### Title

In vivo $^{2}H_{2}O$ administration for the measurement of adipose and hepatic triglyceride synthesis, de novo lipogenesis, adipocyte proliferation and protein synthesis reveals differences in insulin sensitive versus insulin resistant obese humans.




### Author

Allister, Candice



### Publication Date

2014

Peer reviewed|Thesis/dissertation

# *In vivo* $^{2}H_2O$ administration for the measurement of adipose and hepatic triglyceride synthesis, de novo lipogenesis, adipocyte proliferation and protein synthesis reveals differences in insulin sensitive versus insulin resistant obese humans.

By

Candice Alexandria Allister

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Endocrinology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Marc K. Hellerstein, Chair

Professor Tyrone Hayes

Professor Janet King

Spring 2014

Abstract

*In vivo* $^2H_2O$ administration for the measurement of adipose and hepatic triglyceride synthesis, de novo lipogenesis, adipocyte proliferation and protein synthesis reveals differences in insulin sensitive versus insulin resistant obese humans.

By

Candice Alexandria Allister

Doctor of Philosophy in Endocrinology

University of California, Berkeley

Professor Marc K. Hellerstein, Chair

Obesity affects about 34% of adults in the United Sates, and 25% of these individuals will become insulin resistance. Studies have provided evidence to suggest that subcutaneous adipose tissue is protective against insulin resistance and type 2 diabetes. Anti-diabetic drugs, thiazolidinediones (TZDs), improve insulin sensitivity by redistributing fat storage into the subcutaneous fat depots and away from visceral fat depots. Unlike subcutaneous fat, visceral fat is highly lipolytic, releasing elevated concentrations of free fatty acids (FFAs) into circulation. Elevated free fatty acids are a common phenotype of insulin resistance, causing lipotoxicity in tissues and impairing insulin signaling in insulin-responsive cells. For example, hepatic lipotoxicity inhibits insulin's ability to suppress gluconeogenesis, in addition to acetyl-CoA entering into the gluconeogenic pathway. Thus, it is important to prevent the intrusion of lipids into tissues and preserve insulin action. However, not all obese individuals become insulin resistant.

It has been reported that a significant portion of obese individuals are metabolically healthy. The purpose of this dissertation was to investigate potential mechanisms that protect some obese individuals from becoming insulin resistant. Using $^2H_2O$ in BMI-matched obese insulin sensitive (IS) and insulin resistant (IR) individuals, we quantified subcutaneous adipose lipid kinetics, cellular proliferation and the synthesis of proteins involved in metabolic pathways, all of which may play an imperative role in the etiology of insulin resistance.

We hypothesized that dysfunctional subcutaneous adipose triglyceride storage is an initiating factor in the development of systemic insulin resistance in human obesity. We tested this hypothesis using $^2H_2O$ to quantify, *in vivo*, triglyceride (TG) synthesis, *de novo* lipogenesis (DNL), and adipocyte proliferation in subcutaneous adipose cells of insulin-sensitive (IS) and insulin-resistant (IR) subjects. Adipose cell size distribution was quantified using Beckman

Multisizer III. Plasma free fatty acid (FFA) concentrations were measured under fasting and postprandial insulin concentrations during a steady state plasma glucose (SSPG) test as a measurement of insulin suppression of lipolysis. Total TG synthesis and DNL were significantly lower in adipose cells of IR compared to IS subjects, and correlated inversely with the proportion of small adipocytes. FFA concentrations were significantly greater in IR versus IS during fasting and postprandial insulin concentrations. Adipocyte proliferation did not differ significantly between groups. These results strongly support the hypothesis that decreased capacity for lipid storage in subcutaneous adipose tissue is a defining feature of obesity-associated insulin resistance and may contribute to insulin resistance in peripheral tissues due to ectopic fat deposition.

Following our findings in a predominately Caucasian cohort, we sought to determine if adipose dysfunction is predictive of insulin resistance amongst different ethnicities. In the United States, African-Americans (AA) have 51% higher rates of obesity than Caucasians (C), and the highest rates of type 2 diabetes and cardiovascular disease Obesity is associated with insulin resistance, yet the physiological mechanisms of this association and fat regional distribution as a potential contributor is not yet completely understood, particularly in minority populations most affected by this disease. Increased visceral adipose tissue (VAT) mass a significant risk factor for insulin-resistance, but recent studies have discovered that this phenotype may not be a global risk factor. VAT tends to be consistently lower in AA compared to C, yet individuals in this group are more insulin resistant for a given degree of body fat. Thus, we aimed to further investigate differences in adipose lipid synthesis between African-Americans and Caucasians. We administered $^{2}H_2O$ to five African-Americans and seven Caucasians for the quantification of TG synthesis, DNL and adipocyte proliferation

Using $^{2}H_2O$, we aimed to apply dynamic proteomics to quantify protein turnover rates of proteins from isolated subcutaneous mature adipocytes. Dynamic proteomics is the large-scale measurement of protein expression and turnover in a single specimen, biological tissue or bodily fluid. Dynamic proteomics applied to humans may allow us to discover the adipoycte proteome of insulin resistance. We investigated the adipocyte proteome of 3 IS and 3 IR, equally obese subjects at steady state after 4 weeks of $^{2}H_2O$ consumption. Peptides were submitted through LC/MS-MS analysis to separate and identify tryspinized peptides by size and charge, followed by Spectrum Mill analysis to organize peptides based on their corresponding protein. A total of 620 proteins were identified amongst the six subjects, but after filtering for criteria (1<peptide and at least 1 duplicate per group), 438 proteins were identified and included in the final analysis. Proteins were then organized by pathway via KEGG analysis, resulting in a total of 218 pathways. We only focused on pathways known to directly affect or be affected by insulin, and of these, the majority of pathways showed greater protein fractional synthesis rates in IR subjects compared to IS subjects. Interestingly, the synthesis rates of proteins involved in lipid metabolism were greater in IR compared to IS, seemingly contradicting previous findings of reduced TG synthesis in this group. Similarly, proteins involved in cellular growth and proliferation had higher synthesis rates in IR vs IS, whereas adipocyte and stromal vascular cell proliferation were not different between groups. Our findings here reveal differential within tissue and cellular pathways for resistance or sensitivity to insulin in states characterized as insulin resistant based on altered glucose metabolism.

## Table of Contents

**Acknowledgements**

Thank you to my Principal Investigator, Dr. Marc Hellerstein, for providing me this opportunity, guiding me along the way and for always believing in me. Thank you to my collaborators in the Laboratory of Dr. Tracey McLaughlin at Stanford University School of Medicine and Dr. Samuel Cushman at the National Institute of Diabetes and Digestive Kidney Diseases.

I would like to thank my laboratory colleagues and friends, Donald (DJ) Roohk, Airlia Thompson, Candice Herber and Chelsea Bidlow and Mark Fitch, as well other fellow PhD students and undergraduate research assistants for your support and friendship.

## Dedications

This dissertation is dedicated to my Mom, Dad, and my sister, Sonja, for your unconditional love and support throughout all of my endeavors;
and to the Price Family for being a second family away from home

A special dedication goes to my fiancé, Jerome Price, for without you I would not have made it through this journey. This is for you.

## Chapter 1

## Introduction: A review of obesity-induced insulin resistance

*Insulin resistance*

Insulin resistance is defined as impaired insulin action in major insulin-responsive tissues, and serves as the precursor to type 2 diabetes (T2D) (**Figure 1**). An influx of glucose into the pancreas triggers the release of insulin into circulation to push glucose into cells to be metabolized or stored. However, when these tissues fail to respond to insulin, leaving elevated concentrations of glucose in circulation, the pancreas responds by secreting more insulin. In order to meet the demands of continuous resistance to insulin in tissues, β-cells within the pancreas being to proliferate, increasing insulin release into the blood. This enhanced release of insulin is termed "β-cell compensation". In some individuals, β-cell compensation can be maintained throughout their lifetime, but in the majority, β-cells become exhausted and are unable to compensate for hyperglycemic conditions. As a result, hyperglycemia is paired with hypoinsulinemia, as type 2 diabetes develops (1).

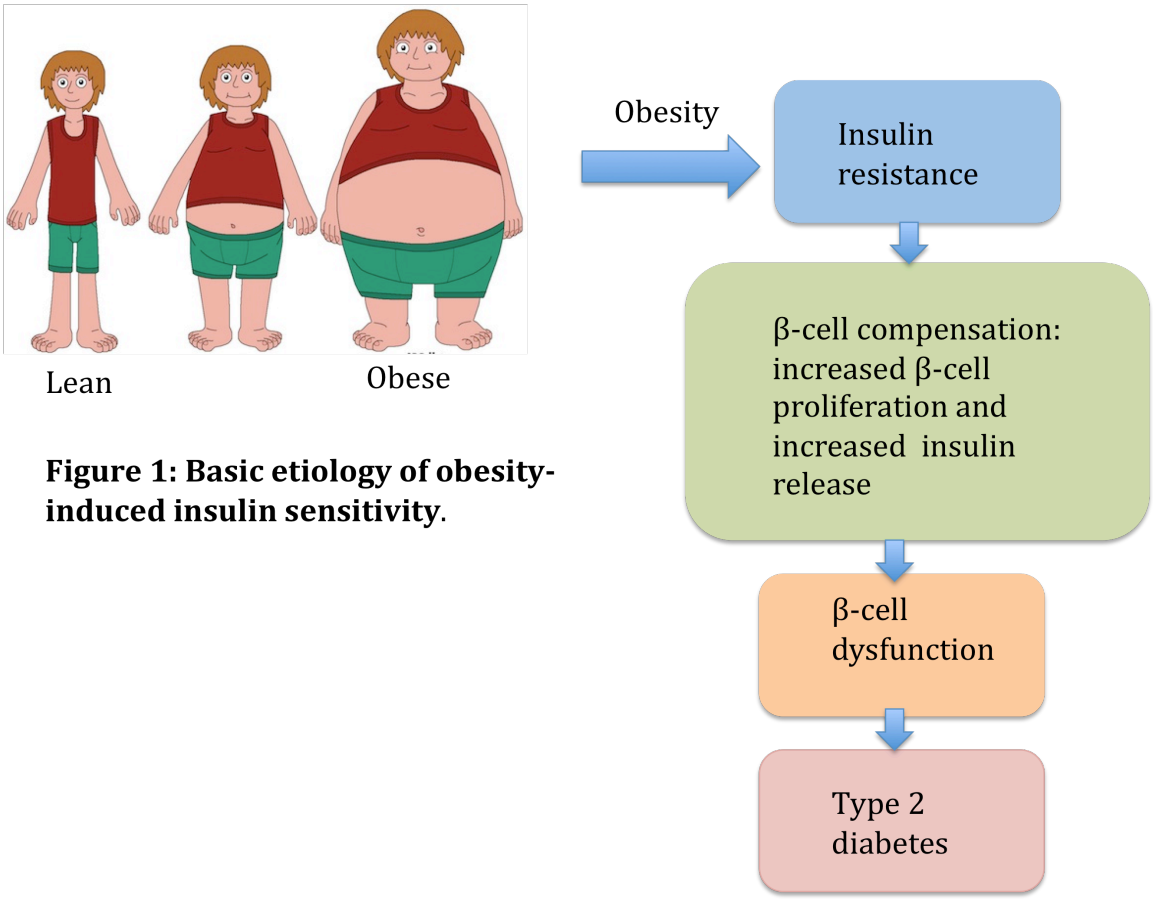



**Figure 1: Basic etiology of obesity-induced insulin sensitivity.**

The etiology of insulin resistance is complex due to insulin's dominating role in metabolism regulating several pathways predominately in muscle, adipose, liver, pancreas, and brain. In the muscle, insulin stimulates glucose uptake through glucose transport 4 (GLUT4) for glycogen storage; in the liver, insulin stimulates lipogenesis through sterol regulatory element binding protein (SREPB1c); in the adipose, insulin stimulates glucose uptake, triglyceride (TG) synthesis, *de novo* lipogenesis, and lipid storage. In addition to its stimulatory responses, insulin also inhibits lipolysis in the adipose and liver, and inhibits gluconeogenesis in the liver and muscle in the fed state. A basic depiction of insulin's action in various tissues is displayed in **Figure 2**. Impairment of any one of these metabolic responses can be deemed insulin resistance (IR), yet the cause of impaired insulin action may vary and is contributed by many various factors, still being investigated today. One of the major risk factors for IR is obesity, a condition that affects about 33.8% of adults in the U.S. (2). Twenty-five percent of these obese patients become insulin resistant (3). The continued efforts towards understanding the role obesity plays in the development of these conditions are pertinent for the treatment and more importantly, prevention of type 2 diabetes.

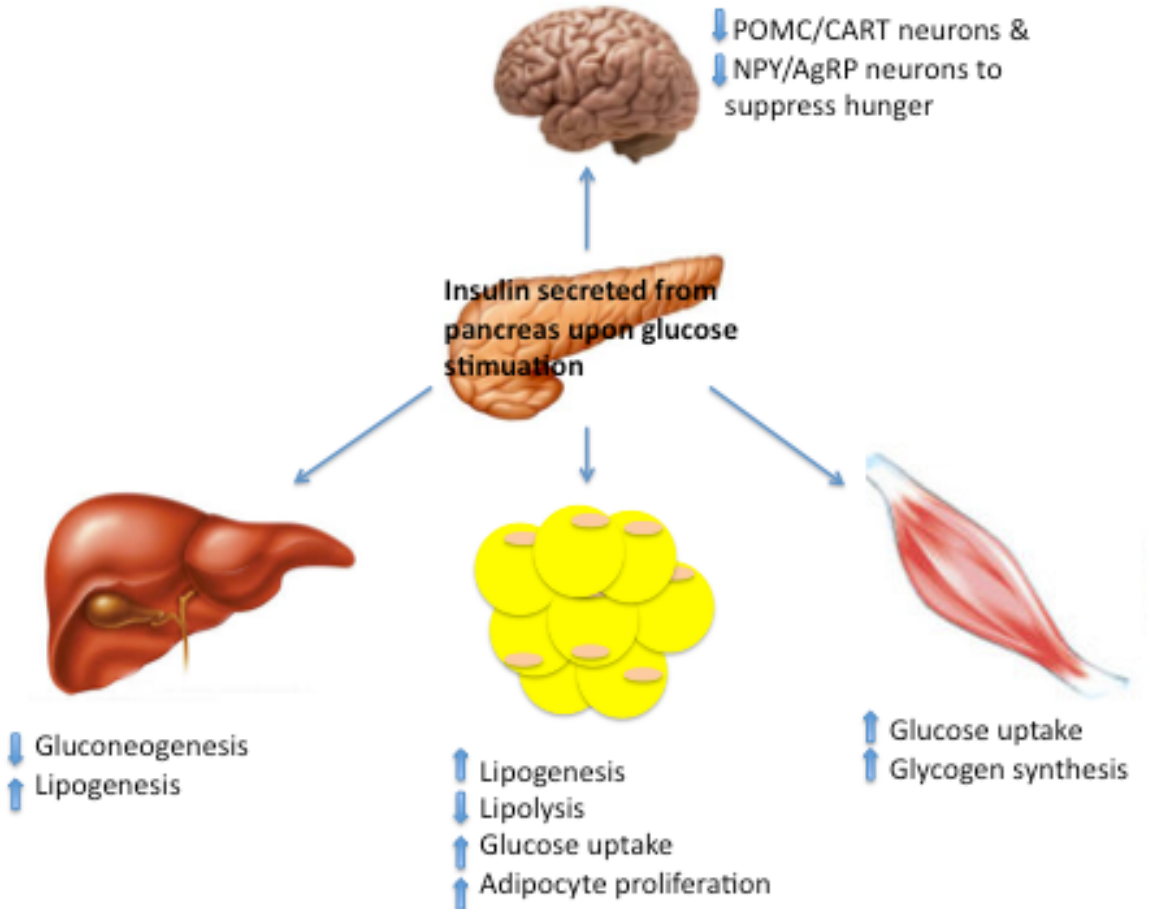



**Figure 2:** A basic schematic of insulin's affects on important metabolic tissues. Insulin is produced and released from pancreatic β-cells in the post-prandial state to stimulate glucose uptake in muscle and adipose tissue, and lipogenesis in adipose and liver. Insulin also suppresses lipolysis in the adipose tissue, and gluconeogenesis in the liver. Adipocyte proliferation in adipose tissue is stimulated by insulin.

*Obesity as a risk factor for insulin resistance*

Obesity is defined as excessive adipose mass caused by both hyperplasia (fat cell proliferation) and hypertrophy (fat cell size expansion) of adipocytes, or fat cells (4*)*. This excess storage is a result of chronic positive energy balance, in which the intake of postprandial lipids and glucose is greater than the amount utilized. The adipose is able to accommodate for this excess energy intake by undergoing hypertrophy or hyperplasia. It is thought that hypertrophy is the primary response to excess storage; while hyperplasia is the secondary response once adipocytes have reached their hypertrophic capacity. This idea is supported by studies showing a greater number of large versus small adipocytes in obese patients and enlargement of already-existing adipocytes but no change in cell number in overfed patients (5,6). How the enlargement of an adipocyte versus the proliferation of new adipocytes effects proper fat cell function is not completely understood. The observation of obesity-associated metabolic consequences, such as insulin resistance, type 2 diabetes and cardiovascular disease suggests there is a limitation to the ability to store excess energy. Examining the physiology of adipose tissue may help us to understand how obesity affects downstream consequences of insulin resistance. Obesity may begin as a compensatory mechanism for excess storage, but it transforms into an endocrine disruptor that interrupts the healthy physiological homeostasis of the human body. It does this by causing lipotoxicity in insulin-responsive tissues, disrupting insulin signaling, enhancing inflammatory responses, and preventing proper uptake of nutrients. However, further research has discovered that it is not simply a matter of being lean versus obese, but rather how an individual stores their excess energy.

*Fat distribution: subcutaneous versus visceral adipose tissue*

Increased adiposity alone may not lead to insulin resistance, but rather it is the distribution of adipose that impacts insulin action. There are many different adipose storage sites, classified into either subcutaneous or visceral fat. Subcutaneous fat includes, gluteal, femoral, epidydimal and superficial abdominal fat (above the abdominal cavity). Visceral fat lies within the abdominal cavity, surrounding major organs of the body, and includes intrabdominal, mesenchymal, perirenal. Measuring the volume of these depots requires the use of a computed topography (CT) or magnetic resonance imaging (MRI) scan, but due to the costs and necessary facilities, many investigators use waist-to-hip ratio (WHR) circumference as a measure of abdominal fat content. WHR has been shown to correlate well with insulin resistance (7), however it is not always an accurate depiction of fat storage. For example, MRI scan results from a human study demonstrated that men with similar WHR's have varying degrees of visceral fat content (8). Thus, the feasibility to quantify these depots in patients provides more information regarding potential risks for developing insulin resistance.

It is well established that an increased visceral to subcutaneous ratio is a major risk factor for insulin resistance, suggesting a significant role for adipose distribution in insulin sensitivity (9,10). There is evidence of physiological and molecular differences between visceral adipose tissue (VAT) versus subcutaneous adipose tissue (SAT), explaining the link between fat deposition and insulin resistance. Subcutaneous fat is thought to be protective against insulin resistance and molecular and physiological characteristics support this notion.

Subcutaneous adipose storage makes up the majority of fat storage in lean individuals, with only 5 to 20% being visceral fat, depending on sex (11). Adipocytes found in SAT are thought to be more sensitive to the uptake and storage of free fatty acids, and thus more responsive to insulin in the postprandial period (12). Subcutaneous adipocytes have been shown to be more responsive to PPAR-γ agonist-stimulation (13,14). PPAR-γ is responsible for up-regulating lipid synthesis and storage and in the adipose, and therapeutic drugs against T2D are known to increase SAT fat storage while simultaneously improving insulin sensitivity (15). More specifically, these drugs, called thiazolidinediones (TZDs) actually decrease VAT storage and redistribute it to SAT by stimulating PPAR-γ in SAT. Such findings propose reasons for fat distribution during weight gain and adaptation to excess lipid storage.

Visceral fat produces more pro-inflammatory cytokines than subcutaneous adipose (16,17). Cytokines, such as tumor necrosis factor-alpha (TNF-α), interlukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), adiponectin and leptin, hinder insulin action. TNF-α is increased in insulin resistance and is thought to have an inhibitory effect on insulin and stimulatory effects on lipolysis (18). Studies in humans have demonstrated that different depots have different adipogenic profiles with varying storage capacities and varying rates of precursor cell proliferation and/or differentiation into mature adipocytes (19,6). VAT is also more lipolytic than SAT, making it the more harmful fat depot. Increased lipolysis is often used as a metric of insulin resistance in the adipose tissue and larger adipocytes have greater rates of lipolysis compared to smaller adipocytes (20,21). NEFAs are released into circulation from the adipose through lipolysis and in the fed state, these fatty acids are re-esterified and stored back into the adipose if they have not been oxidized or stored elsewhere. The constant uptake of lipids into the adipose contributes to obesity, but the inability for this to occur is a common consequence of obesity. This increase in lipolysis releases free fatty acids (FFA) into circulation to reach the liver and muscle, causing lipotoxicity in these tissues. This influx in FFA in the liver and muscle impair insulin signaling, and may partially explain the consequences of obesity (22). Hepatic fat content and ectopic fat storage is a significant issue in the development of diabetes. Its link to insulin resistance is caused by the interference of diacylglycerol (DAG) in the insulin-signaling cascade. As more DAG is synthesized but released into circulation, rather than being stored in the adipose, DAG can enter into insulin-responsive tissues, triggering the activation of PKC-ε, which phosphorylates Ser307 on IRS-1, inhibiting insulin action (23).

