remained stable between 1999-2000 and 2005-2006. In 2005-2006, the proportion of Americans engaged in regular leisure-time physical activity declined with increasing age from 36% in young adults (18-29 years old) to 22% among older adults (65 years and over) (22). The 1996 Surgeon General's report on physical activity and health (31) emphasized the health benefits of moderate-intensity physical activities, especially everyday activities. In 2001 a new set of questions designed to measure occupational, household, and leisure-time physical activity with a special emphasis on moderate-intensity activities was included in the Behavioral Risk Factor Surveillance System (BRFSS), which is a population-based, random-digit-dialed telephone survey administered to U.S. civilian non-institutionalized adults. The prevalence of meeting the criteria (32) for moderate activity was similar for both men and women (32%), but was lower at older ages. The difference between the youngest (18 to 29 years) group and oldest (>75 years) group in meeting recommendations was slightly greater among women than men: 50% of women aged 18 to 29 vs 27% of women aged 75 or older, and 58% of men aged 18 to 29 vs 38% for men aged 75 or older (33). Population based studies in the USA (34) and in Europe (35) showed that a high level of physical activity is associated with a decreased likelihood of being obese.

2.2 INSULIN RESISTANCE IS RELATED TO OBESITY

Insulin resistance (IR) can be seen as a state in which a cell, an organ, or the whole body, do not respond to normal amounts of insulin and thus requires higher than average levels of circulating insulin to elicit its normal physiological response (36). The staging of type 2 diabetes (T2DM) has been viewed as a continuum beginning with peripheral IR and ending with a loss of insulin secretion (1).

IR involves defects in multiple organ systems - muscle, the liver and adipose tissue- act together to produce abnormal glucose and lipid metabolism (5). Whole body IR can be measured in humans as well as specifically in the muscle, liver and in adipose tissue. Skeletal muscle accounts for approximately 80% of the insulin stimulated glucose uptake. IR in the muscle (37) and in the liver (38) can be present and precede for many years the onset of T2DM (5, 37). IR is the principal mechanism by which obesity is considered to increase the risk for T2DM and is a key feature of the metabolic syndrome.

Research towards understanding how obesity leads to these chronic diseases has commonly included examination of regional adipose tissue depositions in addition to considering generalized adiposity influences on IR. Multiple cross-sectional and longitudinal studies have reported that fat accumulation in different adipose tissue locations have distinct metabolic consequences. Abdominal adiposity has been shown to be positively associated with IR and the metabolic syndrome in obese subjects (39) independently of total adiposity (40). Other adipose tissues depositions have also been shown to correlate with IR, among them the thigh adipose tissue (14). More recently, considerable attention has been paid to lipid accumulation in non adipose cells, i.e.. ectopic fat (41, 42).

2.3 INSULIN RESISTANCE AND ECTOPIC FAT DEPOSITION

Excess accumulation of triglycerides within the muscle fibers (intramyocellular triglycerides, IMTG) is associated with obesity, IR, T2DM and the metabolic syndrome. This ectopic fat deposition (fat that is contained within non adipose tissue such as muscle) have also been associated with aging and physical inactivity. These lipid depositions are not inert; they are metabolically active and may be modified by diet and physical activity.

Skeletal muscle is considered one of the most important sites of IR. In this context, IMTG accumulation has been associated with systemic IR (11, 13, 43). Among sedentary subjects, there is an inverse association between IMTG and insulin sensitivity, furthermore, in one study, IMTG accounted for 32% of the variance of insulin sensitivity (18). Multivariate regression revealed that the association between IMTG and IR was independent of total adiposity and age. Other studies have reported associations between IMTG and obesity (44), with T2DM (15), aging (16) and physical inactivity (17).

2.4 EXERCISE INCREASES INSULIN SENSITIVITY AND INCREASES THE CAPACITY FOR FAT OXIDATION

Fat and carbohydrate oxidation are the dominant sources of aerobic energy production. The proportion of fat or carbohydrate utilization depends on many factors including the metabolic demand, e.g., fasting or exercise, intensity of exercise, the fitness level of the subject, and the availability of exogenous carbohydrates. Chronic physical activity (i.e. exercise training) can enhance skeletal muscle insulin action through many potential mechanisms: increases in glucose

transporters (45), in enzymatic activities (46), in fatty acid metabolism (47) and in mitochondria enzyme activity and number (8).

2.5 EXERCISE, ECTOPIC FAT AND INSULIN RESISTANCE

2.5.1 Intramyocellular triglycerides (IMTG)

Although the basal content of IMTG has been shown in sedentary subjects to be related to IR, chronic exercise has been shown to increase IMTG in parallel with improved IR (18, 48); thus indicating an apparent paradox in the association between IMTG and IR (18). This has led to a view that IMTG accumulation per se does not directly affect insulin action but rather, under certain conditions, may act as a surrogate for other potentially harmful lipid metabolites, diacylglycerols (DAG) (19) and ceramides (Cer) (20), which have been identified as playing an important role in mediating fatty acid induced IR in muscle. It is possible that previous observed associations between IMTG and IR are influenced by the utilization of fatty acids within muscle as is likely the case in athletes. Thus under conditions of high fatty acid utilization, such as chronic exercise, the pool of fatty acids is in constant remodeling, thus preventing the accumulation of more damaging fatty acid metabolites within the cell (49).

Data examining the effects of exercise on IMTG in previously sedentary adults who are at risk for T2DM are limited. Pruchnic et al. demonstrated that previously sedentary older men and women performing 12 weeks of exercise had significant increases in IMTG, although IR was not directly measured in that study (50).

The observation that IMTG are markedly higher in endurance-trained athletes, who are at the same time much more insulin sensitive than in sedentary subjects (18), led to the hypothesis that “*the capacity to use fatty acids, ..., during period of physical activity may mediate the influence of muscle triglycerides on insulin sensitivity*” (12), thus the utilization of IMTG during acute exercise may play an important role in IR. IMTG content in the *tibialis anterior* and the *soleus* muscles is significantly reduced in endurance-trained men (51) and women (52) during 90 to 120 minutes of moderate intensity exercise. A recent study confirmed that IMTG are utilized during exercise in proportion to their content (53). Multiple studies confirmed the decrease in IMTG content after acute exercise in humans (51, 54-56). Replenishment of these stores has also been shown in the post-exercise state, particularly with high fat diets (57, 58). These studies support the concept that skeletal muscle lipids are a source of energy through oxidative metabolism during moderate physical activity (59).

2.5.2 Potential role of mitochondria in the association between intramyocellular lipids (IMCL) and IR

A potential mechanism for IMCL accumulation is a diminished fatty acid oxidation by muscle mitochondria (60). Many studies have reported a reduced rate of fat oxidation in the muscle of IR subjects (61), with a reduction of the enzyme complex transporting activated long chain fatty acids from the sarcoplasm into the mitochondria (carnitine palmitoyl transferase, CPT I and CPT II), whose activity is a key step in the regulation of fatty acid oxidation in muscle. A lower CPT activity has been shown to be proportional to an overall reduction in activities of diverse mitochondrial enzymes participating in the tricarboxylic acid cycle, electron transport chain and beta oxidation (10), thus this may reflect a reduced mitochondrial content, resulting in a reduced

capacity for lipid oxidation (12). Recently, we showed that obese sedentary older individuals could increase fat oxidation during a moderate exercise bout (62). Furthermore, exercise training increased insulin sensitivity, oxidative capacity and IMCL in these previously sedentary older subjects, while at the same time decreasing the fatty acid metabolites DAG and Cer (9), thus suggesting that it may not be IMTG per se but possibly these other lipid metabolites that mediate IR.

2.6 AGE INCREASES INSULIN RESISTANCE

There is a widespread assertion that aging leads to IR (6, 63) which is in turn fundamental to the etiology and higher prevalence of T2DM in older adults (4, 5, 28). The evidence behind the concept of age-associated IR, however, is conflicting. Studies supporting the ‘aging theory,’ which state that age-related insulin resistance is associated with mitochondrial defects (7, 8) are contradicted by reports demonstrating that insulin resistance may not be associated with aging per se but rather with lifestyle patterns linked with aging, such as a reduced physical activity (9) and obesity (10). Thus it is not clear whether insulin resistance is characteristic of aging, or alternatively, whether obesity and/or physical inactivity underlie this ‘aging’ effect.

2.7 AGE, ECTOPIC FAT AND INSULIN RESISTANCE

The relationship between intracellular fat accumulation and age-related IR has been examined in cross-sectional studies. Petersen et al. (7) found that higher IMTG levels in older compared to

younger subjects explained more severe insulin resistance in the former. Two studies (16, 64) suggested that higher IMTG and intrahepatocellular lipids in older men and women may explain age-related IR. This led multiple groups (65, 66) to hypothesize that these ectopic fat stores could explain age-related IR. This theory advocates that IR in the elderly is related to increases in IMTG and potentially other lipid metabolites that may be a result of a reduction in mitochondrial oxidative phosphorylation activity due to a reduction in mitochondrial number and/or function (67). What is not clear is whether this reduction of oxidative capacity is purely age-related or is secondary to acquired conditions that come with age, such as a decrease in physical activity and/or body composition changes.

2.7.1 Age, body composition and insulin resistance

In the Health, Aging and Body Composition (Health ABC) Study, an observational cohort of 3,075 men and women aged 70-79 years, the prevalence of T2DM was 24% and an additional 21% of subjects had impaired glucose tolerance (15). In this cohort, BMI was positively associated with IR. Furthermore two adipose tissue depositions were associated with IR independently of total body obesity: visceral adipose tissue and adipose tissue within the muscle (15). This led to the conclusion that IR in older men and women is strongly influenced by regional fat distribution, and specifically, abdominal fat and accumulation of fat within skeletal muscle. Additional studies in this cohort revealed that the association between body fat distribution and IR was strongest in those of normal weight (68). Similarly, a cross-sectional comparison between young and old subjects showed that the age-related decrease in insulin sensitivity was more strongly correlated with abdominal adiposity than to chronological age

(69). This begs the question of whether or not obesity or fat distribution may be underlying the age-related insulin resistance.

2.7.2 Age, exercise and insulin resistance

Mitochondrial dysfunction has been observed in the elderly compared to younger subjects in terms of reductions of decreased mitochondrial protein synthesis (70) and expression (67), oxidative phosphorylation capacity (7) and mitochondrial enzyme activity (71). However when comparing older to young athletes, some of these differences were no longer observed, with some mitochondrial enzymes actually being higher in master athletes compared with matched young runners despite a higher maximal aerobic capacity in younger athletes (72). Lower maximum heart rates and oxygen uptake in Masters athletes were also found in studies where athletes were matched for training programs (73). In another cross-sectional comparison (74), glucose tolerance and insulin levels were similar in master athletes and in young athletes, which led the authors to conclude that “deterioration of glucose tolerance and insulin sensitivity is not an inevitable concomitant of the aging process and can be prevented by means of regularly performed exercise.” Other studies based on glucose tolerance tests confirmed that regular vigorous exercise can protect against the development of glucose intolerance with advancing age (75, 76).

When given an adequate training stimulus, the skeletal muscle of older previously sedentary subjects undergo adaptations including increased mitochondrial enzymes such as succinate deshydrogenase and citrate synthase (77, 78), increased mitochondrial respiratory capacity (79) and mitochondrial content (8). Similar training programs have also increased similarly aerobic capacity in younger and older former sedentary subjects (80). The effect of

exercise training on IMTG and oxidative capacity has also been shown in older previously sedentary adults. Pruchnic and al. (50) demonstrated that 12 weeks of moderate exercise increased IMTG in older previously sedentary subjects and at the same time increased their oxidative capacity with a shift in muscle fiber type toward more oxidative type I fibers. In this same population, the authors showed that exercise enhanced mitochondrial capacity (8, 81). Recently we found that moderate increases in physical activity in a similar population of older subjects were sufficient to increase IMTG and decrease both DAG and Cer in conjunction with improved insulin sensitivity (9). This begs the question whether or not physical inactivity may be underlying the age-related insulin resistance.

2.8 ADVANCES IN INTRACELLULAR FAT DEPOSITION MEASUREMENT TECHNIQUES

Until recently, the technique of choice to investigate intramyocellular lipid content was the biopsy by biochemical analysis or histochemistry (Oil red O staining) that measure specifically intramyocellular triglycerides (IMTG). However, drawbacks to this procedure include its invasive nature, along with the limitation that the measure is restricted to few muscle groups. Computed tomography (CT) has also been used to quantify adipose tissue infiltration within skeletal muscle (82), but has the limitations of its inability to quantify intramyocellular lipids (IMCL) and its use of ionizing radiation. Magnetic Resonance Proton Spectroscopy (^1H -MRS) is a noninvasive in vivo method that allows quantification of intracellular lipid depositions. MRS directly quantifies the chemical composition of a tissue (55), and can be used in combination with Magnetic Resonance Imaging (MRI) to obtain IMCL using image-guided, selective voxel

MRS. Validation of MRS techniques against direct tissue biopsy measures (biochemical and histological assays) in the muscle (83) has confirmed that ^1H -MRS can quantitatively measure IMCL in vivo. Reproducibility with test/retest protocols has also been documented (84). Due to the advantage of MRS regarding multiple testing and its minimal risk, it is the technique of choice for repeated in vivo measurements of IMCL.

2.9 UNANSWERED QUESTIONS

Based on the above, it is not clear whether the obesity and the physical inactivity effects on IR are underlying any aging effect. In recent (unpublished) data, we found that the insulin sensitivity of obese middle age subjects is not different than obese old subjects ($p=0.54$). Although obesity may be a confounding effect on IR in aging, no studies have yet looked at the effect of age on insulin resistance independently of obesity (Specific Aim 1).

While the association between obesity and insulin resistance is consistent in younger subjects, this may not be true in older adults. Indeed, our recent data indicate that the amount of body fat is not associated with insulin resistance in older subjects ($R^2=0.000001$, $p=0.99$). This lack of association strongly suggests that other factors have an important role in insulin resistance in this particular population. To our knowledge no studies have yet examined the effect of obesity, independently of age, in the older population (Specific Aim 2).

Chronic physical activity (i.e. exercise training) can enhance skeletal muscle insulin action through many potential mechanisms: increases in glucose transporters, in enzymatic

activities, in fatty acid metabolism and in mitochondria enzyme activity and content. Prior studies have shown that young athletes are more insulin sensitive than young sedentary subjects, but the effect of chronic exercise on insulin resistance, independently of obesity, has not been explored in older subjects (Specific Aim 3).

2.10 SUMMARY OF LITERATURE REVIEW

Insulin resistance is a common state associated with acquired conditions such as obesity, physical inactivity and aging. A possible mechanism underlying all of these conditions is the accumulation of lipid depots within muscle. This lipid deposition may be metabolically deleterious only in situations where they are inactive. Furthermore, only certain fatty acid metabolites seem to have a particularly negative effect on insulin action. Studies supporting an age-related mitochondrial decline in conjunction with insulin resistance are in contrast with data showing that obesity and inactivity, that in turn impact body composition and fat depositions, may be underlying this age effect. These controversial findings call for the need to examine the separate effects of aging, obesity and chronic exercise on insulin sensitivity, as well as a key correlates and potential mechanisms of insulin resistance, that is, intramyocellular lipid content.

3 RESEARCH DESIGN AND METHODS

3.1 OVERVIEW OF THE STUDY

3.1.1 Study design

This study was designed to compare and contrast insulin resistance and potential underlying factors for insulin resistance in four groups of subjects that differed according to age, obesity and chronic physical activity levels (Table 1). To examine potential differences in insulin resistance according to age, younger (25 to 45 years of age) and older (60 to 75 years of age) were compared (Comparison 1). To examine potential differences in insulin resistance according to obesity within older subjects, two groups of older (60 to 75 years of age) subjects, one group of normal weight ($\text{BMI} < 26 \text{ kg/m}^2$) and one group of obese ($\text{BMI} > 30 \text{ kg/m}^2$) were compared (Comparison 2). To examine potential differences in insulin resistance according to chronic physical activity (exercise training) levels within older subjects, two groups of older (60 to 75 years of age) normal weight ($\text{BMI} < 26 \text{ kg/m}^2$) subjects, one group of sedentary and one group of competitive athletes were compared (Comparison 3).

Table 1 Group matrix

Middle age Athletes	Middle age normal weight sedentary	Middle age obese sedentary
<i>Older Athletes</i>	<i>Older normal weight sedentary</i>	<i>Older obese sedentary</i>

Diagram illustrating the group matrix with arrows and numbers indicating relationships:

- Arrow 1: Vertical double-headed arrow between Middle age normal weight sedentary and Older normal weight sedentary.
- Arrow 2: Horizontal double-headed arrow between Older normal weight sedentary and Older obese sedentary.
- Arrow 3: Horizontal double-headed arrow between Older Athletes and Older normal weight sedentary.

3.1.2 Subjects

3.1.2.1 Subject recruitment and characteristics

Data from younger normal weight subjects included for this project were obtained from a previous study conducted by Dr. Goodpaster and colleagues. Data obtained in the obese older subjects for this project were obtained from baseline assessments from ongoing weight loss and exercise intervention trials in which I conducted alongside Dr. Goodpaster and his research team. I specifically recruited the older normal weight sedentary subjects and athletes for this project.

The inclusion/exclusion criteria applied to the two newly recruited groups (older normal weight sedentary and older athletes) were generally the same as the two groups that had been previously recruited. To qualify as ‘sedentary’, subjects were currently participating in one or less days per week of a structured exercise session of less than 20 minutes. To qualify as ‘athletes’, subjects were currently participating in five or more structured aerobic exercise session per week for more than one year and competing in their sports at regional or national level. To qualify as normal weight, subjects BMI were $<26 \text{ kg/m}^2$. To qualify as obese, subjects were $> 30 \text{ kg/m}^2$. To qualify as ‘younger’, subjects were between the ages of 25 and 45. To

qualify as ‘older’, subjects were between 60 and 75 years of age. All volunteers were in good general health, nonsmokers, with stable weight, were recruited to satisfy the inclusion/exclusion criteria (see appendix B) and based on their willingness to participate.

Recruitment was performed differently for the sedentary subjects and the athletes. For the older normal weight sedentary adults we used four recruitment methods: IRB approved study fliers (see appendix C) were posted on campus, 1,752 IRB approved letters (see appendix D) were sent to subjects on the ‘Pepper Center Registry’ and to University of Pittsburgh faculty and staff in the 60+ age range, three ads were posted in the ‘UPMC extra’ newsletter and ‘Audix messages’ were posted on two different occasions. For the athletes, 89 IRB approved letters (see appendix D) were sent to athletes on the ‘PA seniors athletes registry’, IRB approved fliers (see appendix C) were posted on campus, in multiple sports (running and bike) shops and were distributed on three local sporting events and expositions (the ‘Great race’, the ‘Tour of PA’ and the ‘Pittsburgh triathlon’), as well as in local cycling and running clubs.

Older obese sedentary subjects were selected among baseline data available from subjects recruited from 2005 to 2007 of the SHELL (*‘Study of Health and Exercise in Later Life’*) and the MIRA (*‘Muscle Insulin Resistance in Aging’*) studies. As the MIRA is an ongoing study, modifications of the IRB protocol, new informed consents, letters and flyers were created and approved to accommodate the two newly recruited groups (see IRB protocol letters of acceptance for modifications and informed consents in appendix E-G). The selection criteria for the older obese sedentary subjects in this cross-sectional comparison are described below (chapter 3.1.2.3).

Younger normal weight sedentary subjects were selected among data from Dr. Goodapster’s earlier study (10, 85). In the inclusion/exclusion criteria the only difference with

the other three groups were that women on oral contraceptives were excluded. The selection criteria for the younger normal weight sedentary subjects in this cross-sectional comparison are described below (chapter 3.1.2.2).

3.1.2.2 Specific Aim 1: To examine the effects of older age on insulin resistance in sedentary subjects.

The subjects for the first cross-sectional comparison are presented in the figure 1. Older normal weight sedentary subjects were compared. Younger normal weight sedentary subjects were selected based on 1) their BMI (>18 and <26 kg/m²), 2) availability of their clamp data, 3) their gender and BMI (± 1.5 kg/m²) for matching with the older normal weight subjects on a 1:1 ratio.

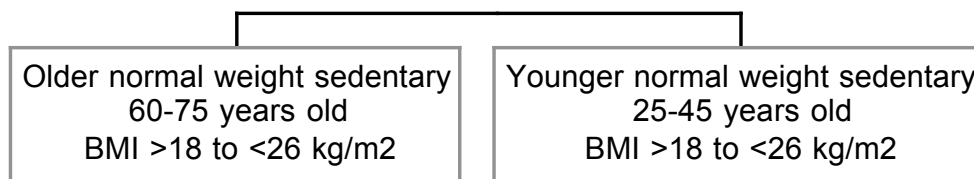


Figure 1 Cross-sectional comparison for Specific Aim 1

3.1.2.3 Specific Aim 2: To examine the effects of obesity on insulin resistance and intramyocellular lipids in sedentary older subjects

The subjects for the second cross-sectional comparison are presented in the figure 2. The same group of newly recruited older normal weight sedentary subjects was used in this comparison. Older obese sedentary subjects were selected based on 1) their BMI (>30 kg/m²), 2) availability of their clamp data, 3) to match gender and age (± 2 years old) with the older normal weight subjects on a 2:1 ratio.

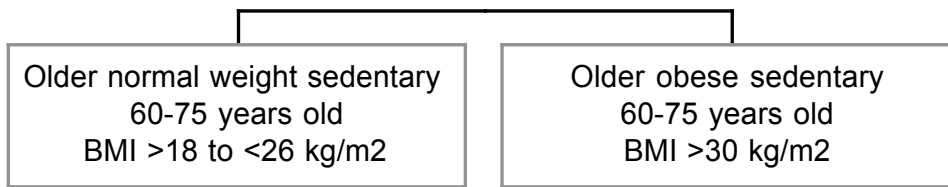


Figure 2 Cross-sectional comparison for Specific Aim 2

3.1.2.4 Specific Aim 3: To examine the effects of chronic exercise on insulin resistance and intramyocellular lipids in older subjects.

The subjects for the third comparison are presented in the figure 3. Older athletes and older normal weight sedentary subjects were recruited. This later group was the same used in the two prior comparisons.

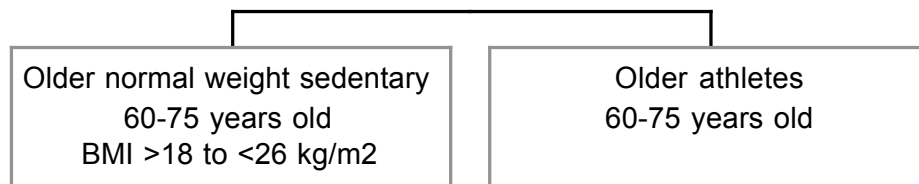


Figure 3 Cross-sectional comparison for Specific Aim 3

3.2 VISITS AND OUTCOME MEASURES

3.2.1 Overall protocol and Visits

Subjects recruited from letters, fliers and ads, were asked to call our research coordinator. After explaining the study, a first consent was solicited thought the phone to perform a 10-minute phone screening including age, height, weight, current level of physical activity and major

illnesses. A description of the study was given. If potentially eligible after the phone screening, the subject was scheduled for the screening visit at the Clinical Translational Research Center (CTRC) that included a history and physical examination (HP), oral glucose tolerance test (OGTT), weight and height. After a detailed explanation of the study and providing written informed consent, and if none of the exclusion criteria were met, the subject was enrolled. The three visits outlined in table 2 were scheduled within the month following the screening visit. Subjects were asked not to modify their physical activity pattern or their diet during this period. Visit 1 and 3 were outpatients visits. Visit 2 was an overnight visit.

Table 2 Plan of visits and measures

Recruitment and phone screening	Screening visit and Consent	Visit 1	Visit 2	Visit 3
Phone interview	HP	DXA	Glucose Clamp	MR Imaging
Phone consent for Screening visit	OGTT	VO ₂ peak	Muscle biopsy	¹ H-MRS
	Consent		Indirect calorimetry	

Visit 1 included body composition and physical fitness measures. Visit 2 included insulin sensitivity measure, indirect calorimetry and the muscle biopsy was performed. Visit 3 included the magnetic resonance imaging (MRI) and spectroscopy (MRS) measures. All these visits were exactly the same for all groups except for visit 3. The 2 newly recruited groups had a magnetic resonance scan for MRI to measure regional fat distribution and MRS, while the other two groups recruited in the past years had their adipose tissue distribution determined by computed tomography.

3.2.2 Endpoint measures

An hyperinsulinemic euglycemic clamp was performed to measure basal insulin sensitivity normalized by fat free mass (FFM) obtained by dual-energy X-ray absorptiometry (DXA). Intramyocellular triglyceride (IMTG) was measured histochemically in the muscle obtained by percutaneous biopsy. The biopsies also served for the measure of other intramyocellular lipids (IMCL), namely DAG and Cer; these were quantified using tandem mass spectrometry. For the newly recruited subjects, the IMCL measure will also be obtained in the *tibialis anterior* with Magnetic resonance spectroscopy (^1H -MRS). Indirect calorimetry was used to derive whole body fat and carbohydrate oxidation. The main outcomes measures obtained are summarized in table 3.