Although much evidence indicates that subcutaneous fat storage is more beneficial than visceral fat, recent data has discovered that not all subcutaneous adipose tissue is made equally. Recent evidence shows that deep subcutaneous adipose tissue has similar characteristics as visceral adipose tissue, presenting itself as a hidden risk factor for insulin resistance. The amount of deep subcutaneous tissue is only significant in obese subjects, perhaps lending to the cause of obesity-induced insulin resistance (24). Superficial SAT has larger fat cells, potentially indicating a greater ability to store fat compared to deep SAT. Microarray analysis showed significant differences in genes expressed in deep SAT versus superficial SAT, suggesting differential functions of the two depots (24). This same study also reported reduced gene expression of PPAR-γ in deep SAT, and thus this depot likely has a reduced ability to synthesize and store lipids. Together with visceral fat, deep SAT contributes to the onset of insulin resistance by enhancing plasma FFAs, leading to inhibition of the insulin-signaling cascade.

*Adipocyte cell size and number*

Adipose tissue was once viewed as an irrelevant organ, but has since been discovered to be of great importance in the development of many obesity-related diseases. The primary function of adipocytes (fat cells of the adipose) is to take up triglycerides for storage, explaining why about 90% of the adipocyte is lipid-filled, with the remaining 10% comprising of intercellular compartments (25). Adipocytes are formed from pre-adipocytes, which become lipid-filled as they differentiate into mature adipocytes (**Figure 3**).

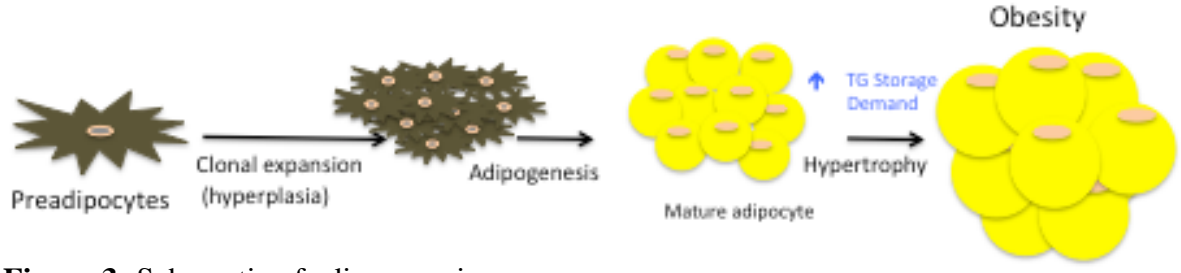



**Figure 3:** Schematic of adipogenesis.

Some investigators have shown that obese IR subjects have more visceral fat storage than obese IS subjects, suggesting that the distribution of fat, rather than simply the accumulation of total fat is key to the development of insulin resistance. Therefore, it has been proposed that mature adipocytes expand in size first in the setting of excess lipid uptake, and once capacity is reached, adipogenesis is stimulated to make more mature adipocytes for storage (26,27,28). The role of adipogenesis in obesity has not been thoroughly explored *in vivo,* but a few studies show reduced expression of adipogenic genes and impaired adipocyte proliferation in IR subjects (29,30). It has been proposed by the McLaughlin group (31) and others that one major cause to the onset of insulin resistance may be impaired adipocyte function, leading to ectopic fat storage and thus, lipotoxicity in insulin-responsive tissues. Previous studies by this group have shown that compared to insulin sensitive subjects, insulin resistance subjects had an enhanced proportion of small adipocytes (31). Investigators suggested this indicated impaired adipogenesis, which is required under conditions of weight gain (ie. obesity). A greater portion of enlarged mature adipocytes versus small mature adipocytes is associated with insulin resistance and type 2 diabetes (32, 33). Most studies focus on the significance of large adipocytes, however, the finding of large portions of small adipocytes in the IR state suggest that larger adipocytes may be reflective of the inability to produce new fat stores, leaving the current cells to continue to become filled with lipids. However, if there in fact exists a capacity at which fat cells can store lipids, these lipids are likely to be stored ectopically, and not in the subcutaneous adipose depot.

*Insulin-sensitive obesity*

As stated earlier, obesity is a major determinant for insulin resistance, however not all obese patients become insulin resistant. Of this group, about 75% become IR, while the other 25% remain insulin sensitive (IS) due to unknown factors (34). There is limited data on metabolically healthy individuals, but the evidence that does exist supports the principal of beneficial fat

distribution and adipocyte function. The main function of adipocytes is to store lipids. As an individual increases caloric intake, fat cells must be able to accommodate for increased storage demands by under going hyperplasia, hypertrophy, or both. Quantification of subcutaneous adipocytes by size in moderately-obese humans revealed a greater portion of small-to-large adipocytes in IR patients, and while IR subjects had fewer large adipocytes, they were bigger than those of IS patients (35). This finding indicates two things: 1) the current population of fat cells in obese IR likely reached their maximum lipid storage capacity and 2) adipogenesis is impaired in these subjects, as the small adipocyte population indicates failure to fully differentiate into mature adipocytes. This phenotype likely leads to increases in visceral fat storage and increases the potential for ectopic fat storage in other tissues. Evidence from two studies show that the most distinguishing phenotype between equally obese IS and IR subjects is the amount of visceral fat storage. Metabolically healthy obese patients have significantly less visceral fat compared to their IR counterparts (36,37). Therefore, the distribution of fat, rather than simply the accumulation of total fat is key to the development of insulin resistance.

The goal of this dissertation was to investigate potential differences in adipocyte function of equally obese IS versus IR to better understand the association of fat distribution with insulin resistance. Using $^{2}H_{2}O$ *in vivo*, we were able to directly quantify the rate at which subcutaneous adipocytes synthesized and stored triglycerides and fatty acids *de novo,* the rate of new fat cell production for increased lipid storage, and the rate at which proteins in various insulin-regulated metabolic pathways are synthesized in response to impaired insulin action. Our results reveal new insights into the etiology of obesity-induced insulin resistance, providing evidence of adipocyte dysfunction as a driving factor of insulin resistance.

**References**


1. Kahn SE. The relative contribution of insulin resistance and beta cell dysfunction to the pathophysiology of type 2 diabetes. Diabetologia 2003, 46:3-19.

2. Flegal KM, Carroll MD, Ogden CL, Curtin LR. Prevalence and trends in obesity among US adults, 1999-2008.

3. McLaughlin T, Allison G, Abbasi F, Lamendola C, Reaven G. Prevalence of insulin resistance and associated cardiovascular disease risk factors among normal weight, overweight, and obese individuals. Metabolism 2004, 53:495-499.

4. Salans, LB, Cushman SW, Weismann RE. Studies of human adipose tissue. Adipose cell size and number in nonobese and obese patients. J Clin Invest. 1973, Vol. 52:929-41, 1973.

5. Björntorp P, Bengtsson C, Blohme G, Johnsson A, Sjöström L, Tibblin E, Tibblin G, Wilhelmsen L. Adipose tissue fat cell size and number in relation to metabolism in randomly selected middle-aged men and women. Metabolism 1971, 20:927-935.

6. Tchoukalova YD, Koutsari C, Vortuba SB, Tchkonia T, Giorgadze N, Thomou T, Kirkland JL, Jensen MD. Sex-and depot-dependent differences in adipogenesis in normal-weight hmans. Obesity 2010, 18:1875-1880.

7. Evans DJ, Hoffman RG, Kalkhoff RK, Kissebah AH. Relationship of body fat topography to insulin sensitivity and metabolic profiles in premenopausal women. Metabolism 1984, 3:68-75.

8. Thomas, EL, Parkinson JR, Frost GS, Goldstone AP, Doré CJ, McCarthy JP, Collins AL, Fitzpatrick JA, Durighel, Taylor-Robinson SD, Bell JD. The missing risk: MRI and MRS phenotyping of abdominal adiposity and ectopic fat. Obesity 2012, 20:76-87.

9. Klöting N, Berthold S, Kovacs P Schön MR, Fasshauer, Ruschke K, Stumvoll M, Blüher M. Insulin-sensitivity obesity. Am J Physiol Endocrinol Metab 2010, 299:E506-515.

10. Johanson, EH, Jansson, PA, Lonn, L Matsuzawa Y, Funahashi T, Taskinen MR, Smith U, Axelsen M. Fat distribution, lipid accumulation in the liver, and exercise capacity do not explain the insulin resistance in healthy males with a family history for type 2 diabetes. *J Clin Endocrinol Metab*, 2003, 88, 4232-4238.

11. Wajchenber BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocr Rev 2000, 21:679-738.

12. Freedland ES. Role of critical visceral adipose tissue threshold in metabolic syndrome: implications for controlling dietary carbohydrates: a review. Nutr Metab 2004 1:1-24.

13. Adams, M, Montague, C.T., Prins, JB, Holder JC, Smith SA, Sanders L, Digby JE, Sewter CP, Lazar MA, Chatterjee VK, O'Rahilly S. Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation. *J Clin Invest* 1997, 100, 3149-3153.

14. Virtanen KA, Hällsten K, Parkkola R, Janatuinen T, Lönngvist F, Vijanen T, Rönnemaa T, Knuuti J, Huupponen R, Lönnroth P, Nuutila P. Differential effects of rosiglitazone and metformin on adipose tissue distribution and glucose uptake in type 2 diabetic subjects. Diabetes 2003, 52, 283-290.

15. De Souza CJ, Eckhardt M, Gagen K, Dong M, Chen W, Laurent D, Burkey BF. Effects of pioglitazone on adipose tissue remodeling within the setting of obesity and insulin resistance. Diabetes 2001, 50:1863-1871.

16. Havel, P.J. Update on adipocyte hormones: regulation of energy balance and carbohydrate/lipid metabolism. *Diabetes* 2004, 53, S143-S151.

17. Fain JN, Mada AK, Hiler ML, Cheema P, Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology 2004, 145:2273-2282.

18. Borst SE. The role of TNF-alpha in insulin resistance. Endocrine 2004, 23:177-182.

19. Tchkonia T, Giorgadze N, Pirtskhalava T, Tchoukalova Y, Karagiannides I, Forse RA< DePonte M, Stevenson M, Guo W, Han J, Waloga G, Lash TL, Jensen MD, Kirkland JL. Fat depot origina affects adipogenesis in primary cultured and cloned human preadipocytes. Am J Physiol Regul Integr Comp Physiol 2002, 282:1286-1296.

20. Zimmerman R, Strauss JG, Haemmerie G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A, Zechner R. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science 2004, 306: 1383-1386.
21. Östman J, Arner P, Engfeldt P, Kager L. Regional differences in the control of lipolysis in human adipose tissue. Metabolism 1979, 28:1198-1205.

22. Thomas EL, Hamilton G, Patel N, O'Dwyer R, Doré RD, Bell JD, Taylor-Robinson SD. Hepatic triglyceride content and its relation to body adiposity: a magnetic resonance imaging and protin magnetic resonance spectroscopy study. Gut 2005, 54:122-127.

23. Nagle CA, An AJ, Shiota M, Torres P, Cline GW, Liu ZX, Wan S, Catlin RL, Shulman GI, Newgard CB, Coleman RA. Hepatic overexpression of glycerol-sn-3-phosphate acyltransferease 1 in rats causes insulin resistance. J Biol Chem 2007, 282:14807-14815.

24. Cancello R, Zulian A, Gentilini D, Maestrini S, Barba AD, Invitti C, Corà D, Caselle M, Liuzzi A, Di Blasio AM. Molecular and morpholic characterization of superficial and

deep-subcutaneous adipose tissue subdivisions in human obesity. Obesity 2013, 21:2562-2570.

25. Baker, George. Human adipose tissue composition and age. Am J Clin Nutri 1969, 2:829-835.

26. Ailhaud G. Adipose tissue as a secretory organ: from adipogenesis to the metabolic syndrome. C R Biologies 2006, 329:570-577.

27. Prins JB and O'Rahilly S. Regulation of adipose cell number in man. Clin Sci (Lond) 1997, 3-11.

28. Jo J, Gavrilova O, Pack S, Jou W, Mullen S, Sumner AE, Cushman SW, Periwal V. Hypertrophy and/or hyperplasia: dynamics of adipose tissue growth. PLoS Comput Biol 2009, 1-11.

29. MacLaren R< Cui W, Simard S, Cianflone K. Influence of obesity and insulini sensitivity on insulini signaling genes in human omental and subcutaneous adipose tissue. J Lipid Res 2008, 49:308-323.

30. Weyer C, Foley JE, Bogardus c, Tataranni PA, Pratley RE. enlarged subcutaneous abdominal adipocyte size,but not obesity itself, predicts type ii diabetes independent of insulin resistance. Diabetologia 2000, 43:1498-506.

31. McLaughlin, T., Sherman A, Tsao P, Gonzalez O, Yee G, Lamdendola C, Reaven GM, Cushman SW. Enhanced proportion of small adipose cells in insulin-resistance vs insulin sensitive obese individuals implicates impaired adipogenesis. Diabetologia. 2007; 50(8): 1707-15.

32. Bahceci M, Gokalp D, Bahecici S, Tuzcu A, Atmaca S, Arikan S. The correlation between adiposity and adiponectin, tumor necrosis factor alpha, interleukin-6 and high sensitivity C-reactive proteins levels. Is adipocyte size associated with inflammation in adults? J Endocrinol Invest 2007, 30:210-214.

33. Ravussin E and Smith SR. Increased fat intake fat oxidation, and failure of fat cell proliferation results in ectopic fat storage, insulin resistance, and type 2 diabetes mellitus. Annals of the New York Academy of Sciences 2006, 363-378.

34. McLaughlin T, Allison G, Abbasi F, Lamendola C, Reaven G. Prevalence of insulin resistanceand associated cardiovascular risk factors among normal, overweight, and obese individuals. Metabolism 2004, 53:495-499.

35. McLaughlin T, Lamendola C, Coghlan N, Liu TC, Lerner K, Sherman A, Cushman SW. Subcutaneous adipose cell size and distribution: relationship to insulin resistance and body fat. Obesity 2014, 22:673-680.

36. Klöting, N, Fasshauer M, Dietrich A, Kovacs P, Schön MR, Kern M, Stumvoll M, Blüher M. Insulin-sensitivity obesity. Am J Physiol Endocrinol Metab 2010, 299:E506-E515.

37. Stefan N, Kantartzis K, Machann J, Schick F, Thamer C, Rittig K, Balletshofer B, Machicao F, Fritsche A, Häring H. Identification and characterization of metabolically benign obesity in humans. Arch Intern Med 2008, 168:1609-1616.

## Chapter 2: Methods

*Subjects*: Healthy individuals with BMI 25-35 kg/m$^2$ were recruited. Subjects with prior bariatric surgery, liposuction, > 2 pound change in body weight in month prior to evaluation, alcohol use in excess of 3 drinks per day, pregnancy/lactation, or use of diabetogenic or weight loss medications were excluded. The study was approved by the Stanford University Human Subjects Committee and all subjects gave written, informed consent during screening in the Stanford University Clinical and Translational Research Unit (CTRU). *African-American subjects* recruited based on self-identification as African-American/Non-hispanic black.

*Measurement of insulin-mediated glucose uptake (IMGU) and insulin-suppression of lipolysis*: Subjects were admitted to the CTRU after a 12-hour fast for quantification of IMGU and insulin-suppression of lipolysis via the octreotide-modified two-stage insulin-suppression test (1,2,3). Briefly, insulin was infused at two concentrations: 12 uU/mL from 0-120 min, representing a "basal" (non-fed) state, in which differences in suppression of lipolysis are greatest, and 60 uU/mL (fed state) from 120-240 min in which differences in IMGU are greatest. Blood was sampled at 90-120 min for steady-state FFA (SSFFA) concentrations, and 210-240 min for steady-state plasma glucose (SSPG) concentrations. Subjects in the lowest and highest 40 percentile of SSPG were defined as IS and IR, respectively, based on prior studies (4).

*Abdominal and femoral subcutaneous fat distribution*: Abdominal and femoral subcutaneous adipose tissue (SAT) volumes were measured by computed tomography (CT) scan done a L4-5 (California Advanced Imaging, Atherton, CA) (3).

*Hepatic fat content:* Intrahepatic triglyceride (IHTG) was quantified using proton magnetic resonance spectroscopy (1H-MRS) and run by Dr. Dan Spielman at Lucas Center. Percent IHTG content was be calculated as the ratio of the area under the resonance peak for methylene groups in fatty acid chains of intrahepatic triglycerides.

*Adipose Tissue Biopsy and Analysis of Cell Size Distribution:* Subcutaneous periumbilical adipose tissue biopsies were performed and prepared as previously described (5). Two 25 mg samples of tissue were immediately fixed in osmium tetroxide and incubated in a water bath at 37°C for 48 h, after which adipose cell size was determined via a Beckman Coulter (Miami, FL, USA) Multisizer III with a 400μm aperture and effective cell-size range of 20 to 240μm.

*Isolation of Adipose Cells for Measurement of Lipid Dynamics:* Adipose cells were isolated from fresh adipose tissue using a modification of the original protocol of Rodbell (6). Fat was mechanically minced in Modified Eagle's Medium containing 0.3% collagenase for 45 min at 37°C in a shaking water bath. Digested solution was passed through 450um nylon filter and floating adipocytes were resuspended 3 times in HEPES-Krebs buffer.

*$^2H_2O$ Body Water Enrichment:* Subjects consumed a total of 150 ml of 70% deuterated water per day for the first 7 days (taken as 50 ml in three individual doses), followed by 100ml/day (50 ml twice a day) for the remaining 21 days prior to adipose tissue biopsy to achieve and maintain a total body water (TBW) enrichment of 1.0-2.0%, as previously validated (7). Compliance was

checked by counting number of empty vials returned at weekly visits and TBW enrichment was measured from intravaneous blood draws at weeks 0, 2 and 4.

*Lipid Dynamics Measured with* $^2H_2O$: Triglycerides were separated from isolated adipocytes or plasma using the Folch technique (8). Fractional triglyceride ($f_{TG}$) synthesis and de novo lipogenesis ($f_{DNL}$) were calculated based on the deuterium ($^2H$) incorporation into TG-glycerol and TG-palmitate, respectively:

$$f_{TG\ syn.} = EM_{1\ glycerol/} A_1^{\infty}{}_{glycerol} \text{ or } \quad f_{DNL} = EM_{1\ palmitate} A_1^{\infty}{}_{palmitate}.$$

$EM_1$ represents the measured mass + 1-labeled species of TG-glycerol or -palmitate in excess of natural abundance, and $A_1\infty$ represents the theoretical plateau value for fully labeled TG-glycerol or TG-palmitate. To account for slight variances in labeling times amongst subjects, all fractional measures are expressed as the rate of synthesis per day, or $k_s$, calculated using both the fractional value (f) and labeling time (t) for each as previously described (9): $k$= -ln(1-f)/days of labeling (or f/day for plasma lipid kinetics). Proliferation of mature adipocytes was measured via incorporation of deuterium into the deoxyribose moiety of extracted mature adipocyte DNA (Qiagen) (10):

$$f_{DNA} = EM_{1deoxyribose}/A_1^{\infty}{}_{deoxyribose.}$$

*Peptide trypsinization for dynamic proteomics*
Isolated adipocytes were sonicated in at 30Hz for 20 minutes for complete homogenization in Mammalians protein extraction reagent (M-PER) by Thermo Pierce, to which a protease inhibitor cocktail was added (Set 1, by Calbiochem). Protease inhibitor cocktail contained AEBFSH, aprotinin, E-64, and leupeptin. Following homogenization, cell lysate was centrifuged at 20,000 x g for 10 minutes at 4°C. Supernatant was removed using 23G needle and 1ml syringe to remove supernatant from lipid layer. Protein concentration was determined using a Bicinchononic Acid (BCA) kit to determine lysate volume for 500ug of protein. Lysate was prepared with NuPage LDS 4X loading buffer, 125mM of iodoacetemide and TCEP. Proteins were denatured at 70°C and 100ug of protein was loaded onto an SDS-page gel. Gel was run at 200 volts for 50 minutes and viewed on and stained in Simply Blue Biosafe Coosmassie dye for 1 hour. Following several washes, the gel was imaged using the Odyssey LCz infrared technology instrument for fluorescence imaging and densitometry quantification of gel bands at various molecular weights (Li-Cor). Gel pieces were cut for each individual subject based on molecular weight, and dye was removed using a solution of 25mM $NH_4HCO_3$/50% Acetonitrile. De-dyed gel pieces were then trypsinized overnight in 125ul of 1mg/1ml Promega Gold trypsin and 25mM $NH_4HCO_3$. Overnight trypsizination released peptides, which were then desalted for LC-MS/MS submission.

*LC-MS/MS for peptide analysis and kinetic calculations*
Trypsin-digested peptides were analyzed on an Agilent 6550 Q-TOF (quadrupole time-of-flight) mass spectrometer with 1260 Chip Cube nano ESI source (Agilent Technologies, Santa Clara, CA). Peptides were separated chromatographically using a Polaris HR chip (Agilent #G4240-62030) consisting of a 360 nL enrichment column and a 0.075 x 150 mm analytical column, each

packed with Polaris C18-A stationary phase with 3 $\mu$m particle size. Mobile phases were (A) 5% v/v acetonitrile and 0.1% formic acid in deionized water and (B) 95% acetonitrile and 0.1% formic acid in deionized water. Peptides were eluted at a flow rate of 350 nL/min during a 27 min nano LC gradient (2%B at 0 min, 5%B at 1 min, 30%B at 18 min, 50%B at 22 min, 90%B at 22.1-27 min, 2%B at 27.1 min; stop time: 41 min).

Each sample was analyzed twice, once for protein/peptide identification in data-dependent MS/MS mode and once for peptide isotope analysis in MS-only mode. Acquisition parameters were: MS/MS acquisition rate = 6 Hz MS and 4 Hz MS/MS with up to 12 precursors per cycle, MS acquisition rate = 0.9 Hz, ionization mode = positive electrospray; capillary voltage = 1980 V; drying gas flow = 9 L/min; drying gas temperature = 290 °C; fragmentor = 360 V; skimmer = 45 V; maximum precursor per cycle = 12; scan range = 100-1700 m/z (MS), 50-1700 m/z (MS/MS); isolation width (MS/MS) = medium (~4 m/z); collision energy (V) = -4.8+3.6*(precursor m/z/100); active exclusion enabled (exclude after 1 spectrum, release after 0.12 min); charge state preference = 2, 3, >3 only, sorted by abundance; TIC target = 25,000; reference mass = 1221.990637 m/z.

Acquired MS/MS spectra were extracted and searched using Spectrum Mill Proteomics Workbench software (version B.04.00, Agilent Technologies, Santa Clara, CA) and a UniProtKB/Swiss-Prot human protein database (20,265 proteins, UniProt.org, release 2013_05). Data files were extracted with the following parameters: fixed modification = carbamidomethylation of cysteine, scans with same precursor mass merged by spectral similarity within tolerances (retention time +/- 10 sec, mass +/-1.4 m/z), precursor charge maximum z = 6, precursor minimum MS1 S/N = 10, and 12C precursor m/z assigned during extraction. Extracted files were searched with parameters: enzyme = trypsin, species = *Homo sapiens*, fixed modification = carbamidomethylation of cysteine, variable modifications = oxidized methionine + pyroglutamic acid + hydroxylation of proline, maximum missed cleavages = 2, minimum matched peak intensity = 30%, precursor mass tolerance = 10 ppm, product mass tolerance = 30 ppm, minimum detected peaks = 4, maximum precursor charge = 3. Search results were validated at the peptide and protein levels with a global false discovery rate of 1%.

Proteins with scores greater than 11.0 were reported and a list of peptides with scores greater than 6 and scored peak intensities greater than 50% was exported from Spectrum Mill and condensed to a non-redundant peptide formula database using Excel. This database, containing peptide elemental composition, mass, and retention time was used to extract MS spectra (M0-M3) from corresponding MS-only acquisition files with the Find-by-Formula algorithm in Mass Hunter Qualitative Analysis software (version B.05.00, Agilent Technologies, Santa Clara, CA). MS spectra were extracted with parameters: EIC integration by Agile integrator, peak height > 10,000 counts, include spectra with average scans > 12% of peak height, no MS peak spectrum background, unbiased isotope model, isotope peak spacing tolerance = 0.0025 m/z plus 12.0 ppm, mass and retention time matches required, mass match tolerance = +/- 12 ppm, retention time match tolerance = +/- 0.8 min, charge states z = +2 to +4, chromatogram extraction = +/-12 ppm (symmetric), EIC extraction limit around expected retention time = +/- 1.2 min.

Details of FSR calculations were described previously (11). Briefly, software was developed to calculate peptide elemental composition and curve fit parameters for predicting isotope

enrichments of peptides in newly synthesized proteins based on precursor body water enrichment (p) and the number (n) of amino acid C–H positions per peptide actively incorporating H and 2H from body water. Incorporation of 2H into tryptic peptides decreases the relative proportion of M0 within the overall isotope envelope spanning M0-M3. Fractional synthesis was calculated as the ratio of excess %M0 (EM0) for each peptide compared to the maximal absolute EM0 possible at the measured body water enrichment. Data from individual biological samples were filtered to exclude protein measurements with fewer than two peptide spectra measurements per protein.

*KEGG analysis*
An in-house analytical tool was developed to query and statistically analyze pathway terms, accessed programmatically from Kyoto Encyclopedia of Genes and Genomes (KEGG) (12-13). KEGG terms were retrieved based on an initial search of the proteins identified in the experimental datasets. The median, standard deviation and number of matching proteins were calculated for each pathway identified, based on the corresponding proteins from experimental data, and used for statistical analysis.

*Statistical Analysis*: Data are presented as mean $\pm$ standard error of the mean (SEM) unless otherwise noted. The majority of variables were normally distributed, although in the case of TG synthesis, DNL, adipocyte proliferation for the heterogeneity study, values were log-transformed due to non-normal distribution. A standard unpaired, two-way t-test was used to determine statistical differences between groups for the heterogeneity studies (Chapters 1 and 2). In addition, analysis of covariance was used to compare IR vs IS subjects with regard to dependent variables (lipid dynamics), adjusting for BMI. *Overfeeding study:* Differences at baseline versus post-overfeeding were statistically evaluated using a two-way ANOVA. P-values presented for two-way ANOVA analyses are reflect differences based on insulin sensitivity as the determining factor behind the difference in baseline versus post-overfeeding outcomes, unless otherwise noted. The change in variables (delta) after overfeeding were statistically analyzed by a paired, two-way t-test. General linear regression for all studies was utilized to assess independent associations between lipid dynamics measures, insulin sensitivity, fat mass distribution and $P<0.05$ was considered statistically significant. *Proteomics study:* Protein synthesis differences for all pathways represented were statistically analyzed by two-way ANOVA with p-values expressed based on mean protein synthesis differences between IS and IR.

**References**


1. Pei D, Jones CN, Bhargava R, Chen YD, Reaven GM 1994 Evaluation of octreotide to assess insulin-mediated glucose disposal by the insulin suppression test. Diabetologia 1994, 37:843-5.
2. Greenfield MS, Doberne L, Kraemer F, Tobey TA, Reaven GM 1981, Assessment of insulin resistance with the insulin suppression test and the euglycemic clamp. Diabetes 1981, 30:387-92.
3. McLaughlin T, Yee G, Glassford A, Lamendola C. Use of a Two-stage Insulin Suppression Test to Assess the Relationship Between Insulin-Suppression of Lipolysis and Insulin-Mediated Glucose Uptake in Overweight/Obese, Nondiabetic Women. Metabolism 2011, 60:1741-7.
4. Yeni-Komshian H, Carantoni M, Abbasi F, Reaven GM. Relationship between several surrogate estimates of insulin resistance and quantification of insulin-mediated glucose disposal in 490 healthy nondiabetic volunteers. Diabetes Care. 2000; 23:171.-5.
5. McLaughlin, T., Sherman A, Tsao P, Gonzalez O, Yee G, Lamdendola C, Reaven GM, Cushman SW. Enhanced proportion of small adipose cells in insulin-resistance vs insulin sensitive obese individuals implicates impaired adipogenesis. Diabetologia. 2007; 50(8): 1707-15.
6. Rodbell M. Metabolism of isolated fat cells. Effects of hormones on glucose metabolism and lipolysis. J Biol Chem 1964, 239:375-380.
7. Strawford A, Antelo F, Christiansen M, Hellerstein MK. Adipose tissue triglyceride turnover, de novo lipogenesis, and cell proliferation in humans measured with 2H2O. Am J Physiol Endocrinol Metab. 2004, 286:E577-88.
8. Folch J, Lees M, Sloane Stanley GH : A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 1957, 226: 497– 509.
9. Turner SM, Murphy EJ, Neese RA, Antelo F, Thomas T, Agarwal A, Go C, Hellerstein MK. Measurement of TG synthesis and turnover in vivo by 2H2O incorporation into the glycerol moiety and application of MIDA. Am J Physiol Endocrinol Metab 2003, 285:E790-E803.
10. Neese, RA., Misell LM ,Turner S, Chu A, Kim J, Cesar D, Hoh R , Antelo F, Strawford A, McCune JM, Christiansen M and Hellerstein MK. Measurement in vivo of proliferation rates of slow turnover cells by $^{2}$H2O labeling of the deoxyribose moiety of DNA. Proc Natl Acad Sci USA 99:15345-50, 2002.
11. Price JC, Holmes WE, Li KW, Floreani NA, Neese RA, Turner SM, Hellerstein MK. (2012) Measurement of human plasma proteome dynamics with 2H2O and liquid chromatography tandem mass spectrometry. Anal Biochem 420: 73–83.
12. Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M.; Data, information, knowledge and principle: back to metabolism in KEGG. Nucleic Acids Res. 42, D199–D205 (2014).
13. Kanehisa, M. and Goto, S.; KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res. 28, 27-30 (2000).

# Chapter 3

## *In vivo* $^{2}H_2O$ administration directly reveals impaired triglyceride storage in subcutaneous adipose tissue of insulin-resistant, but not of insulin-sensitive, moderately-obese humans

**Abstract**

We hypothesized that dysfunctional subcutaneous adipose triglyceride storage is an initiating factor in the development of systemic insulin resistance in human obesity. Here, we tested this hypothesis using $^2H_2O$ to quantify, *in vivo*, triglyceride (TG) synthesis, *de novo* lipogenesis (DNL), and adipocyte proliferation in subcutaneous adipose cells of insulin-sensitive (IS) and insulin-resistant (IR) subjects. Moderately-obese humans classified as IS or IR on the basis of the modified insulin-suppression test, were given oral $^2H_2O$ for 4 weeks. Lipid dynamics and adipocyte proliferation were quantified, *in vivo*, by measuring deuterium incorporation into newly synthesized lipid moieties and DNA in mature adipose cells. Adipose cell size distribution was quantified using Beckman Multisizer III. Plasma free fatty acid (FFA) concentrations were measured under fasting and postprandial insulin concentrations during a steady state plasma glucose (SSPG) test. Total TG synthesis and DNL were significantly lower in adipose cells of IR compared to IS subjects, and correlated inversely with the proportion of small adipocytes. FFA concentrations were significantly greater in IR versus IS during fasting and postprandial insulin concentrations. Adipocyte proliferation did not differ significantly between groups. These results strongly support the hypothesis that decreased capacity for lipid storage in subcutaneous adipose tissue is a defining feature of obesity-associated insulin resistance.

**Introduction**

We hypothesized that impaired triglyceride (TG) storage in subcutaneous adipose tissue in the setting of obesity may be the prominent determinant for the onset of human obesity-induced insulin resistance (1). McLaughlin and colleagues were one of the first groups to propose this potential etiological characteristic of insulin resistance (IR) (1,2,3), and with the use of deuterated water ($^2H_2O$) in the current study, we are the first to address this hypothesis directly *in vivo*. The incorporation of deuterium into newly synthesized glycerol, palmitate, and deoxyribose for the measurement of total TG synthesis, DNL, and adipose cell proliferation, respectively, allows us to supplement our previous indirect measurements of adipose tissue function (1), such as greater visceral relative to subcutaneous fat storage, decreased expression of differentiation and fat storage genes, and an accumulation of very small adipose cells in the subcutaneous adipose tissue of IR (1,4) and type 2 diabetic subjects (5,6). We can use this stable isotope technique to directly address an important gap in the current understanding of the mechanisms underlying obesity-induced IR.

The percentage of obese individuals that develop insulin resistance are at a substantially greater risk to developing other obesity-related conditions (7). A small fraction of obese subjects will not develop IR and will remain healthy individuals, yet evidence is lacking to allow scientists to understand the physiological differences between these two populations. Our hypothesized inability for IR subjects to store TG in the subcutaneous adipose may be a result of impaired proliferation of early preadipocytes, differentiation of committed preadipocytes, or reduced capacity for TG uptake and storage in differentiated preadipocytes Support for this hypothesis can be found in mouse models in which elevated visceral fat and severe metabolic dysfunction in lipodystrophic mice was reversed by subcutaneous fat implants (8). Unlike animal models, *in vivo* evidence in humans is lacking. In common obesity, an observed increased in the number, rather than the size, of adipocytes in those with a BMI beyond 25 kg/m$^2$ implies that adipose cell enlargement is nearly maximal by the time BMI has reached 25 kg/m$^2$ (5). Potentially, either cell proliferation or maturation of committed preadipocytes is required for continued expansion of fat mass. Investigators previously demonstrated an accumulation of small adipose cells in insulin-resistant (IR) patients as compared with insulin-sensitive (IS) moderately-obese individuals who were matched for BMI (5), supporting the hypothesis that impaired triglyceride storage in committed adipocytes may contribute to human insulin resistance. The current study was designed to directly test this hypothesis *in vivo* by quantifying lipid dynamics and cell proliferation through incorporation of deuterated water into newly synthesized triglyceride and DNA in mature adipocytes. Administration of $^2H_2O$ in overweight IS and IR adults allows us to address an important gap in the current understanding of the mechanisms underlying obesity-associated insulin resistance.

**Results**

Eight males and three females were studied. All except one were Caucasian. BMI was slightly higher in the IR group; otherwise groups were NS different (**Table 1**). Insulin sensitivity measured by SSPG test was significantly different between IS and IR ($p<0.0001$), while fasting glucose was did not differ. Subcutaneous adipose tissue (SAT) did not differ between groups, however there was significantly greater visceral adipose tissue (VAT) in the IR group (**Figure 1A-B**). There was a trend towards a higher ratio of VAT to SAT in the IR group, but this was difference was not significant (**Figure 1C**). Combined, total abdominal fat volume was greater in IR subjects (**Figure 1D**). To further demonstrate, linear regression analysis shows a positive correlation between subcutaneous fat and insulin sensitivity, $p=0.00394$ (**Figure 2A**). A similar, yet stronger, relationship was shown between fat visceral volume and insulin sensitivity with a p-value of 0.0003 (**Figure 2B**). Femoral fat was also measured using CT scan, however there were no differences in fat volume within this depot between groups (**Figure 3**). **Figure 4A** shows that the rate of TG synthesis, expressed as $\%*day^{-1}$ in log transformation, was 33% higher in the IS as compared with the IR subgroup ($p<0.001$). Obese IS subjects had a TG replacement rate of 0.3% per day, while for IR subjects it was 0.17% per day. **Figure 4B** shows the rate of DNL, expressed as $\%*day^{-1}$ in log-transformation was two-fold greater in the IS as compared to the IR group ($p=0.009$), with rate constants of 0.09% per day (IS) and 0.04% per day (IR). DNL accounted for 31 and 23% of total TG-palmitate synthesis, respectively, in these subgroups, but did not differ significantly between groups ($p=0.007$). Mature adipocyte proliferation, measured by $^2H$-incorporation in newly synthesized deoxyribose of isolated cells, was 0.6 and 0.8% per day (NS), respectively, in the IS and IR subgroups (**Figure 5A**). There was no difference in SVC proliferation (**Figure 5B**).

**Figure 6A-C** depicts the relationships among the lipid dynamic variables. We observed a positive relationship between adipose DNL and TG, $p=0.02$. To determine a potential relationship between the ability to synthesize lipids, a linear regression analysis was performed comparing and DNL to adipocyte proliferation. No relationship existed between adipocyte proliferation and DNL, $p=0.84$ (**Figure 6B**). **Figure 6C** shows a negative, although, not significant, relationship between adipocyte proliferation and TG synthesis ($p=0.098$). Furthermore, **Figure 7A** demonstrates a trend towards a negative relationship between TG synthesis and insulin sensitivity, $p=0.0905$. A similar trend was observed between DNL and insulin sensitivity, $p=0.1095$, while no relationship existed between adipocyte proliferation and SSPG (**Figures 7B-C**).

Interestingly, TG synthesis and percent number small adipose cells, measured by our collaborators at Stanford and NIDDK using the Beckman Multisizer, were significantly correlated, (data not shown here) ($r= -0.90$, $p=0.01$). In other words, the greater the fraction of small cells, the lower the TG synthesis. Percent small adipose cells also negatively correlated with adipocyte DNL, although p-value was of borderline significance ($r= -0.77$, $p=0.06$). Percent small adipose cells did not correlate significantly with adipocyte proliferation (data not shown). Peak diameter of adipose cells was not correlated with any of the lipid dynamic measures. Analyses here represent only 8 male subjects. No relationship was observed when women were included, yet the power was not great enough to do a female-only regression analysis.

Plasma FFAs measured under both fasting and experimentally-controlled basal and postprandial concentrations (referred to as Steady State 1 (SS1) and Steady State 2 (SS2) when insulin is infused at concentrations of 12 and 60 uU/mL, respectively) during the SSPG test were significantly higher in IR versus IS subjects (**Figure 8A-C**), consistent with impaired insulin-suppression of lipolysis (10). Fasting plasma lipids, including cholesterol, triglycerides (TG), HDL and LDL were also measured. There was no difference in cholesterol (p=0.0021), but fasting TG was significantly higher in IR subjects, leading a significant difference in the ratio of TG/cholesterol, p=0.0247 (**Figure 9A-C**). No differences were observed between fasting HDL and LDL, nor in the ratios of LDL/HDL or Total cholesterol/HDL, p=0.3720 and p=0.2012 (**Figure 9F-G**). **Figure 10A** shows significantly greater hepatic DNL, quantified using $^{2}H$-enriched plasma palmitates, p=0.0141. Plasma cholesterol and TG were also quantified by our stable isotope method, and no differences were observed between groups (**Figure 10B-C**). Adipose cholesterol synthesis was also no different between groups (**Figure 10D**). **Figure 11** shows hepatic fat content was measured by MRI scan and showed a treated towards greater hepatic fat in IR subjects compared to IS, p=0.1399. To demonstrate the relationship between visceral fat and hepatic fat measurements, linear regression analyses show no relationship between hepatic fat measured by MRI and visceral depot volume, but there was borderline significance between visceral depot volume and hepatic DNL quantified by $^{2}H_{2}O$ use *in vivo*, p=0.0530 (**Figure 12A-B**). **Figure 12C** shows a significant relationship between visceral depot volume and fasting plasma TG, p=0.0079.

**Discussion**

As an endocrine classified cell type, the adipocyte has recently gained much attention in its role in the development of insulin resistance. The current study represents the first controlled study to directly test the hypothesis that TG uptake and storage is impaired in IR individuals. Body fat distribution analysis, measured by CT scan, shows similar volumes of abdominal subcutaneous fat, but greater visceral fat storage in IR patients. When expressed as the portion of visceral fat versus subcutaneous fat ratio, IR subjects show a greater VAT/SAT ratio, which is consistent with previous observations (10). No differences were observed in femoral fat between the two groups, suggesting that any metabolic differences may be due to differences in abdominal SAT storage, rather than femoral SAT storage.

It is widely accepted that an altered distribution of fat into the visceral cavity is a major risk for type 2 diabetes, potentially due to the greater lipolytic activity observed in this depot paired with increased inflammation within the visceral depot (11,12). Therefore, inhibiting visceral fat storage may protect from inflammation and in turn prevent insulin resistance. In fact, thiazolidinediones (TZDs), a class of PPAR-γ agonists, are insulin-sensitizing drugs whose actions prevent increases in visceral fat storage by increasing fat storage in the subcutaneous depot (13). Although the main function of the adipose is to serve as reservoirs for excess fat storage, the impact of fat storage on insulin resistance is not completely understood. One key characteristic of fat storage is the ability of the adipocyte to expand with increases in accumulating lipids, which make up 60-90% of the adipocyte (14). It has been hypothesized that adipocytes have a maximum limit of expansion and once this limit is reached, excess fat must be stored elsewhere (15). This hypothesis is supported by findings in insulin resistant lean and obese humans showing a decreased lipogenic gene expression paired with an accumulation of small fat cells (1,3,4,16). Our results here suggest that insulin resistance may be driven by the inability for adipocytes to function properly and store excess lipids in the subcutaneous depot.

Measurement of total subcutaneous adipose TG synthesis is quantified by incorporation of $^{2}H$ (deuterium) into newly synthesized glycerol. Because any newly formed glycerol has only one fate, to become the backbone of a triglyceride, any glycerol containing at least one carbon attached to a $^{2}H$ represents a newly synthesized triglyceride molecule (17). A triglyceride is composed of three C:16 palmitate fatty acids, however each of these fatty acids that attaches to a glycerol may be formed from several different sources: from the diet, *in vivo*, or may be re-esterified. For this reason, newly synthesized palmitates are used for quantified newly synthesized fatty acids, but cannot be used to determine total TG synthesis.

In this study, mature adipocytes were isolated to quantify both total TG synthesis and DNL to investigate potential differences in lipid metabolism in healthy vs IR obese subjects. Use of a stable isotope, *in vivo*, revealed a 33% lower rate of subcutaneous adipose TG synthesis in the IR individuals. This was matched with 50% less adipose DNL. These results indicate adipose dysfunction in two aspects of TG storage within the adipose, both of which are insulin-stimulated processes. The observed TG synthesis rate of 0.3% per day in IS obese individuals is similar to that observed in lean healthy individuals in a prior study (6). Therefore, these data

suggest that a TG synthesis rate of approximately 0.3% per day reflects a healthy state, regardless of body weight.

In the healthy state, insulin stimulates lipid synthesis via up-regulation of diacylglyceride acyl transferase (DGAT) and glycerol synthase in adipose tissue (18,19). Carbohydrate responsive-element binding protein (ChREBP), a key regulator of DNL in adipose tissue, correlates with insulin sensitivity (20). Investigators have therefore proposed that this protein may be protective against insulin sensitivity, in that in promote lipids storage in adipose tissue (20). Rather than measuring lipogenic genes, we measured TG synthesis directly *in vivo,* providing information that gene or protein expression may not necessarily reveal. Our measurements of lipogenesis indicate that IS subjects are sensitive to insulin in their adipose tissue, while IR subjects are resistant to insulin's actions in subcutaneous adipose tissue. This resistance to insulin may be causing a shift in fat distribution from subcutaneous depots into visceral depots, supported by greater visceral fat content in IR patients. As will be discussed later, influx of lipids to visceral depots leads to metabolic dysfunction (21, 22, 23). Therefore, the ability to prevent fat deposition in this cavity may be crucial in the prevention of insulin resistance. We measured another subcutaneous depot, femoral fat content using CT scan, a depot that has been shown to correlate with insulin resistance (24). While we did not quantify TG synthesis in this location, CT scan results were not different between groups. This suggests that the key site responsible for preventing obesity-induced insulin resistance is likely the abdominal subcutaneous depot, at least in Caucasian males. Contrary to our results here, past investigators have suggested a stronger relationship between abdominal subcutaneous fat (compared to visceral fat) and insulin resistance in subjects with a range of body weights (24). Ample research in the past decade leads us to believe this may not the case for the majority of patients. Furthermore, despite our small sample size, there existed a stronger relationship between SSPG and visceral fat compared to SSPG and subcutaneous fat, in our cohort. Visceral fat is significantly more lipolytic than subcutaneous fat, leading to spillover of free fatty acids through the portal vein (25). This characteristic of visceral fat is proposed to be one of the mechanisms by which obesity triggers insulin resistance.