Table 3 Outcome measures

Major Outcome Variables (procedures)	Covariates
Insulin sensitivity (glucose clamp)	Total body fat (DXA)
Intramyocellular lipids (biopsy, ^1H -MRS)	Total lean mass (DXA)
Diacylglycerol and Ceramide (biopsy)	Visceral Adipose Tissue (MRI or CT)
Fat and carbohydrate oxidation (indirect calorimetry)	Proportion of fiber type and oxidative capacity (biopsy)

3.2.3 Details of the visits and measurements

3.2.3.1 Initial Screening Visit

The screening visit took place after an overnight fast at the Clinical Translation Research Center (CTRC) located on the 6th floor of Montefiore Hospital (Pittsburgh, PA). A thorough screening visit description was given to the subject and time was given to ask questions about the screening

procedure. A screening visit written consent was obtained before starting the screening procedures. The following measures were performed:

1) Medical Health History and Physical conducted by a study physician. The physical element of the exam included a standard physical exam including general assessment of the following: Head, ears, nose, eyes, throat, heart, lungs breast, abdomen, genitalia/rectal, extremities, skin, neurological functioning, and any other impressions or problems noted by the physician at the time of exam. Weight was measured on a calibrated medical digital scale (BWB-800; Tanita Corporation, Tokyo, Japan) fasted without shoes and subjects wearing minimal clothing. Height was measured at the same time with a wall-mounted stadiometer. Waist circumference was measured on the subjects standing and relaxing their abdomen, with a tension regulated tape at the midpoint between the lowest rib and the iliac crest on the mid-axillary line. This measurement was performed twice, and the average of the two readings was used. A third measurement was performed if the difference between the two readings was larger than 1 cm.

2) A fasting blood draw for determination of lipid profiles (cholesterol, HDL, LDL, VLDL, triglycerides); electrolytes (sodium, potassium, chloride, carbon dioxide); blood glucose, CBC (Complete blood count); platelet count, HbA1c, kidney function (BUN, creatinine, and urinalysis); TSH, liver function (ALT, AST, Alk. phos). All of these were processed through standard hospital-certified laboratory protocols. Only subjects with values within the exclusion/inclusion criteria (see appendix B) were included in the study.

3) Oral Glucose Tolerance Test - Subjects ingested (within a two minute time period) a 75 gram glucose solution (Glucola). Blood samples were obtained at time 0 (prior to ingestion), 30, 60, 90, and 120 min following glucose ingestion. Sterile saline (1-2 ml) were infused periodically between samples to keep the catheter line patent. Subjects remained at rest at the CTRC until completion of

the test. Plasma glucose was measured in duplicate immediately following sampling using an automated glucose oxidase reaction (YSI 2300 Glucose Analyzer, YSI inc, Yellow Springs, OH). Plasma insulin was measured in batches with an Elisa kit (LINCO Research, St. Charles, MO).

Following this screening visit, subjects were eligible to enter the study if no exclusion criteria were evidenced during the health and physical exam or on the results of the lab tests (see addendum B). Following the American Diabetes Association diagnostic criteria (86), only subjects with normal glucose tolerance (2h OGTT glycemia $<140\text{mg/dl}$) or impaired glucose tolerance (2h OGTT glycemia $\geq 140\text{ mg/dl}$ but $<200\text{mg/dl}$) were included in this study, subjects with type 2 diabetes (2h OGTT glycemia $\geq 200\text{mg/dl}$) were excluded (see consent form for screening in addendum F). Subjects with medical or laboratory abnormalities were referred to their Primary Care Physician for follow-up (see exclusion criteria- appendix B).

A complete study description was given to eligible subjects. Subjects had time to ask questions, and these were answered by the research coordinator, a study physician or the primary investigator (PI). Upon signature, the patient was entered into the study.

3.2.3.2 Dual Energy X-Ray Absorptiometry (DXA) (Visit 1)

At the first testing visit, research subjects were scheduled to have a body composition measurement. This test took place at the Obesity and Nutrition Research Center (ONRC) using a Lunar prodigy scanner and enCORE software 2005 version 9.30 (GE Healthcare, Milwaukee, MI). This DXA scanner is calibrated weekly. A whole body scan was acquired with the subject supine and aligned on the scanner table as prescribed by the manufacturer. The DXA was performed by a certified technician. This test allowed the measurement of their overall body fat mass, lean body mass and bone mineral content. No particular regions of interest were

analyzed. Fat free mass (FFM) was computed by adding their mineral content to the lean body mass, and was expressed in Kg. This was used to express insulin sensitivity relative to FFM.

3.2.3.3 Peak Aerobic Capacity (VO_{2peak}) (Visit 1)

All subjects performed a cycle ergometer peak aerobic capacity test (VO_{2peak}) to determine physical fitness. This test took place at the ONRC. An exercise physiologist with training in Advance Cardiac Life Support conducted all graded exercise tests (GXT). A physician was present during the test. Each subject performed a GXT, lasting 8 to 12 minutes, on an electronically braked cycle ergometer (Sensormedics, Yorba Linda, CA) using a protocol well-suited for elderly individuals with a wide range of exercise capacities. The test consisted of an initial warming-up of 2 min with no load pedaling. The GXT began at 25 Watts for females and 50 Watts for males as used in prior studies (50). Every two minutes the resistance was increased by 25 Watts until volitional exhaustion and one of the established criteria for VO_{2max} had been reached (87). For the athletes, the resistance was increased by 50 Watts for the first three stages followed by 25 Watts increments until the same criteria as above were reached. Heart rate, blood pressure and ECG were recorded before the test, at the end of each two minutes stages and during recovery for at least six minutes. Subjects breathed through a mouthpiece connected to a two-way breathing valve (Hans Rudolph, Kansas City, MO) during the test, and expired air was collected via an open-circuit spirometry to a computerized metabolic cart (Sensor Medics CS 2900, Yorba Linda, CA) to determine oxygen consumption (VO_2) and carbon dioxide production (VCO_2). The metabolic cart analyzed the data every 30 seconds. For safety purposes, our study cardiologist examined all the ECG. Subjects with ECG abnormalities were referred to their Primary Care Physician for follow-up and were excluded from the study if cardiovascular disease was confirmed (see exclusion criteria- appendix B).

3.2.3.4 The Hyperinsulinemic Euglycemic Glucose Clamp (Visit 2)

An hyperinsulinemic euglycemic glucose clamp was performed to determine rates of insulin-stimulated glucose disposal (Rd). This is the gold standard to measure in vivo insulin sensitivity (88). When coupled with stable isotopes it can distinguish and quantify skeletal muscle and hepatic insulin resistance. This procedure was performed at the CTRC under the supervision of the PI. On the evening before the clamp, subjects were admitted in the CTRC. They received a standard dinner (7.5 kcal/kg of body weight, 50% carbohydrates, 30% fat, 20% proteins) and then fasted until completion of the glucose and insulin infusion in the following day. Subjects were instructed not to perform physical exercise 48 hours before the clamp to avoid the acute effects of exercise on insulin sensitivity. On the day of the clamp, at ~5:30 A.M. a catheter was inserted in a prominent forearm vein for later infusion of glucose and insulin. A second catheter was inserted in a retrograde fashion into a dorsal hand or wrist vein of the opposite arm. The hand was warmed by a heating pad to 70°C to “arterialize” the blood. After obtaining basal blood samples, a primed constant infusion of stable isotope of glucose [6,6-2H²] glucose (0.22 µmol/kg, 17.6 µmol/kg prime) 99% enriched (Cambridge Isotopes Laboratories, Andover, MA) was started with a calibrated syringe pump (Harvard Apparatus, Natick, MA), allowing 2 hours for isotopic equilibration before the initiation of the insulin infusion. This was continued during the insulin infusion to determine rates of systemic glucose utilization and to determine insulin suppression of hepatic glucose production. A continuous infusion of insulin (Humulin, Eli Lilly, Indianapolis, IN) was started at ~8 A.M. given at a rate of 40 mU/m²/min for 4 hours, and euglycemia (Target Glucose = 90 mg/dl) was maintained using an adjustable infusion of 20% dextrose (with [6, 6-2H²] additive). Blood samples were obtained every 5 minutes thereafter to determine plasma glucose levels and in order to adjust the infusion rate of the 20% dextrose. Blood samples for measurement of [6, 6-2H²] glucose enrichment were collected every 10 minutes during the

last 40 minutes of the clamp (210, 220, 230 and 240 min). A total of about 300cc of blood (per subject) were taken during each CTTC overnight stay. Following completion of the clamp at ~12 P.M., all subjects were given a lunch.

Plasma glucose and insulin were measured using the same techniques and tools as described for the OGTT. Plasma [6, 6-2H²] glucose enrichment was determined by gas chromatography (Agilent 6890 Series, Agilent Technologies, Santa Clara, CA) coupled to a Mass Selective Detector (Agilent 5973 Network, Agilent Technologies, Santa Clara, CA). The analysis of the glucose penta-acetate derivatives was performed selectively monitoring ions at mass-to-charge 200 and 202.

Rate of plasma glucose appearance and utilization were calculated using the Tracer-Tracee method with the Steele equations (89), as modified for variable rate glucose infusions which contain isotopes (90, 91). As at steady state, the rate of glucose disposal (Rd) is the sum of the hepatic glucose output (HGO) and the glucose infusion rate (GIR). The HGO and the percent suppression of the HGO (from baseline to the end of clamp) were also computed, allowing a measure of liver insulin sensitivity.

3.2.3.5 Indirect Calorimetry (Visit 2)

Indirect calorimetry was used to determine resting energy expenditure (REE), fat and carbohydrate oxidation after an overnight fast and in the insulin stimulated state (i.e. at the end of the hyperinsulinemic euglycemic clamp). Indirect calorimetry, used to measure oxygen consumption (VO₂) and carbon dioxide production (VCO₂), was performed for 30 minutes using an open canopy system (Moxus, AEI Technologies, Pittsburgh, PA). Prior to every test, calibration of both gas and pressure were completed. After the overnight fast at the CTTC, at ~6:00 A.M. subjects were placed under the canopy while resting in bed. They were told to close their eyes and try to sleep, refrain from fidgeting or to perform any type of activity (i.e. watching

TV or reading). The first five minutes of data collection were discarded to allow the computations to be performed on steady state measures. The same protocol was repeated at the end of the hyperinsulinemic euglycemic clamp (minutes 200 to 230).

Systemic fat (Fat-ox) and carbohydrate (Cho-ox) oxidation rates were calculated using the stoichiometric equations of Frayn (92).

Equation 1 Systemic fat and carbohydrate oxidation

$$\text{Fat-ox}_{(\text{mg/min})} = 1.67 \text{ VO}_{2(\text{ml/min})} - 1.67 \text{ VCO}_{2(\text{ml/min})}$$

$$\text{Cho-ox}_{(\text{mg/min})} = 4.55 \text{ VCO}_{2(\text{ml/min})} - 3.21 \text{ VO}_{2(\text{ml/min})}$$

The Fat-ox value were then transformed into kcal/min and expressed as a proportion of energy derived from fat (EF).

Equation 2 Proportion of Energy derived from fat

$$\text{EF}_{(\%)} = (\text{Fat-ox}_{(\text{kcal/min})} / \text{REE}_{(\text{kcal/min})}) * 100$$

Protein oxidation rates were not included based on our prior work demonstrating that rates of urinary nitrogen excretion were similar in lean and obese subjects during resting conditions (93) and on the assumptions that the amount of proteins oxidized and other metabolic processes (such as gluconeogenesis from proteins, ketone body formation and lipogenesis) during resting and insulin stimulation (i.e. in the fed state) are quantitatively negligible compared to glucose and fatty acid oxidation (94).

3.2.3.6 Percutaneous Muscle Biopsy (Visit 2)

Percutaneous muscle biopsies were obtained at the CTRC at ~7 A.M. thus following the overnight fast and in the same standardized conditions as described for the clamp, with a standardized meal for dinner and no exercise in the last 48 hours to prevent acute effects of exercise on IMCL (59, 95). Muscle biopsies were obtained using 2% buffered lidocaine for anesthetic, a ~ 0.5 cm incision was made in the skin from the middle region of the vastus lateralis (15 cm above the Patella) and ~2 cm away from the fascia by percutaneous needle biopsy technique using previously published methods (10, 14, 18) adapted from Evans (96). About 100-150 mg of muscle were obtained. The incision was closed with sterile adhesive strips, and a pressure bandage was applied for a period of 24 hours.

Specimens were trimmed of any visible adipose tissue, mounted in OCT mounting-medium (Miles, Inc., Elkhart, IN), and frozen in isopentane cooled at -160°C by liquid nitrogen and stored at -80°C for histochemical analysis. All specimens were stored at -80° C refrigerators.

Histochemical analyses were performed at the ONRC as previously described (97, 98). Frozen tissue blocks were sectioned at -20°C (Cryotome E; Shandon Scientific, England) 10 µm thick and placed on individual pre-cleaned glass slides. Initial sections from each frozen muscle block were inspected without stain to ensure that proper cross-sectional cuts were being obtained. Muscle sectioning, staining and image analysis were performed by the same technician and done in a blinded manner with respect to subject. Each analysis included data from 80 to 300 fibers, and intra-assay variability was <5% (9).

IMCL was stained with Oil Red O soluble dye, which stains neutral lipid (mainly intramyocellular triglycerides; IMTG) (14, 18). After staining, a light microscope (Leica DM 4000B, Leica Microsystems, Wetzlar, Germany) was used to examine the stained muscle

sections, using a x40 oil immersion objective and bright field settings. Images were digitally captured using a charge-coupled device camera (Retiga 2000R camera; Q Imaging, Surrey, BC, Canada) and converted to 16-bit gray-scale images. Contiguous fields of view within the biopsy section that were free from artifact were analyzed for lipid content. Semi-quantitative image analysis (Northern Eclipse software v.6.0, Empix Imaging, North Tonawanda, NY) was then carried out on at least 80 fibers, or ~10 contiguous fibers/field and normalized to background. A control section treated with acetone and subsequently stained revealed no visible background staining. After normalization to the background, the ORO value was expressed as an arbitrary unit (AU).

Equation 3 Arbitrary unit for ORO

$$\text{ORO}_{(\text{AU})} = (\text{ORO} / \text{mean ORO for all subjects in the analysis})$$

Succinate dehydrogenase (SDH) staining (99, 100) was used as a marker for oxidative capacity of muscle. A stock solution was made with PBS, nitro blue tetrazolium, KCN, MgCL and distilled water as described elsewhere (50). This stock solution was then added into a working solution containing sodium succinate and menadione. Sections were incubated for 45 minutes at room temperature then washed in distilled water. Quantification of SDH staining was performed using image analysis of staining intensity and normalized to background in the same manner as ORO staining. As a negative control, sections were incubated in media without the enzyme substrate succinate. SDH values were expressed in AU as described for ORO.

The proportion of type I slow oxidative and type II fast glycolytic muscle fibers were determined using immunohistochemistry as described elsewhere (9, 50). The slides were incubated with antibodies specific for type I and type IIa fibers (Santa Cruz Biotechnology,

Santa Cruz, CA). Signals for fiber specific were recorded with sulforhodamine 101 acid chloride (Texas Red) (type I) and fluoresceine isothiocyanate (FITC) excitation filters (type IIa). Approximately 100-300 total fibers were manually counted and relative proportions were calculated.

Quantification of intramuscular lipid metabolite diacylglycerols (DAG) and ceramides (Cer) were performed using high-performance liquid chromatography tandem mass spectrometry (101) at the Lipidomics Core, Medical University of South Carolina. This allows quantitative analysis of different species of DAG and Cer. Tissue homogenates (in buffer containing 0.25 M sucrose, 25 mM KCl, 50 mM Tris, 0.5 mM EDTA, pH 7.4) were fortified with internal standards and extracted into a one-phase neutral organic solvent system (ethyl acetate/isopropyl alcohol/water; 60:30:10 v/v/v), evaporated and reconstituted in methanol and analyzed by a surveyor/TSQ 7000 liquid chromatography/mass spectrometry system (Thermo Finigan, Thermo Fisher Scientific Inc., Waltham, MA) (102). Quantitative analysis was performed in a positive multiple-reaction monitoring mode, based on calibration curves generated by adding to an artificial matrix known amounts of target analytes, synthetic standards, and an equal amount of internal standard. The DAG and Cer levels were normalized to total protein levels (1mg of protein/sample).

3.2.3.7 Magnetic resonance spectroscopy ($^1\text{H-MRS}$)(visit 3)

The two newly recruited groups (older normal weight sedentary and older athletes) underwent $^1\text{H-MRS}$ to measure intra-myocellular lipid (IMCL). Scanning was performed on the 3.0T scanner (Siemens 3T Trio, Siemens Medical Systems, Erlanger, Germany) at the University of Pittsburgh Magnetic Resonance Research Center (MRRC) located on the 8th floor of the Presbyterian hospital.

IMCL was measured using the extremity coil. The inferior extremity of patella served as landmark for the placement of the coil. IMCL concentration levels were measured in a single $11 \times 12 \times 18 \text{ mm}^3$ Voxel placed in the right *tibialis anterior* in a position such as to limit as much as possible visible interstitial tissue or fat (figure 4).

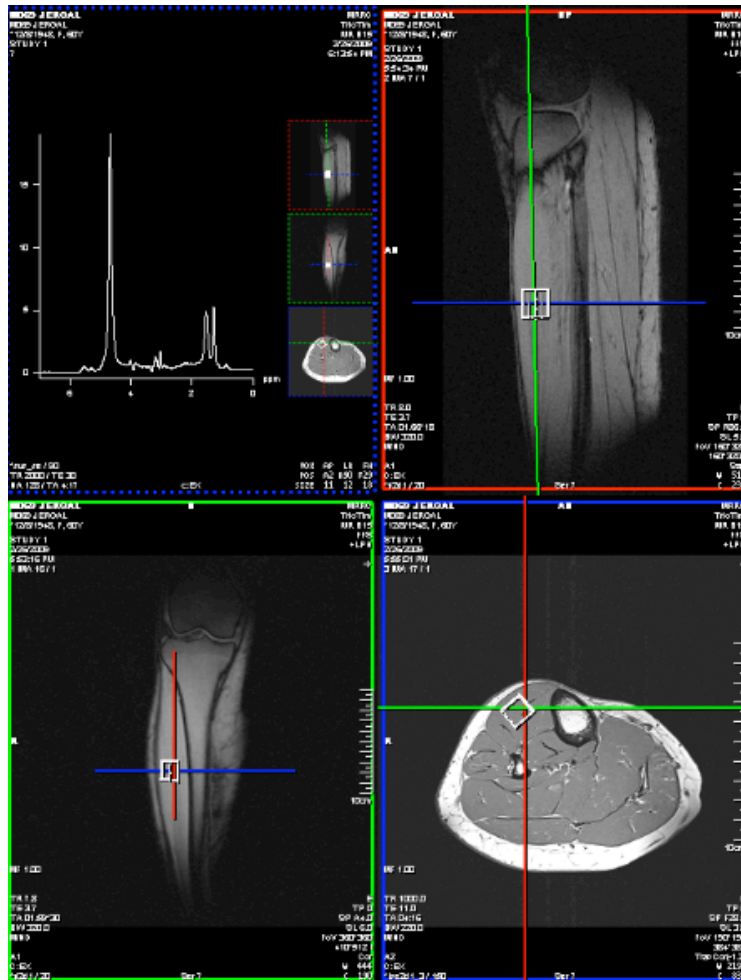


Figure 4 Example of voxel placement in the tibialis anterior (Siemens 3Tesla, PRESS TR3s TE 30 ms $11 \times 12 \times 18 \text{ mm}^3$, extremity coil)

The acquisition used an optimized PRESS sequence (repetition time = 3 s, echo time = 20 ms, 128 acquisitions, water presaturation) (53, 103). To assess the quality of the acquisition, the following criteria were used (figure 5): 1) the splitting of resonances of creatine CH₂ at 3.96 ppm (Cr2, peaks 14 and 15) (104), 2) presence of trimethylammonium (TMA, peak 12) and of the central peak of creatine CH₃ (peak 10), 3) the separation of EMCL-CH₂ and IMCL-CH₂ (respectively peaks 4 and 3) (105).

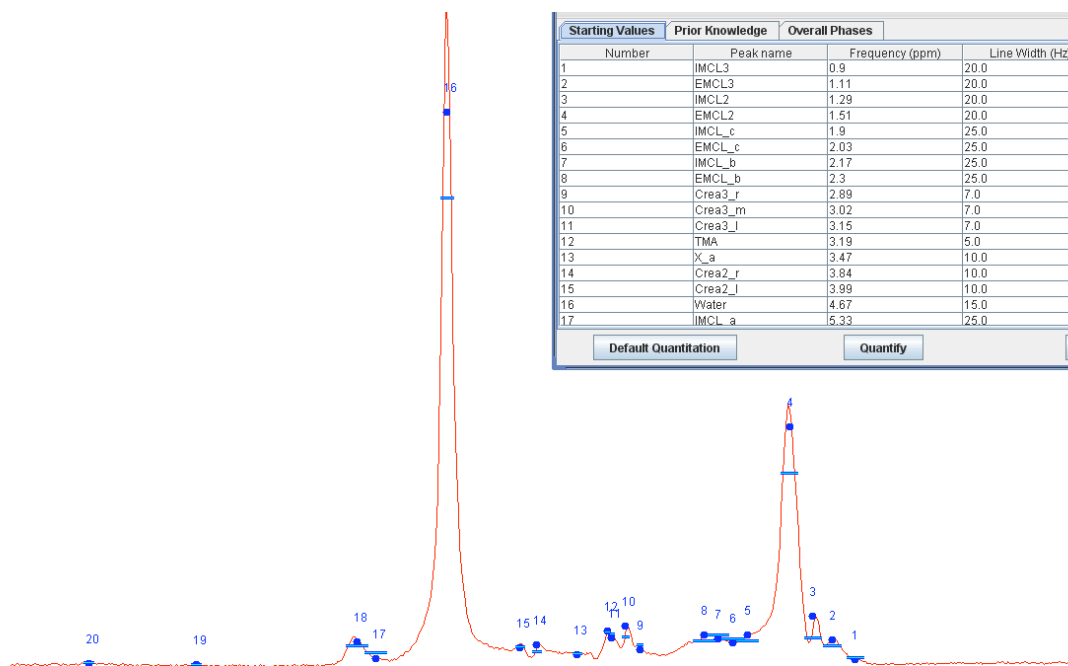


Figure 5 ¹H-MRS Spectrum of a single voxel in the muscle tibialis anterior (Siemens 3Tesla, PRESS TR3s TE 30 ms 11x12x18mm³, extremity coil)

After phasing and referencing the raw spectra (to the water peak at 4.7 ppm), IMCL was quantified by using the unsuppressed water signal as internal concentration standard. Fitting of the spectra was done with jMRUI software version 2.2 (<http://www.mrui.uab.es/mrui/>), using published prior knowledge (106) calibrated via the unsuppressed water signal. To assess the

quality of the fit, the following criteria were used 1) the smallest residues possible and 2) the width of the IMCL-CH₂.

Absolute IMCL levels in millimoles per kilogram muscle wet weight were calculated using the area of the IMCL resonance, the area of H₂O resonance and a correction factor based on the concentration of water in the muscle, the correction for different voxel sensitivity, the correction for relaxation, the number of resonating protons as described in table 4.

Table 4 Numbers used for the determination of absolute concentrations of IMCL

Concentration of H₂O muscle		
MR Visibility (106)	76%	[%]
Density of tissue [wet weight] (106)	1.06	[g/ml]
Density of H ₂ O muscle	1	[g/ml]
Molecular weight of H ₂ O muscle	18	[g/mol]
Concentration of H₂O muscle	39832	[mmol/kg ww]

Correction for different voxel sensitivity		
Sensitivity in H ₂ O voxel	1	[au]
Sensitivity in IMCL voxel	1	[au]
Correction factor for H₂O/IMCL sensitivity	1	

Correction for relaxation and number of resonating protons		
Number of protons of resonating in H ₂ O signal	2	
Number of protons of resonating in IMCL signal	62	
Repetition time TR	2000	[msec]
Echo time TE	30	[msec]
Relaxation time T1 of H ₂ O signal(107)	900	[msec]
Relaxation time T2 of H ₂ O signal(107)	29	[msec]
Relaxation time T1 of IMCL signal(107)	382	[msec]
Relaxation time T2 of IMCL signal(107)	68	[msec]
Correction factor for H ₂ O decay/saturation	0.317	
Correction factor for IMCL decay/saturation	0.640	
Correction factor for relaxation and number of protons	0.016	

Overall correction factor	636	[mmol/kg ww]
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3.2.3.8 Magnetic resonance imaging (MRI) or Computed Tomography (CT) (visit 3)

The two newly recruited groups (older normal weight sedentary and older athletes) underwent MRI to measure visceral adipose tissue (VAT). Scanning was performed during the same MR session than the ¹H-MRS at the University of Pittsburgh Magnetic Resonance Research Center (MRRC). The two groups recruited in the prior years (older obese sedentary and middle age normal weight sedentary) had their imaging performed with CT. These scans were performed with a GE Hi-speed multiple slice scanner (GE Healthcare, Waukesha, WI, USA) at Center Commons MRI and CT office offsite in Shadyside.

For the MRI, VAT and total abdominal fat were measured from a single slice abdominal T1-weighted image at the L3-L4 level acquired during a breath hold. MIPAV version 1.2 (Medical Image Processing, Analysis and Visualization, Center for Information Technology, National Institute of Health, Bethesda, MD) was used to analyze the central slice. First the different perimeters (whole abdomen, visceral area) were drawn and the surfaces were recorded. Then the solid organs (liver and kidneys) were removed if present on the slice of interest. To separate adipose tissue from lean tissue, an automated fuzzy c means algorithm (figure 6) was used in conjunction to inhomogeneities corrections (108, 109).