A previous study measuring TG synthesis in obese humans used isotopic method $^{14}C$ quantified from exposure to nuclear energy in the environment over each individual's lifetime. In this environmentally uncontrolled study, TG synthesis was shown to be greater in obese subjects compared to lean, and was also positively correlated with insulin resistance as estimated by HOMA-IR (26). However, when the obese subset was considered alone, the relationship between TG synthesis and HOMA-IR disappeared. Thus it seems that TG synthesis in this study was related to obesity, rather than insulin resistance, per se. Furthermore, this study was conducted under conditions in which diet, weight and physical activity were not necessarily held constant as done so in our study, potentially accounting for differences in results. Very few studies investigating the physiological mechanisms of insulin resistance examine obese-only cohorts (ie. obese IS in addition to obese IR) in efforts to remove weight differences as a confounding factor in outcome variables. This study here, achieves an *in vivo* examination of lipid dynamics in the IR state by controlling for the potential influence of body weight. The use of $^{2}H_2O$ *in vivo* in BMI-matched subjects allows us to demonstrate impaired lipogenesis in the IR state, regardless of body weight. A very similar and recently published study by Tuvdendorj and colleagues verify this phenotype of reduced TG synthesis in obese patients with the

metabolic syndrome (27). While insulin resistance is one criterion for the metabolic syndrome, not all patients with this condition are insulin resistant. Therefore, our results are specific to adipose function in the insulin resistant state. While further research in a larger cohort would confirm our results, our data suggests that the ability to maintain a normal rate of TG synthesis of 0.3% per day in the subcutaneous adipocyte, as observed in our IS patients, may protect from the onset of insulin resistance.

DNL, TG synthesis and suppression of lipolysis are all insulin-stimulated processes through phosphorylation of acetyl co-A carboxylase (ACC) (28), activation of glycerol-3-phosphate acyltransferase and inhibition of hormone-sensitive lipase (HSL) (29,30,31), respectively. This inability of insulin to stimulate TG synthesis in the adipose may explain the elevated FFA being shunted to other ectopic sites of storage, causing lipotoxicity and insulin resistance (32,33). Our concurrent finding of elevated plasma FFAs, widely considered a systemic manifestation of dysfunctional TG storage and impaired insulin-suppression of lipolysis (34) also provides a link between adipose tissue and peripheral insulin resistance. Indeed, FFA shunting from adipose tissue to ectopic sites such as liver and skeletal muscle has not only been associated with insulin resistance, but molecular pathways have been identified (35). Re-esterification of FFA released from lipolysis is dependent on the availability of glycerol in adipocytes. As previously mentioned, IR patients had a reduction in the synthesis and retention of glycerol in the abdominal adipose tissue, providing an explanation for the inability for insulin to suppress lipolysis. If insulin cannot stimulate the production of glycerols, FFAs released during lipolysis are unable to be stored back into these adipocytes and are forced to be stored elsewhere. In addition, insulin's ability to stimulate lipoprotein lipase (LPL), which is responsible for the clearance of FFA from circulating into the adipocyte, is reduced in obesity and T2D patients (36,37). Our results provide evidence not only for decreased TG uptake and storage in adipose tissue, but also for lipotoxicity via systemic circulation of FFA, in association with insulin resistance independent of degree of obesity. Taken together with evidence from PPAR-γ studies, showing improvements in insulin sensitivity depots despite weight gain (38), insulin sensitivity may be improved through drugs promoting fat storage in subcutaneous adipose cells.

TG synthesis, DNL and lipolysis are some of insulin's major actions in adipose tissue, and thus quantifying these processes have revealed adipose-specific insulin resistance. Proliferation of mature adipocytes, which includes proliferation of preadipocytes and differentiation into mature adipocytes, is an insulin-stimulated process and may provide further insight into the mechanisms of obesity-induced insulin resistance. Quantifying deuterium incorporation into DNA of mature adipocytes provided additional information about potential sources of TG synthesis in the current study. Surprisingly, despite differences in TG synthesis, proliferation of adipocytes did not differ between the IR and IS groups. This observation suggests that it is not adipocyte proliferation that is impaired in IR, but rather, the ability of committed adipocytes to mature into TG-storing cells. We also did not observe any differences in SVC proliferation between groups. The SVC fraction contains many cell types, including macrophages, T cells, fibroblasts and preadipocytes (39). Given that we did not specifically isolate preadipocytes, conclusions regarding the significance of cell proliferation in the SVC fraction cannot be made. Committed adipocytes, derived from pre-adipocytes, increase in size, as they become mature, TG-storing cells.

The observation of a greater portion of small adipocytes quantified by Beckman Multisizer in IR patients may suggests impaired differentiation in these cells. Impaired differentiation likely leads to continuous proliferation of preadipocytes in efforts to compensate for dysfunctional, yet pre-existing adipocytes. This may be at least in part related to decreased TG synthesis, but unrelated to adipocyte proliferation in the steady state. The correlation between decreased TG synthesis and small cells was robust, and thus validates this measure as an estimate of impaired TG synthesis. McLaughlin and colleagues previously showed that an accumulation of small adipose cells in IR, as compared with equally-obese IS individuals, was associated with decreased expression of adipogenic genes (1), suggesting that impaired adipocyte differentiation/fat storage might explain differential risk for insulin resistance in the setting of excess body weight. Others have hypothesized the same (40,41,42), but none have provided direct results showing decreased TG synthesis/storage in vivo in association with systemic insulin resistance. Molecular mechanisms should be further sought in future studies to identify targets for treatment to enhance TG storage in an effort to reduce insulin resistance in overweight/moderately-obese individuals.

Elevated hepatic lipid content is another risk factor for T2D and is observed in many IR patients (43,44). While MRI results did not show a significant difference in hepatic fat content, there was a very significant difference in hepatic lipids between IS and IR groups. Using $^{2}H_{2}O$, we measured the synthesis rates of lipids circulating in plasma, the majority of which originate from the liver, and thus represent hepatic newly synthesized fatty acids. One potential mechanism by which obesity may cause IR could be an up-regulation of hepatic insulin response as a consequence of filling subcutaneous fat storage capacities. Free fatty acids released into circulation can be taken up by the adipose or by the liver due to insulin up-regulating lipoprotein lipase (LPL) expression. It is possible that a portion of hepatic DNL quantified here originated from the adipose tissue; however, our isotope labeling method cannot directly determine this. While it is possible that DNL measured in adipose may include newly synthesized fatty acids from other sources, such as the liver, it is very unlikely that this is the case here. We detected high rates of incorporation of deuterium into plasma TG-palmitate, representative of elevated hepatic DNL, in IR compared to IS subjects (data unpublished). This would indicate that adipose DNL quantified here is in fact adipose-derived in that high rates of hepatic newly synthesized fatty acids and thus would not account for low rates of DNL in the adipose. It is very unlikely that a significant portion of adipose DNL quantified here originated from the liver. The liver is the dominant source of DNL production in humans, unlike in murine models, producing approximately 1-3% newly synthesized fatty acids per day (45). In adipose, on the other hand, fractional DNL over 4 weeks is approximately 2% in a healthy individual, and constitutes only 20% of all newly made TG. The liver produces 20g of fat per day in the form of VLDL-TG, 20% of which is TG palmitate. Thirty percent of this 4g of palmitate is newly synthesized, equaling just about 1g of DNL coming from the liver (45). The adipose produces 100g in a healthy individual, 20% (or 20g) of which palmitate, but only 4g are newly synthesized palmitate. Therefore, at least 75% of DNL originated from the adipose, and any lipids from other sources is minimal in comparison.

In addition to it's effects on LPL, activation of the insulin receptor leads to downstream activation of PKCλ\ζ, which stimulates sterol regulatory element binding protein 1c (SREBP1c) and fatty acid synthase (FAS), leading to hepatic DNL and TG synthesis (46,47). SREBP1-c is

also responsible for cholesterol synthesis, yet we observed no differences in hepatic cholesterol synthesis measured by $^2H_2O$, or in circulating fasting cholesterol concentrations. There was, however, a trend of greater adipose cholesterol synthesis in IR versus IS. Interestingly, fasting cholesterol, HDL and LDL were no different between groups, but there was significantly higher hepatic TG synthesis and hepatic DNL in IR measured by $^2H_2O$, indicating hepatic insulin sensitivity. It has been demonstrated in mouse models that up-regulation of SREBP1c, leading to increased TG synthesis and DNL, is dependent on PKCλ\ζ, not Akt, which is also activated by insulin (48). Therefore, it can be hypothesized that the elevated hepatic DNL observed in IR patients may be due to an up-regulation of PKCλ\ζ, particularly given that IR patients are hyperinsulinemic. Together, our data suggests differential insulin signaling in the liver in the insulin resistant state, as insulin continues to stimulate lipogenesis, but fails to maintain the ability to suppress gluconeogenesis.

The use of $^2H_2O$ *in vivo* in equally obese insulin sensitive versus insulin resistant subjects has allowed us to reveal differences in both adipocyte function and hepatic function in IR compared to healthy individuals. We have shown that IS individuals had greater triglyceride synthesis and DNL in subcutaneous adipocytes, whereas IR individuals showed greater lipogenesis occurring in the liver and significantly less occurring in the adipose, compared to IS individuals. Such findings suggest a differential insulin resistance, in that adipose tissue becomes resistant to insulin's actions, while the liver may have an enhanced response to insulin as a result of peripheral resistance in other tissues. We demonstrate that loss of adipocyte function may be the driving force of insulin resistance developing in obese individuals. Future interventions should focus on increasing adipocyte fat storage, while simultaneously decreasing hepatic lipogenesis.

**Study Limitations**

This study has several limitations, including a small number of female and non-Caucasian subjects. Future studies should include women and other races to evaluate possible differences in lipid dynamics between groups. Secondly, we sampled only subcutaneous adipose tissue, so it remains to be seen if the differences observed between IR and IS subjects are concentrated in a single adipose depot, or are more diffuse. TG synthesis was not measured in lean IR subjects, so we cannot comment on whether decreased TG synthesis might also account for insulin resistance in non-obese individuals. Despite these limitations, the results generated from the current study represent the first clear evidence in support of the increasingly popular hypothesis that impaired fat storage in the subcutaneous depot is related to insulin resistance.

In conclusion, the current findings provide direct evidence that impaired TG synthesis and storage is a central feature of insulin resistance in the setting of excess body weight. Future studies linking low lipogenesis in adipose tissue to muscle insulin resistance via increased ectopic lipid deposition and lipotoxicity would be illuminating, as would be identification of molecular targets responsible for decreased lipogenesis in subcutaneous adipose cells. Clarification of these points may ultimately lead to new treatments for systemic insulin resistance and prevention of type 2 diabetes.

**References**


1. McLaughlin, T., Sherman A, Tsao P, Gonzalez O, Yee G, Lamdendola C, Reaven GM, Cushman SW. Enhanced proportion of small adipose cells in insulin-resistance vs insulin sensitive obese individuals implicates impaired adipogenesis. Diabetologia 2007; 50(8): 1707-15.

2. Danforth E Jr. Failure of adipocyte differentiation causes type II diabetes mellitus? Nat Genet 2000, 26:13.

3. Gray S and Vidal-Puig AJ. Adipose Tissue Expandability in the Maintenance of Metabolic Homeostasis. Nutrition Reviews 2007, 65: S7-S12.

4. Weyer C, Foley JE, Bogardus c, Tataranni PA, Pratley RE. enlarged subcutaneous abdominal adipocyte size,but not obesity itself, predicts type ii diabetes independent of insulin resistance. Diabetologia 2000, 43:1498-506.

5. Yang J, Eliasson B, Smith U, Cushman SW, Sherman AS. The size of large adipose cells is a predictor of insulin resistance in first-degree relatives of type 2 diabetic patients. Obesity 2012, 20: 932-938.

6. Dubois SG, Heilbronn LK, Smith SR, Albu JB, Kelley DE, Ravussin E, and the Look AHEAD Research Group. Decreased expression of adipogenic genes in obese subjects with type 2 diabetes. Obesity 2006, 14:1543-1552.

7. National Lung, Heart and Blood Institute. What are the health risks of overweight and obesity? http://www.nhlbi.nih.gov/health/health-topics/topics/obe/risks.html. Updated July 13, 2012. Searched August 2013.

8. Tran TT, Yamamoto Y, Gesta S, Kahn R. Beneficial effects of subcutaneous fat transplantation on metabolism. Cell Metabolism 2008, 7:410-420.

9. Weyer C, Foley JE, Bogardus c, Tataranni PA, Pratley RE. Enlarged subcutaneous abdominal adipocyte size,but not obesity itself, predicts type ii diabetes independent of insulin resistance. Diabetologia 2000, 43:1498-506.

10. Kloting N, Fassahauer M, Dietrich A, Kovacs P, Schon MR, Kern M, Shumvoll M, Bluher M. Insulin-Sensitive Obesity. Am J Physiol Endocrinol Metab, 2010; 299:E506-515.

11. Alvehus, M, Burén J, Sjöström, Goedecke J, Olsson T. The human visceral fat depot has a unique inflammatory prolife. Obesity 2010, 18:879-883.

12. Kintscher U, Hartge M, Hess K, Foryst-Ludwig A, Clemenz M, Wabitsch M, Fischer-Posovszky, Pamela, Barth TFE, Dragun D, Skurk T, Hauner H, Blüher M, Wolf AM, Knippschild W, Homback V, Marx N. T-lymphocyte infiltration in visceral adipose

tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. Arteriosclerosis, Thrombosis, and Vacsular Biology 2008, 28:1304-1310.

13. Adams M, Montague CT, Prins JB, Holder JC, Smith SA, Sanders L, Digby JE, Sewter CP, Lazar MA, Chatterjee VK, O'Rahilly S. Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation. J Clin Invest 1997, 100:3149-3153.

14. Baker, George. Human adipose tissue composition and age. Am J Clin Nutri 1969, 2:829-835.

15. Tan CY, Vidal-Puig A. Adipose tissue expandability: the metabolic problems of obesity may arise from the inability to become more obese. Biochem Soc Trans. 2008;36:935–940.

16. Wang, M, Grayburn P, Chen Shuyuan, Ravzzola M, Orci L, and Unger RH. Adipogenic capacity and the susceptibility to type 2 diabetes and metabolic syndrome. PNAS 2008; 6139-6144.

17. Turner SM, Murphy EJ, Neese Ra, Antelo F, Thomas T, Agarwal A, Go C, Hellerstein MK. Measurement of TG synthesis and turnover in vivo by 2H2O incorporation into the glycerol moiety and application of MIDA. Am J Physiol Endocrinol Metab 2003, 285:E790-E803.

18. Rossetti L, Giaccari A. Relative contribution of glycogen synthesis and glycolysis to insulin-mediated glucose uptake: A dose-response euglycemic clamp study in normal and diabetic rats. J Clin Invest 1990;85:1785–82.

19. Farese Jr RV, Cases S, Smith SJ. Triglyceride synthesis: insights from the cloning of diacylglycerol acyltransferase. Curr Opin Lipidol 2000, 11:229-234.

20. Herman, MA, Peroni OD, Villoria J, Schon MR, Abmurad NA, Bluher M, Klein S, Kahn B. A novel ChREBP isoform in adipose tissue regultes systemic glucose metabolism. Nature 2012, 484: 333-338.

21. Després JP, Lemieux I, Prud'homme D. Treatment of obesity: need to focus on high risk abdominally obese patients. BMJ 2001, 322: 716–720.

22. Grundy SM, Brewer HB, Cleeman JI, Smith SC, Lenfant C. Definition of metabolic syndrome: Report of the National Heart, Lung and Blood Institute/American Heart Association conference on scientific issues related to definition. Circulation 2004, 109:433-438.

23. Gabrierly I. Surgical removal of visceral adipose tissue: effects on insulin action. Curr Diab Rep 2003: 3, 201-206.

24. Goodpaster BH, Thaete FL, Simoneau JA, Kelley DE. Subcutaneous abdominal fat and thigh muscle composition predict insulin sensitivyt independently of visceral fat. Diabetes 1997, 46:1578-1585.

25. Frayn, KN. Visceral fat and insulin resistanc—causative or correlative? Br J Nutri 2000, 83:S71-S77.

26. Arner P, Bernard S, Salehpour M, Possnert G, Liebl J, Steier P, Buccholz BA, Eriksson M, Arner E, Hauner H, Skurk T, Rydén M, Frayn KN, Spalding KL. Dynamics of human adipose lipid turnover in health and metabolic disease. Nature 2011, 25:110-3.

27. Tuvdendorj D, Chandalia M, Batbayar T, Saraf M, Beysen C, Murphy EJ, Abate N. Altered subcutaneous abdominal adipose tissue lipid synthesis in obese, insulin-resistant humans. Am J Physiol Endocrinol Metab 2013, 305:E999-E1006.

28. Witters, LA, Watts TD, Daniel DL and Evan JL. Insulin stimulates the dephosphorylation and activtion of acetyl-CoA carboxylase. Proc Natl Acad Sci 1988, 85:5473-5477.

29. Choi SM, Tucker DF, Gross DN, Easton RM, DiPilato LM, Dean AS, Monks BR, Bimbaum MJ. Insulin regulates adipocyte lipolysis via an Akt-independent signaling pathway. Mol Cell Biol 2010, 30:5009-5020.

30. Mulder H, Holst L, Svensson H, Degerman E, SUndler F, Ahren B, Rorsman P, Holm C. Hormone-sensitive lipase, the rate-limiting enzyme in triglyceride hydrolysis, is expressed and active in beta-cells. Diabetes 1999, 48:228-232.

31. Belfrage P, Fredrikson G, Nilsson NÖ, and Stralfors P. Regulation of adipose tissue lipolysi: phosphorylation of hormone-sensitive lipase in intact rat adipocytes. FEBS letters 1980, 111: 120-124.

32. McLaughlin TM, Liu T, Yee G, Abbasi F, Lamendola C, Reaven GM, Tsao P, Cushman SW, Sherman A. Pioglitazone increases the proportion of small cells in human abdominal subcutaneous adipose tissue. Obesity 2010, 18:926-931.

33. Sadur CN, Tost TG, Eckel RH. Insulin reponsiveness of adipose tissue lipoprotein lipase is delayed but preserved in obesity. J Clin Endocrinol Metab, 1984; 59:1176-1182.

34. McLaughlin T, Lamendola C, Liu A, Abbasi F. Preferential fat deposition in subcutaneous versus visceral depots is associated with insulin sensitivity. J Clin Endocrinol Metab 2011, 96:E1756-60.

35. Robbins DC, Horton ES, Tulp O, Sims EA. Familial partial lipodystrophy: complications of obesity in the non-obese? Metabolism. 1982. 31:445-452.

36. Shi Y and Burn P. Lipid metabolic enzymes: emerging drug targets for the treatment of obesity. Nature Reviews 2004, 3; 695-710.

37. Yu YH and Ginsberg HN. Adipocyte signaling and lipid homeostasis. Circulation Research 2005, 96:1042-1052.

38. Adams M, Montague CT, Prins JB, Holder JC, Smith SA, Sanders L, Digby JE, Sewter CP, Lazar MA, Chatterjee VK, O'Rahilly S. Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentitation. J Clin Invest 1997, 100:3149-3153.

39. Riordan NH, Ichim TE, Min, WP, Want H, Solano F, Lara F, Alfaro M, Rodriguez JP, Harman RJ, Patel AN, Murphy MP, Lee RR, Minev B. Non-expanded adipose stromal vascular fraction cell therapy for multiple sclerosis. J Trans Med 2009, 7:1-9.

40. Danforth E Jr. Failure of adipocyte differentiation causes type II diabetes mellitus? Nat Genet 2000, 26:13.

41. Gray S and Vidal-Puig AJ. Adipose Tissue Expandability in the Maintenance of Metabolic Homeostasis. Nutrition Reviews 2007, 65: S7-S12.

42. Iozzo, Patricia. Myocardial, perivascular, and epicardial fat. Diabetes Care 2011, 34:S371-S379.

43. Bergman R, Kim S, Catalano K, Hsu I, Chiu J, Kabir M, Hucking K, Ader M. Why Visceral Fat is Bad: Mechanisms of the Metabolic Syndrome. Obesity 2006, 14:16S-19S.

44. Nagale C, Klett E, Coleman R. Hepatic triglyceride accumulation and insulin resistance. J Lip Res 2009, 50:S74-S79.

45. Hellerstein MK, Schwarz J, Neese RA. Regualtion of hepatic de novo lipogenesis in humans. Annual Reviews 1996, 16:523-557.

46. Kim JB, Sarraf P, Wright M, Yao KM, Mueller E, Solanes G, Lowell BB, Spiegelman B: Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. J Clin Invest 1998;101:1–9.

47. Commerford SR, Peng L, Dube JJ, O'Doherty RM: In vivo regulation of SREBP-1c in skeletal muscle: effects of nutritional status, glucose, insulin, and leptin. Am J Physiol Regul Integr Comp Physiol 2004;287:R218–R227.

48. Taniguchi CM, Kondo T, Sajan M, Luo J, Bronson R, Asano T, Farese R, Cantely LC, Kahn CR. Divergent regulation of hepatic glucose and lipid metabolicm by phosphoinositide 3-kinase via Akt PKClambda/zeta. Cell Metab 2006, 3:343-353.