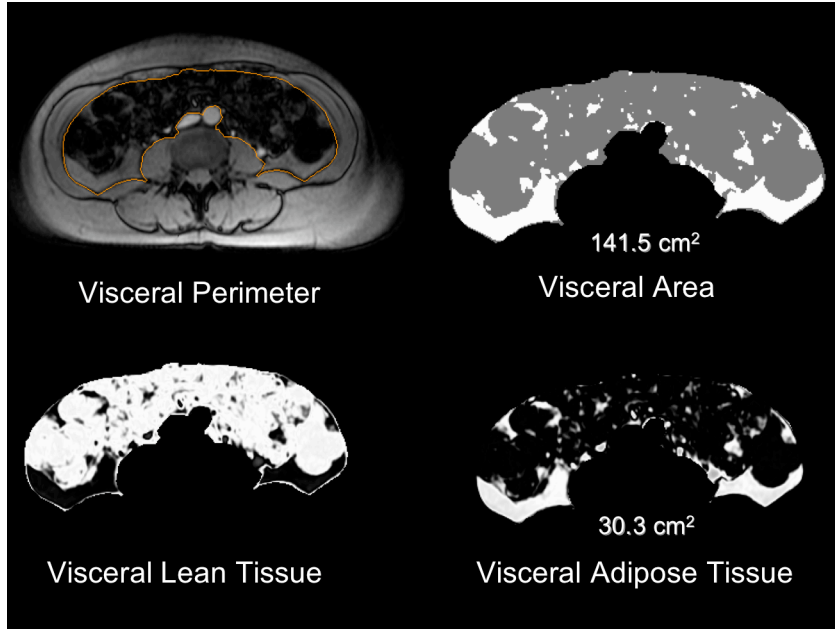


Figure 6 Example of Fuzzy C means algorithm for Abdominal MRI analysis (Siemens 3T, body coil, TR 34ms, TE 3.69ms, slice thickness 10 mm)

This automated pixel clustering algorithm identified light and dark pixels by comparing pixel intensities defined by a specified number of classes. We used two classes: adipose tissue (light pixels) and muscle (dark pixels). This approach removed subjectivity and enhanced reproducibility (110).

The CT images were analyzed using SliceOmatic software version 3 (Tomovision, Montreal, QC, Canada). Adipose tissue was measured by selecting the regions of interest as defined by Hounsfield Units of attenuation (HU) which are based on a linear attenuation coefficient scale using water as a reference (0 HU), thus allowing a quantitative scale for radiodensity (111).

Due to the difference of technique, the following ratios were computed to compare CT and MR data: visceral adipose tissue/total abdominal adipose tissue (VAT/TAAT) and subcutaneous abdominal adipose tissue/total abdominal adipose tissue (ScAAT/TAAT).

3.3 DATA ANALYSIS

3.3.1 Data Collection, management and quality control

After all of the analysis (histology, Mass spectroscopy), readings (MRI), post-processing (MRS) and computations (stable isotopes and clamps) were performed, all data was entered in a main database and de-identified to protect subject identity. Data management and quality control were thoroughly performed by comparing results of the different measures to literature results or when not available (^1H -MRS) by discussing the methods and results with experts in the field.

3.3.2 Descriptive and exploratory analysis

Frequencies and percentages were used to summarize the dichotomous and categorical variables. Continuous variables were summarized by the mean, standard error of the mean, minimum and maximum values. The distributions of each key variables were examined for differences, to identify outliers and to determine whether transformation were required. The distributions were viewed using histograms for general shape and skewness. Boxplots were used to identify outliers. Bivariate correlations and scatterplots were also examined.

3.3.3 Statistical analysis Specific Aim 1: To examine the effects of older age on insulin resistance in sedentary subjects

This aim compared normal weight sedentary older subjects vs. normal weight sedentary younger subjects in their insulin sensitivity. It was hypothesized that the older normal weight sedentary

subjects would be more insulin resistant, i.e., have lower insulin sensitivity than younger normal weight sedentary subjects.

The main analysis for this aim was based on an independent t test with rate of glucose disposal (Rd) as the dependent variable of insulin sensitivity and group as the independent variable.

Other group differences were also studied with independent t test. These included subjects' characteristics (i.e. age, weight, BMI, systolic and diastolic blood pressure), blood results (lipid panel, fasting glucose), body composition data (i.e. FFM, FM, fat%), VO_{2peak} , HGO, REE and the abdominal imaging ratios (i.e VAT/TAAT and ScAAT/TAAT). If the assumption of equal variances (Bartlett test) were not met, comparisons between groups for these specific variables were performed using the Welch ANOVA for the means test, which is equivalent to an unequal variance t test. Although this test is robust for non-normality, if the assumption of normality (Shapiro-Wilk test) was not met, the results of the Welch test were compared to the non parametric Median test.

3.3.4 Statistical analysis Specific Aim 2: To examine the effects of obesity on insulin resistance and intramyocellular lipids in sedentary older subjects

This aim compared insulin sensitivity and IMCL content in normal weight sedentary older subjects vs. obese sedentary older subjects. It was hypothesized that the obese subjects would be more insulin resistant compared to normal weight subjects matched by age and physical activity. It was also hypothesized that the lower skeletal muscle insulin sensitivity in the obese subjects would be observed in the context of higher IMCL, including IMTG, DAG and Cer.

The main analysis for the first hypothesis of this aim was based on an independent t test with Rate of glucose disposal (Rd) as the dependent variable and group as the independent variable. Other group differences were also studied with independent t test. These included subjects' characteristics (i.e. age, weight, BMI, systolic and diastolic blood pressure), blood results (lipid panel, fasting glucose, HbA1c), body composition data (i.e. FFM, FM, fat%), VO_2peak , HGO, REE, fat- and cho-ox and the abdominal imaging ratios (i.e VAT/TAAT and ScAAT/TAAT). If the assumption of equal variances (Bartlett test) were not met, comparisons between groups for these specific variables were performed using the Welch ANOVA for the means test, which is equivalent to an unequal variance t test. Although this test is robust for non normality, if the assumption of normality (Shapiro-Wilk test) was not met, the results of the Welch test were compared to the non parametric Median test. In addition, to look at the possible covariance effect of VAT, an analysis of covariance on Rd x group was performed with VAT as the covariable and another one with VAT/TAAT as the covariable.

For categorical dependent variables such as gender, metabolic status (normal glucose tolerance vs. impaired glucose tolerance) and impaired fasting glucose vs. normal fasting glucose, contingency analyses with the Pearson Chi-square test were performed to assess for differences of proportions between the two groups.

As REE, Fat-ox and Cho-ox were assessed in the fasting condition and at the end of the glucose clamp, the switch from the fasting to the insulin-stimulated condition for each of these variables was assessed with one tail paired t test for each group and with a 2x2 repeated measures ANOVA (group x time).

For the second hypothesis of this aim, we performed separate independent t tests to compare IMTG, total DAG and Cer between the two groups. Other group differences were also

studied with independent t test. These included percentage of fiber types, SDH, and the different species of DAG and Cer. The assumptions were checked as described above and the same statistical tests were performed if the assumptions were not met.

3.3.5 Statistical analysis Specific Aim 3: To examine the effects of chronic exercise on insulin resistance and IMCL in older subjects

This aim compared insulin sensitivity and IMCL content in older endurance trained Masters athletes vs. normal weight sedentary older subjects. It was hypothesized that the athletes would have greater insulin sensitivity compared to sedentary subjects matched by age and total body fatness. It was also hypothesized that the higher skeletal muscle insulin sensitivity in the athletes would be observed in the context of higher IMTG content but with lower levels of DAG and Cer.

The main analysis for the first hypothesis of this aim was based on an independent t test with Rate of glucose disposal (Rd) as the dependent variable and group as the independent variable. Other group differences were also studied with independent t test. These included subjects' characteristics (i.e. age, weight, BMI, systolic and diastolic blood pressure), blood results (lipid panel, fasting glucose, HbA1c), body composition data (i.e. FFM, FM, fat%), $\text{VO}_{2\text{peak}}$, HGO, REE, fat- and cho-ox, VAT and the abdominal imaging ratios (i.e VAT/TAAT and ScAAT/TAAT). The assumptions were checked as described for the second specific aim and the same statistical tests were performed if the assumptions were not met. In addition, to look at the possible covariance effect of body fatness, an analysis of covariance on Rd x group was performed with fat mass as the covariable and another one with percent body fat as covariable. As described for the specific aim 1, the categorical variables (gender, metabolic status, fasting

glucose status) were analyzed with contingency tables. In addition, the switch from fasting to insulin-stimulated condition (REE, Fat-ox, Cho-ox) was assessed by paired t test and 2x2 mixed ANOVA as described for the specific aim 2.

For the second hypothesis of this aim, we performed separate independent t tests to compare IMCL measured by ¹H-MRS, IMTG measured by ORO staining, total DAG and Cer between the 2 groups. Other group differences were also studied with independent t test. These included percentage of fiber types, SDH, and the different species of DAG and Cer. As for the previous analyses, the assumptions were checked as described above and the same statistical tests were performed if the assumptions were not met.

3.3.6 Secondary analyses – Differences in insulin sensitivity and intramyocellular lipids among older athletes, normal weight and obese sedentary subjects.

Differences in Rd between the three older groups (athletes, normal weight sedentary and obese sedentary subjects) were performed with a one-way between subjects ANOVA with post hoc analysis (Tukey HSD adjustment).

To examine patterns of differences of the molecular species of DAG and Cer, one-way between subjects ANOVAs were performed for each molecular species. This allowed us to combine species in three primary sub-groups: “group I” were the species found significantly higher in the obese group, “group II” were the species found to be significantly higher in the athletes and “group III” were not significant or neither higher in the obese nor in the athletes.

3.3.7 Statistical tools

The descriptive and exploratory examination was performed using JMP for Macintosh version 5.2.1.2 (SAS Institute Inc., Cary, NC), which allows the identification of each point in the scatterplots and histograms. Parametric tests and covariates analysis were also performed with JMP. The non-parametric tests and transformations were performed using Stata for Macintosh version 10.0 (StataCorp, College Station, TX). Analyses of covariance (ANCOVA) were performed using manually entered syntax with SPSS for Macintosh version 16.0.2 (SPSS inc. Chicago, IL).

For all analyses, the α level was set *a priori* at 0.05. All statistics were carried out in a 2-tailed manner.

3.3.8 Sample size and Power calculations

All the aims in this study included the primary dependent variable of insulin sensitivity. Based on previous studies we were able to estimate sample sizes for the specific aims 2 and 3. It is important to note that our focus was on the recruitment needs for the two newly recruited subject groups: sedentary normal weight older subjects and older athletes.

For the second comparison, sedentary normal weight older subjects vs. sedentary obese older subjects, we based our power calculations on results of a former published study of Dr. Goodpaster (85) comparing young sedentary obese subjects (N=32, mean Rd 5.87, SD 3.11 mg/min/kg_{FFM}) to young sedentary lean subjects (N=15, mean Rd 9.39, SD 2.75 mg/min/kg_{FFM}). Based on a paired t test with a two-sided significance level set at P=0.05, with a power of 0.80, we estimated that we needed to recruit 9 subjects in both groups. With an estimation of 25%

drop-out rate during the measurement period, we projected to recruit 11 subjects for the sedentary normal weight older group. The sedentary obese older subjects were matched as described in chapter 3.1.2.3. These calculations were only based on insulin sensitivity measures.

For the third comparison, sedentary normal weight older subjects vs. Masters athletes, we based our power calculations on results of a former published study (112) comparing young sedentary normal weight subjects (N=13, whole body insulin sensitivity index 5.49, SD 2.46) to young athletes (N=23, whole body insulin sensitivity index 9.77, SD 3.87). Based on a paired t test with a two-sided significance level set at $P=0.05$ and a power of 0.80, we estimated the need to recruit 7 subjects in both groups. With an estimation of 25% drop-out rate during the measurement period, we anticipated to recruit 9 subjects for the master athletes. These calculations were only based on insulin sensitivity measures.

For the first comparison, sedentary normal weight older subjects vs. sedentary normal weight younger subjects, we decided to use the sample size estimated for the specific aim 2 (see above) and match the number of subjects with an equivalent number of the sedentary normal weight younger subjects (see chapter 3.1.2.2).

4 RESULTS

4.1 RECRUITMENT

As described in chapter 3.1.2.1., I specifically recruited the older normal weight sedentary subjects and the athletes for this project. Figure 7 presents the exact numbers and exclusion reasons for the older normal weight sedentary subjects. It is important to note that in order to maintain gender balance in this group, recruitment was stopped earlier for women.

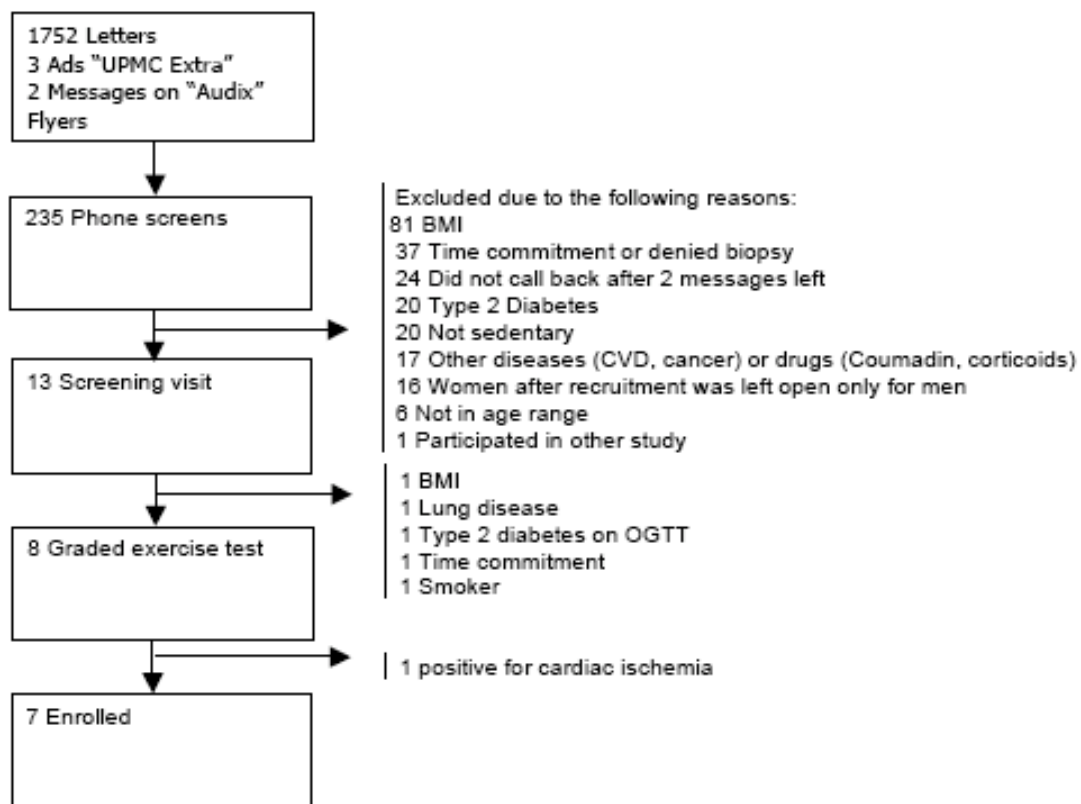


Figure 7 Recruitment flow chart for the older normal weight sedentary subjects

Figure 8 presents the exact numbers and exclusion reasons for the athletes. In order to maintain gender balance across comparison groups, potential male athletes responding to recruitment ads were not enrolled for the last 6 months of the recruitment period.

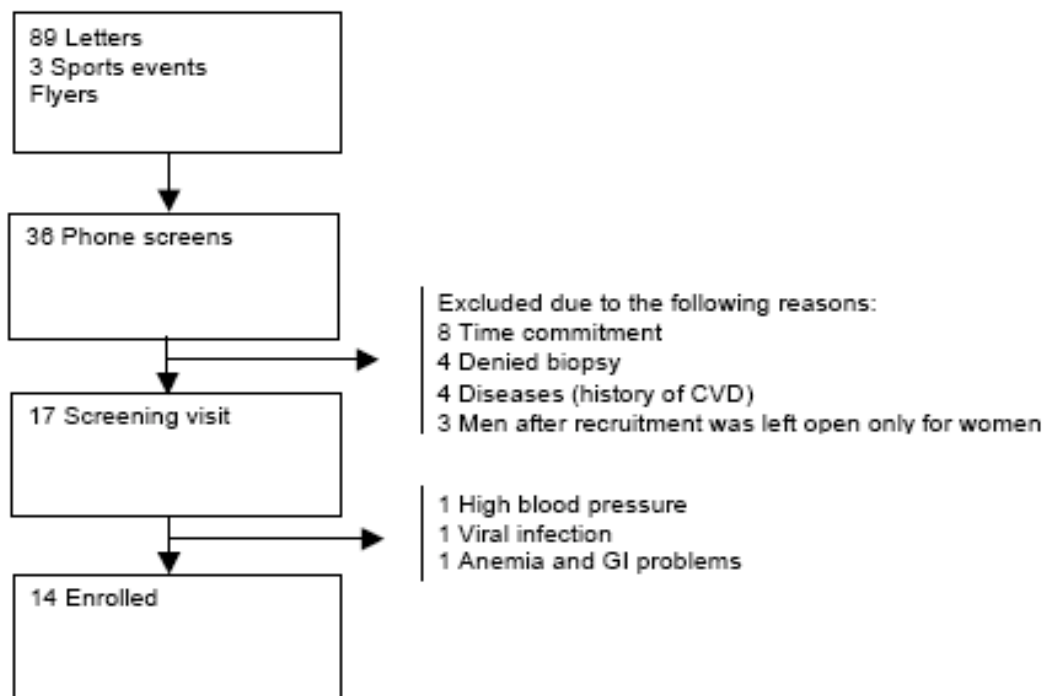


Figure 8 Recruitment flow chart for the athletes

4.2 RESULTS FOR SPECIFIC AIM 1: TO EXAMINE THE EFFECTS OF OLDER AGE ON INSULIN RESISTANCE IN SEDENTARY SUBJECTS

4.2.1 Subjects

A total of seven (4 women and 3 men) older normal weight sedentary subjects were enrolled and completed the testing procedure. Seven younger normal weight sedentary subjects were selected among data from Dr. Goodpaster's earlier study matched on gender and BMI to the older group as described in chapter 3.1.2.2. The characteristics for these two groups are presented in table 5.

Table 5 Specific aim 1 - Subject characteristics

	Older normal weight sedentary	Younger normal weight sedentary
N	7	7
Age (years)	66.5 ± 1.6	34.6 ± 2.4 *
Gender	4W/3M	4W/3M
BMI (kg/m ²)	24.5 ± 0.4	23.6 ± 0.6
Weight (kg)	70.4 ± 2.9	67.5 ± 3.1
Data expressed as mean ± SE. * P<0.05.		

The older and younger groups did not differ with respect to physical fitness, fasting glucose and insulin or lipid profile (table 6). On average both groups had a normal fasting glucose, although two subjects in each group were identified as having impaired fasting glucose (fasting glucose > 100 mg/dl), and one subject in the older group had impaired glucose tolerance (see chapter 3.2.3.1). The older subjects had on average more body fat than the younger subjects. Abdominal fat distribution was similar between the two groups. Interestingly, the fat free mass was not significantly different between the two groups. The younger subjects had higher resting

energy expenditure values than the older subjects, but this was not significantly different when expressed relative to fat free mass. Systolic blood pressure was elevated in the older compared to the younger, but diastolic blood pressure was similar.

Table 6 Specific aim 1 - Physical fitness, body composition, blood pressure, fasting glucose and insulin, lipid panel, resting energy expenditure and abdominal fat distribution

	Older normal weight sedentary	Younger normal weight sedentary
VO ₂ peak (l/min)	1835.3 ± 270.6	2041.4 ± 149.6
VO ₂ peak (ml/min/kg _{FFM})	39.1 ± 3.5	41.1 ± 1.4
Fat% (%)	34.6 ± 3.6	25.6 ± 2.6 [#]
Fat mass (kg)	23.7 ± 2.1	16.8 ± 1.5 [*]
FFM (kg)	46.12 ± 4.2	49.7 ± 3.5
SBP (mmHg)	141.0 ± 4.1	112.9 ± 4.1 [*]
DBP (mmHg)	76.7 ± 2.5	74.2 ± 2.7
Fasting glucose (mg/dl)	82.2 ± 4.2	88.0 ± 3.1
Fasting insulin (μU/ml)	4.5 ± 0.9	6.3 ± 0.8
NFG/IFG	5/2	5/2
NGT/IGT	6/1	7/0
Total cholesterol (mg/dl)	180.1 ± 7.8	200.0 ± 15.9
HDL cholesterol (mg/dl)	55.3 ± 4.7	51.5 ± 4.0
LDL cholesterol (mg/dl)	110.6 ± 7.6	126.2 ± 16.1
VLDL cholesterol (mg/dl)	14.3 ± 1.9	20.7 ± 4.7
Triglycerides (mg/dl)	91.0 ± 11.9	111.5 ± 26.3
REE (kcal/24h)	1271.9 ± 74.7	1557.1 ± 46.9 [*]
REE/FFM (kcal/24h/kg _{FFM})	28.3 ± 1.6	31.9 ± 1.4
VAT (cm ²)	105.3 ± 30.7	67.4 ± 11.2
TAAT (cm ²)	359.5 ± 64.3	262.9 ± 25.8
VAT/TAAT	0.28 ± 0.06	0.27 ± 0.06
ScAAT (cm ²)	254.2 ± 50.2	196.0 ± 26.5
ScAAT/TAAT	0.72 ± 0.06	0.74 ± 0.05
Data presented as mean ± SE. [*] P<0.05, [#] P 0.05 to <0.1. Abbreviations in Appendix A.		

4.2.2 Insulin sensitivity

Insulin sensitivity was not different between the older and the younger normal weight sedentary subjects (figure 9). It is important to note that one subject from the older group was identified as a possible outlier (far outside the upper whisker of the boxplot) with an Rd of 14.29

ml/min/kg_{FFM}, The range of Rd for the other subjects in the older normal weight sedentary group was from 4.62 to 9.84 ml/min/kg_{FFM}, and for the younger normal weight sedentary group Rd ranged going from 5.7 to 9.59 ml/min/kg_{FFM}.. The outlier was confirmed by a Grubbs outlier test (P<0.05). All of the analyses revealed that the results were similar with or without this outlier (for all the specific aims). Therefore, this data point was maintained in the dataset. To illustrate how this data point influences the mean of the older group, figure 6 presents the comparison with and without this outlier.

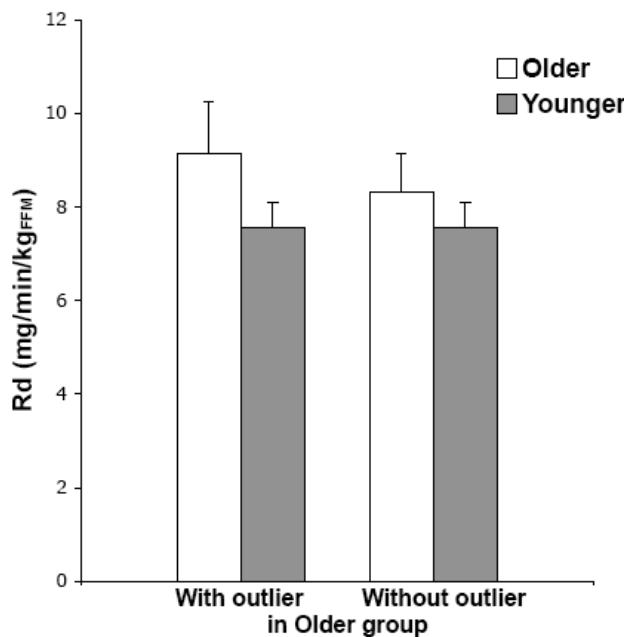


Figure 9 Specific aim 1 - Insulin sensitivity (Rd = rate of glucose disposal, FFM = fat free mass)

4.3 RESULTS FOR SPECIFIC AIM 2: TO EXAMINE THE EFFECTS OF OBESITY ON INSULIN RESISTANCE AND INTRAMYOCYLLUYLAR LIPIDS (IMCL) IN SEDENTARY OLDER SUBJECTS

4.3.1 Subjects

The same group of seven (4 women and 3 men) older normal weight sedentary subjects was used for this comparison ('Normal weight'). Fourteen older obese sedentary ('Obese') subjects were selected among the baseline data available from the SHELL and the MIRA studies as described in chapter 3.1.2.3. The subject characteristics for these two groups are presented in table 7.

Table 7 Specific aim 2 - Subject characteristics

	Older normal weight sedentary	Older obese sedentary
N	7	14
Age (years)	66.5 ± 1.6	67.2 ± 1.3
Gender	4W/3M	8W/6M
BMI (kg/m ²)	24.5 ± 0.4	33.6 ± 0.5 **
Weight (kg)	70.4 ± 2.9	93.3 ± 2.5 **
Data expressed as mean ± SE. * P<0.05, ** P<0.01		

There were no differences in peak oxygen uptake between the two groups when the data were expressed in absolute values (L/min) (table 8). However, when expressed to more physiologically relevant values relative to FFM to account for the muscle mass, normal weight subjects had on average a higher oxygen uptake than the obese subjects (P=0.012). Neither the

peak work output nor the total time of the test were statistically different between the two groups: 138.6 ± 22.3 Watts and 9.37 ± 1.3 minutes for the normal weight group, 102.2 ± 8.1 Watts and 7.31 ± 0.6 minutes for the obese group ($P=0.17$ and 0.11 respectively). Peak heart rate tended to be higher in the normal weight group (160.6 ± 5.9 bpm) compared to the obese group (147.3 ± 3.6 bpm, $P=0.057$).

Table 8 Specific aim 2 - Physical fitness, body composition, blood pressure, blood labs and abdominal fat distribution

	Older normal weight sedentary	Older obese sedentary
VO ₂ peak (l/min)	1835.3 ± 270.6	1574.6 ± 106.6
VO ₂ peak (ml/min/kg _{FFM})	39.1 ± 3.5	$30.1 \pm 1.5^*$
Fat% (%)	34.6 ± 3.6	$43.6 \pm 1.9^*$
Fat mass (kg)	23.7 ± 2.1	$40.3 \pm 1.8^{**}$
FFM (kg)	46.1 ± 4.2	52.3 ± 2.1
SBP (mmHg)	141.0 ± 4.1	142.1 ± 3.3
DBP (mmHg)	76.7 ± 2.5	75.2 ± 2.1
Fasting glucose (mg/dl)	82.2 ± 4.2	$91.7 \pm 2.0^*$
HbA1c (%)	5.6 ± 0.1	5.7 ± 0.1
Fasting insulin (μ U/ml)	2.7 ± 0.5	$6.1 \pm 0.6^{**}$
NFG/IFG	5/2	9/4
NGT/IGT	6/1	5/9 [*]
Total cholesterol (mg/dl)	180.1 ± 7.8	189.0 ± 8.2
HDL cholesterol (mg/dl)	55.3 ± 4.7	50.7 ± 3.8
LDL cholesterol (mg/dl)	110.6 ± 7.6	113.5 ± 5.9
VLDL cholesterol (mg/dl)	14.3 ± 1.9	$24.8 \pm 2.0^{**}$
Triglycerides (mg/dl)	91.0 ± 11.9	$155.4 \pm 12.6^{**}$
WC (cm)	85.5 ± 4.0	$109.5 \pm 3.9^{**}$
VAT (cm ²)	105.3 ± 30.7	$209.2 \pm 17.5^{**}$
TAAT (cm ²)	359.5 ± 64.3	$608.3 \pm 38.3^{**}$
VAT/TAAT	0.28 ± 0.06	0.35 ± 0.02
ScAAT (cm ²)	254.2 ± 50.2	$379.0 \pm 34.6^{\#}$
ScAAT/TAAT	0.72 ± 0.06	0.62 ± 0.04
Data presented as mean \pm SE. * $P<0.05$, ** $P<0.01$ [#] $P 0.05$ to <0.1 . Abbreviations in Appendix A.		

Body composition and fat distribution were significantly different between the two groups, the obese having more fat mass and greater abdominal fat than the normal weight subjects (table 8). Fasting glucose, fasting insulin or lipid profile also differed significantly between the two groups, towards a less metabolically favorable profile in the obese group.

4.3.2 Insulin sensitivity

The normal weight group had a higher rate of insulin-stimulated glucose disposal (Rd) than the obese group (figure 10). This difference remained after adjusting for VAT and of VAT/TAAT as potential covariates. When examining the two components that account for insulin-stimulated glucose disposal, the difference in Rd was explained by a higher capacity for glucose storage ($P=0.006$) in the normal weight subjects, but not in glucose oxidation ($P=0.43$).

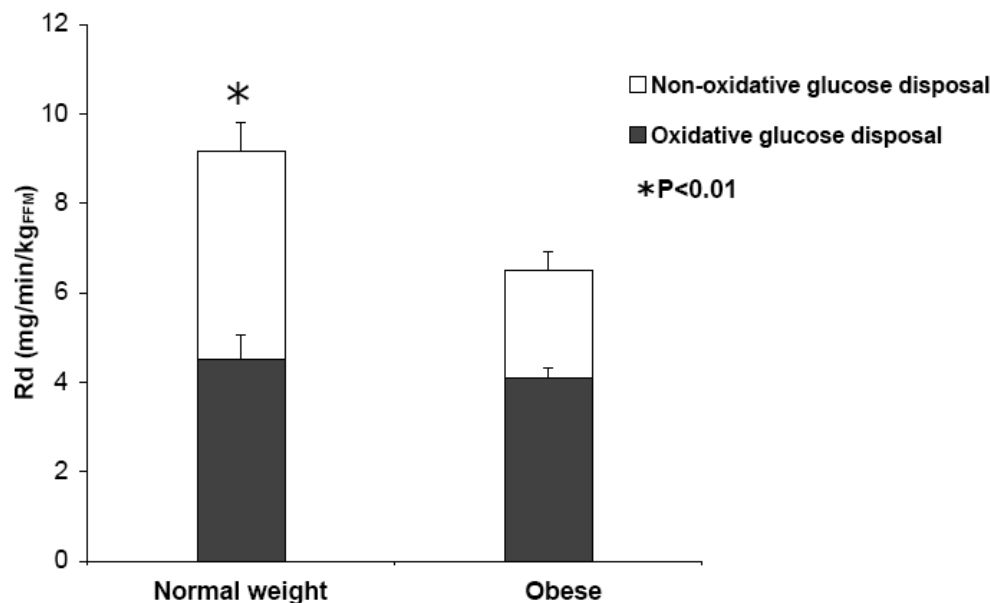


Figure 10 Specific aim 2 - Insulin sensitivity (Rd = rate of glucose disposal, FFM = fat free mass)

Hepatic insulin sensitivity can be expressed as insulin-stimulated hepatic glucose output (HGO), or as percent of HGO suppression by insulin. As presented in figure 11, the obese subjects had significantly lower HGO suppression than the normal weight subjects, indicating their lower hepatic insulin sensitivity.

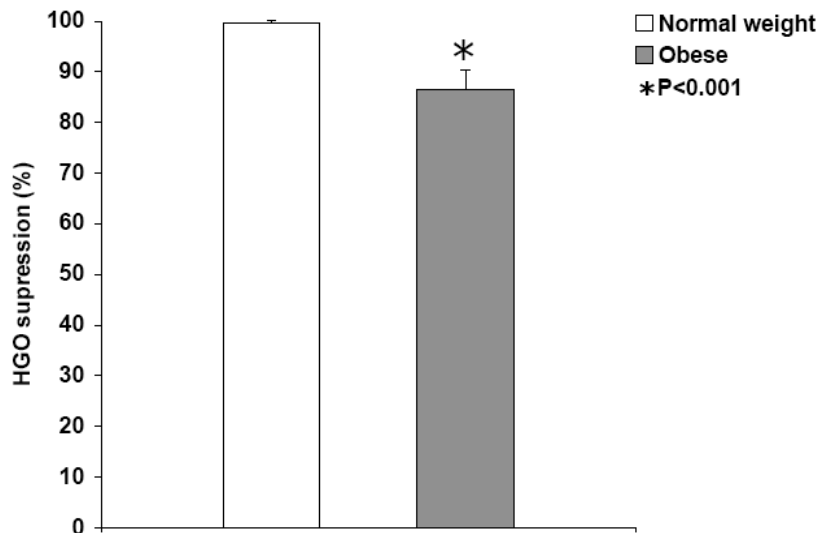


Figure 11 Specific aim 2 - Hepatic glucose output (HGO)

4.3.3 Systemic substrate oxidation and energy expenditure

The obese group had on average a higher REE (1634.5 ± 128.3 kcal/24h) than the normal weight group (1271.9 ± 74.7 kcal/24h, $P=0.022$) (figure 12). This difference disappeared when REE was normalized for FFM. There were no significant differences in RQ, CHO-ox or Fat-ox in fasting or in insulin-stimulated conditions either in absolute (figure 12) or relative units (table 9) between normal weight and obese groups..

Table 9 Specific aim 2 - Substrate oxidation and energy expenditure

	Older normal weight sedentary	Older obese sedentary
Fasting RQ	0.80 ± 0.01	0.84 ± 0.03
Insulin stimulated RQ	0.93 ± 0.02	0.91 ± 0.01
Fasting REE (kcal/24h/kg _{FFM})	28.26 ± 1.59	31.36 ± 1.89
Insulin stimulated REE (kcal/24h/kg _{FFM})	30.90 ± 1.69	31.49 ± 1.57
Fasting Fat-ox (kcal/24h/kg _{FFM})	17.81 ± 1.76	19.16 ± 1.99
Insulin stimulated Fat-ox (kcal/24h/kg _{FFM})	6.65 ± 1.94	9.41 ± 1.52
Fasting Cho-ox (kcal/24h/kg _{FFM})	10.53 ± 0.91	12.32 ± 1.19
Insulin stimulated Cho-ox (kcal/24h/kg _{FFM})	25.97 ± 3.04	23.51 ± 1.43
Data expressed as mean ± SE.		

Both normal weight and obese subjects increased their CHO-ox and decreased their Fat-ox significantly when going from fasting to insulin-stimulated conditions, without differences between groups (Paired t test $P < 0.001$, significant effect of time $P < 0.001$) (figure 12). Only the normal weight group increased significantly their total energy expenditure in response to insulin (Paired t test $P < 0.05$ and a trend towards interaction effect $P = 0.06$ for REE/FFM and $P = 0.07$ for REE).

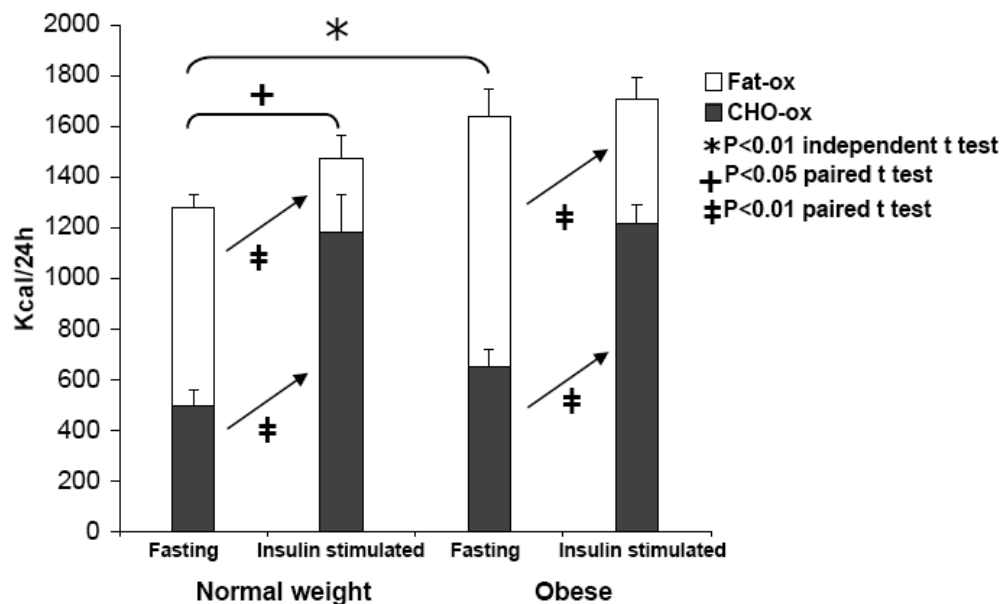


Figure 12 Specific aim 2 - Metabolic flexibility

4.3.4 Intramyocellular lipids

Surprisingly, we did not observe, as hypothesized, a higher content of IMTG measured with histologic ORO staining in the obese group compared to the normal weight group (figure 13). To the contrary, the total amount of IMTG tended to be lower in the obese subjects.

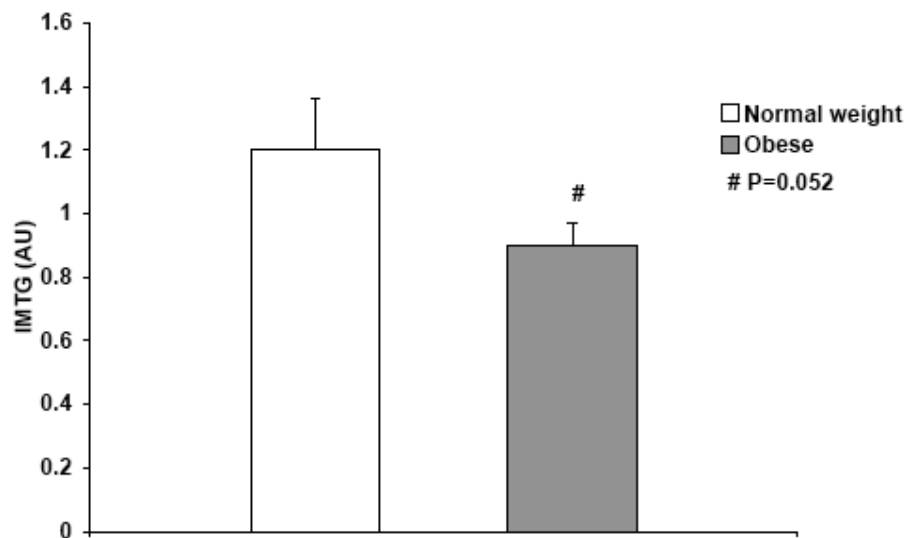


Figure 13 Specific aim 2 - Intramyocellular triglycerides (IMTG)

Differences between groups in total Cer and total DAG are depicted in figure 14. Obese subjects had significantly higher total Cer than normal weight subjects. No differences were found in DAG.

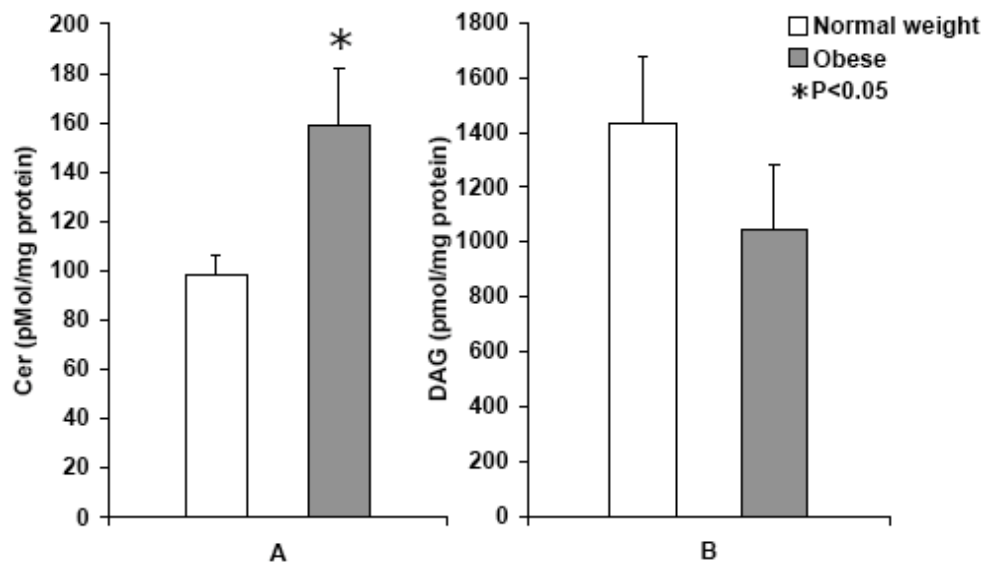


Figure 14 Specific aim 2 - Total ceramides (Cer, panel A) and total diacylglycerols (DAG, panel B)

The concentrations of the various species of Cer and other sphingolipids are presented in table 10. The majority of sphingolipids (18:1, 24:0, 24:1, Sphingosine-1P) were significantly higher in the obese group, while 14:0 Cer and Sphingosine were higher in the normal weight group.

Table 10 Specific aim 2 - Ceramides

	Older normal weight sedentary	Older obese sedentary
Cer 18:1	6.88 ± 0.99	10.91 ± 3.41 [§]
Cer 14:0	1.87 ± 0.18	0.80 ± 0.15 **
Cer 16:0	9.55 ± 0.99	14.50 ± 6.20
Cer 18:0	27.20 ± 4.59	25.94 ± 8.14
Cer 20:0	4.62 ± 0.55	5.54 ± 1.05
Cer 24:0	24.22 ± 1.89	45.21 ± 8.69 [#]
Cer 24:1	24.13 ± 2.69	55.57 ± 11.86 *
DHC16 Cer	0.57 ± 0.14	0.96 ± 0.25
Sphingosine	2.22 ± 0.33	0.81 ± 0.16 **
Sphingosine-1P	0.22 ± 0.05	12.41 ± 7.37 [§]
Data presented as mean ± SE. All units are pMol/mg protein. * P<0.05, ** P<0.001 [#] P 0.05 to <0.1, [§] P<0.05 only with non parametric test.		

The concentrations of the different species of DAG are presented in table 11. Some species of DAG (16:1/18:1, DiC 14:0, DiC 18:1) were significantly higher in the obese group, while others (14:0/18:0, 16:0/18:0, DiC18:0) were higher in the normal weight group.

Table 11 Specific aim 2 - Diacylglycerols

	Older normal weight sedentary	Older obese sedentary
DAG C14:0/16:0	1.68 ± 0.27	4.48 ± 1.90
DAG C14:0/18:0	6.55 ± 1.37	2.90 ± 1.03 ^{#§}
DAG C14:0/18:1	3.66 ± 1.15	18.19 ± 7.12 [#]
DAG C16:0/18:0	496.75 ± 91.42	267.86 ± 78.98 [#]
DAG C16:0/18:1	114.92 ± 17.45	131.82 ± 50.13
DAG C16:1/18:0	1.82 ± 0.28	3.39 ± 0.84
DAG C16:1/18:1	17.54 ± 4.42	68.76 ± 16.11 [*]
DAG C18:0/18:1	23.84 ± 4.11	49.79 ± 18.78 [§]
DAG DI-C14:0	0.83 ± 0.24	5.32 ± 0.98 [*]
DAG DI-C16:0	40.83 ± 7.72	43.93 ± 12.33
DAG DI-C16:1	1.11 ± 0.95	11.63 ± 4.00 [*]
DAG DI-C18:0	689.57 ± 118.58	309.73 ± 109.02 [*]
DAG DI-C18:1	34.98 ± 12.90	125.43 ± 42.26 [#]
Data presented as mean ± SE. All units are pMol/mg protein. [*] P<0.05, ^{**} P<0.001 [#] P 0.05 to <0.1. [§] P<0.05 only with non parametric test.		

The oxidative capacity of muscle and the proportion of the more oxidative type I fibers are potential covariates to explain differences in intramyocellular lipids and insulin resistance. We did not observe any significant differences in the proportion of type I fibers or in oxidative capacity assessed by succinate dehydrogenase (SDH) staining in these two groups (figure 15).

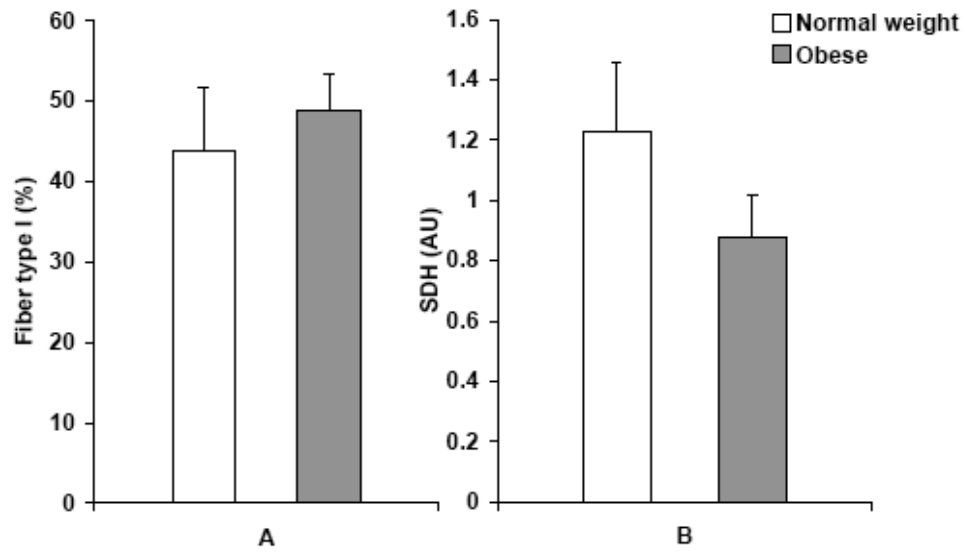


Figure 15 Specific aim 2 - Proportion of fiber type I (panel A) and oxidative capacity (SDH, panel B)

4.4 RESULTS FOR SPECIFIC AIM 3: TO EXAMINE THE EFFECTS OF CHRONIC EXERCISE ON INSULIN RESISTANCE AND IMCL IN OLDER SUBJECTS

4.4.1 Subjects

A total of fourteen (4 women and 10 men) Masters athletes ('Athletes') were enrolled and completed the testing procedures. The same group of seven (4 women and 3 men) older normal weight sedentary subjects ('Normal weight') described above was used for this comparison. Table 12 presents the subject characteristics for these two groups. These groups were similar age, weight and BMI.

Table 12 Specific aim 3 - Subject characteristics

	Masters athletes	Older normal weight sedentary
N	14	7
Age (years)	65.1 ± 1.3	66.5 ± 1.6
Gender	4W/10M	4W/3M
BMI (kg/m ²)	23.7 ± 0.6	24.5 ± 0.4
Weight (kg)	68.1 ± 2.6	70.4 ± 2.9
Data expressed as mean ± SE.		

The athletes had higher peak aerobic capacity (table 13) than the normal weight sedentary subjects. The peak work output was higher in the athletes (205.1 ± 15.6 vs. 138.6 ± 22.3 Watts, $P=0.02$). Peak heart rate was above age-predicted and was similar in both groups (161.4 ± 3.4 bpm vs. 160.6 ± 5.9 bpm, $P=0.90$) indicating that both groups gave a similar maximal voluntary effort.

Body composition and fat distribution (table 13) were significantly different between the two groups, the athletes having less fat mass, less overall abdominal fat and less subcutaneous abdominal fat than the normal weight sedentary subjects. Regarding visceral abdominal fat (VAT), one outlier was identified in the athlete group, but no significant difference was observed with or without this outlier, therefore the data was kept in the comparison. FFM tended to be higher in the athletes compared to their sedentary counterparts. Fasting glucose, fasting insulin or lipid profile did not differ between the two groups.

Table 13 Specific aim 3 - Physical fitness, body composition, blood pressure, blood labs and abdominal fat distribution

	Masters athletes	Older normal weight sedentary
VO ₂ peak (l/min)	2799.6 ± 206.7	1835.3 ± 270.6 *
VO ₂ peak (ml/min/kg _{FFM})	50.9 ± 2.7	39.1 ± 3.5 **
Fat% (%)	20.3 ± 2.8	34.6 ± 3.6 **
Fat mass (kg)	13.9 ± 2.0	23.7 ± 2.1 **
FFM (kg)	54.3 ± 2.5	46.1 ± 4.2 [#]
SBP (mmHg)	135.2 ± 3.7	141.0 ± 4.1
DBP (mmHg)	76.0 ± 2.0	76.7 ± 2.5
Fasting glucose (mg/dl)	87.2 ± 2.5	82.2 ± 4.2
HbA1c (%)	5.4 ± 0.1	5.6 ± 0.1
Fasting insulin (μU/ml)	2.6 ± 0.3	2.7 ± 0.5
NFG/IFG	9/2	5/2
NGT/IGT	12/2	6/1
Total cholesterol (mg/dl)	186.3 ± 5.2	180.1 ± 7.8
HDL cholesterol (mg/dl)	64.4 ± 3.0	55.3 ± 4.7
LDL cholesterol (mg/dl)	109.4 ± 5.3	110.6 ± 7.6
VLDL cholesterol (mg/dl)	12.6 ± 1.1	14.3 ± 1.9
Triglycerides (mg/dl)	77.8 ± 6.6	91.0 ± 11.9
WC (cm)	79.5 ± 1.9	85.5 ± 4.0
VAT (cm ²)	68.3 ± 6.7	105.3 ± 30.7
TAAT (cm ²)	223.5 ± 25.1	359.5 ± 64.3 *
VAT/TAAT	0.32 ± 0.03	0.28 ± 0.06
ScAAT (cm ²)	155.3 ± 20.6	254.2 ± 50.2 *
ScAAT/TAAT	0.68 ± 0.03	0.72 ± 0.06
Data presented as mean ± SE. * P<0.05, ** P<0.01, [#] P 0.05 to <0.1. Abbreviations in Appendix A.		

4.4.2 Insulin sensitivity

The athletes had a higher rate of insulin-stimulated glucose disposal (Rd) than the normal weight group (figure 16). This difference remained after taking into account the amount and proportion of body fat as potential covariates. The higher Rd in athletes was explained by a higher capacity for non-oxidative glucose disposal, or glucose storage ($P=0.002$), but not by insulin-stimulated glucose oxidation ($P=0.91$). Liver insulin sensitivity assessed by insulin-stimulated hepatic glucose output (HGO) was similar in both groups (figure 17).