**Figures**

**Table I**. Demographic and Clinical Characteristics (mean ± SD) of Insulin Resistant and Insulin Sensitive Moderately-obese Human Subjects

| Characteristic | Insulin Sensitive (n=8) | Insulin Resistant (n=7) | p-value* |
|---|---|---|---|
| Age (years) | 56.9 ± 8.0 | 54.7 ± 4.9 | 0.77 |
| Sex (M/F) | 6/2 | 6/1 | 0.63 |
| Race (white/non-white) | 8/0 | 4/3 | 0.08 |
| BMI ($kg/m^2$) | 28.5 ± 1.7 | 31.3 ± 3.2 | 0.07 |
| SSPG (mg/dL) | 83.5 ± 18.3 | 195.3 ± 29.1 | <0.001 |
| Fasting Glucose (mg/dL) | 100.3 ± 9.2 | 98.4 ± 5.0 | 0.63 |
| Fasting TG (mg/dL) | 86.5 ± 15.1 | 141 ± 37.2 | 0.007 |
| HDL (mg/dL) | 56.1 ± 10.8 | 48.6 ± 14.4 | 0.28 |
| LDL (mg/dL) | 114.9 ± 16.5 | 116.0 ± 30.6 | 0.93 |
| SAT ($cm^3$) | 113.0 ± 38.3 | 135.3 ± 31.1 | 0.24 |
| VAT ($cm^3$) | 46.9 ± 23.7 | 70.3 ± 25.7 | 0.09 |
| VAT/SAT ratio | 0.43 ± 0.22 | 0.52 ± 0.16 | 0.20 |
| Thigh fat ($cm^3$) | 52.8 ± 21.0 | 44.7 ± 15.3 | 0.40 |

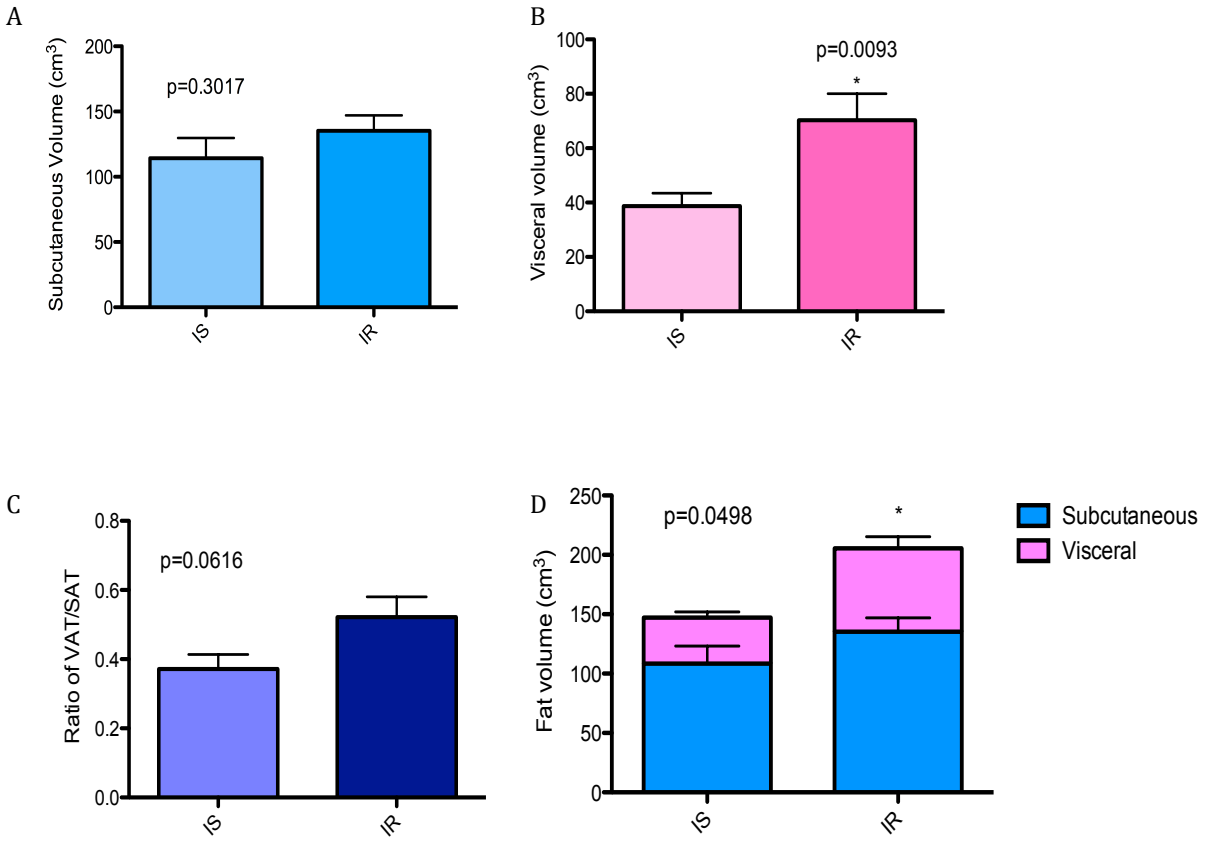



**Figure 1:** Fat distribution in IS vs IR measured by CT scan. A) Subcutaneous fat volume, p=0.3017; B) Visceral fat volume, p=0.0093; C) Ratio of VAT/SAT, p=0.062; D) Total abdominal fat volume, p=0.0498.

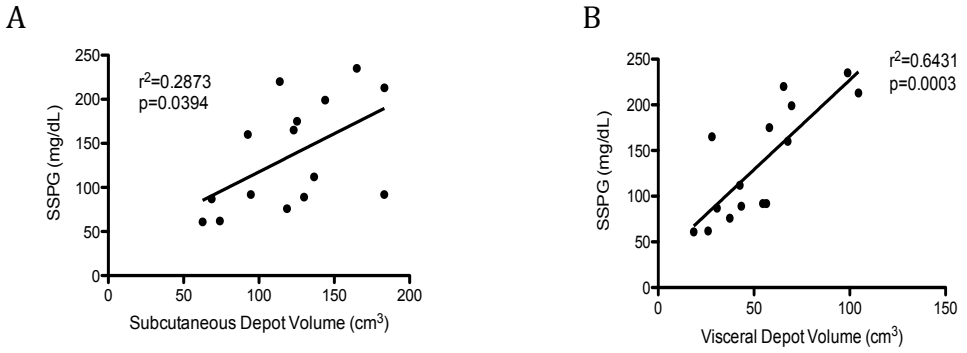



**Figure 2:** Relationship between fat depot and insulin sensitivity. A) Subcutaneous fat volume vs SSPG, p=0.0394; B) Viscereal fat volume vs SSPG, p=0.0003.

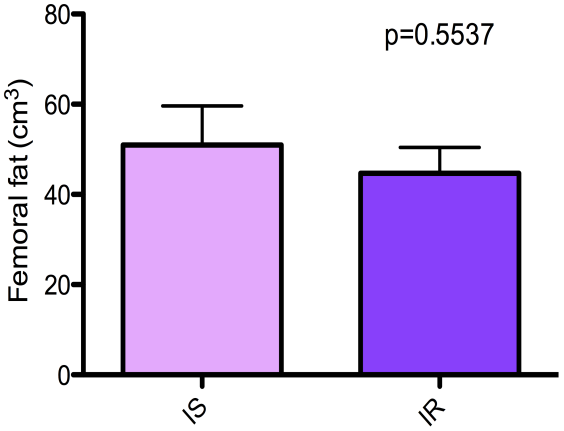



**Figure 3:** Femoral fat volume measured by CT scan, p=0.5537.

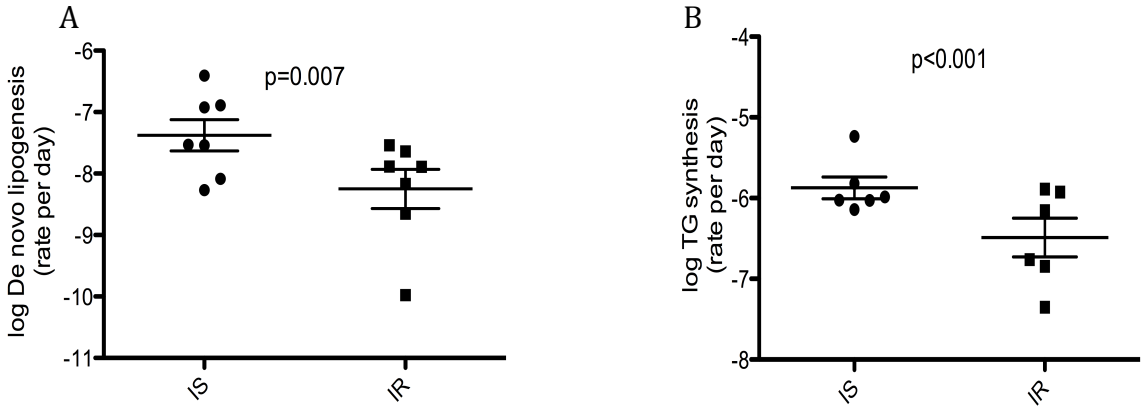



**Figure 4:** Subcutaneous adipocyte TG synthesis and DNL in IS vs IR expressed as log-transformed values based on fractional synthesis per day (*k*). A) log TG synthesis, p<0.001; B) log DNL, p=0.007.

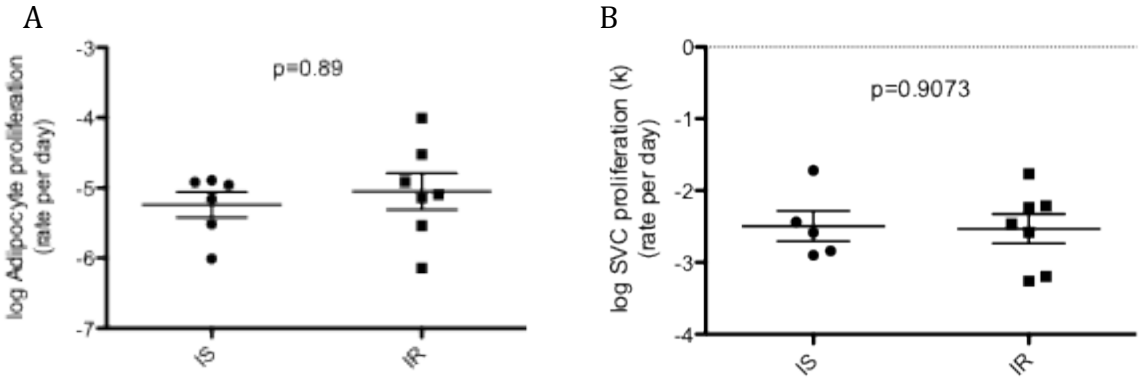



**Figure 5:** Subcutaneous adipocyte and stromal vascular cell (SVC) proliferation in IS vs IR expressed as log-transformed values based on fractional synthesis per day (*k*). A) log adipocyte proliferation, p=0.89; B) log SVC proliferation, p=0.91.

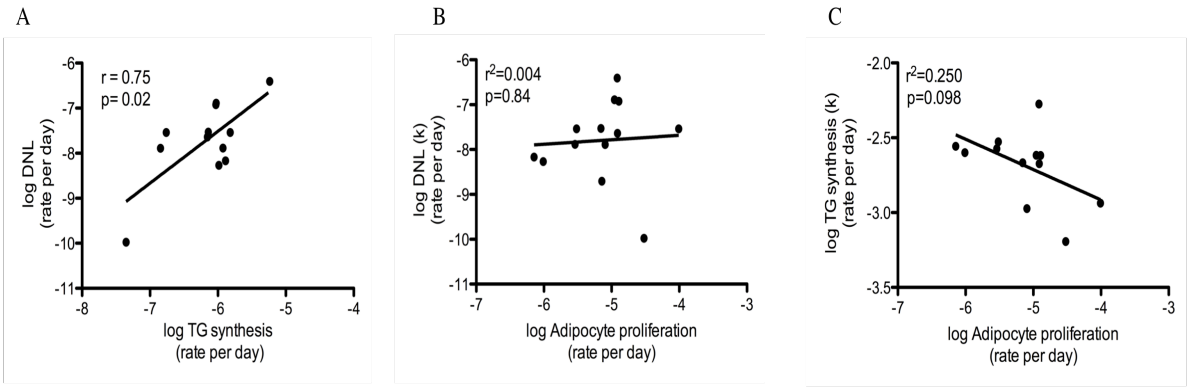



**Figure 6:** Relationships between adipose lipid and cell proliferation kinetics expressed as log-transformed values. A) log TG synthesis vs log DNL, p=0.02; B) log adipocyte proliferation vs log DNL, p=0.084; C) log adipocyte proliferation vs log TG synthesis, p=0.98.

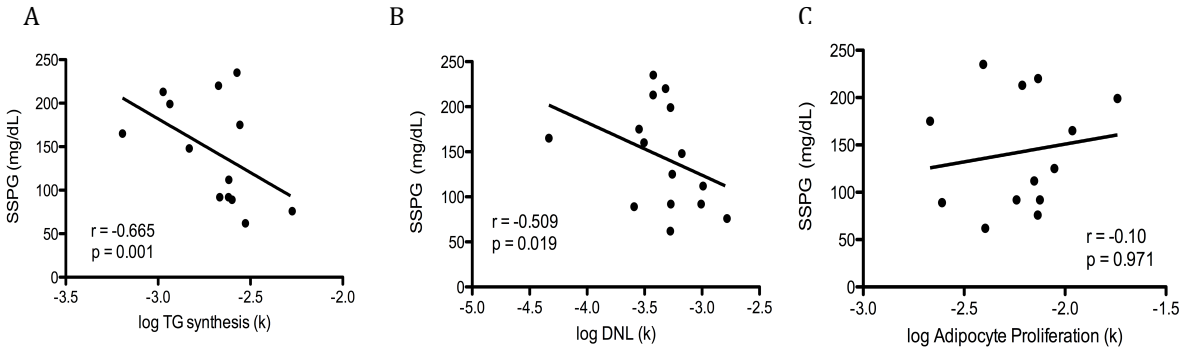



**Figure 7:** Relationship between adipose function and insulin sensitivity. A) log TG synthesis vs SSPG, p=0.001; B) log DNL vs SSPG, p=0.019; C) log adipocyte proliferation, p=0.971.

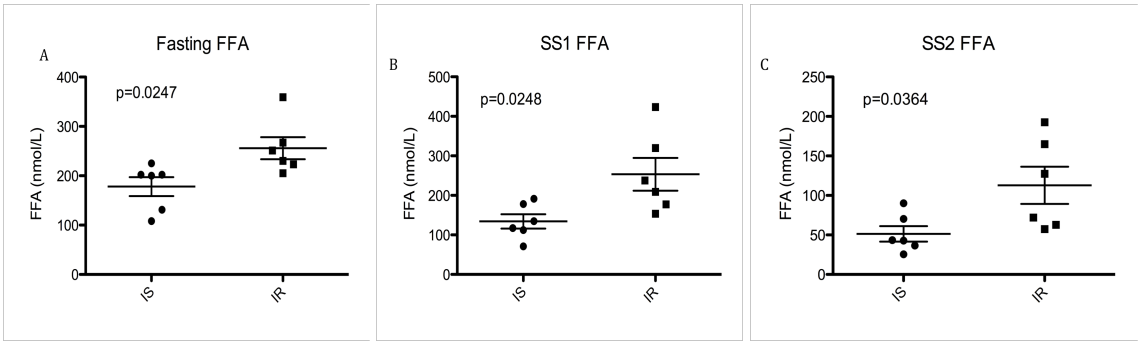



**Figure 8:** Insulin-suppression of lipolysis determined by the measurement of free fatty acids (FFAs) during SSPG test. A) FFA at baseline, p=0.247; B) FFAs at first steady state (SS1) when insulin infusion concentration is low, p=0.0248; C) FFAs at second states sate when insulin infusion concentration is high, p=0.0364.

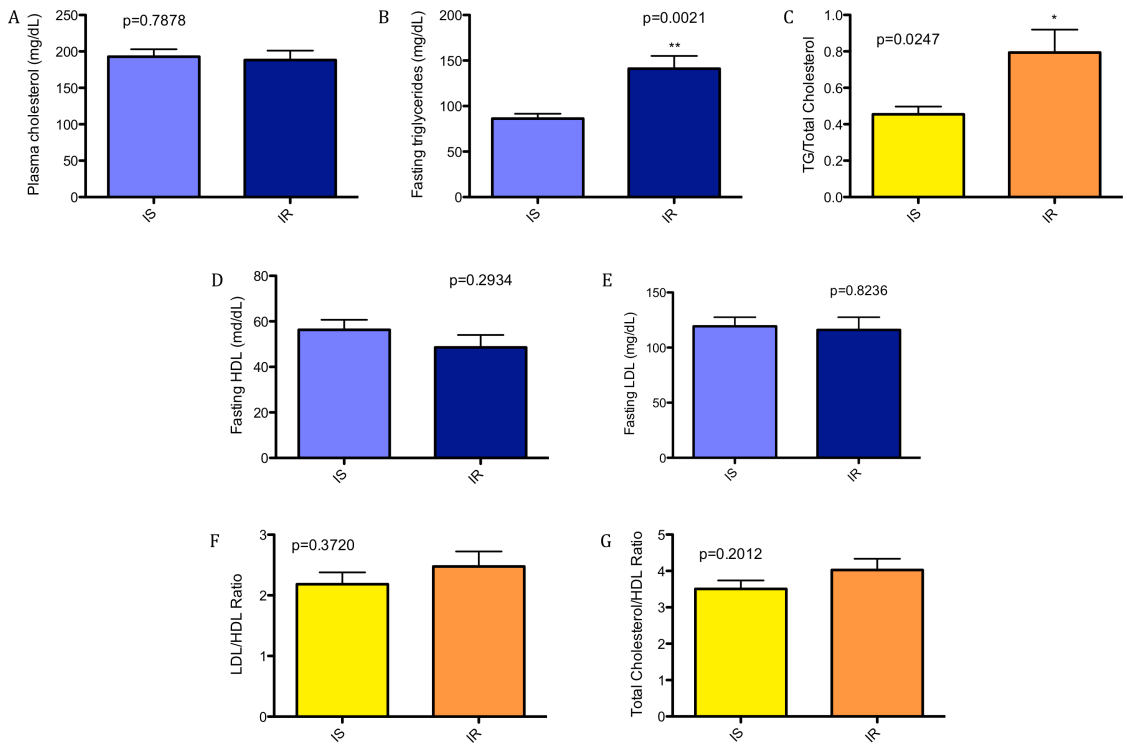



**Figure 9:** Fasting plasma lipids. A) Total cholesterol, p=0.79; B) triglycerides, p=0.0021; C) Ratio of TG/total cholesterol, p=0.025; D) HDL, p=0.023; E) LDL, p=0.82; E) LDL/HDL ratio, p=0.37; F) total cholesterol/HDL, p=0.2012.

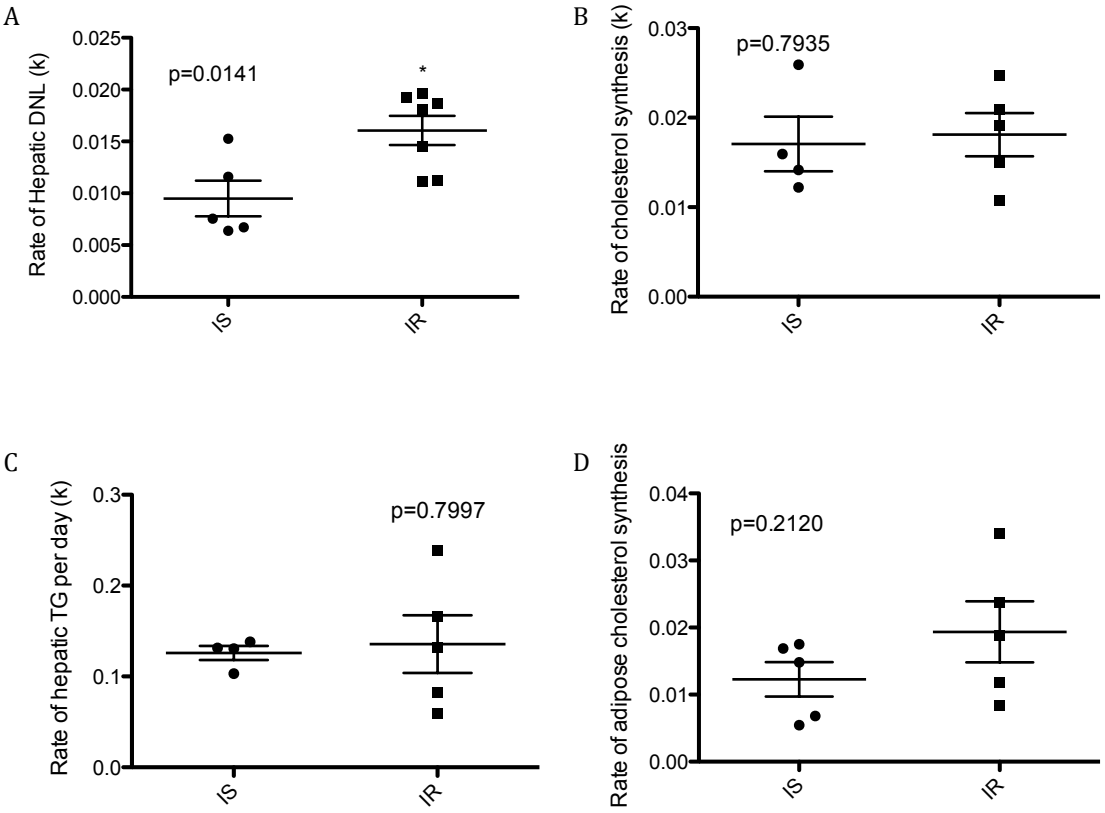



**Figure 10:** Hepatic lipid kinetics measured through $^{2}H$-enriched plasma lipids, and adipose cholesterol synthesis, expressed as fractional synthesis per day (f/day). A) Rate of hepatic DNL, p=0.0141; B) Rate of cholesterol synthesis, p=0.0794; C) rate of hepatic TG synthesis, p=0.7997; D) Rate of adipose cholesterol synthesis, p=0.2120.

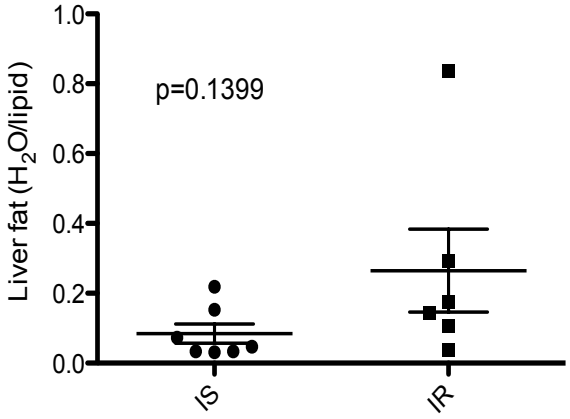



**Figure 11:** Liver fat measured by MRI scan, p=0.1399.

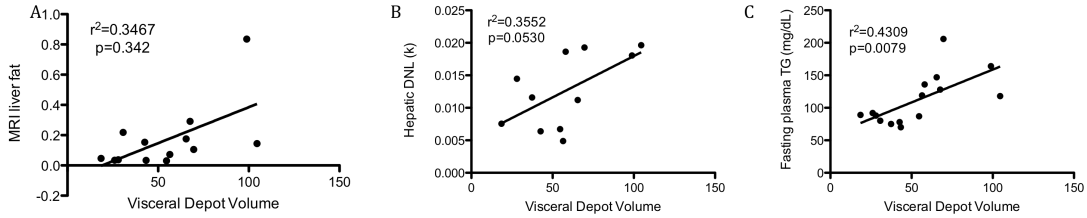



**Figure 12:** Relationship between visceral fat volume and hepatic fat. A) Visceral fat volume vs liver fat ($H_2O$/lipid ratio), p=0.342; B) visceral fat volume vs hepatic DNL, p=0.0530; C) visceral fat volume, p=0.0079.

# CHAPTER 4

## *In vivo* $^{2}H_2O$ administration reveals impaired triglyceride storage in subcutaneous adipose tissue of moderately-obese African-American relative to Caucasians

**Abstract**
African-Americans are one of the most at-risk groups for developing type 2 diabetes and is the leading group for obesity rates in the United States. Our previous study consisting of a predominately Caucasian population, suggested that maintenance of adipocyte function to synthesize and store lipids may protect obese individuals from becoming insulin resistant. However, studies suggest that the physiological mechanisms behind the etiology of insulin resistance may differ amongst racial groups. While African-Americans are at a greater risk for developing insulin resistance, they tend to have less visceral and hepatic fat as a group, which strongly correlated to insulin resistance. The adipose has not been thoroughly investigated in African-Americans, and may reveal further insights into the mechanism causing insulin resistance in this population. Thus, we aimed to investigate potential differences in adipocyte function between African-Americans and Caucasians. We administered $^{2}H_{2}O$ to five African-Americans and seven Caucasians for the quantification of TG synthesis, DNL and adipose proliferation in isolated subcutaneous adipocytes. We found African-Americans showed a trend towards lower TG synthesis, while DNL remained similar between African-Americans and Caucasians. No difference was observed in adipocyte proliferation. There were no differences in hepatic lipid kinetics, and the African-American subjects in this cohort showed a healthier plasma lipid profile compare to Caucasians.

**Introduction**

The previous study, conducted in a predominately Caucasian population, demonstrated lower rates of newly synthesized triglycerides and fatty acids in the abdominal subcutaneous adipose tissue of obese insulin resistant (IR), compared to insulin sensitive (IS), indicating impaired adipocyte dysfunction in the insulin resistant state. While these recent findings contribute a potential mechanism linking adipose tissue to insulin resistance, further investigation is required to better cellular mechanisms and to determine if adipose dysfunction is predictive of insulin resistance amongst different ethnicities.

In the United States, African-Americans (AA) have 51% higher rates of obesity than Caucasians (C), and the highest rates of type 2 diabetes and cardiovascular disease (1,2). Obesity is associated with insulin resistance, yet the physiological mechanisms of this association and fat regional distribution as a potential contributor is not yet completely understood, particularly in minority populations most affected by this disease. We, and others, have shown that visceral adipose tissue (VAT) in C is associated with insulin resistance, whereas SAT (adjusted for BMI) is protective (1,3). However, this is not the case for African-Americans. Paradoxically, VAT mass is consistently lower in AA compared to C, yet are more insulin resistant for a given degree of body fat (4-9). Despite lower absolute and relative VAT mass, studies have shown that after adjustment for BMI or body fat, VAT and/or VAT/ SAT ratio correlates with insulin resistance in AA (6,7,10). Intrahepatic fat is also decreased in AA relative to C and correlates with VAT, yet no correlations were observed with insulin resistance (10,11). Fat distribution showing greater SAT and lower VAT and its association with insulin resistance in the African-American population is not known. Whether or not this pattern of distribution is protective against or predictive of insulin resistance is yet to be determined. Greater SAT relative to VAT may be related to enhanced adipogenesis, increased triglyceride synthesis and storage, and/or decreased lipolysis in SAT.

**Results**

The African-American (AA) cohort consisted of both females (*n*=4) and males (*n*=1), compared to a Caucasian subjects with 2 females and 6 males. Due to the small sample size, groups are not stratified by insulin sensitivity, with AA consisting of 2 IR and 3 IS, and Caucasians consisting of 3 IR and 5 IS. Insulin sensitivity was not significantly different between the minority and Caucasian cohorts, and fasting glucose was very similar between groups (**Table 1**). Fat distribution differs between the minority cohort and Caucasians, with significantly greater abdominal SAT (p=0.0082) in the minorities (**Figure 1A**). There is a trend towards greater visceral fat in the Caucasian cohort, but this difference was not significant, p=0.2350 (**Figure 1B**). The ratio of VAT versus SAT is significantly different between groups, p=0.0139 (**Figure 1C). Figure 1D** demonstrates differences in abdominal fat distribution measured by CT scan. There was a trend towards greater thigh fat in AA's, but the difference was not significant, p=0.0804 (**Figure 2**).

The primary aim of this pilot study was to investigate potential differences in lipid metabolism in abdominal SAT of African-Americans versus Caucasians using $^2H_2O$ *in vivo*. Consisting of 5 subjects, the African-Americans cohort showed a lower rate of TG synthesis in minorities compared to Caucasians, but a p-value bordering significance (p=0.0695) (**Figure 3A**). In the absence of the outlier in the Caucasian cohort, the difference between groups becomes significant, with a p-value of 0.0334. **Figure 3B** shows that the rate of adipose DNL and adipocyte proliferation were very similar between groups (p=0.8437 and 0.6564, respectively). No differences were observed in the rate of adipocyte nor stromal vascular cell proliferation (SVC), p=0.4994 (**Figure 4A-B**).

Fasting plasma lipids were measured as a metric of cardiovascular and hepatic health. Fasting cholesterol was significantly different between groups (p=0.0124), while fasting plasma TG was not, p=0.07347 (**Figure 5A-B**). Plasma HDL, an outcome often used to evaluate atherosclerosis risk, was significantly greater in African-Americans, compared to Caucasians (**Figure 5C)**. Fasting plasma LDL, however, was not different between groups (**Figure 5D**). This finding was confirmed by greater ratios of LDL/HDL and total cholesterol/HDL, p=0.0271 and p=0.0010, respectively (**Figure 6A-B**). The ratio of TG/cholesterol was not significantly different with a p-value of 0.2234. To further evaluate lipid metabolism between minorities and Caucasians, DNL was quantified by $^2H$ incorporation into newly synthesis palmitate in plasma, representative of hepatic DNL. Surprisingly, hepatic DNL was similar between groups (**Figure 7A**), with a p-value of 0.7981. Hepatic TG synthesis, represented by $^2H$-enriched plasma glycerol was slightly higher in Caucasians, but this difference was not significant (**Figure 7B**). Linear regression analysis shows an inverse relationship between hepatic DNL, quantified by $^2H_2O$, and fasting plasma TG in African-Americans, although no relationship existed between these variables in Caucasians (**Figure 8A-B**). **Figure 9** demonstrates no significant difference in hepatic lipid content, measured by MRI, observed between groups, p=0.4067. To confirm that the relationship between visceral fat and insulin sensitivity exists even amongst BMI-matched obese patients, a linear regression model was calculated. Results show a significant positive correlation between visceral fat content and insulin sensitivity, p=0.0011 (**Figure 10**). Linear regression analysis showed no relationship between visceral fat content measured by CT scan and hepatic DNL in Caucasian subjects, but a positive trend in African-Americans, p=0.1036 (**Figure 11A-B**).

**Discussion**

Previous studies have demonstrated that while African-Americans are at a greater risk for developing insulin resistance and T2D, they display fewer symptoms of the metabolic syndrome (11,12). The physiological mechanisms that differ between African-Americans and Caucasians needs further attention given the paradoxical phenotypes associated with insulin resistance amongst African-Americans.

African-Americans as a group tend to have less intra-abdominal adipose tissue than whites (13,5,6,7), yet are more insulin resistant. A study by Hyatt and colleagues, found significantly lower visceral fat in African-Americans women compared to Caucasian women, but similar areas of SAT were observed between groups (14). This differs from our results, although our study compares two cohorts with similar insulin sensitivities (determined by SSPG). The Hyatt study showed significantly lower insulin sensitivity in the African-American cohort compared to Caucasians, which may have confounded results. Although, a previous study by Katzmarzyk and colleagues showed that both Caucasian men and women have lower SAT compared to African-American men and women (15). We found significantly greater subcutaneous volumes in our minority cohort, compared to Caucasians. It is possible that this may be due to the disproportionate number of females versus males between the two cohorts, although other studies have shown less SAT in both male and female African-Americans compared to Caucasians (16,17).

Our findings of higher subcutaneous depot volume conflict with our findings of lower TG synthesis in African-Americans compared to Caucasians. One potential explanation for such confounding results may be that subcutaneous fat storage measured by CT scan includes both superficial and deep subcutaneous depots, which are not distinguished in our analysis. Unlike CT scan analyses, fat biopsies in our subjects were taken only from the superficial portion of the SAT. The metabolic profile of deep subcutaneous adipose tissue closely resembles that of visceral adipose tissue, and thus correlated with insulin resistance (18,19). However, given that minorities whose SSPG test identified them as being IS had greater SAT volume, this explanation is unlikely. Another potential explanation may be enhanced lipolysis in the minority subjects, compared to Caucasians. Given that these subjects are in steady state during the course of the study, it is possible that the majority of newly synthesized glycerol was not retained in the superficial SAT, and may instead be stored ectopically or be re-distributed to other depots. The high SAT volume measured by CT scan versus the low TG synthesis rate measured by $^{2}H$-incorporation into newly synthesized glycerol sounds contradicting. A plausible explanation can be considered, however others have hypothesized that there may be a maximal capacity for lipid storage in subcutaneous adipocytes (20,21,22). The greater SAT volume compared in AA's compared to Caucasians, suggests that perhaps the maximum lipid storage was already reached prior to participation in the study. This is supported by the low rate of TG synthesis observed in these subjects, indicating storage in other depots or even ectopic storage. However, one major flaw in this study that may explain our confounding results is that we observed significant differences in fat distribution, which we did not match for in this study. Since visceral fat is not elevated in the AA cohort, as shown by us and others (6,17), any excess lipids may be distributed in the thigh, which although not significant, was higher in AA's. Lipid synthesis measurements in this study may suggest that the abdominal subcutaneous adipose of African-Americans has a low threshold for maximal lipid storage, regardless of insulin sensitivity and body weight. While adipose TG differs between groups, adipose DNL remains similar. This finding may indicate a

differential response to insulin in the adipose tissue of African-Americans. The synthesis glycerol and palmitate are both up-regulated by insulin, at different steps. Insulin pushes glucose into insulin-sensitive tissues, where glucose undergoes glycolysis. Glycerol-3-phosphate is synthesized by the up-regulation of glycerol-3-phosphate dehydrogenase, and in response to insulin stimulating acyl-transferases, it continues on to form triglyceride molecules. Palmitate, on the other hand, is synthesized after the oxidative portion of the glycolytic pathway and through the production of acetyl-CoA and up-regulation of fatty acid synthase (FAS) by insulin (23). It appears that insulin-stimulated DNL is maintained, while insulin-stimulation of TG synthesis (ie. via up-regulation of proteins, such as diacyltransferease, DGAT), is impaired in obese African-Americans. As demonstrated in our previous study consisting of predominately Caucasian subjects, the ability to maintain TG synthesis in the SAT, may be protective against insulin sensitivity. This may be the case for African-Americans, however, the etiology of insulin resistance in this population seems to be deeper than subcutaneous adipose tissue function.

Many investigators have reported that despite having lower visceral fat, African-Americans are at greater risk for developing insulin resistance (Liu, 2014, Hoffman). Studies by Goran and others, demonstrate that African-Americans have less hepatic fat stores, yet are more insulin resistant compared to their Caucasian counterparts (24,25). It is widely accepted that visceral fat is highly correlated with insulin sensitivity, however to demonstrate that this relationship is maintained in BMI-matched patients, we performed a linear regression analysis. Administration of $^{2}H_2O$ to quantify hepatic DNL may reveal an important relationship between visceral fat and hepatic lipid metabolism. Linear regression analysis showing no relationship between visceral fat content measured by CT scan and hepatic DNL in Caucasian subjects, but a positive trend in African-Americans suggests that visceral fat content may contribute a spillover of free fatty acids into the liver through the portal vein to a greater extent in African-Americans. In another study, it was shown that visceral adiposity and hepatic fat may contribute to increased inflammation in the subcutaneous depots or impaired beta-cell function, in obese Hispanics and African-Americans (26). Fasting circulating lipids, such as cholesterol, TG, HDL, LDL and TG-HDL ratios are used as markers of cardiovascular function and the metabolic syndrome (27). As the liver produces TG, VLDL production is up-regulated for TG export from the liver. The lipid-rich TG-VLDL particles transport TGs into other tissues, such as the adipose, where it is lypholized by lipoprotein lipase (LPL) before being transported across the membrane for storage in adipocytes. In doing so, the amount of plasma TG is reduced. Thus, measuring fasting plasma TG is used as a marker of peripheral TG uptake and catabolism. Aarsland and colleagues, 1996 used tracer methods to determine that in the hyperinsulinemic state, the availability of FA's (already formed in vivo or from diet) was the major determinant of hepatic VLDL-TG secretion (28). This would explain why hypertriglyceridemia is a common phenotype of insulin resistance (29) and the LDL/HDL ratio in conjunction with hypertriglyceridemia is associated with high cardiovascular disease risk. This, however, is not the case in African-Americans.

In agreement with previous studies, our African-American cohort displayed not only healthy levels of fasting plasma lipids, but a healthier overall lipid profile than the Caucasian subjects (30,31,32). Results such as this, encouraged investigators to conclude that fasting TG and TG-HDL ratios are not suitable markers for insulin sensitivity in African-Americans. In our study, fasting cholesterol levels were significantly lower in AA's compared to Caucasians, as further shown by a significantly lower cholesterol/HDL ratio, $p=0.0010$ in this group. However, if the

IS and IR patients in the African-Americans are examined individually, the two IR patients in this group have higher ratios than the IS subjects. Nonetheless, the higher ratios observed in IR AA patients are lower than those observed in IR Caucasians ($2.78 \pm 0.29$ vs. $4.34 \pm 0.87$, respectively). This further supports previous findings that these measurements are not proper markers, or alternately, different criteria for identifying cardiovascular risk in African-Americans may need to be adjusted (30).

Insulin's inability to suppress adipose tissue lipolysis may directly stimulate insulin's promotion of VLDL-TG secretion in the liver (33,34). This may very likely be the case in Caucasian patients, as we previously observed reduced rates of adipose TG synthesis in congruence with elevated hepatic DNL in IR compared to IS (refer to Chapter 1). Similarly, in this study, those with the highest rates of hepatic DNL, measured by $^2$H-enriched plasma palmitate, are all IR patients, in both the African-American and Caucasian cohorts. Hepatic DNL was accompanied by elevated fasting plasma TG in Caucasian IR patients. This supports the notion of hepatic insulin sensitivity in these patients, perhaps enhanced by resistance to insulin action in the adipose tissue causing an elevation in circulating free fatty acids. Interestingly, despite greater hepatic DNL in IR African-Americans, this same pattern of plasma TG with hepatic DNL is not observed in our African-Americans patients. While hepatic DNL is greater in the IR African-American subjects, fasting plasma TG is actually lower in the IR patients within this group (those with the highest fasting TG levels are IS). In fact, regardless of the insulin sensitive state, African-Americans' TG levels are within a normal healthy range, based on standards established by the American Diabetes Association (35). Further analysis by linear regression shows a trend towards a negative correlation between hepatic DNL, measured by $^2H_2O$ and fasting plasma TG. This relationship did not exist in Caucasians. Hepatic DNL and plasma TG were not different between African-Americans and Caucasians, so this difference in lipid dynamics is suggestive of differences in hepatic function between the two groups. As hepatic DNL increases, fasting plasma TG decreases, potentially indicating greater uptake of TG into the liver of African-Americans, but not in Caucasians. Our use of a stable isotope reveals information concerning hepatic function in a select few insulin resistant African-Americans that cannot be identified using standard methods of care. Future use of $^2H_2O$ to measure hepatic lipogenesis may uncover the mystery behind the strong association between low levels of hepatic fat and insulin resistance in African-Americans.

**Study Limitations**

There were several limitations in this study. Our study consisted of mostly women in the minority cohort, and mostly men in the Caucasians cohort. This presents a cofounding variable that may also suggest sex differences in adipose storage as it relates to insulin sensitivity. Sex differences paired with a small sample size calls for further investigation, however due to funding, this endeavor was not possible. Lastly, subcutaneous fat volume from which fat biopsies were taken, were significantly higher in our African-American subjects. If this study were to be repeated, subjects should be matched by subcutaneous adipose tissue volume in order to better interpret differences in TG synthesis measured by $^2H_2O$. Future and larger studies are needed to further investigate differences in adipose lipid metabolism between African-Americans.

**References**


1. Harris MI, Flegal KM, Cowie CC, Eberhardt MS, Goldstein DE, Little RR, Wiedmeyer HM, Byrd-Holt DD. Prevalence of diabetes, impaired fasting glucose, and impaired glucose tolerance in US adults. The Third National Health and Nutrition Examination Survey, 1988-1994. Diabetes Care 1998, 21:518-524.

2. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Soliman EZ, Sorlie PD, Sotoodehnia N, Turan TN, Virani SS, Wong ND, Woo D, Turner MB; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Executive summary: heart disease and stroke statistics--2012 update: a report from the American Heart Association. Circulation. 2012 Jan 3;125(1):188-97.

3. McLaughlin T, Allison G, Abbasi F, Lamendola C, Reaven G. Prevalence of insulin resistanceand associated cardiovascular risk factors among normal, overweight, and obese individuals. Metabolism 2004, 53:495-499.
4. Goedecke JH, Levitt NS, Lambert EV, Utzschneider KM, Faulenbach MV, Dave JA, West SW, Victor H, Evans J, Olsson T, Walker BR, Seckl JR, Kahn SE. Differential effects of abdominal adipose tissue distribution on insulin sensitivity in black and white South African women.Obesity 2009, 17:1506-12.

5. Conway JM, Yanovski SZ, Avila NA, Hubbard VS. Visceral adipose tissue differences in black and white women. Am J Clin Nutr 1995, 61:765-771.

6. Lovejoy JC, de la Bretonne JA, Klemperer M, Tulley R. Abdominal fat distribution and metabolic risk factors: effect of race. Metabolism 1996, 45:1119-1124.

7. Albu JB, Murphy L, Frager DH, Johnson JA, Pi-Sunyer FX. Visceral fat and race-dependent health risks in obese nondiabetic premenopausal women. Diabetes 1997, 46:456-46.

8. Perry AC, Applegate EB, Jackson ML, Deprima S, Goldberg RB, Ross R, Kempner L, Feldman BB. Racial differences in visceral adipose tissue but not anthropometric markers of health-related variables. J Appl Physiol 2000, 89:636-643.

9. Hill JO, Sidney S, Lewis CE, Tolan K, Scherzinger Al, Stamm ER. Racial differences in amounts of visceral adipose tissue in young adults: the CARDIA study. Am J Clin Nutr 1999, 69:381-387.

10. Guerrero R, Vega GL, Grundy SM, Browning JD. Ethnic differences in hepatic steatosis: an insulin resistance paradox? Hepatology 2009, 49:791-801.

11. DeFronzo RA, Ferrannini E. Insulin resistance: a multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care* 1991, 14:173–94.

12. Meis SB, Schuster D, Gaillard T, Osei K. Metabolic syndrome in nondiabetic, obese, first-degree relatives of African American patients with type 2 diabetes: African American triglycerides-HDL-C and insulin resistance paradox. Ethn Dis 2006, 16:830-860.

13. Després JP, Lemieux I, Prud'homme D. Treatment of obesity: need to focus on high risk abdominally obese patients. BMJ 2001, 322: 716–720
14. Hyatt TC, Phadke RP, Hunter GR, Bush NC, Muñoz AJ, Gower BA. Insulin sensitivity in African-American and White women: Association with inflammation. Obesity 2009, 17:276-282.

15. Katzmarzyk PT, Bray GA, Greenway FL, Johsnon WD, Newton RL Jr, Ravussin E, Ryan DH, Smith SR, Bouchard C. Racia differences in adboinal depot-specific adiposity in white and African-American adults. Am J Clin Nutr 2010, 9:7-15.

16. Hoffman DJ, Wang Z, Gallagher D, Heymsfield SB. Comparison of Visceral Adipose Tissue Mass in Adult African Americans and Whites. Obesity Research 2005, 13:66-74.

17. Liu J, Coady S, Carr J, Hoffmann U, Taylor HA, Fox CS. Differential associations of abdominal visceral, subcutaneous adipose tissue with cardiometabolic risk factors between African and European Americans. Obesity 2013, 000:1-8.

18. Walker GE, Verti B, Marzullo P, Savi G, Mencarelli M, Zurleni F, Liuzzi A, Di Blasio AM. Deep subcutaneous adipose tissue: a distinct abdominal adipose depot. Obesity 2007, 15:1933-1943.

19. Kelley DE, Thaete FL, Troost F, Huwe T, Goodpaster BH. Subdivisions of subcutaneous abdominal adipose tissue and insulin resistance. Am J Physiol Endocrinol Metab 2000, 278:E941-E948.