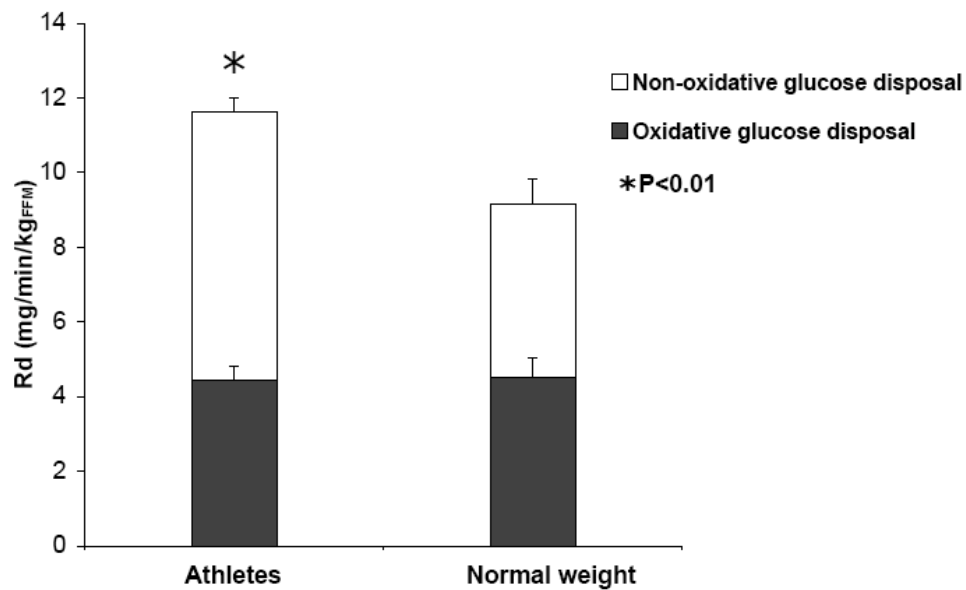


Figure 16 Specific aim 3 - Insulin sensitivity (Rd = rate of glucose disposal, FFM = fat free mass)

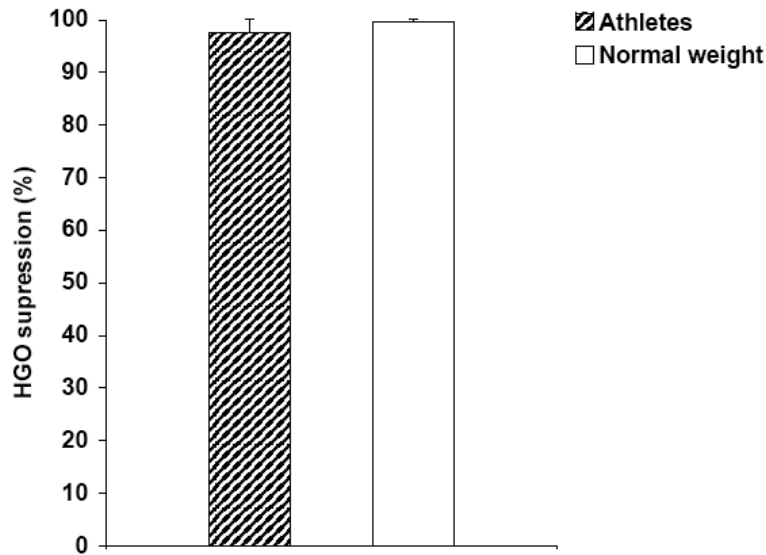


Figure 17 Specific aim 3 - Hepatic glucose output (HGO)

4.4.3 Systemic substrate oxidation and energy expenditure

The athletes had on average a higher REE (1456.9 ± 45.3 kcal/24h) than the normal weight group (1271.9 ± 74.7 kcal/24h, $P=0.04$) (figure 18), but this difference disappeared when adjusting for FFM. In the fasting condition, absolute rates for Fat-ox was higher in the athletes compared to the normal weight subjects (1080.5 ± 95.8 vs. 785.3 ± 50.1 kcal/24h respectively, $P=0.01$), but these differences also disappeared when normalized for FFM (table 14). There were no significant differences between the two groups in what regards RQ and CHO-ox in fasting condition in either absolute (figure 18) or relative (to FFM) units (table 14).

Table 14 Specific aim 3 - Substrate oxidation and energy expenditure

	Masters athletes	Older normal weight sedentary
Fasting RQ	0.77 ± 0.02	0.80 ± 0.01
Insulin stimulated RQ	0.93 ± 0.02	0.93 ± 0.02
Fasting REE (kcal/24h/kg _{FFM})	26.96 ± 1.16	28.26 ± 1.59
Insulin stimulated REE (kcal/24h/kg _{FFM})	30.78 ± 1.11	30.90 ± 1.69
Fasting Fat-ox (kcal/24h/kg _{FFM})	20.39 ± 2.24	17.81 ± 1.76
Insulin stimulated Fat-ox (kcal/24h/kg _{FFM})	7.14 ± 1.80	6.65 ± 1.94 *
Fasting Cho-ox (kcal/24h/kg _{FFM})	6.61 ± 1.27	10.53 ± 0.91 *
Insulin stimulated Cho-ox (kcal/24h/kg _{FFM})	25.54 ± 2.09	25.97 ± 3.04
Data expressed as mean ± SE. * P<0.05		

When switching from the fasting to the insulin-stimulated condition, both groups increased their REE and CHO-ox and decreased their Fat-ox significantly (Paired t tests P<0.05, figure 18). The magnitude of this change in substrate selection was significantly greater in the athlete group (significant interaction effect for Fat-ox P=0.01 and for CHO-ox P=0.03).

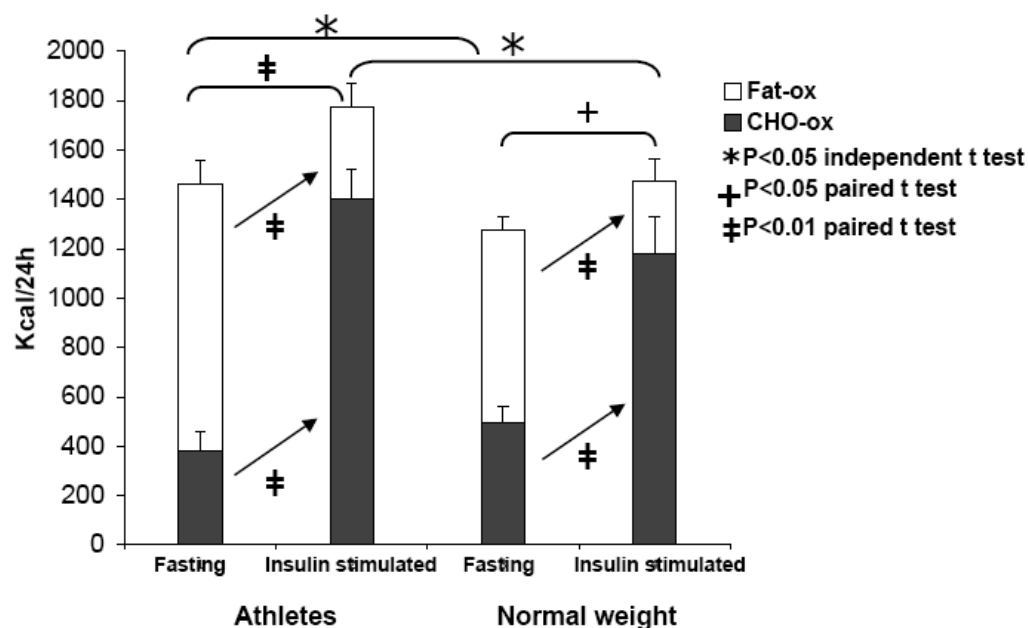


Figure 18 Specific aim 3 - Metabolic flexibility

4.4.4 Intramyocellular lipids

The athletes tended to have a higher content of IMTG in the *vastus lateralis* measured with the ORO staining compared to the normal weight group (figure 19). These results obtained with the ex-vivo invasive method were concordant with the results obtained from the in-vivo non-invasive method in the *tibialis anterior* with ^1H -MRS (figure 20), which show that IMCL was also higher in the athletes compared to the normal weight sedentary subjects.

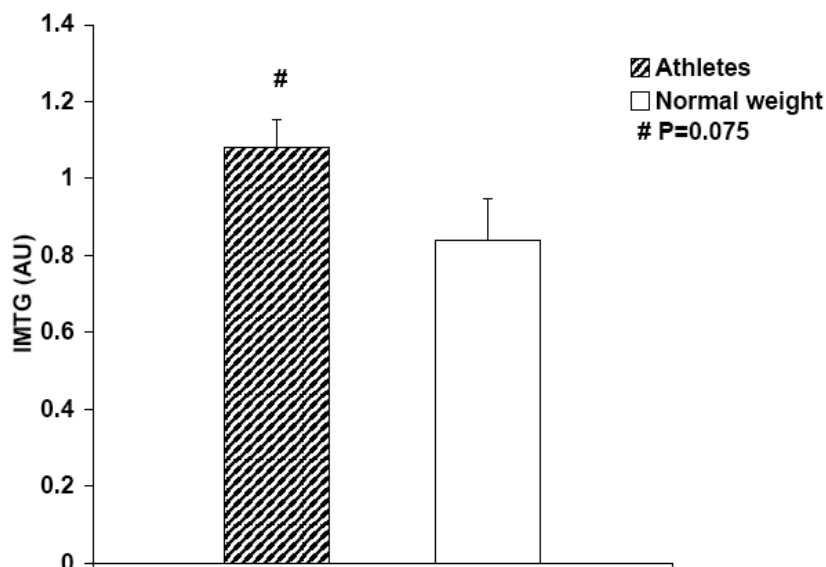


Figure 19 Specific aim 3 - Intramyocellular triglycerides (IMTG) by ORO staining

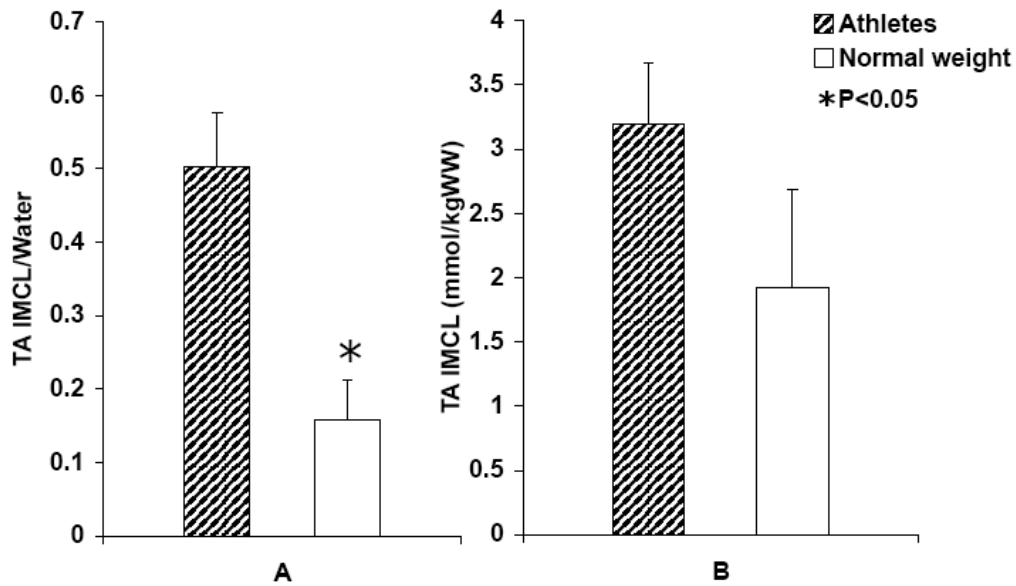


Figure 20 Specific aim 3 - Intramyocellular lipids (IMCL) by ^1H -MRS relative to the water content (panel A) and in absolute concentrations (panel B). (TA = *tibialis anterior*, WW = wet weight). The test-retest reliability for the measure of IMCL via ^1H -MRS was acceptable with an $r = 0.932$, $P = 0.011$ (N=5)

Differences in total Cer and total DAG within skeletal muscle *vastus lateralis* biopsy specimens are depicted in figure 21. No significant differences were observed between the athletes and the normal weight sedentary subjects. The concentrations of the different species of Cer and other sphingolipids are presented in table 15. Only Sphingosine-1P tended to be higher in the athletes than in the normal weight subjects.

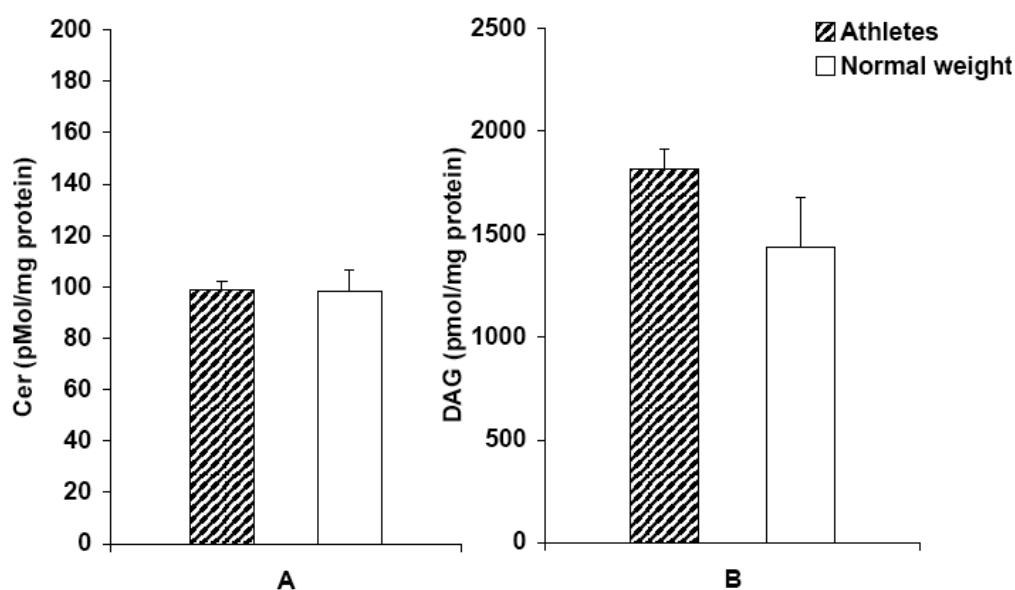


Figure 21 Specific aim 3 - Total ceramides (Cer, panel A) and total diacylglycerols (DAG, panel B)

Table 15 Specific aim 3 - Ceramides

	Masters athletes	Older normal weight sedentary
Cer 18:1	5.82 ± 0.44	6.88 ± 0.99
Cer 14:0	1.99 ± 0.33	1.87 ± 0.18
Cer 16:0	8.15 ± 0.76	9.55 ± 0.99
Cer 18:0	23.38 ± 1.94	27.20 ± 4.59
Cer 20:0	5.55 ± 0.58	4.62 ± 0.55
Cer 24:0	27.98 ± 2.21	24.22 ± 1.89
Cer 24:1	25.91 ± 1.25	24.13 ± 2.69
DHC16 Cer	0.41 ± 0.08	0.57 ± 0.14
Sphingosine	2.33 ± 0.24	2.22 ± 0.33
Sphingosine-1P	0.43 ± 0.08	0.22 ± 0.05 ^{#§}
Data presented as mean ± SE. All units are pMol/mg protein. [#] P 0.05 to <0.1. [§] P<0.05 only with non parametric test.		

The concentrations of the different species of DAG are presented in table 16. DAG species of 14:0/18:0, 16:0/18:1, 16:1/18:1, 18:0/18:1, DiC 16:0 were significantly higher in the athletes group. We did not find any species that were significantly higher in the normal weight group.

Table 16 Specific aim 3 - Diacylglycerols

	Masters athletes	Older normal weight sedentary
DAG C14:0/16:0	2.99 ± 0.84	1.68 ± 0.27
DAG C14:0/18:0	10.45 ± 0.98	6.55 ± 1.37 *
DAG C14:0/18:1	5.28 ± 1.25	3.66 ± 1.15
DAG C16:0/18:0	565.20 ± 34.62	496.75 ± 91.42
DAG C16:0/18:1	242.84 ± 29.43	114.92 ± 17.45 **
DAG C16:1/18:0	3.90 ± 0.49	1.82 ± 0.28 **
DAG C16:1/18:1	23.78 ± 7.23	17.54 ± 4.42
DAG C18:0/18:1	49.10 ± 7.20	23.84 ± 4.11 **
DAG DI-C14:0	1.50 ± 0.50	0.83 ± 0.24
DAG DI-C16:0	56.26 ± 4.40	40.83 ± 7.72 #
DAG DI-C16:1	0.25 ± 0.13	1.11 ± 0.95
DAG DI-C18:0	801.26 ± 51.30	689.57 ± 118.58
DAG DI-C18:1	55.99 ± 7.80	34.98 ± 12.90
Data presented as mean ± SE. All units are pMol/mg protein. * P<0.05, ** P<0.01, # P 0.05 to <0.1.		

The oxidative capacity of muscle and the proportion of the more oxidative type I fibers are potential covariates to explain differences in intramyocellular lipids and insulin resistance. We observed significant differences in the proportion of type I fibers or in oxidative capacity assessed by succinate dehydrogenase (SDH) staining in these two groups (figure 22).

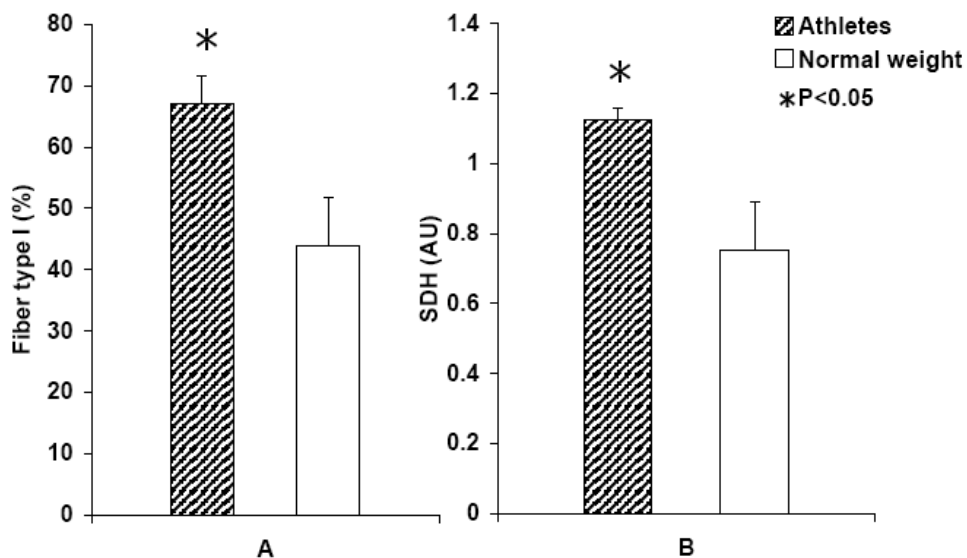


Figure 22 Specific aim 3 - Proportion of fiber type I (panel A) and oxidative capacity (SDH, panel B)

4.5 DIFFERENCES IN INSULIN SENSITIVITY AND INTRAMYOCELLULAR LIPIDS AMONG OLDER ATHLETES, NORMAL WEIGHT AND OBESE SEDENTARY SUBJECTS

To gain a better perspective on differences in insulin sensitivity and intramyocellular lipids according to obesity and chronic physical activity among older adults, additional three-group analyses were performed. Differences in Rd among the three older groups matched by age (Masters athletes, normal weight and obese sedentary) are presented in figure 23. Athletes were more insulin sensitive than normal weight sedentary subjects, who in turn were more insulin sensitive than obese sedentary subjects. Athletes did not, however, have higher hepatic insulin sensitivity (HGO suppression) than normal weight sedentary subjects, although both of these groups had higher HGO suppression than obese subjects.

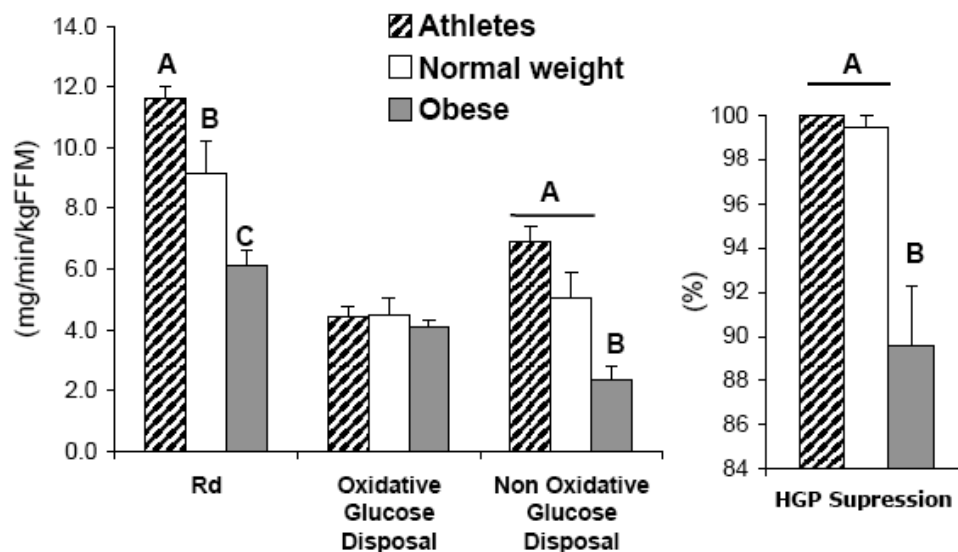


Figure 23 Three older groups comparison - insulin sensitivity (A, B, and C = significant statistical differences in the one-way between subjects ANOVA, Rd = rate of glucose disposal, FFM = fat free mass, HGP = hepatic glucose production)

IMTG, oxidative capacity and the proportion of type I fibers measured by histochemistry are presented in figure 24. Athletes had higher IMTG than obese subjects. Athletes also had higher oxidative capacity measured by SDH staining compared to both normal weight and obese subjects, and normal weight subjects had higher oxidative capacity than obese subjects. Athletes had higher proportion of type I fibers than both the normal weight and the obese sedentary groups.

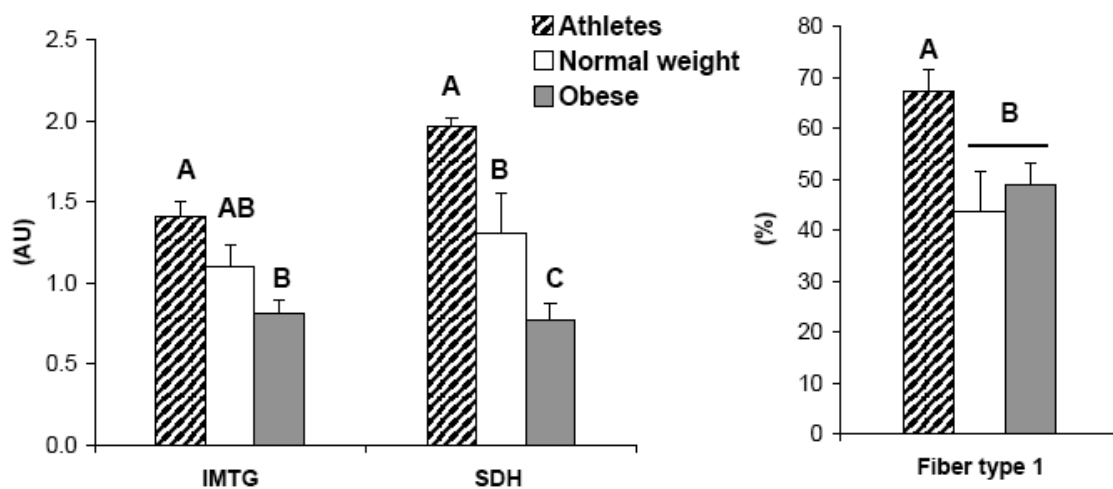


Figure 24 Three groups comparison – IMTG, oxidative capacity and proportion of type I fibers (A, B, and C = significant statistical differences in the one-way between subjects ANOVA, IMTG = intramyocellular triglycerides, SDH = succinate deshydrogenase)

Total Cer were significantly higher in the obese than in the two other groups (figure 25, panel A). Total DAG were significantly higher in the athletes compared to the obese, but did not reach significance compared to the normal weight subjects (figure 25, panel B). Bivariate correlations showed that Rd was negatively associated with the total content of Cer ($R^2=0.23$, $P=0.03$) but positively associated with DAG ($R^2=0.22$, $P=0.03$).

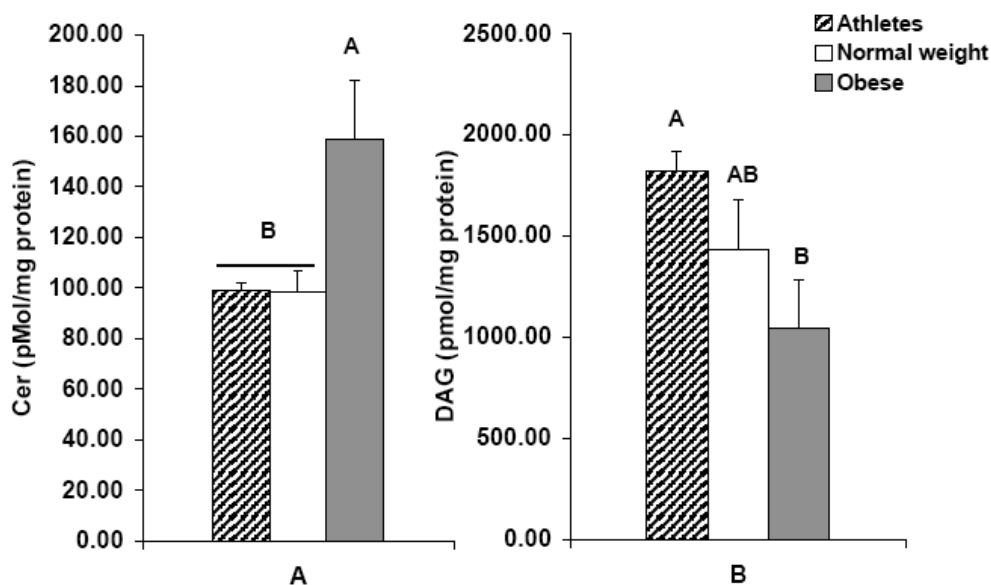


Figure 25 Three groups comparison - Total ceramides (Cer, panel A) and total diacylglycerols (DAG, panel B) (A, B, and C = significant statistical differences in the one-way between subjects ANOVA)

To examine possible patterns of lipid species distribution across the three subject groups, one-way ANOVAs were performed for each molecular species of Cer and DAG. Based on the results of these tests, we separated the species in three groups (table 17). Those species that were significantly higher in the athletes were put together in a group that was labeled “Group I”. Those that were significantly higher in the obese subjects were put together in a group labeled “Group II”. Those that were neither higher in the athletes nor in the obese were labeled “Group III”.