20. McLaughlin, T., Sherman A, Tsao P, Gonzalez O, Yee G, Lamdendola C, Reaven GM, Cushman SW. Enhanced proportion of small adipose cells in insulin-resistance vs insulin sensitive obese individuals implicates impaired adipogenesis. Diabetologia. 2007; 50(8): 1707-15.

21. Yang J, Eliasson B, Smith U, Cushman SW, Sherman AS. The size of large adipose cells is a predictor of insulin resistance in first-degree relatives of type 2 diabetic patients. Obesity 2012, 20: 932-938.

22. Gray S and Vidal-Puig AJ. Adipose Tissue Expandability in the Maintenance of Metabolic Homeostasis. Nutrition Reviews 2007, 65: S7-S12.

23. Sul HS, Latasa MJ, Moon Y, Kim KH. Regulation of the fatty acid synthase promoter by insulin. Ju Nutr 2000, 130:315S-320S.

24. Goran, MI, Bergman RN, Cruz ML and Watanabe, R. Insulin Resistance and Associated Compensatory Responses in African-American and Hispanic Children. *Diabetes Care* 2002, 25:2184-2190.

25. Alderete TL, Toldedo-Corral CM, Desai P, Weigensberg MJ, Goran MI. Liver fat has a stronger association with risk factors type 2 diabetes in African-Americans compared with Hispanice adolescents. *J Clin Endocrinol Metab* 2013, 98:3748-3754.

26. Lé KA, Mahurkar S, Alderete TL, Hasson RE, Adam TC, Kim JS, Beale E, Xie C, Greenber AS, Allayee H and Goran MI. Subcutaneous adipose tissue macrophage infiltration is associated with hepatic and visceral fat deposition, hyperinsulinemia, and stimulation of NF-ϰB stress pathway. *Diabetes* 2011, 60:2802-2809.

27. Lemieux I, Lamarche B, Coulillard C, Pascot A, Cantin B, Bergeron J, Dagenais GR, Després JP. Total cholesterol/HDL cholesterol ratio vs LDL cholesterol/HDL cholesterol ratio as indices of ischemic heart risk in men: the Quebec Cardiovascular Study. Med 2001, 16:2685-2592.

28. Aarsland A, Wolfe RR. Hepatic secretion of VLDL fatty acids during stimulated lipogenesis in men. J Lipid Res 1998, 39:1280-1286.

29. Steiner, G. The dyslipoproteinemias of diabetes. Ahtherosclerosis 1994, 110:S27-S33.

30. Sumner AE, Vega GL, Genovese DJ, Finley KB, Bergman RN, Boston RC. Normal triglyceride levels despite insulin resistance in African Americans: role of lipoprotein lipase. Metabolism 2005; 54:902-909.

31. Foster T, Anania FA, Li D, Katz R, Rudoff M. The prevalence and clinical correlates of nonalcoholic fatty liver disease (NAFLD) in African-Americans: the multiethnic study of atherosclerosis (MESA). Dig Dis Sci 2013, 58:2392-2398.

32. Yu SS, Ramsey NL, Castillo DC, Ricks M, Sumner AE. Triglyceride-based screening tests fail to recognize cardiometabolic disease in African immigrant and African-American men. Metab Syndr Relat Disord 2013, 11:15-20.

33. Sørensen LP, Søndergaard E, Nellemann B, Christiansen JS, Gormsen LC, Nielsen S. Increased VLDL-Triglyceride secretion precedes impaired control of endogenous glucose production in obese, normoglycemic men. Diabetes 2011, 60:2257-2264.

34. Sørensen LP, Andersen IR, Søndergaard E, Gormsen LC, Schmitz O, Christiansen JS, Nielsen S. Basl and insulin medicated VLDL-triglyceride kinetics in type 2 diabetic men. Diabetes 2011, 60: 88-96.

35. Solano MP, Goldberg RB. Lipid Management in Type 2 Diabetes. Clinical Diabetes 2006, 24:27-32.

**Figures**

**Table 1:** Subject Characteristics

| | Caucasian *n*= 9 | African-American *n*= 5 | p-value |
|---|---|---|---|
| **Age (yrs)** | 56 ± 10 | 57.8 ±7 | 0.6624 |
| **Body Weight (kg)** | 95.2 ± 4.0 | 87.0 ± 7.4 | 0.3009 |
| **BMI (kg/m$^2$)** | 30.4 ± 2.8 | 31.0 ± 2.3 | 0.5561 |
| **SSPG (mg/dL)** | 94.75 ± 15 | 96 ± 45 | 0.6972 |
| **Fasting glucose (mg/dL)** | 99.80 | 100.6 | 0.8996 |

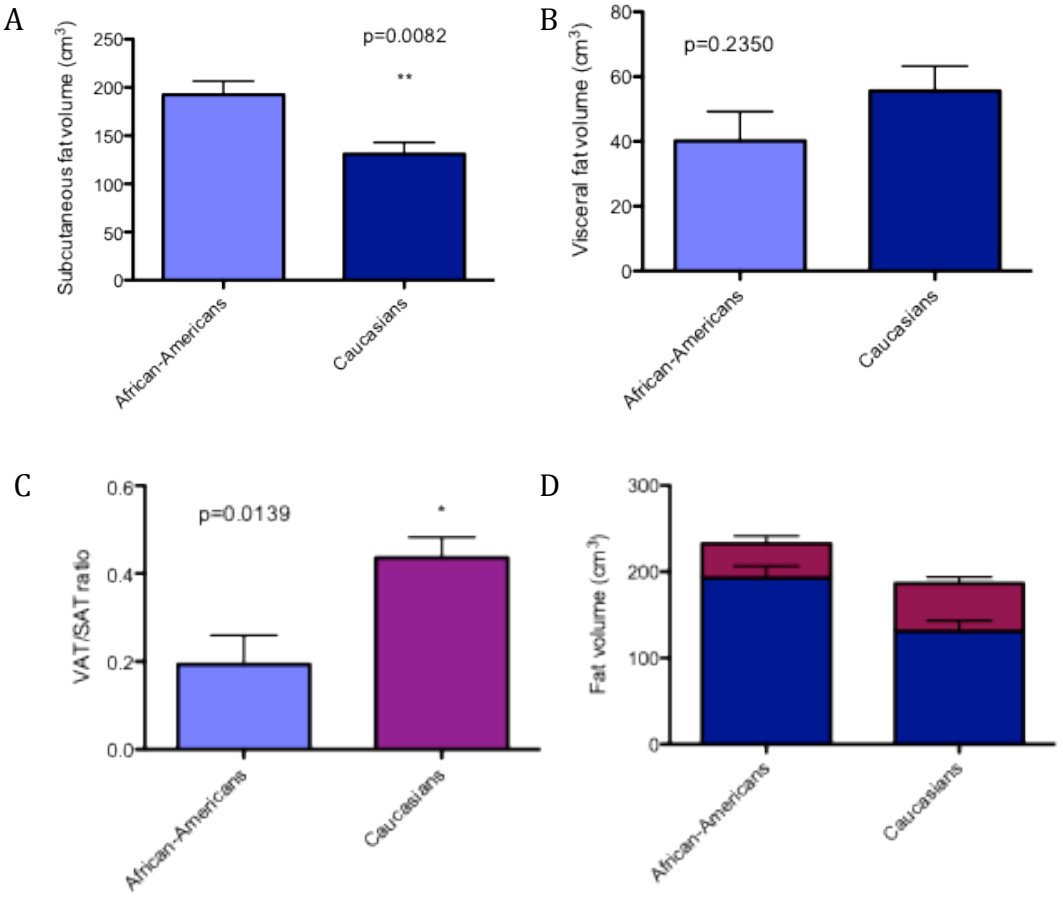



**Figure 1:** Fat distribution in African-Americans vs. Caucasians. A) Subcutaneous fat volume, p =0.0082; B) Visceral fat volume, p=0.2350; C) VAT/SAT ratio, p=0.139; D) Fat volume distribution, p=0.0563;

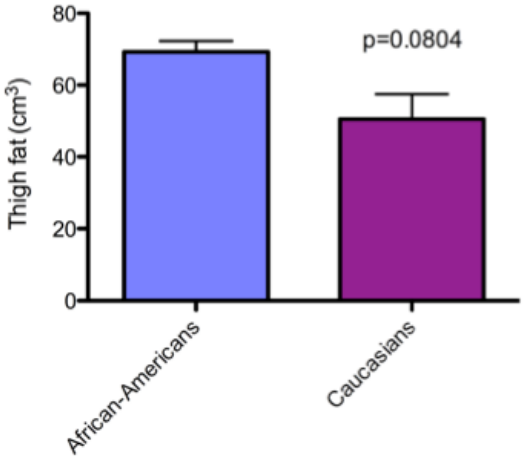



**Figure 2:** Thigh fat measured by CT scan, p=0.0804.

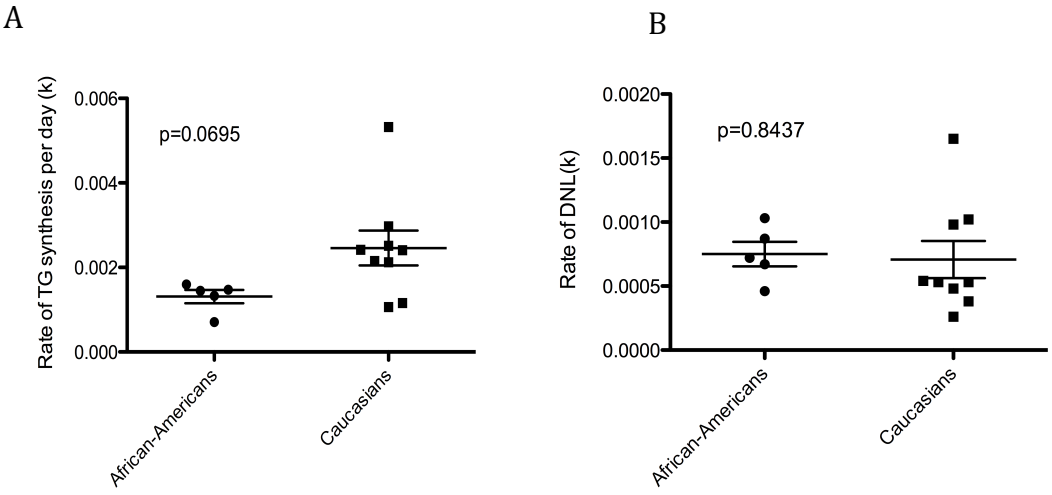



**Figure 3:** Subcutaneous adipose lipid kinetics A) Rate of TG synthesis, p=0.0695; B) Rate of DNL, p=0.8437.

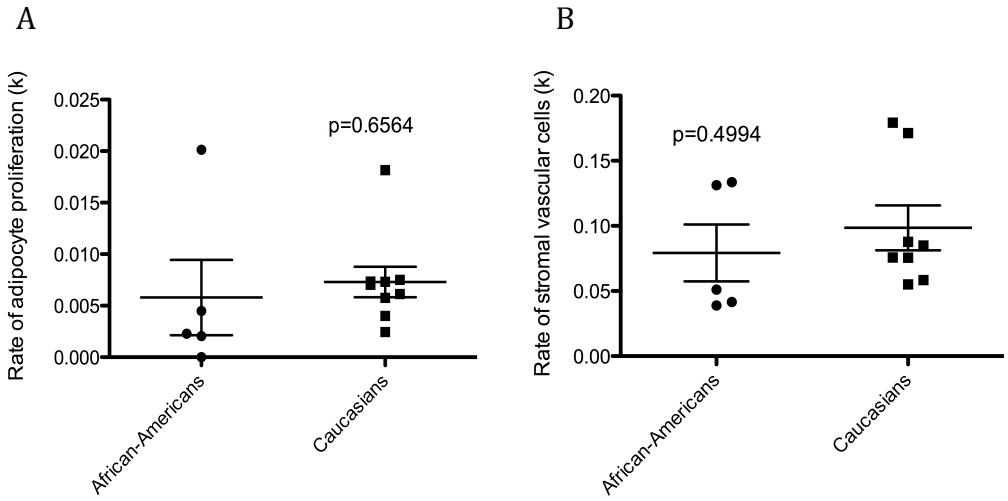



**Figure 4:** Cell proliferation measured by $^{2}H$-incorporation into deoxyribose moieties. A) Rate of adipocyte proliferation, p=0.6564; B) Rate of stromal vascular cell proliferation, p=0.4994.

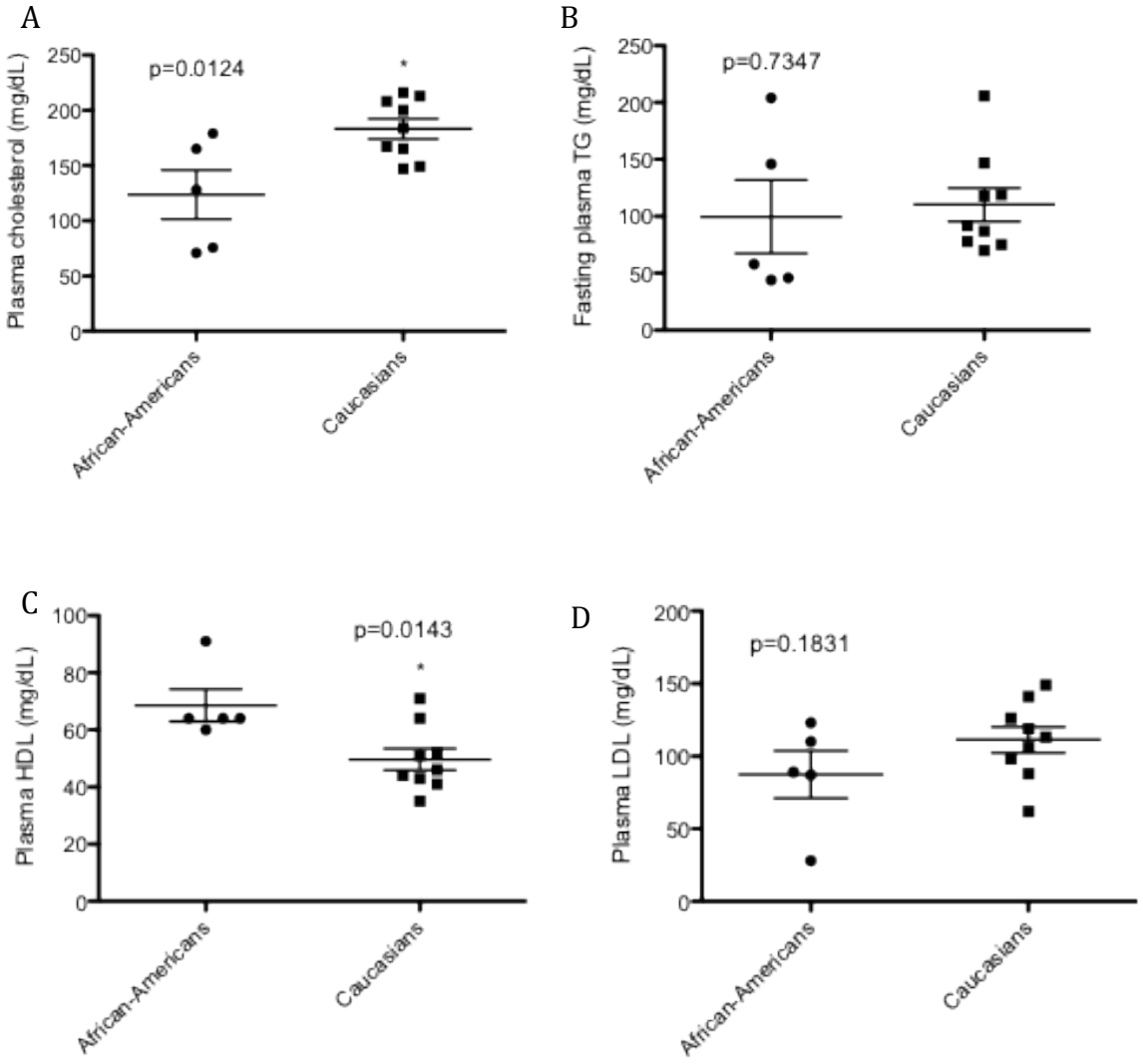



**Figure 5:** Fasting plasma lipids. A) Plasma cholesterol, p=0.0124; B) plasma TG, p=0.7347; C) plasma HDL, p=0.0143; D) plasma LDL, p=0.1831.

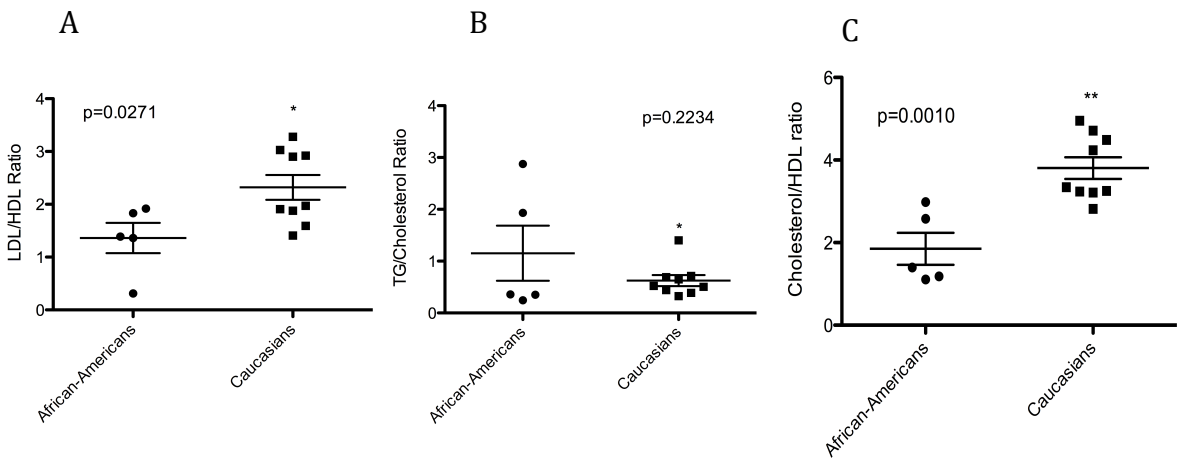



**Figure 6:** Fasting plasma lipids ratios. A) LDL/HDL ratio, p=0.0271; B) Ratio total cholesterol/HDL ratio, p=0.0010; C) TG/total cholesterol ratio, p=0.2234.

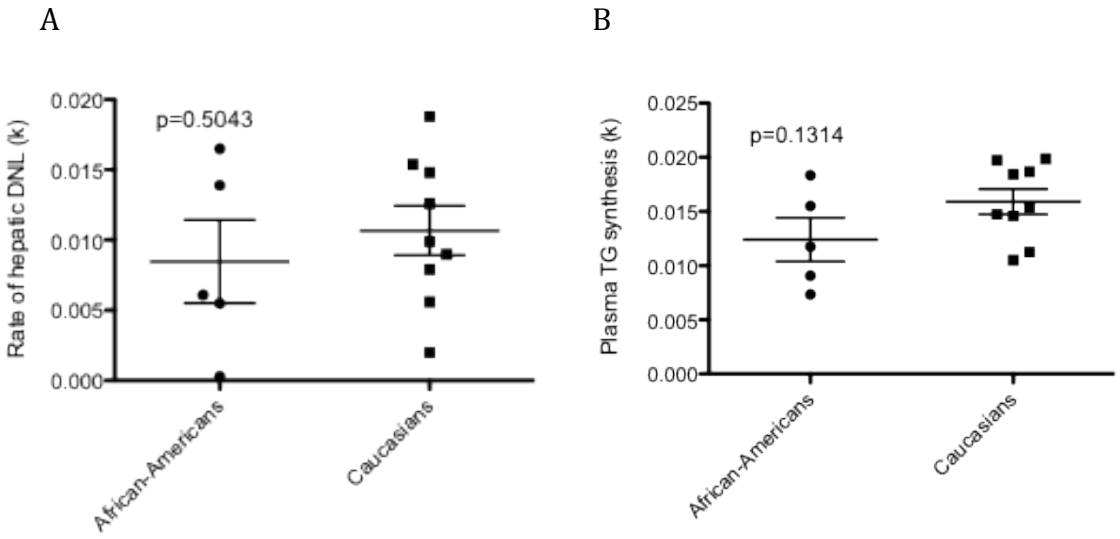



**Figure 7:** Hepatic lipid content. A) Rate of hepatic DNL represented by $^{2}H$-incorporation into newly synthesized plasma palmitate, p=0.5043; B) Rate of hepatic TG synthesis represented by $^{2}H$-incorporation in newly synthesized glycerol in plasma, p=0.1314.

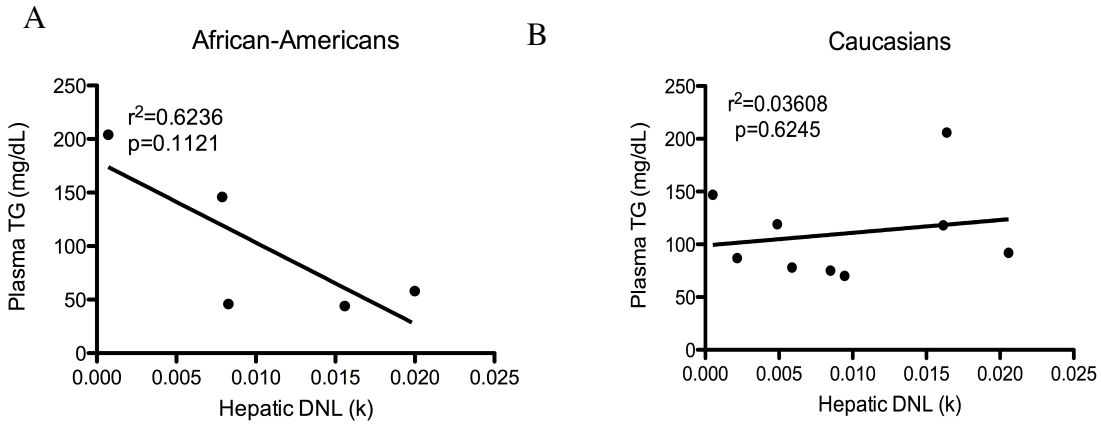



**Figure 8:** The relationship between hepatic DNL measured by $^2H_2O$ and plasma TG. A) Rate of hepatic DNL vs. fasting plasma TG in African-Americans, $r^2$=0.6236, p=0.1121; B) Rate of hepatic DNL vs. fasting plasma TG in Caucasians, $r^2$=0.036, p=0.6245.