Table 17 Three groups comparison – DAG and Cer species

<i>"Group I"</i>			
	Masters athletes	Older normal weight sedentary	Older obese sedentary
Cer 14:0	1.99 ± 0.33 ^A	1.87 ± 0.18 ^{AB}	0.80 ± 0.15 ^B
Sphingosine	2.33 ± 0.24 ^A	2.22 ± 0.33 ^A	0.81 ± 0.16 ^B
DAG C14:0/18:0	10.45 ± 0.98 ^A	6.55 ± 1.37 ^{AB}	2.90 ± 1.03 ^B
DAG C16:0/18:0	565.20 ± 34.62 ^A	496.75 ± 91.42 ^{AB}	267.86 ± 78.98 ^B
DAG C16:0/18:1	242.84 ± 29.43 ^A	114.92 ± 17.45 ^B	131.82 ± 50.13 ^B
DAG C16:1/18:0	3.90 ± 0.49 ^A	1.82 ± 0.28 ^B	3.39 ± 0.84 ^{AB}
DAG DI-C18:0	801.26 ± 51.30 ^A	689.57 ± 118.58 ^A	309.73 ± 109.02 ^B

<i>"Group II"</i>			
Cer 24:0	27.98 ± 2.21 ^B	24.22 ± 1.89 ^B	45.21 ± 8.69 ^A
Cer 24:1	25.91 ± 1.25 ^B	24.13 ± 2.69 ^B	55.57 ± 11.86 ^A
DHC16 Cer	0.41 ± 0.08 ^B	0.57 ± 0.14 ^{AB}	0.96 ± 0.25 ^A
DAG C14:0/18:1	5.28 ± 1.25 ^B	3.66 ± 1.15 ^B	18.19 ± 7.12 ^A
DAG C16:1/18:1	23.78 ± 7.23 ^B	17.54 ± 4.42 ^B	68.76 ± 16.11 ^A
DAG DI-C14:0	1.50 ± 0.50 ^B	0.83 ± 0.24 ^B	5.32 ± 0.98 ^A
DAG DI-C16:1	0.25 ± 0.13 ^B	1.11 ± 0.95 ^B	11.63 ± 4.00 ^A
DAG DI-C18:1	55.99 ± 7.80 ^B	34.98 ± 12.90 ^B	125.43 ± 42.26 ^A

<i>"Group III"</i>			
Cer 18:1	5.82 ± 0.44	6.88 ± 0.99	10.91 ± 3.41
Cer 16:0	8.15 ± 0.76	9.55 ± 0.99	14.50 ± 6.20
Cer 18:0	23.38 ± 1.94	27.20 ± 4.59	25.94 ± 8.14
Cer 20:0	5.55 ± 0.58	4.62 ± 0.55	5.54 ± 1.05
Sphingosine-1P	0.43 ± 0.08	0.22 ± 0.05	12.41 ± 7.37
DAG C18:0/18:1	49.10 ± 7.20 ^A	23.84 ± 4.11 ^B	49.79 ± 18.78 ^A
DAG C14:0/16:0	2.99 ± 0.84	1.68 ± 0.27	4.48 ± 1.90
DAG DI-C16:0	56.26 ± 4.40	40.83 ± 7.72	43.93 ± 12.33

Data presented as mean ± SE. A>B>C: different letters denote significant differences between groups 1 way ANOVA with Tukey adjustments (P<0.05).

5 DISCUSSION

5.1 EFFECTS OF OLDER AGE ON INSULIN RESISTANCE IN SEDENTARY SUBJECTS

The first specific aim was to determine whether or not older age is associated with insulin resistance independently of obesity and of chronic exercise. We hypothesized that normal weight younger subjects would be more insulin sensitive than normal weight older subjects, when matched by BMI and by physical activity level (all sedentary subjects).

The key finding of the first specific aim was that insulin sensitivity was not different in older normal weight sedentary subjects compared to younger normal weight sedentary subjects. Although our sample size was limited to adults from middle-age to those in their 70's, this is an important argument supporting that insulin resistance may not be characteristic of aging per se.

We recently reported that, similar to observed for normal weight subjects, insulin sensitivity is also similar in sedentary obese older vs. obese younger adults (Amati, Diabetes Care, in press). Our results are consistent with former studies comparing younger and older normal weight individuals with sedentary lifestyles (113), but are in contrast with other studies not controlling for patterns of physical activity (6, 63, 114).

5.2 EFFECTS OF OBESITY ON INSULIN RESISTANCE AND INTRAMYOCYELLULAR LIPIDS IN SEDENTARY OLDER SUBJECTS

The primary purpose of the second specific aim was to determine whether or not obesity or generalized adiposity is associated with insulin resistance independently of physical activity specifically within older subjects. We first hypothesized that sedentary obese subjects would have lower insulin sensitivity compared to age matched sedentary normal weight subjects.

A primary finding was that obesity was associated with lower insulin sensitivity (thus a greater insulin resistance) in sedentary older adults. Furthermore, the effect of obesity on insulin sensitivity was independent of visceral adiposity. This was true not only for overall insulin sensitivity but also for hepatic insulin sensitivity as the obese subjects had an incomplete glucose output suppression compared to their normal weight counterparts. Thus after accounting for both age and level of chronic physical activity, obesity per se was associated with insulin resistance in sedentary subjects. These results are in accord with a number of studies demonstrating an effect of obesity (10, 115) and weight loss (85, 116) on insulin sensitivity in younger adults. Although there are fewer studies examining insulin resistance specifically in older adults, our results also confirm that obesity but not aging is primarily associated with insulin resistance.

We also examined markers of metabolic flexibility, corresponding to the ability to switch from mostly fat oxidation in the fasting condition to carbohydrate oxidation in the insulin-stimulated condition. We found that obese subjects were no less metabolically flexible than normal weight older adults, as both groups increased their reliance on carbohydrates and decreased their reliance on fat going from the fasted to the insulin-stimulated condition. This is in apparent contrast to the seminal work by Kelley and colleagues (10) who reported that fat oxidation after an overnight fast was impaired in obesity. However, in that study, metabolic

flexibility was assessed more specifically in skeletal muscle using limb balance methods to examine substrate metabolism in skeletal muscle. The current study relied on whole body measurements of substrate oxidation, which could explain the discrepancy due to the fact other tissues and organs are also taken into account in the current study. Therefore, the possibility remains that skeletal muscle fatty acid oxidation during fasting conditions is indeed impaired in these obese older subjects.

We also hypothesized that lower skeletal muscle insulin sensitivity in sedentary obese older subjects would be observed in the context of higher intramyocellular triglycerides (IMTG) and with higher levels of potentially harmful lipid metabolites, including diacylglycerols and ceramides, compared to sedentary normal weight older subjects. Surprisingly, we did not find higher IMTG in the obese insulin resistant group as described in previous studies with younger subjects (44). It is important to note that none of our subjects had type 2 diabetes. This is further evidence supporting the notion that it may not be IMTG per se that directly confers insulin resistance.

An important finding of this study was that obese older subjects had nearly two-fold higher ceramide levels within muscle compared to either athletes or normal weight sedentary subjects. These results agree with a study by Adams et al (20) who found higher ceramide content in skeletal muscle of obese younger subjects. Strackowski et al (117) also found the inverse relationship between ceramide content and insulin sensitivity. Bruce et al (118) found that eight weeks of training tended to reduce both the total ceramide content and the saturated ceramide species in young obese insulin-resistant subjects without significant weight loss.

We observed that total diacylglycerol content within muscle was not significantly different between obese and normal weight groups. Contrary to what we hypothesized, we did

not observe an association between the total content of diacylglycerols (DAG) and either obesity or with insulin resistance. This is in contradiction with animal studies (119) as well as human studies with lipid infusion showing the relationship between DAG content and insulin resistance (19). However, Straczkowski et al (117) did not find increased levels of DAG in obese younger subjects. Our results are in contradiction with Bruce et al (118) who observed a trend towards the reduction both in the total DAG content and the saturated DAG species in young obese insulin-resistant subjects after eight weeks of moderate exercise training. To the contrary, athletes had a two-fold higher concentration of total DAG within skeletal muscle than the obese subjects. Ours is the first study to report that total DAG content is actually higher in athletes.

We did not find significant differences in muscle oxidative capacity and fiber type distribution between normal weight and obese groups. This is in accord with a prior study by Kelley and colleagues (120) who observed no significant differences in fiber-type proportions between obese and lean middle-aged subjects. On the other hand our results contradict former studies showing that lean subjects had significantly higher proportions of type I fibers than obese (121) or that the degree of obesity was related with the proportion of fiber type (122), although both these studies did not control for habitual physical activity.

5.3 EFFECTS OF CHRONIC EXERCISE ON INSULIN RESISTANCE AND IMCL IN OLDER SUBJECTS

The primary purpose of the third specific aim was to determine whether or not chronic exercise training is associated with insulin resistance in older subjects independently of obesity. We first hypothesized that endurance-trained athletes would have greater skeletal muscle insulin sensitivity compared to age matched sedentary normal weight subjects independently of their total body fat. This hypothesis was confirmed as endurance-trained athletes had higher insulin sensitivity compared to the sedentary subjects even after accounting for the residual effect of the last acute bout of exercise on insulin sensitivity. This was true only for peripheral insulin sensitivity as both groups presented similar hepatic insulin sensitivity. Thus after accounting for both age and total body fatness, chronic physical inactivity was associated with insulin resistance in older subjects. These data are in accord with many prior studies demonstrating an effect of chronic exercise to improve insulin sensitivity in younger and older subjects (9, 18). Furthermore, we recently reported that masters athletes had comparable levels of insulin sensitivity to younger athletes (Amati, Diabetes Care in press). Thus after accounting for both obesity and level of chronic physical activity, aging per se may not be associated with insulin resistance. These findings corroborate a recent study by Lanza and al (123) that suggested that *“reduced insulin sensitivity is likely related to changes in adiposity and to physical inactivity rather than being an inevitable consequence of aging”*.

We also looked at markers of metabolic flexibility, corresponding to the ability to switch from mostly fat oxidation in the fasting condition to carbohydrate oxidation in the insulin stimulated condition. We found that master athletes were significantly more metabolically flexible than normal weight sedentary subjects, as the magnitude of their switch from more fat

oxidation to carbohydrate oxidation within the two conditions was higher for both substrates. These data are also consistent with our observation that the athletes had higher oxidative capacity in muscle, which is supported by extensive previous research demonstrating that exercise training in younger and older subjects increases the capacity for skeletal muscle fat oxidation (47, 50, 77, 124). The athletes had higher proportion of type I fibers, which is also supported by many reports in the literature (125, 126, 127).

We also hypothesized that higher skeletal muscle insulin sensitivity in master athletes would be observed in the context of higher intramyocellular triglycerides but with lower levels of lipid metabolites, including diacylglycerol and ceramides, compared to sedentary normal weight older subjects. Both intramyocellular lipids measured in-vivo in the calf muscle and intramyocellular triglycerides measured ex-vivo in the thigh muscle were higher in the athletes than in their sedentary counterparts. This is the first study to confirm the athletes paradox (18) in older subjects.

Surprisingly, we did not find significant differences between the athletes and normal weight sedentary older subjects with respect to total muscle content of ceramides or DAG. This is in accord with Helge and colleagues who observed similar muscle ceramide content in endurance trained compared with untrained normal weight young men (128). These same investigators found similar ceramide concentration in skeletal muscle from middle aged men over a wide variety of insulin sensitivities including type 2 diabetics, impaired glucose tolerant and normal glucose tolerant subjects (129).

5.4 INSULIN SENSITIVITY AND PATTERN OF DISTRIBUTION OF THE DIFFERENT SPECIES OF CERAMIDES AND DIACYLGLYCEROLS

A novel aspect of this study is the description of virtually all of the molecular species of both diacylglycerol and ceramides in muscle of normal weight, obese and athletic human subjects. Our data strongly suggest that not all diacylglycerols or ceramides are the same with respect to how they relate to insulin resistance. We could hypothesize that based on their biochemical and molecular structure some of these fatty acid metabolites interact with the insulin signaling cascade while others not, therefore having different impacts on insulin resistance. The different subgroups of these complex lipids within muscle were based on whether they were higher in obese subjects or in athletes. Thus, one could view these subgroups as “favorable” or “unfavorable” according to their specific associations with insulin resistance. These arbitrary classifications did not seem to be related to the level of fatty acid saturation or of length of the carbon chains. Furthermore, we demonstrated that the proportion of the “favorable” ceramide and diacylglycerols to the total amount of intramyocellular triglycerides was negatively associated with insulin resistance ($R^2=0.39$, $P=0.002$ and $R^2=0.30$, $P=0.009$ respectively). More research needs to be performed to examine possible reasons to explain why some species of diacylglycerols and ceramides are related to insulin resistance while others not or may even confer higher insulin sensitivity.

5.5 LIMITATIONS

This study was not without limitations. First our sample sizes are relatively small. Based on our power computations, our desired sample size for the normal weight sedentary older subjects was 9, therefore we achieved a recruitment of 78%. In part this limited recruitment was due to the fact that, in the attempt to have a balanced gender ratio, we closed earlier the recruitment to women. Although this calls for a limited power in the first specific aim, it is important to note that our results for that comparison are confirmed by another recent study (123).

The second limitation is related to the use of retrospective data for two of the four groups. In order to limit as much as possible the bias in the selection of the subjects in the retrospective data, the selection criteria were define in advance and subjects were selected in a blind manner.

An important element to take into account is the amount of physical activity performed by the Masters athletes. These were enrolled if they were doing at least five sessions per week of endurance exercise. Nearly all of these subjects were training for competition at the time of testing and were performing much more exercise than is generally prescribed for the average population (31) . Therefore this study does not allow us to extrapolate these findings in athletes to more moderate physical activity as emphasized for general health. Thus the possibility remains that there is some threshold level or dose-response effect of physical activity on insulin sensitivity.

This study also does not allow us to address an important and controversial question in the field of exercise physiology: “ Is fitness or fatness more important in determining cardiometabolic risk”? (130-132). On one side of the controversy it is hypothesized that it is not obesity per se that is deleterious for health but rather due to the likelihood that obese persons exercise less or are limited to perform the same amount of physical activity. Our study provided

important clues in a cross-sectional design that probably both fitness and fatness are important determinants of insulin resistance. To more fully answer this question, we have data to support that exercise and weight loss interventions each independently improve insulin sensitivity in previously sedentary obese older adults (9, 133) (and unpublished data).

It is also important to note that all studies recruiting older subjects through advertisement are prone to some level of selection bias in the fact that the responders to the ads are typically subjects receiving medical care and that are interested in their health. One example to illustrate this point is that 100% of the recruited subjects in this study had regular check-ups with their primary care physicians. In addition, many of these subjects were being treated with lipid lowering medications (statins), which may explain that we did not see any differences between younger and older subjects in regards of their lipid panel. Therefore, a generalization of these conclusions to other populations, such as underserved or under medicated, needs to be avoided.

5.6 CONCLUSIONS

We conclude that age per se does not influence insulin sensitivity in subjects matched by body fatness and physical inactivity. We can also deduce that obesity is associated with insulin resistance among older subjects after accounting for chronic physical inactivity. We can also conclude that chronic exercise positively influences insulin sensitivity after accounting for age and body fatness. Therefore, by carefully controlling age, chronic exercise (sedentary vs. athletes) and total body fat, the present investigation indicates that insulin resistance is associated with acquired conditions, including obesity and physical inactivity, but not with aging per se.

Another key aspect of this study was to describe for the first time in subjects with widely different levels of insulin sensitivity the content and composition of intramyocellular lipids and their potential link to insulin resistance. We clearly demonstrated that ceramides within skeletal muscle are higher in obesity. However, the distribution of the various molecular species of these complex lipids is different in obese insulin resistant subjects compared to insulin sensitive normal weight athletes. We found that athletes have a different molecular species distribution of diacylglycerols and ceramides within the intramyocellular lipid pool. This suggests that ectopic fat deposition within muscle may be dependent on the oxidative capacity or metabolic flexibility of the individual or on the dynamic state, i.e., conditions of low vs. high energy flux or mobilization.

5.7 RECOMMENDATIONS FOR FUTURE RESEARCH

Based on the findings of the present investigation and its limitations, future studies on the effects of exercise, obesity and aging on insulin sensitivity and skeletal muscle lipid content should focus on the following:

- Although our first comparison between younger and older normal weight sedentary subjects showed that insulin resistance was not influenced by age per se, this does not rule out possible differences in the etiology of insulin resistance between older and younger persons which need to be examined. In other words, it is possible that younger and older people develop insulin resistance in different ways according to some aging effect. Furthermore, differences in the extent or degree to which insulin resistance can be improved in both younger and older adults needs further investigation.

- A possible dose-response effect on the amount of physical activity to achieve improvements in sedentary subjects has not yet been identified, either in younger or older subjects. Therefore, further investigations on the threshold or dose-response effects of physical activity on younger and older adults are warranted. Comparisons of insulin sensitivity improvements due to more moderate exercise programs to that of Masters athletes who have been exercising for many years would be a good start to address this question.

- An increased capacity for fat oxidation has been associated with insulin sensitivity and possibly to a greater utilization of IMCL, but the dynamic aspect of the content of IMCL related to the acute utilization of this substrate store has not been adequately explored. Observational studies examining the partitioning and alterations in the different species of fatty acid metabolites before and after an acute bout of exercise would shed light on how quickly muscle ectopic stores are remodeled and the impact of this remodeling on insulin sensitivity.

- In vivo non invasive techniques such as ^1H -MRS are still limited in the quality of the spectra depending on particular characteristics of the subjects. Obesity and older age are characteristics that contribute to the poor quality of ^1H -MRS spectra to quantify IMCL in vivo. The exploration and quantification of IMCL and other metabolites in higher field MR scanners may overcome these anatomical and physical limitations. Therefore, the further development of non invasive MRS methods should aid in this endeavor.

APPENDIX A

ABBREVIATIONS LIST

Abbreviation	Definition
M	Men
W	Women
BMI	Body mass index
DXA	Dual-energy X-ray absorpiometry
FFM	Fat free mass
FM	Fat mass
Fat%	Proportion of body fat
Rd	Rate of glucose disposal
HGO	Hepatic glucose output
OGTT	Oral glucose tolerance test
NGT	Normal glucose tolerance
IGT	Impaired glucose tolerance
T2DM	Type 2 diabetes
NFG	Normal fasting glucose
IFG	Impaired fasting glucose
IR	Insulin resistance
IMCL	Intramyocellular lipids
IMTG	Intramyocellular triglycerides
DAG	Diacylglycerols
Cer	Ceramides
ORO	Oil red O staining
SDH	Succinate deshydrogenase staining
MR	Magnetic resonance
MRI	Magnetic resonance imaging
¹ H-MRS	Proton magnetic resonance spectroscopy
CT	Computed tomography
VAT	Visceral adipose tissue
ScaAT	Subcutaneous abdominal adipose tissue
TAAT	Total abdominal adipose tissue
WC	Waist circumference (tape measured)
VO ₂ peak	Peak oxygen uptake
RQ	Respiratory quotient
REE	Resting energy expenditure
Cho-ox	Systemic carbohydrate oxidation
Fat-ox	Systemic fat oxidation
EF	Energy derived from fat
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
HbA1c	Glycosylated hemoglobin
IRB	Institutional review board

APPENDIX B

INCLUSION/EXCLUSION CRITERIA

INCLUSION CRITERIA

INCLUSION CRITERIA	YES	NO	Notes
60-75 years of age			
Stable weight (No Gain/Loss of > 10 lbs in 6 months)			
Sedentary (≤ 1 continuous exercise/week) Or highly trained (>5 exercise sessions/week since ≥ 1 year)			
Non-smoker			
BMI 18-38.0 KG/M ²			
Resting Blood Pressure ≤ 150 mmHg systolic and ≤ 95 mmHg diastolic			
Normal Glucose Tolerance: Fasting Glucose < 100 mg/dl or 2 hour glucose from OGTT < 140 mg/dl			
Note from PCP/Cardiologist for exercise clearance if positive stress test symptoms were observed from GXT			

EXCLUSION CRITERIA

EXCLUSION CRITERIA	YES	NO	Notes
Clinically significant CVD including h/o MI			
Peripheral Vascular Disease			
Hepatic, renal, muscular/neuromuscular, or active hematologic/oncologic disease			
Clinically diminished pulse			
Presence of bruits in lower extremities			
Previous history of pulmonary emboli			
Peripheral Neuropathy			
Currently not engaged in a regular program and have a VO ₂ max pre-training value > 55 ml/kg-fat free mass-min., indicative of moderate fitness. Or Currently engaged in regular program and having a VO ₂ max value ≤ 55 ml/kg-fat free mass-min.			
Anemia (Hematocrit $< 34\%$)			
Any contraindications to moderate exercise (Please specify)			
Inability and/ or willingness to comply with the protocol as written			
Active alcohol or substance abuse (Past 5 Years)			
Total cholesterol > 300 mg/dL			

Triglyceride >350 mg/dL			
ALT >80, AST>80, Alk Phos >240			
Proteinuria (defined as > 1+ on routine dipstick)			
hypothyroidism (sTSH>8)			
Therapeutic Doses of Nicotinic Acid			
Impaired Glucose Tolerance (fasting glucose \geq 100 mg/dl \leq 126 mg/dl or 2 hour glucose from OGTT \geq 140mg/dl but less than 200 mg/dl or Type 2 Diabetes: Fasting Glucose \geq 126 mg/dl or 2 hour glucose \geq 200 mg/dl			
Oral glucocorticoids			
Females currently on hormone replacement therapy (HRT) less than 6 months			
Claustrophobia			
Previous difficulty with lidocaine or other local anesthetic			
Stress test symptoms: <ul style="list-style-type: none"> ∞ Positive ECG (> 2mm ST segment depression) <u>without</u> PCP cardiologist permission to participate ∞ Signs or symptoms of cardiovascular decomposition (hypotensive response to exercise) ∞ Onset of angina or angina like symptoms, shortness of breath, change in heart rhythm, signs of poor perfusion (light-headedness), tightness, ∞ Hypotension ∞ Other 			

STRESS TEST RESULTS	YES	NO	Notes
Clearance obtained by study cardiologist MD Name: _____			
Clearance obtained by Participant's PCP/Cardiologist MD Name: _____			

Participant Eligible? _____ Yes _____ No

If NO, note specific exclusion criteria: _____

Study Coordinator _____ Date _____ Initials _____

Study Physician _____ Date: _____ Initials _____

APPENDIX C

IRB APPROVED FLYERS

C.1 RECRUITMENT FLYER FOR ATHLETES



University of Pittsburgh
School of Medicine
Department of Medicine
Division of Endocrinology & Metabolism

Do you want to participate in a research study?

**Masters Athletes Wanted for Research Study
CONDUCTED AT UNIVERSITY OF PITTSBURGH
MEDICAL CENTER**

Where: University of Pittsburgh, Montefiore Hospital

Who:

- Men and Women between the ages of 60-75
- Currently exercising regularly and training for competition
- Healthy
- Nonsmoker

Purpose: To study the role of exercise and/or weight loss in seniors at a higher risk of developing type 2 diabetes

Visits in Oakland:

- Three outpatient visits and one overnight visits to Montefiore Hospital
- Compensation: up to \$150 and free parking at Montefiore Hospital

If you are interested in participating, or if you have questions, please call the study coordinator at 412-692-2415

C.2 RECRUITMENT FLYER FOR SEDENTARY SUBJECTS



University of Pittsburgh
School of Medicine
Department of Medicine
Division of Endocrinology & Metabolism

Do you want to exercise?

**Volunteers Wanted for Research Study
CONDUCTED AT UNIVERSITY OF PITTSBURGH MEDICAL
CENTER**

What: 16 week Exercise Program

Where: University of Pittsburgh, Montefiore Hospital

Who:

- Men and Women between the ages of 60-75
- Healthy
- Don't exercise often
- Nonsmoker

Purpose: To study the role of exercise in seniors at a higher risk of developing type 2 diabetes

Visits in Oakland:

- Six outpatient visits and two overnight visits to Montefiore Hospital
- 16 week Exercise Program with exercise specialists
- Compensation: up to \$300 and free parking at Montefiore Hospital

If you are interested in participating, or if you have questions, please call the study coordinator at 412-692-2415

APPENDIX D

IRB APPROVED RECRUITMENT LETTERS



University of Pittsburgh School of Medicine

*Department of Medicine
Division of Endocrinology & Metabolism*

3459 Fifth Ave.
UPMC Montefiore, N807
Pittsburgh, PA 15213
412-692-2848

Dear Friend,

If you, a spouse, or a friend

- are between 60-75 years of age
- exercise 5 times or more per week and/or are preparing for a competition

We have a program that is designed for you.

The American Diabetes Association reports that 10.3 million people age 60 years or older have diabetes. We know that many people are interested in losing weight and exercising because this can help lower the risk of type 2 diabetes.

You are being asked to participate in this study so that your results can be compared to those that are at higher risk to get diabetes.

Study features:

- physical exams and medical tests at no cost to you (this will not replace medical care by your doctor), including among other things a maximal exercise test and body composition assessment.
- support from doctors, nurses and exercise specialists

The study will last about 1 month. A complete assessment will involve 4 outpatient visits and 1 overnight visit. You will be asked not to change your training routine during this time. This study is voluntary.

We hope you consider joining this study. Your participation can help us answer important questions about the role of exercise in preventing type 2 diabetes.

Parking is validated for all study visits to Oakland. Participants will receive up to \$150 upon completion of the study.

If you are interested in learning more about this study, please contact Francesca Amati, MD at 412-692-2415.

Sincerely,

A handwritten signature in black ink, reading "Bret Goodpaster". The signature is written in a cursive, slightly slanted style.

Bret H. Goodpaster, PhD
Associate Professor of Medicine
Division of Endocrinology & Metabolism
Obesity Nutrition Research Center
University of Pittsburgh School of Medicine



University of Pittsburgh School of Medicine

*Department of Medicine
Division of Endocrinology & Metabolism*

3459 Fifth Ave.
UPMC Montefiore, N807
Pittsburgh, PA 15213
412-692-2848

Dear Friend,

If you, a spouse, or a friend

- are between 60-75 years of age
- are not exercising regularly

We have a research program that is designed for you.

The American Diabetes Association reports that 10.3 million people age 60 years or older have diabetes. We know that many people are interested in losing weight and exercising because this can help lower the risk of type 2 diabetes.

The University of Pittsburgh is inviting tri-state residents to participate in this research study. This study will look at the role of regular exercise in the development of type 2 diabetes.

Study features:

- regular exercise under the supervision of exercise specialists
- physical exams and medical tests at no cost to you (this will not replace medical care by your doctor)
- support from doctors, nurses and exercise specialists

The study will last about 4 months. You will first be asked to come to 4 outpatient visits and one overnight visit. If you are eligible to participate, you will be assigned to the exercise program. At the end of the study, 3 outpatient visits and the overnight visit will be repeated. We will compare what kind of changes you have had in your risk for type 2 diabetes. This study is voluntary.

We hope you consider joining this study. Your participation can help us answer important questions about the role of exercise in preventing type 2 diabetes.

Parking is validated for all study visits to Oakland. Participants will receive up to \$300 upon completion of the study.

If you are interested in learning more about this study, please contact Francesca Amati, MD at 412-692-2415.

Sincerely,

A handwritten signature in black ink that reads "Bret Goodpaster". The signature is written in a cursive, slightly slanted style.

Bret H. Goodpaster, PhD
Associate Professor of Medicine
Division of Endocrinology & Metabolism
Obesity Nutrition Research Center
University of Pittsburgh School of Medicine

APPENDIX E

IRB LETTERS OF ACCEPTANCE



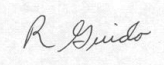
University of Pittsburgh

Institutional Review Board

3500 Fifth Avenue
Ground Level
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)

MEMORANDUM

TO: Bret Goodpaster, PhD

FROM: Richard Guido, MD, Chair 

DATE: April 4, 2008

SUBJECT: IRB #0406003: Skeletal Muscle Lipid and Insulin Resistance: Effects of Physical Activity and Weight Loss

The Institutional Review Board reviewed and approved your renewal with modifications at the Full Board Meeting (Committee A) on March 4, 2008.

Level of Risk: Greater than Minimal

Please include the following information in the upper right-hand corner of all pages of the consent form:

Approval Date: April 4, 2008
Renewal Date: March 3, 2009
University of Pittsburgh
Institutional Review Board
IRB #0406003

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1504.

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month prior** to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

If this research study is subject to FDA regulation, please forward to the IRB all correspondence from the FDA regarding the conduct of this study.

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

RG:dj



University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Ground Level
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)

MEMORANDUM

TO: Bret H. Goodpaster, PhD
FROM: Christopher Ryan, PhD, Vice Chair *Chris*
DATE: October 22, 2008
SUBJECT: IRB #0405764: Muscle Lipid and Insulin Resistance in the Elderly

The Institutional Review Board reviewed the recent modifications to your protocol and consent form(s) and find them acceptable for expedited review. These changes, noted in your submission of September 18, 2008, are approved.

Please include the following information in the upper right-hand corner of all pages of the consent form(s), if modifications were made to the consent form(s):

Current Approval Date: April 04, 2008
Modification Approval Date: October 22, 2008
Renewal Date: March 3, 2009
University of Pittsburgh
Institutional Review Board
IRB #0405764

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month prior** to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

If this research study is subject to FDA regulation, please forward to the IRB all correspondence from the FDA regarding the conduct of this study.

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

CR:kh



University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
<http://www.irb.pitt.edu/>

Memorandum

To: Bret Goodpaster, MD
From: Ron Shapiro, MD, Vice Chair
Date: 2/12/2009
IRB#: [IRB0405764](#)
Subject: Muscle lipid and insulin resistance in the elderly

At its full board meeting on 2/3/2009, the University of Pittsburgh Institutional Review Board, Committee A, reviewed the above referenced research study and approved it pending minor modifications. Your responses to these comments have been reviewed and the research submission, in its currently modified form, adequately addresses the concerns of the IRB and is therefore approved.

Please note the following information:

Please note that the waiver for the requirement to obtain a written informed consent for telephone screening has been previously approved.

Please note that the advertisements that were submitted for review have been previously approved as written. As a reminder, any changes to the wording of the approved advertisements would require IRB approval prior to distribution.

The risk level designation is Greater Than Minimal Risk.

Approval Date: 2/11/2009

Expiration Date: 2/2/2010

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least one month prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh

APPENDIX F

IRB APPROVED INFORMED CONSENT FOR SCREENING



University of Pittsburgh

*School of Medicine
Department of Medicine
Division of Endocrinology & Metabolism*

CONSENT TO ACT AS A SUBJECT IN AN EXPERIMENTAL STUDY

TITLE: Screening Consent Form for Eligibility to Participate in Studies of Physical Activity and/or Weight Loss Programs for Older Adults

Investigators:

Bret Goodpaster, PhD (PI)
Associate Professor of Medicine
Division of Endocrinology/Metabolism
Obesity Nutrition Research Center
University of Pittsburgh
Phone: (412) 692-2437

Jolene Brown, MD (Co-I)
University of Pittsburgh Dept of Medicine
Division of Endocrinology & Metabolism
(412) 692-2285 (office)
(412) 958-4288 (Pager)

Jennifer Gabany MSN, CRNP-C, CCRC (Co-I)
Division of Endocrinology/Metabolism
University of Pittsburgh
Phone: (412) 578-9259

Francesca Amati, MD (Co-I)
Research Fellow
University of Pittsburgh
Ph: (412) 692-2415

Jason Ng, MD (Co-I)
Department of Medicine
Division of Endocrinology & Metabolism
Ph: (412) 692-2973

SOURCE OF SUPPORT: American Diabetes Association (BG)

Why is this research being done?

The Obesity and Nutrition Research Center at the University of Pittsburgh Medical Center is conducting multiple investigations assessing how exercise and weight loss affect how your body responds to the hormone insulin. Insulin is an important hormone secreted by your body that affects how your body uses sugar for energy. The way your body responds to insulin can determine whether you may be at high risk for the development of type 2 diabetes. The results from our investigations will provide further direction on the cause and treatment for individuals who may be at risk for developing Type 2 Diabetes.

Page 1 of 11

Subject's Initials _____



University Of Pittsburgh
Institutional Review Board

Approval Date: 2/11/2009
Renewal Date: 2/2/2010

IRB #: IRB0405764

Who is being asked to take part in our research studies?

You are being asked to take part in our investigations because you are:

- Between the ages of 60-75,
- Normal weight OR Slightly or moderately overweight, based on a calculation of your height and weight called a Body Mass Index (BMI),
- Not currently participating in any exercise or weight loss programs.

These risk factors (age, weight, and level of physical activity) increase an individual's risk for the development of type 2 diabetes.

What studies can I be eligible for?

The "*Muscle Lipid and Insulin Resistance in the Elderly (MIRA)*" and "*Skeletal Muscle Lipid and Insulin Resistance: Effects of Physical Activity and Weight Loss (SHELL)*" studies will examine the effects of weight loss and/or physical activity on the risk for the development of type 2 diabetes in older adults.

If an individual has a decreased ability to use sugar for energy, that person is commonly described as being "insulin resistant". As you age, you are at an increased risk for insulin resistance and the development of type 2 diabetes. How much fat an individual has stored in their body also plays a role in how that fat is used for energy. The primary goal of the MIRA and SHELL studies is to examine the role of fat tissue stored in the bodies of older adults that are insulin resistant. The MIRA and SHELL studies are exercise and/or weight loss intervention studies designed to examine the effect of physical activity and weight loss on the body's ability to use muscular and abdominal fat in relation to an individual's level of insulin resistance.

What procedures in the screening process will be performed for research purposes?

The Screening Process:

If you agree to find out if you are eligible for our studies, you will be asked to complete a set of screening procedures that will give researchers information about your health.

The investigators ask that you read through and discuss this consent form with a member of our study staff. This consent form explains all aspects of the screening portion of our studies. This consent form also briefly explains the nature of the investigations that you may be eligible to participate in. After learning about the studies that are being performed as well as the screening procedures that need to be performed, you will be asked to decide whether you are interested in participating in these studies. A copy of this consent document and all study results will be given to you. You are free to share these records with anyone of your choosing.



The Screening Procedures:

If you decide that you are interested in participating in our research, you will be asked to complete a few screening procedures that will help researchers determine if you are actually eligible to participate in our studies. Since there are multiple studies that you may be eligible for, the results of the screening procedures will also help researchers more accurately decide exactly which specific study you are able to participate in.

We will ask you to complete the following procedures within 1 to 2 visits.

1) General Medical History and Physical Exam: A comprehensive medical history (a thorough review of your health history) and physical exam will be obtained by one of the study physicians or a cardiologist. The physical element of the exam includes a standard physical exam including general assessment of the following: Head, ears, nose, eyes, throat, heart, lungs breast, abdomen, genitalia/rectal, extremities, skin, neurological functioning, and any other impressions or problems noted by the physician at the time of exam. This procedure will take approximately 30 minutes to complete.

2) Blood and Urine Analysis: In order to complete these tests, you will be asked to not eat or drink anything except for water for 12 hours. An analysis of your blood will be performed on a small sample of blood that will be drawn from a vein in your arm. About 2 tablespoons (one ounce) of blood will be drawn to complete the screening lab work. The tests that will be performed on your blood sample will include a complete blood count, electrolytes, liver function tests, thyroid function tests, and blood sugar levels (fasting blood sugar and HbA1c – a number that tells us approximately what your blood sugar has been for the past three months), and blood fat levels (cholesterol, HDL, LDL, VLDL, triglycerides). A small sample of your urine will also be collected and analyzed. The tests on your urine sample will examine how well your kidneys function. This procedure will take approximately 15 minutes to complete.

3) Oral Glucose Tolerance Test (OGTT): In order to complete this test, you will be asked to not eat or drink anything except for water for 12 hours. For this test, a nurse will insert an IV into your arm and a small amount of blood will be drawn every 30 minutes for 2 hours after you drink a sugary drink. The amount of blood taken during this test is about two tablespoons. The purpose of this test is to determine how well your body used sugar for energy. This procedure will take approximately 2 1/2 hours to complete.

What medical conditions could exclude me from participating in the studies?

In order to be eligible to participate in any of our studies, you must be free of clinical evidence of heart, kidney, liver, and blood vessel disease, or any other major medical problems that would endanger your health or compromise the scientific results of this study. In the event that a significant, unknown disease or condition is discovered during the screening process, you will be made aware of all results. You will be given a copy of the screening lab work and will be instructed to contact your primary care physician for follow-

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up. If the screening examination confirms your good health, you will be asked to participate in the study.

How will I know if I am eligible to participate in a study?

The researchers will review the results of your blood and urine tests, your OGTT and medical history to determine if you are eligible for the next visit. If you are eligible for the next visit, you will be asked to ride a stationary bike to determine that you are safe to participate in exercise. We want to be certain that you are safe to exercise. Your group assignment is determined by chance (like the flip of a coin) and you may be assigned to the exercise group.

Researchers will use the explanation of the results from the OGTT to determine which study you may be eligible for (MIRA or SHELL). The specific results that will be used to make that decision include your blood sugar level after you have not eaten or drank anything except for water for 12 hours as well as your blood sugar level 2 hours after having a sugary drink. Your fasting and 2-hour blood sugar measurement must be classified as non diabetic in order to be eligible for the MIRA study. If either your fasting or 2-hour blood sugar measurement is classified as Type 2 diabetes, you are eligible for the SHELL study. The following table helps to explain the potential results from the OGTT as well as the study that you may be eligible to participate in:

Fasting Blood Sugar Measurements (mg/dL)

Fasting Blood Sugar Measurement (mg/dL)	Explanation	Study You May Be Eligible For
Less than 100 mg/dL	Normal	MIRA Study
Greater than or equal to 100, but less than 126 mg/dL	Impaired Sugar Tolerance	SHELL or MIRA Study
Greater than or equal to 126 mg/dL	May Have Type 2 Diabetes	SHELL Study



OGTT 2-Hour Blood Sugar Measurements (mg/dL)

2-Hour Blood Sugar Measurement (mg/dL)	Explanation	Study You May Be Eligible For
Less than 140 mg/dL	Normal	MIRA Study
Greater than or equal to 140, but less than 200 mg/dL	Impaired Sugar Tolerance	SHELL or MIRA Study
Greater than or equal to 200 mg/dL	May Have Type 2 Diabetes	SHELL Study

Could I be a Diabetic?

The American Diabetes Association uses the OGTT and the blood sugar values listed above to determine if an individual may have type 2 diabetes. If your fasting blood sugar levels at the time of screening are between 126 and 200 mg/dl, or if your 2-hour glucose value during the oral glucose tolerance test is greater than 200 mg/dl, you will be referred to your Primary Care Physician with a possible diagnosis of type 2 diabetes. Only your physician can determine if you actually have type 2 diabetes and your physician will determine the appropriate course of treatment for you.

If after the OGTT is completed you have a blood sugar level suggesting that you may have type 2 diabetes, with your approval, we will send a letter to your doctor describing the study that you may be eligible for as well as the results of our tests. The letter will ask your physician whether or not he/she provides permission for you to be in our studies. If your doctor allows you to begin the study without medications to treat your diabetes, you can still be included in the study.

What are the possible risks, side effects, and discomforts of this research study?

Participation in the screening procedures for these investigations may entail some risks. Information on the frequency of possible risk has been categorized using the following categories: Likely – occurs in more than 25% of people (more than 25 out of 100 people); Common – occurs in 1% out of 25% of people (1 to 25 out of 100 people); Rare – occurs in less than 1% of people (less than 1 out of 100 people). *As with any investigational study, there may be adverse events or side effects that are currently unknown and it is possible that certain of these unknown risks could be permanent, serious or life threatening.* The known risks are:

1) Blood sampling: The risks of blood sampling are *common (occurs in 1% to 25% of people)* and may include bleeding, bruising, and soreness. Infection from the blood sampling is rare (less

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than 1% of people). In our experience using similar protocols, research subjects have not experienced adverse effects from these procedures other than a small amount of residual localized soreness at the blood sampling area.

2) Vessel Cannulation: Venipuncture, or insertion of an IV catheter, in the antecubital area (where the arm bends) and in the hand vein may cause bruising, bleeding, hematoma (a bleed under the skin) or soreness (all common) or infection (rare).

“What are possible benefits from taking part in the screening process for these research studies?”

There may be no benefit to participating in this screening process. The only potential benefit from participation in the screening portion of these studies includes access to health related information obtained by the results of the screening procedures.

“What treatments or procedures are available if I decide not to take part in part in the screening process for these research studies?”

Alternative methods available for the determination of general health and glucose tolerance would include requesting a general health physical exam be performed by your own physician or medical practitioner. Additional tests could be performed at the discretion of your own physician or medical practitioner in order to address any health concerns.

“If I agree to take part in the screening process for these research studies, will I be told of any new risks that may be found during the course of the study?”

You will be promptly notified if any new information develops during the conduct of this research study, which may cause you to change your mind about continuing to participate.

“Will I or my insurance provider be charged for the costs of any procedures performed as part the screening process for these research studies?”

There are no costs to you or to your insurance provider for the screening evaluation, the screening lab work, the Obesity Nutrition Research Center visits, or laboratory tests. You and/or your insurance provider will be responsible for any routine care costs, including any applicable copays, coinsurances and deductibles.

“Will I be paid if I take part in the screening process for these research studies?”

There will be no monetary reimbursement for the screening procedures (i.e. lab work, OGTT) however, transportation/parking will be reimbursed up to \$10 and you will receive a copy of all results. If your screening tests reveal that you are eligible to participate in one of the investigations, compensation is available for further participation in the various studies.



“Who will pay if I am injured as a result of taking part in these studies?”

University of Pittsburgh researchers and their associates who provide services at the UPMC recognize the importance of your voluntary participation in their research studies. These individuals and their staffs will make reasonable efforts to minimize, control, and treat any injuries that may arise as a result of this research. If you believe that you are injured as a result of the research procedures being performed, please contact immediately the Principal Investigator or one of the co-investigators listed on the first page of this form.

Emergency medical treatment for injuries solely and directly related to your participation in this research study will be provided to you by the hospitals of the UPMC. It is possible that the UPMC may bill your insurance provider for the costs of this emergency treatment, but none of these costs will be charged directly to you. If your research-related injury requires medical care beyond this emergency treatment, you will be responsible for the costs of this follow-up care unless otherwise specifically stated below. There is no plan for monetary compensation. You do not, however, waive any legal rights by signing this form.

“Who will know about my participation in these research studies?”

Any information about you obtained from or for this research study will be kept as confidential (private) as possible. You will not be identified by name in any publication of research results unless you sign a separate form giving your permission (release).

All records related to your involvement in this research study will be stored in a locked file cabinet. Your identity on those research records will be indicated by a case number as well as your name. Access to your research records will be limited to the researchers listed on the first page of this form and to authorized representatives of the Food and Drug Administration and the study sponsor (the National Institutes of Health), who may need to review the records for accuracy and completeness. Representatives of the study sponsor may also be present during your participation in the research study. The fact that you are participating in a research study and that you are undergoing certain research procedures (but not the results of the procedures) may also be made known to individuals involved in insurance billing and/or other administrative activities associated with the conduct of the study. University of Pittsburgh policy states that your research records must be maintained for at least five years after study completion. The researchers conducting this study have chosen to maintain your research records indefinitely.

“Will these research studies involve the use or disclosure of my identifiable medical record information?”

This research study will involve the recording of current identifiable medical information from your hospital records. The information that will be recorded will be limited to information concerning your screening laboratory work and study participation. This information will be used for the purpose of determining whether you qualify, based on study specific inclusion/exclusion criteria, for study participation. This research study will result in identifiable information that will be placed into your medical records held at the University of Pittsburgh

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Medical Center. The nature of the identifiable information resulting from your participation in this research study that will be recorded in your medical record possibly includes your screening laboratory work, information related to the insulin infusion and the muscle biopsy.

“Who will have access to identifiable information related to my participation in these research studies?”

In addition to the investigators listed on the first page of this authorization (consent) form and their research staff, the following individuals will or may have access to identifiable information (which may include your identifiable medical record information) related to your participation in this research study:

- 1) Authorized representatives of the University of Pittsburgh Research Conduct and Compliance Office may review your identifiable research information (which may include your identifiable medical record information) for the purpose of monitoring the appropriate conduct of this research study.
- 2) In unusual cases, the investigators may be required to release identifiable information (which may include your identifiable medical record information) related to your participation in this research study in response to an order from a court of law. If the investigators learn that you or someone with whom you are involved is in serious danger or potential harm, they will need to inform, as required by Pennsylvania law, the appropriate agencies.
- 3) Authorized representatives of the sponsor of this research study, (the American Diabetes Association), will review and/or obtain identifiable information (which may include your identifiable medical record information) related to your participation in this research study for the purpose of monitoring the accuracy and completeness of the research data and for performing required scientific analyses of the research data. Authorized representatives of the study sponsor may also be present during *your* participation in certain research procedures. While the study sponsor understands the importance of maintaining the confidentiality of your identifiable research and medical record information, the UPMC and University of Pittsburgh cannot guarantee the confidentiality of this information after it has been obtained by the study sponsor.
- 4) The investigators involved in the conduct of this research study may receive funding from the sponsor to perform the research procedures and to provide the sponsor with identifiable research and medical record information related to your participation in the study.
- 5) Authorized representatives of the U.S. Food and Drug Administration may review and/or obtain identifiable information (which may include your identifiable medical record information) related to your participation in this research study for the purpose of monitoring the accuracy of the research data. While the U.S. Food and Drug Administration understands the importance of maintaining the confidentiality of your identifiable research and medical record information, the University of Pittsburgh and the UPMC cannot guarantee the confidentiality of this information after it has been obtained by the U.S. Food and Drug Administration.



- 6) Authorized representatives of the UPMC hospitals or other affiliated health care providers (such as the Clinical Translational Research Center) may have access to identifiable information (which may include your identifiable medical record information) related to your participation in this research study for the purpose of (a) fulfilling orders, made by the investigators, for hospital and health care services (e.g., laboratory tests, diagnostic procedures) associated with research study participation; (b) addressing correct payment for tests and procedures ordered by the investigators; and/or (c) for internal hospital operations (i.e. quality assurance).

“For how long will the investigators be permitted to use and disclose identifiable information related to my participation in the screening process for these research studies?”

The investigators may continue to use and disclose, for the purposes described above, identifiable information (which may include your identifiable medical record information) related to your participation in this research study indefinitely following study completion.

“May I have access to my medical record information that results from my participation in the screening process for these research studies?”

In accordance with the UPMC Notices of Privacy Practices document that you have been provided, you are permitted access to information (including information resulting from your participation in this research study) contained within your medical records filed with your health care provider unless otherwise specifically stated below.

“Is my participation in the screening process for these research studies voluntary?”

Your participation in this research study, including the use and disclosure of your identifiable information for the purposes described above, is completely voluntary. (Note, however, that if you do not provide your consent for the use and disclosure of your identifiable information for the purposes described above, you will not be allowed, in general, to participate in the research

study.) Whether or not you provide your consent for participation in this research study will have no effect on your current or future relationship with the University of Pittsburgh. Whether or not you provide your consent for participation in this research study will have no effect on your current or future medical care at a UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

“May I withdraw, at a future date, my consent for participation in the screening process for these research studies?”

You may withdraw, at any time, your consent for participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above. (Note, however, that if you withdraw your consent for the use and disclosure of your identifiable information for the purposes described above, you will also be withdrawn, in general, from further participation in this research study.) Any identifiable research or medical record

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information recorded for, or resulting from, your participation in this research study prior to the date that you formally withdrew your consent may continue to be used and disclosed by the investigators for the purposes described above.

To formally withdraw your consent for participation in this research study you should provide a written and dated notice of this decision to the principal investigator of this research study at the address listed on the first page of this form.

Your decision to withdraw your consent for participation in this research study will have no effect on your current or future relationship with the University of Pittsburgh. Your decision to withdraw your consent for participation in this research study will have no effect on your current or future medical care at a UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

“If I agree to participate in the screening process for these research studies, can I be removed from the study without my consent?”

You may be removed from this research study by the investigators in the event that the investigators feel that the study may adversely influence your health, if you don't comply with study requirements.

If you do not qualify for this particular study, would you like to be contacted for future research studies? They would explain what any additional project studies involve before you would agree to volunteer. (Please check one)

☐ I do not agree to be contacted for future studies.

☐ I do agree to be contacted for future studies.

If you agree to be contacted, please provide a day time phone number where you can be reached:

_____ Daytime phone number



VOLUNTARY CONSENT

All of the above has been explained to me and all of my current questions have been answered. I understand that I am encouraged to ask questions about any aspect of this research study during the course of this study, and that such future questions will be answered by the researchers listed on the first page of this form.

Any questions I have about my rights as a research participant will be answered by the Human Subject Protection Advocate of the IRB Office, University of Pittsburgh (1-866-212-2668)

By signing this form, I agree to participate in this research study. A copy of this consent form will be given to me.

Participant's Signature

Date

CERTIFICATION OF INFORMED CONSENT

I certify that I have explained the nature and purpose of this research study to the above-named individual, and I have discussed the potential benefits and possible risks of study participation. Any questions the individual has about this study have been answered, and we will always be available to address future questions as they arise.

Printed Name of Person Obtaining Consent

Role in Research Study

Signature of Person Obtaining Consent

Date



APPENDIX G

IRB APPROVED INFORMED CONSENT FOR ATHLETES



University of Pittsburgh

*School of Medicine
Department of Medicine
Division of Endocrinology & Metabolism*

ATHLETE CONSENT TO ACT AS A SUBJECT IN AN EXPERIMENTAL STUDY

TITLE: The MIRA Study: Muscle Lipid and Insulin Resistance in the Elderly

INVESTIGATORS:

Bret Goodpaster, PhD (PI)
Associate Professor of Medicine
Director of the Exercise Laboratory in the
Obesity Nutrition Research Center
Division of Endocrinology/Metabolism
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Ph: (412) 692-2437
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CO-INVESTIGATORS:

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University of Pittsburgh
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Professor of Epidemiology
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Maja Stefanovic-Racic MD, PhD
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Jennifer Gabany MSN, CRNP-C, CCRC
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Jolene Brown, MD
Department of Medicine
Division of Endocrinology & Metabolism
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Jason Ng, MD
Department of Medicine
Division of Endocrinology & Metabolism
Ph: (412) 692-2973

Mark Simon, MD
Cardiology Fellows University of Pittsburgh
Department of Medicine
Ph: (412) 692-2370

SOURCE OF SUPPORT: NIH -RO1 (BG)

Why is this research being done?

Based on the results from your screening procedures, you have been asked to participate in a research study. This study will help us learn whether physical activity (exercise) changes the amount of fat contained within your muscle, and changes the amount of fat you burn. This study will also help us learn whether these changes are related to how your body uses the hormone insulin (which controls blood sugar) and uses blood sugar. These studies should provide new information about the role of exercise to improve how you burn fat in your body and to improve insulin resistance (your body's ability to use blood sugar) We are studying older adults who are at high risk for the development of type 2 diabetes(condition of high blood sugar). This may help us learn more about what causes type 2diabetes.

Who is being asked to take part in this research study?

You are being asked to participate in this study because you are between the ages of 60-75 years and currently performing at least five days per week of exercise and are training for competition in an endurance sport, for example, cycling, rowing, running, triathlon. You will have a physical examination to determine if you are in good general health for participating in this study, and these results will be given to you for your records. If you qualify you will be one of up to 70 subjects in this study. Participation in the study will last 4 weeks and will include one overnight stay in the hospital.

What procedures will be performed for research purposes?

The investigators ask that you read through and discuss this entire consent form which explains the remaining aspects of this study. If, after learning about this research study in detail and reading and signing this consent form, you desire participation, you will be asked to complete a set of procedures to obtain information about your how your body uses sugar and fat for energy. The procedures this research study that are used to collect information about your metabolism are called "Experimental Procedures and Monitoring".

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Experimental Procedures and Monitoring:

Maximal Exercise Test (VO₂max): To measure your fitness level, you will undergo an exercise test on a stationary bicycle called an exercise stress test or VO₂ max. Your heart rate will be monitored by attaching adhesive pads to your chest that are connected to a heart monitor. You will be asked to breathe through a mouthpiece in order to measure how much oxygen your body is using as you pedal the bike. You will be given instructions on how to place the mouthpiece in your mouth. You will be given the opportunity to get used to the mouthpiece, in your mouth prior to the start of the exercise test. Every two minutes, the bike will be adjusted to make pedaling more difficult, and you will be asked to keep pedaling until you are too tired to continue. If you develop a pain in your chest or have particular patterns on the heart monitor, you will be asked to stop pedaling. If your exercise stress test is abnormal, you will be denied entry into the study and will be instructed to follow-up with your primary care physician. This procedure will take approximately 1 hour to complete.

If results from the exercise test exhibit features that place your eligibility for the study into question, you will be recommended for follow-up with your Primary Care Physician (PCP) or cardiologist. If your physician determines that it is safe for you to participate in our study after reviewing the results of your test, we will potentially ask you to repeat the maximal exercise test or to simply continue on in the investigation. If your physician determines that you are not able to safely participate in the investigation, you will be excluded from further involvement in the investigation.

If you are eligible to continue on in the investigation based on the results of the maximal exercise test, you will be next asked to complete the following procedures from which we will obtain more detailed information about how your body uses sugar and fat for energy. The various tests will also give researchers information about what types of substances make up your body (fat, bone, muscle, etc.).

MRI Scan: You will be asked to have magnetic resonance imaging (MRI) scans of your abdomen (belly), and mid-thigh to measure the amount of fat in each location. The MRI scanner is located in the MR Research center in UPMC Presbyterian Hospital and it looks like a donut shaped x-ray machine. The MRI involves lying on a table for about 60 minutes and sliding into a large magnet so that the fat in your calf muscle, in your liver and in your abdomen can be measured. Specifically, only one of your legs will slide into the magnet (a cage like device). You may experience some discomfort associated with noise the machine makes which will be minimized by the use of earplugs and sound padding over the ears. Once the MRI technologist positions you, you cannot move until the scan is finished. You will be provided with information about the progress of the examination via earphones that you will wear throughout the procedure. This procedure will take approximately 90 minutes to complete.

Overnight Visit: Participation will also involve an admission to the University of Pittsburgh Clinical Translational Research Center (CTRC), located on 6 NE Montefiore University Hospital, lasting approximately 20 hours. During this admission, the following will occur.

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You will be admitted to the CTRC at about 5 pm, be served dinner, and then will be instructed not to eat until after the study is completed the following day, probably at about 1 pm, although you can have water. Also, beginning at the time you are admitted to the CTRC, your urine will be collected into a urine collection container. The urine will be sent to the lab for measurements of urea, nitrogen, and creatinine (all end products of your metabolism). The next morning, an intravenous (IV) catheter (plastic needle in your vein) will be placed in the bend of your arm to give insulin (natural hormone in body), glucose (sugar) and a non-radioactive glucose tracer infusion called deuterated glucose. Deuterated glucose is a nonradioactive tracer/marker (has no known harmful effects) that allows the researchers to better evaluate your glucose metabolism by letting them measure how much glucose your liver is making. This naturally occurring nonradioactive marker of glucose is used as a tracer (label) to study the body's processing of sugar. It is safe, non-radioactive, and has no known harmful effects. This non-radioactive tracer is not required to have Food and Drug Administration (FDA) approval. Another intravenous (IV) catheter will be placed in the opposite arm, to sample blood. This IV will be heated with a heating pad to make the blood sampling easier. An insulin infusion, lasting 4 hours, will be given to assess the sensitivity of your glucose metabolism (how well your body burns sugar). Insulin is the hormone made by your body to control your blood glucose levels. During the insulin infusion, your blood glucose level will be closely monitored (every 5 minutes) and kept stable at standard levels (85 – 90 mg/dl) with a glucose infusion (20% dextrose, which is sugar water given through the IV). You will be asked to stay awake during the insulin infusion so that we can be certain that you are not having problems from the infusion. The amount of blood drawn during the insulin infusion part of the study is about 300 milliliters, or 10 ounces. This amount of blood is about one half of what you would donate if you were to go to the blood bank to donate blood. Your blood count is checked during the screening visit to make sure that you can safely donate this amount.

During two 45 minute periods, one at the start of the insulin infusion and one at the end of the insulin infusion, a clear plastic hood will be placed over your face and neck to collect your exhaled air (the air you breathe out) while you lie face up in the bed. This measurement is called indirect calorimetry and is done to measure your metabolic rate (amount of oxygen your body uses and how much fat and glucose it is burning).

Sometime during the last hour of the insulin infusion, you will have a muscle biopsy of your thigh muscle. The purpose of this biopsy is to obtain a small piece of skeletal muscle tissue from your thigh so that the fat and proteins (enzymes) in your muscle cells can be studied. The biopsy is done by a study physician. This biopsy procedure will take approximately 20 minutes. On the outer surface of one thigh, an area the size of a quarter will be numbed by an injected anesthetic (numbing medicine) called lidocaine (such as a dentist might use). Lidocaine is an FDA approved local anesthetic. A small incision (about a quarter of an inch long) will be made and a biopsy needle will be passed into the muscle in order to obtain a small piece of muscle (the size of a pencil tip, equal to 100-150 milligrams). If the first pass of the biopsy needle fails to provide a muscle sample (i.e. if a fat sample is obtained), you will be asked if it would be okay for Dr. Goodpaster, Dr. Toledo or Dr. Maja Stefanovic-Racic to make a second pass through the



same small incision. You can choose to decline a second pass if you wish and this will not affect your compensation or your participation in the study. Betadine ointment and steri-strips will be applied to the muscle biopsy site and this will be covered with a bandage and an elastic wrap. An ice pack will be applied for 20 minutes after the biopsy. You will be given instructions about how to care for the biopsy site and provided with the telephone numbers of the investigators to call if you experience any difficulty. Your muscle sample will be kept for an indefinite length of time for all analyses to be performed. No genetic testing will be performed on your muscle sample. All muscle samples will be stored in -80° C refrigerators located in the Obesity Nutrition Research Center. The Primary investigator and study coordinator will assume overall responsibility for the control of the storage area and your muscle samples. Your muscle specimens identified only by code numbers will be stored in a separate, secure, locked location. This is done to protect your confidentiality. If you decide to withdraw or are withdrawn from the study, your muscle sample will continue to be stored with a linkage code to your identity. Your stored muscle samples will not be available to any investigators who are not listed on this research protocol (front page of this consent). Approximately 100-150mg of your muscle sample will be stored. After the insulin infusion has been completed, the catheters (IV's) will be removed, you will be fed a meal, and this will conclude the overnight study visit. This entire overnight visit will take approximately 20 hours to complete.

Fat Metabolism Test: Participants will be asked to attend an outpatient visit prior to the start of the intervention in which an additional exercise test will be performed. Prior to this visit you will be instructed to avoid strenuous physical activity for two days prior to this study and to eat at least 200 g of carbohydrate per day for the three days preceding the study to make sure that your energy levels are high for the exercise bout. You will have nothing to eat or drink during for 10-12 hours before your fat metabolism test. You will be fed a meal after you have completed your test. The next morning you will perform another exercise test. You will be asked to come to the Obesity Nutrition Research Center Laboratory on the 8th floor Montefiore University Hospital. The Exercise Physiologist will monitor you as you ride a stationary bicycle for exactly one hour. The exercise on the cycle will be relatively easy in the beginning but may become more difficult as your muscles tire. During the exercise, you will be wearing a belt around your chest, which contains a transmitter to measure your heart rate. You will be asked to breathe through a mouthpiece connected to a breathing valve for 5 minutes at four different times during the exercise test in order to collect your expired air for the measurement of your exercising metabolism. During this period you will be breathing in normal room air. You will be allowed to drink water at any time during exercise and cooled by a fan if you request. This entire procedure, including being prepared for the test as well as performing the actual test, will take approximately 90 minutes to complete.

DEXA Scan: You will be scheduled to have a measurement of your body's fat and muscle content. In order to measure this, you will have a non-invasive scan performed which is called a "DEXA" or "dual-energy x-ray absorptiometry" scan. This scan is performed similar to an x-ray study and you will lie on an examination table in a room equipped with the DEXA scanner for approximately 15 minutes. There are no contrast injections (x-ray dye), or blood samples, and the test is painless except for any discomfort you may experience in your back



because of lying on the firm examination table. The DEXA scanner is located on the 8th floor of Montefiore Hospital.

By measuring the amount of fat and muscle tissue you have (by DEXA and MRI scan), the amount of muscle cell lipid (fat) and enzymes you have (determined by the needle biopsy), your body's insulin sensitivity (how well the hormone insulin works in your body), and your exhaled air, the research team is able to perform this comprehensive evaluation.

What are the possible risks, side effects, and discomforts of this research study?

Participation may entail some risks. Information on the frequency of possible risk has been categorized using the following categories: Likely – occurs in more than 25% of people (more than 25 out of 100 people); Common – occurs in 1% out of 25% of people (1 to 25 out of 100 people); Rare – occurs in less than 1% of people (less than 1 out of 100 people). *As with any investigational study, there may be adverse events or side effects that are currently unknown and it is possible that certain of these unknown risks could be permanent, serious or life threatening.* The known risks are:

1) Insulin Infusion: The risk of receiving insulin is that it could cause a low blood sugar reaction (termed hypoglycemia). Hypoglycemia (rare) may cause you to feel shaky, sweaty, hungry, and dizzy and have a rapid heart rate. If severe, hypoglycemia can cause coma, seizure or even death (all rare). It is very unlikely, however, that severe hypoglycemia will occur during the insulin infusion because the blood glucose will be checked every 5 minutes. To minimize the risk of hypoglycemia, you will be given glucose by intravenous infusion to keep your blood glucose at a normal, safe level. As an additional safety measure, you will be kept awake during the insulin infusion so that the study team can be certain that you are not having any kind of unusual or unexpected reaction to the insulin infusion.

2) Exercise Testing/Training: The maximal exercise test or exercise training sessions (unsupervised or supervised sessions) may cause muscle soreness or fatigue (common). If an abnormal rise in blood pressure or changes in the electrical pattern of the heart beats is detected, or if you develop chest pain, the exercise should be stopped immediately. Rarely, exercise may cause moderate to extreme pain which could be due to muscle sprains, muscle strains, broken bones or chest pain. Rarely, exercise tests and exercise training may cause cardiovascular complications and even sudden death. When you perform unsupervised exercise (versus the supervised sessions), you will not have access to the same prompt emergency care that the hospitals of the UPMC offer. You will need to call 911 to activate the emergency medical response. To help you in the event of a medical emergency, the study team will provide you with two handouts of instructions. One handout is on the warning signs of a heart attack and the other is on what to do in the event of having moderate to extreme pain in your legs which could be a muscle pull, strain, or a bone break during exercise. Another risk is redness, skin chafing or irritation from the EKG electrodes used during exercise testing (common).



3) Muscle Biopsy: Muscle biopsy with a needle may cause discomfort, bleeding and bruising at the time of the biopsy and persistent soreness for the next several days (common). There is also the possibility of infection (rare). Lidocaine is used to anesthetize (numb) the muscle and is given just before the biopsy. Lidocaine has risks which are described next. Additional risk includes any unusual reaction to the elastic bandage wrap and ice, i.e. leg numbness which would indicate the elastic bandage had been applied too tightly or the ice left on too long; or any skin redness, irritation, and chafing from the applied antibiotic ointment and/or steri-strips. A portion of this muscle specimen will be sent to the University of Kentucky for analysis but the specimen will be de-identified and then labeled with a unique study code number that can only be linked to your name by the University of Pittsburgh investigators listed at the beginning of this informed consent document. None of your personal identifying information will be shared.

4) Local Anesthetic: A rare, but possible side-effect of lidocaine (xylocaine), which is used for the muscle biopsy, is an anaphylactic reaction (an extreme sensitivity/ strong over-reaction to the lidocaine) that could result in a severe allergic reaction with symptoms such as severe shortness of breath, swelling of the throat, inflammation of the skin, and skin rash. In order to prevent such an occurrence, you will be questioned on screening about prior experiences with the local anesthetic lidocaine. If you have experienced any prior difficulties with this local anesthetic, for your safety, you will be excluded from study participation.

5) Radiation Exposure: Participation in this research study involves a small amount of radiation exposure from the DEXA scans. The amount of radiation exposure you will receive from each DEXA scan is approximately 2 mrem. A rem is a unit of radiation exposure. If you participate in both the pre and post phases of this research study, your exposure to radiation will be double but will still be minimal. For comparison, these radiation exposures are a small fraction of the average whole body radiation exposure (300 mrem) that each member of the public receives per year from radiation sources found in nature. There is no known minimum level of radiation exposure that is recognized as being totally free of the risk of causing genetic defects (cellular abnormalities) or cancer. However, the risk associated with the amount of radiation exposure that you will receive from this procedure is considered to be low and comparable to other everyday risks.

6) MRI Scan: MR imaging has certain conditions that would exclude you from participating in this study. These include cardiac pacer, shrapnel, or other metal devices. Metal objects present in the body could be moved by the large magnet involved in the MRI, and such movement could cause serious injury. Fear of closed spaces are also reasons to be excluded from the study. You may feel anxious in the magnet, but since your head will be outside the scanner, risk of this is rare. No serious biological effects have been reported from being in a magnet. If you experience a fear of the confined space while in the magnet, you can terminate the study. More severe reactions such as respiratory distress or severe drop in blood pressure are rare. Trained medical personnel are always in attendance during these studies. The custom-built extremity coil has not been approved by the FDA for human use. However, this coil has been constructed by a trained radiofrequency engineer using high-performance components, is mechanically and electrically isolated from the patient and has been tested on phantoms and volunteers. It is also mechanically



robust, using the same support system as used by commercial manufacturers. In addition, this extremity coil meets the criteria for 'nonsignificant risk' according to the FDA as having a specific absorption rate less than 12 Watts/kg in tissues of the extremity for periods of 5 minutes.

7) Vessel Cannulation: Venipuncture, or insertion of a catheter, in the antecubital area (where the arm bends) and in the hand vein may cause bruising, bleeding, hematoma (a bleed under the skin) or soreness (all common) or infection (rare).

8) Indirect Calorimetry: Rarely, during the indirect calorimetry testing, individuals may become claustrophobic (fearful of enclosed places) while their head is under this clear plastic hood. If this happens to you, the hood will be removed.

9) Deuterated Glucose: This naturally occurring isotope has been carefully tested in a laboratory and proven to be free of impurities. However, on rare occasions, people may have reactions to the isotope such as back pain, chills, or nausea. If this happens, the test and the isotope infusion will be stopped immediately.

“What are possible benefits from taking part in this study?”

You may benefit personally from participation in this study since you will be given all of your tests results. If you train within a team, you are free to share your results with your coach that will be able to use them for your personal training.

“What procedures are available if I decide not to take part in this research study?”

There are a wide variety of exercise performance labs where you can have some of these tests done. Your primary care physician can give you information about your general health. Please consult your primary care physician or coach to discuss these options.

“If I agree to take part in this research study, will I be told of any new risks that may be found during the course of the study?”

You will be promptly notified if any new information develops during the conduct of this research study which may cause you to change your mind about continuing to participate.

“Will I or my insurance provider be charged for the costs of any procedures performed as part of this research study?”

There are no costs to you or to your insurance provider for the screening evaluation, the screening lab work, the exercise visits at the Obesity Nutrition Research Center, laboratory tests, the EKGs, exercise testing, the MRI scans, DEXA Scan, the muscle biopsies or the overnight visits to the CTRC. You and/or your insurance provider will be responsible for any routine care costs, including any applicable copays, coinsurances and deductibles.



“Will I be paid if I take part in this research study?”

There is no monetary payment for the screening evaluation; however, you will receive a copy of all the screening laboratory testing. Costs related to transportation and parking for the screening visit will be reimbursed up to \$10. You will receive payment for study participation in the amount of \$150 for completing the insulin infusion, the muscle biopsy, the indirect calorimetry testing, and MRI scans. In addition, any parking fees related to your participation in this study will be paid for by the study.

“Who will pay if I am injured as a result of taking part in this study?”

University of Pittsburgh researchers and their associates who provide services at UPMC recognize the importance of your voluntary participation in their research studies. These individuals and their staffs will make reasonable efforts to minimize, control, and treat any injuries that may arise as a result of this research. If you believe that you are injured as a result of the research procedures being performed, please contact immediately the Principal Investigator or one of the co-investigators listed on the first page of this form.

Emergency medical treatment for injuries solely and directly related to your participation in this research study will be provided to you by the hospitals of UPMC. It is possible that UPMC may bill your insurance provider for the costs of this emergency treatment, but none of these costs will be charged directly to you. If your research-related injury requires medical care beyond this emergency treatment, you will be responsible for the costs of this follow-up care unless otherwise specifically stated below. There is no plan for monetary compensation. You do not, however, waive any legal rights by signing this form.

“Who will know about my participation in this research study?”

Any information about you obtained from or for this research study will be kept as confidential (private) as possible. You will not be identified by name in any publication of research results unless you sign a separate form giving your permission (release).

All records related to your involvement in this research study will be stored in a locked file cabinet. Your identity on these records will be indicated by a case number rather than by your name, and the information linking these numbers with your identity will be kept separate from the research records. Access to your research records will be limited to the researchers listed on the first page of this form and to authorized representatives of the Food and Drug Administration and the study sponsor (the National Institutes of Health), who may need to review the records for accuracy and completeness. Representatives of the study sponsor may also be present during your participation in the research study. The fact that you are participating in a research study and that you are undergoing certain research procedures (but not the results of the procedures) may also be made known to individuals involved in insurance billing and/or other administrative activities associated with the conduct of the study. University of Pittsburgh policy states that

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your research records must be maintained for at least five years after study completion. The researchers conducting this study have chosen to maintain your research records indefinitely.

“Will this research study involve the use or disclosure of my identifiable medical record information?”

This research study will involve the recording of current identifiable medical information from your hospital records. The information that will be recorded will be limited to information concerning your screening laboratory work and study participation. This information will be used for the purpose of determining whether you qualify, based on study specific inclusion/exclusion criteria, for study participation. This research study will result in identifiable information that will be placed into your medical records held at the University of Pittsburgh Medical Center. The nature of the identifiable information resulting from your participation in this research study that will be recorded in your medical record possibly includes your screening laboratory work, information related to the insulin infusion and the muscle biopsy.

“Who will have access to identifiable information related to my participation in this research study?”

In addition to the investigators listed on the first page of this authorization (consent) form and their research staff, the following individuals will or may have access to identifiable information (which may include your identifiable medical record information) related to your participation in this research study:

1) Authorized representatives of the University of Pittsburgh Research Conduct and Compliance Office may review your identifiable research information (which may include your identifiable medical record information) for the purpose of monitoring the appropriate conduct of this research study.

2) In unusual cases, the investigators may be required to release identifiable information (which may include your identifiable medical record information) related to your participation in this research study in response to an order from a court of law. If the investigators learn that you or someone with whom you are involved is in serious danger or potential harm, they will need to inform, as required by Pennsylvania law, the appropriate agencies.

3) Authorized representatives of the sponsor of this research study, (the American Diabetes Association), will review and/or obtain identifiable information (which may include your identifiable medical record information) related to your participation in this research study for the purpose of monitoring the accuracy and completeness of the research data and for performing required scientific analyses of the research data. Authorized representatives of the study sponsor may also be present during *your* participation in certain research procedures. While the study sponsor understands the importance of maintaining the confidentiality of your identifiable research and medical record information, the UPMC and University of Pittsburgh cannot guarantee the confidentiality of this information after it has been obtained by the study sponsor.

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4) The investigators involved in the conduct of this research study may receive funding from the sponsor to perform the research procedures and to provide the sponsor with identifiable research and medical record information related to your participation in the study.

5) Authorized representatives of the U.S. Food and Drug Administration may review and/or obtain identifiable information (which may include your identifiable medical record information) related to your participation in this research study for the purpose of monitoring the accuracy of the research data. While the U.S. Food and Drug Administration understands the importance of maintaining the confidentiality of your identifiable research and medical record information, the University of Pittsburgh and the UPMC cannot guarantee the confidentiality of this information after it has been obtained by the U.S. Food and Drug Administration.

6) Authorized representatives of the UPMC hospitals or other affiliated health care providers (such as the General Clinical Research Center at Children's Hospital of Pittsburgh, where the DEXA Scanner is located), may have access to identifiable information (which may include your identifiable medical record information) related to your participation in this research study for the purpose of (a) fulfilling orders, made by the investigators, for hospital and health care services (e.g., laboratory tests, diagnostic procedures) associated with research study participation; (b) addressing correct payment for tests and procedures ordered by the investigators; and/or (c) for internal hospital operations (i.e. quality assurance).

"For how long will the investigators be permitted to use and disclose identifiable information related to my participation in this research study?"

The investigators may continue to use and disclose, for the purposes described above, identifiable information (which may include your identifiable medical record information) related to your participation in this research study indefinitely following study completion.

"May I have access to my medical record information that results from my participation in this research study?"

In accordance with the UPMC Notices of Privacy Practices document that you have been provided, you are permitted access to information (including information resulting from your participation in this research study) contained within your medical records filed with your health care provider unless otherwise specifically stated below.

"Is my participation in this research study voluntary?"

Your participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above, is completely voluntary. (Note, however, that if you do not provide your consent for the use and disclosure of your identifiable information for the purposes described above, you will not be allowed, in general, to participate in the research study.) Whether or not you provide your consent for participation in this research study will have no effect on your current or future relationship with the University of Pittsburgh.

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Whether or not you provide your consent for participation in this research study will have no effect on your current or future medical care at a UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

“May I withdraw, at a future date, my consent for participation in this research study?”

You may withdraw, at any time, your consent for participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above. (Note, however, that if you withdraw your consent for the use and disclosure of your identifiable information for the purposes described above, you will also be withdrawn, in general, from further participation in this research study.) Any identifiable research or medical record information recorded for, or resulting from, your participation in this research study prior to the date that you formally withdrew your consent may continue to be used and disclosed by the investigators for the purposes described above.

To formally withdraw your consent for participation in this research study you should provide a written and dated notice of this decision to the principal investigator of this research study at the address listed on the first page of this form.

Your decision to withdraw your consent for participation in this research study will have no effect on your current or future relationship with the University of Pittsburgh. Your decision to withdraw your consent for participation in this research study will have no effect on your current or future medical care at a UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

“If I agree to participate in this research study, can I be removed from the study without my consent?”

You may be removed from this research study by the investigators in the event that the investigators feel that the study may adversely influence your health, or if you don't comply with study requirements.

If you do not qualify for this particular study, would you like to be contacted for future research studies? The researchers would explain what any additional project studies involve before you would agree to volunteer. (Please check one)

☐ I do not agree to be contacted for future studies.

☐ I do agree to be contacted for future studies.

If you agree to be contacted, please provide a day time phone number where you can be reached:
Daytime phone number _____



VOLUNTARY CONSENT

All of the above has been explained to me and all of my current questions have been answered. I understand that I am encouraged to ask questions about any aspect of this research study during the course of this study, and that such future questions will be answered by the researchers listed on the first page of this form.

Any questions I have about my rights as a research participant will be answered by the Human Subject Protection Advocate of the IRB Office, University of Pittsburgh (1-866-212-2668).

By signing this form, I agree to participate in this research study. A copy of this consent form will be given to me.

Participant's Signature

Date

Printed Name of Participant

CERTIFICATION OF INFORMED CONSENT

I certify that I have explained the nature and purpose of this research study to the above-named individual, and I have discussed the potential benefits and possible risks of study participation. Any questions the individual has about this study have been answered, and we will always be available to address future questions as they arise.

Printed Name of Person Obtaining Consent

Role in Research Study

Signature of Person Obtaining Consent

Date

Printed Name of Physician Obtaining Consent

Role in Research Study

Signature of Physician Obtaining Consent

Date

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