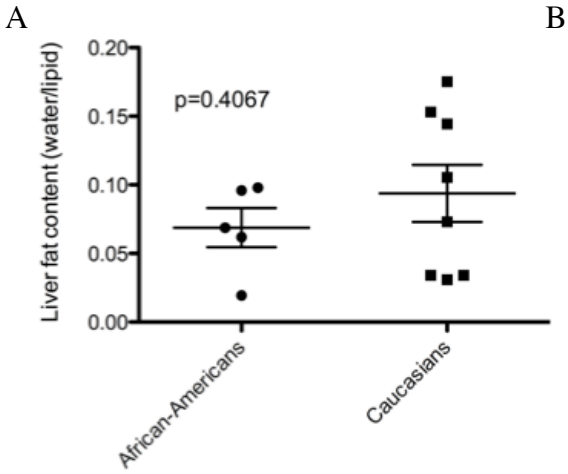



**Figure 9:** Liver fat content in African-Americans vs Caucasians, p=0.4067.

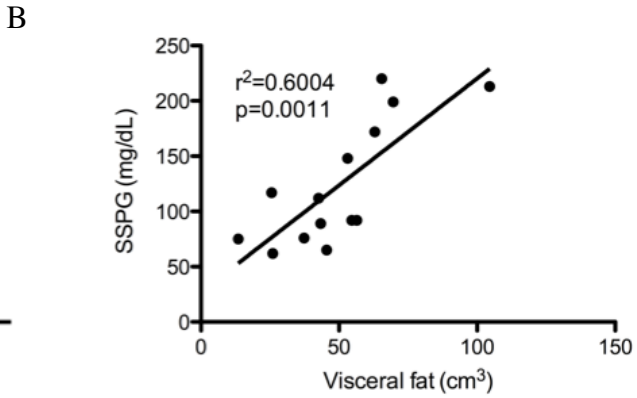



**Figure 10:** The relationship between visceral fat and insulin sensitivity in all subjects, $r^2$=0.6004, p=0.0011.

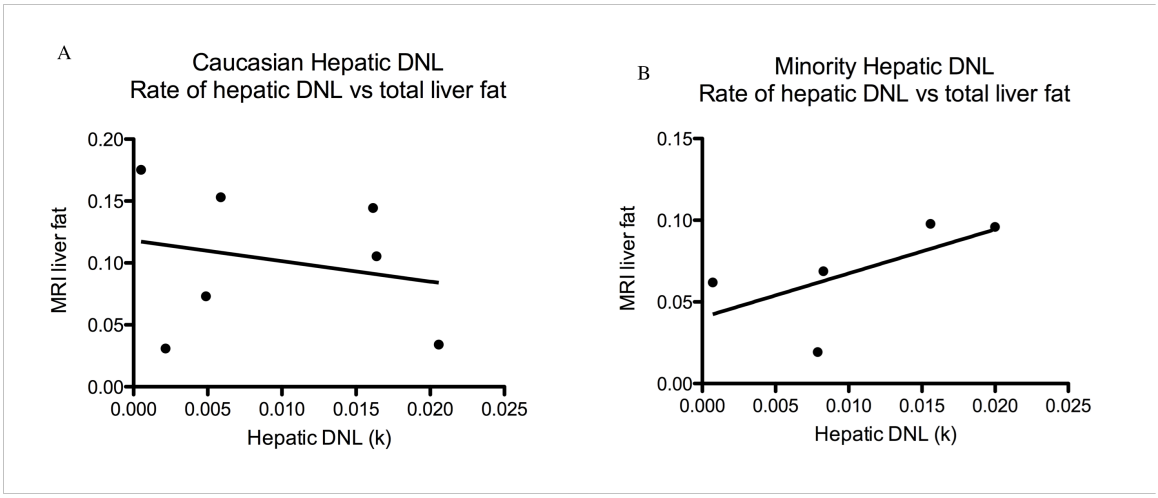



**Figure 11:** The relationship between hepatic DNL measured by $^2H_2O$ and plasma TG. A) Rate of hepatic DNL vs. fasting plasma TG in African-Americans, $r^2$=0.6236, p=0.1121; B) Rate of hepatic DNL vs. fasting plasma TG in Caucasians, $r^2$=0.036, p=0.6245.

# Chapter 5

## *In vivo* $^{2}H_2O$ administration reveals subcutaneous adipose lipid synthesis and storage response to overfeeding and weight-gain in obese-insulin-sensitive individuals.

**Abstract**

Obesity is a result of an overconsumption of calories, leading to weight gain. Approximately 25% of obese people remaining metabolically healthy, but whether or not they can remain healthy with continuous weight gain remain undetermined. Our previous findings of greater TG synthesis and DNL in adipocytes of obese IS patients suggest that maintaining lipid synthesis and storage in the subcutaneous depot protects against the development of insulin resistance. We therefore aimed to determine if under conditions of rapid, short-term weight gain can obese IS subjects remain insulin sensitive. Eleven IS ($n$=7) and IR ($n$=4) obese subjects were recruited to consume an additional 1000 calories a day for 28 days. We hypothesized that obese IS subjects would maintain a healthy metabolic profile based on findings of proper adipocyte function at baseline, whereas IR subjects would have an even further reduction in insulin sensitivity and overall decreased metabolic function. Abdominal subcutaneous fat biopsies were taken at baseline and after overfeeding to quantify potential changes in TG synthesis, DNL and adipocyte proliferation in response to weight gain. Most striking was the metabolic response to overfeeding in IS subjects, whose insulin sensitivity decreased by 33%. IR subjects showed a decrease in insulin sensitivity by only 6%. Surprisingly, IS subjects showed no change in adipose TG synthesis or DNL, while IR subjects actually showed an increase both adipose TG synthesis and DNL after overfeeding. Neither group showed significant changes in adipocyte proliferation, but SVC proliferation went up in IR and down in IS. Our results demonstrate that obese IS cannot remain insulin sensitive in response to overfeeding. Findings here suggest that failure of adipocytes to increase TG synthesis and storage may drive the onset of insulin resistance. Future studies should include a larger sample size to determine if decreased adipocyte function drives ectopic and visceral fat storage, initiating defects in insulin response.

## Introduction

Obesity is characterized by increased stores of at in adipose tissue, which in turn reflect caloric consumption exceeding energy expenditure. Our previous study demonstrated that some individuals are able to maintain insulin sensitivity by maintaining adipocyte's ability to synthesis and store lipids, despite being obese. The majority of obese patients become insulin resistant, but whether or not individuals who are insulin sensitive despite being obese are able to remain healthy for the remainder of their lives if they continue to be obese, has not been thoroughly investigated.

In the postprandial period, macronutrients not yet needed for fuel are sent primarily to the adipose for storage. Carbohydrates are broken down into glucose and are pushed into either the muscle to be stored as glycogen, or are sent to adipose tissue to be stored as triglycerides. Dietary lipids are also stored in the adipose, but their first stop is through the liver. The liver is one of the most important metabolic tissues, serving as the hub of lipid transport of new, recycled and dietary fats. The liver is the one site of DNL in the human body, but produces only about 1g per day, which is released into circulation bound to VLDL (1). VLDL-TG arriving at the adipose is hydrolyzed by lipoprotein lipase (LPL) into free fatty acids, which are able to cross into the membrane of adipocytes for storage in the lipid droplet. This process of lipid uptake and storage in adipocytes is controlled by insulin's up-regulation of LPL and simultaneous down-regulation of hormone sensitive lipase (HSL) (2). During the fasting period, HSL is activated, stimulating lipolysis to release free fatty acids (FFAs) into circulation for fuel use in other tissues, such as the muscle and liver. However, in obesity and insulin resistance, LPL activity is down so less fatty acids are taken up by the adipose and are instead transported back to the liver (3). This excess lipid influx into the liver can have detrimental effects on metabolism. For example, free fatty acid influx into hepatocytes can stimulate gluconeogenesis and impair glucose oxidation, in addition to increasing the production of VLDL-TG (4). Increased hepatic TG synthesis may eventually lead to hepatic steatosis (fatty liver), impairing insulin signaling and leading to insulin resistance (5,6).

Overfeeding is the process of excess consumption of calories and may increase fat synthesis in both the adipose and liver. The type of diet consumed will determine changes in lipid synthesis, rather than simply the amount of calories. Carbohydrate overfeeding diets enhance hepatic DNL, however the majority of DNL would occur in adipose tissue (7,8). However, one study in which subjects were placed on an isocaloric diet showed an increase in hepatic DNL but no change in adipose DNL (9). Another group found an increase in whole body DNL after an isocaloric diet, however the increase was not as great in overweight subjects, compared to lean (10). They also showed significant increases in insulin concentrations following overfeeding in both lean and overweight subjects. Others and we have hypothesized that the ability to remain metabolically healthy, yet obese may be determined by one's ability to maintain efficient storage of lipids in the subcutaneous adipose depot, which is deemed the metabolically protective depot (11,12,13). Overfeeding reduces insulin sensitivity in healthy, lean individuals (14), but the direct effects of overfeeding on adipose total TG synthesis have not been investigated. Our previous data demonstrate that obese individuals with maintained adipocyte function to synthesize triglycerides are protected from insulin resistance. However, will these subjects

remain insulin sensitive after weight-gain? We hypothesized that obese IS subjects will remain insulin sensitive after overfeeding due to their ability to synthesis TG at a normal rate compared to IR subjects at baseline, while IR subject's insulin resistance will worsen due to their inability at baseline to store TG in their subcutaneous adipose tissue. Surprisingly, we found that baseline IS subjects showed a greater reduction in insulin sensitivity than baseline IR subjects, in combination with impaired capacity to store TG in adipose tissue.

**Results**

**Table 1** compares subjects' characteristics between baseline and post-overfeeding intervention. Eleven male insulin sensitive (*n*=7) and insulin resistant (*n*=4) subjects were recruited to determine the effects of overfeeding on adipocyte function and insulin sensitivity. **Table 1** confirms no differences in age or race between groups, p=0.84 and 0.24, respectively. Body weights, BMI and fasting glucose were also not different between groups, nor were the changes in these outcomes after overfeeding significant. The change in body weight was not statistically different between groups: IS mean=3.8 kg; IR mean=2.3kg, p=0.12. Insulin sensitivity in IS subjects decreased by 33.3%, while IR subjects' insulin sensitivity was reduced by only 6%, p=0.05.

Baseline BMI correlated positively with baseline SSPG (p=0.0302), while baseline SSPG was negatively correlated with the percent chance in SSPG, p=0.0.302 (**Figure 1A and B**). Two subjects in our study showed improved insulin sensitivity, so if these subjects are removed from the analysis, the relationship between baseline BMI and baseline SSPG remain the same with a p-value of 0.0349 (**Figure 2A**). However, the relationship between baseline SSPG and percent change in SSPG after weight gain becomes highly significant with a p-value of 0.0014 (**Figure 2B**). The effect of overfeeding on TG synthesis or DNL did not differ based on insulin sensitivity, p=0.3980 and 0.2213, respectively.

There was a trend towards an effect of overfeeding on adipocyte proliferation with a p-value of 0.1091, however the direction of change was different in each group (**Figure 4A**). In IS subjects, adipocyte proliferation decreased, while increasing in IS subjects, although these differences were not significant, p=0.2831 and 0.3126, respectively. Proliferation of stromal vascular cells decreased in IS patients (p=0.0554), but remained unchanged in IR patients, p=0.4287 (**Figure 4B**).

Hepatic DNL, represented by newly synthesized palmitate in plasma, was significantly lower after overfeeding in both groups, p=0.0136 (**Figure 5**). Overall, IR patients displayed greater hepatic fat content, measured by MRI, but this difference did not reach significance, p=0.0868. Although not quite significant, in hepatic fat content increased after overfeeding in either group, with a p-value of 0.0996. The degree of change in hepatic fat content was not dependent on whether subjects were IS or IR, p=0.9448.

**Figure 6A-B** compares differences in subcutaneous and visceral fat content, measured by CT scan before and after the overfeeding intervention. There were no differences in subcutaneous fat volume between IS and IR, p=0.4201. Overfeeding did not have an effect on subcutaneous fat content, p=0.9947. Overall, visceral fat was not different between IS and IR (p=0.2567), nor was there an effect of overfeeding on visceral fat content in either group, p=0.2467. There was significant subject variability in CT scan results for both subcutaneous and visceral depots (p=0.0005), therefore, each individual's abdominal fat content for each depot is displayed separately in **Table 2**. There was even greater variability amongst subjects in thigh fat, p<0.0001, with no differences existing between groups based on insulin sensitivity or overfeeding, p=0.9572 and 0.4958, respectively.

**Figure 7** shows the change in adipose kinetic dynamics induced by overfeeding in IS and IR cohorts. TG synthesis in IS remained unchanged, while IR subjects had an increase in TG synthesis. This change between groups was not significant, p=0.2971 (**Figure 7A**). A difference in the change in DNL between groups mirrors that of TG synthesis with a p-value of 0.2213 (**Figure 7B**). Adipocyte proliferation slightly decreased in IS, but increased in IR subjects (**Figure 7C**). These differences did not reach significance, p=0.3366. Changes in SVC proliferation were very similar between IS an IR, p=0.8913 (**Figure 7D**). While adipocyte proliferation slightly decreased, subcutaneous fat increased by an average of 4.54$cm^3$ in IS, compared to a decrease of 4.47$cm^3$ in IR patients. These changes, however, were not significantly different between groups, p=0.3834. Changes in visceral fat were not different between groups, p=0.3834 (**Figure 7E**). Changes in visceral fat not different between groups, p=0.9685 (**Figure 7F**), although changes were highly variable within each group (refer to **Table 2**). MRI analysis reveals a greater increase in hepatic fat content in IS subjects compared to IR, although this difference was not significant, p=0.6296. Changes in hepatic DNL were also not significantly different between groups, p=0.5289 (**Figure 8B**).

The relationship between BMI and SSPG was even stronger after overfeeding, compared to baseline, p=0.0110 versus 0.0302, respectively (**Figure 9A-B**). Baseline BMI was predictive of insulin sensitivity after overfeeding (p=0.0071) and correlated better than comparing BMI to SSPG at baseline or post-intervention separately (**Figure 9C**). On the other hand, the change in BMI versus the change in SSPG showed a positive relationship, but was not quite significant, p=0.0927.

**Figure 10A** shows there a negative trend demonstrating a potential relationship between the change in BMI and the change in TG synthesis in response to overfeeding, p=0.1191; the greater the increase in BMI, the greater the reduction in the ability to synthesize TG in the abdominal subcutaneous depot. This trend was not observed between delta BMI and delta DNL after overfeeding, p=0.3407. Linear regression analysis suggests a potential for baseline TG synthesis correlating negatively with insulin sensitivity, but unlike in the previous study, this relationship between TG synthesis and insulin sensitivity after overfeeding (**Figure 11B**). The rate of TG synthesis at baseline may be predictive of the rate of TG synthesis after overfeeding, although more subjects are needed to reach significance, p=0.1042 (**Figure 12**). Since weight gain is often a risk factor for worsening insulin sensitivity, a linear regression analysis was calculated to determine if there existed a relationship between the percent weight gain and percent change in insulin sensitivity after overfeeding. When comparing these variables amongst all subjects, there was not relationship between weight gain and change in insulin sensitivity (**Figure 13A**), p=0.3490. However, if these outcomes are evaluated in just IS subjects whose insulin sensitivity decreased, there was a positive relationship between percent weight gain and percent SSPG, nearly reaching significance, p=0.0507 (**Figure 13B**).

**Figure 14** provides a summary of changes in plasma lipids after overfeeding. **Figure 14A through C** shows no significant differences between IS and IR with changes in plasma cholesterol, TG or LDLD levels, p=0.4566, 0.3361 and 0.4354, respectively. Changes in plasma TG were highly variable amongst the four IR subjects, ranging from a 1 to 188mg/dL increase. There was, however, a significant difference between the change in HDL that occurred between

IS and IR, with IR showing a decrease in HDL, by 3.5mg/dL, while IS didn't really change (delta=0.85mg/dL) (**Figure 14D**). IR patients showed a greater change in delta LDL/HDL and HDL/cholesterol ratios compared to IS for both outcomes, but neither difference reached significance, p=0.1061 and p=0.1161, respectively (**Figure 14E and F**). The difference in the ratio of LDL/HDL was three times greater in IR with a 7% decrease in IS compared to a 14% increase in IR.

**Discussion**

Overconsumption of calories without an increase in exercise is what has resulted in an obesity epidemic in the US to affect more than half of the population today. The CDC reports that in the US, 35.7% of adults are obese, while 17% of children are obese (15,16). It is well-documented that obesity leads to many of the potentially life-threatening diseases like type 2 diabetes, cardiovascular disease and cancer (17,18), thus in working to prevent obesity, we are working to prevent the leading causes of death in America every year (19).

Twenty-five percent of obese patients remain healthy and insulin-sensitive, but the majority of obese persons become insulin resistant (20). This fact raises two key questions: 1) What allows select individuals to remain healthy, despite being obese? 2) If these obese, yet healthy individuals continue to gain weight, will they continue to remain insulin sensitive? By investigating the effects of overfeeding and weight gain in already-obese individuals, we can determine if physiological differences allows some obese patients to adapt differently than others in response to excess energy intake. In measuring TG synthesis *in vivo* via $^{2}H_{2}O$ administration, we can determine if the rate at which obese IS individuals store fat changes during short-term, moderate weight gain.

Obesity is the leading risk factor for the development of insulin resistance, in part because increased weight gain leads to increases in visceral fat storage and lipotoxicity, impairing the functions of insulin-responsive tissues (21). It has been proposed that this series of events is a consequence of adipocyte dysfunction in the subcutaneous depot, a depot thought to be protective against whole-body metabolic dysfunction (11,22,23). In fact, metabolically healthy obese individuals have been shown to have lower visceral fat storage compared to metabolically unhealthy persons (24).

We hypothesized that patients who were insulin-sensitive at baseline would remain insulin sensitive even after weight gain if their ability to maintain TG storage in their abdominal subcutaneous depots persisted throughout the stress of excess energy consumption. If subjects are able to increase their TG synthesis rates in response to overfeeding, they are likely to avoid excess lipids spilling into visceral depots or ectopic regions, and are thus more likely to remain obese, but metabolically healthy. Interestingly, 5 out of 7 subjects in our study became insulin resistant after an average weight gain of 4kg. The borderline correlation we observed between baseline SSPG and the percent change in SSPG demonstrates that those who were IS at baseline had the greatest decrease in insulin sensitivity. While weight-gain was similar between IS and IR in our study, there was an inconsistent pattern of fat distribution amongst subjects, likely impacted by genetic differences in the response to weight gain. CT scan analyses revealed no differences in the change in subcutaneous depots between IS and IR, however changes in visceral adiposity were highly variable amongst all subjects. A larger sample size may be necessary to observe changes in visceral fat after overfeeding. Evidence for this exists in a study in which 41 healthy subjects were overfed showed significant increases in visceral fat (25). However, data also shows great variability in changes in both visceral and subcutaneous fat mass amongst all individuals, regardless of total amount of weight gained.

Measuring the effects of very moderate weight gain on fat distribution using a CT scan may not be to provide a clear portrayal of changes in fat storage, and definitely not fat synthesis. Measuring fat synthesis *in vivo* allows for an accurate assessment of adipocyte function in response to energy surplus. In our study, the use of $^2H_2O$ administration *in vivo* revealed changes in TG synthesis, DNL and adipocyte proliferation in our subjects during weight gain. To evaluate measures of changes in TG storage, we determined the relationship between fat storage quantified by CT scan compared to fat storage quantified by $^2H_2O$. CT scan analyses did not agree with changes in TG synthesis in all subjects. Subject 1, for example, had an 85% decrease in TG synthesis after weight gain, yet subcutaneous fat measured by CT scan decreased by just 1%. TG synthesis in this subject represents an 85% loss of newly synthesized TG, indicating that the majority of TG that is newly made in this depot during weight gain was likely hydrolyzed out of the abdominal subcutaneous depot and re-located to other tissues for storage. Since the volume of subcutaneous fat remained the same, any TG stored was likely re-esterified from the adipose after lipolysis or re-esterified from plasma circulation. CT scan analyses provide a measurement of total fat stored, but it cannot provide information regarding the original source of these fats. It is possible that patient 1 had a shift in newly synthesized TG towards other depots, and may explain the increase observed in visceral fat content. Fat storage measured by CT scan may be more reflective of dietary lipid intake, re-etherified lipids, or a combination of the two.

Body weight as it relates to metabolic health is evaluated based on an individual's calculated body mass index (BMI). While the amount of weight gained in our subjects did not result in a significant change in BMI, there was a trend demonstrating that the greater the increase in BMI, the greater the decrease in TG synthesis in the abdominal subcutaneous depot. This suggests that an increase in BMI, albeit small, triggered adipocyte dysfunction, leading to fat storage in other areas. Adipocyte dysfunction is determined by the inability of insulin to stimulate glucose uptake and metabolism, as well as stimulation of lipid synthesis and storage. The SSPG test measures muscle glucose uptake, and while we cannot determine adipose' glucose uptake, we can directly measure TG synthesis and DNL in the adipose. At baseline, we observed a negative correlation trending between TG synthesis and SSPG outcome, meaning the greater the TG synthesis, the greater the insulin sensitivity (lower SSPG outcome). There was, however no evidence of a relationship between these two variables post-weight gain. This may potentially indicate that decreases in insulin sensitivity are triggered by other factors and are not solely dependent on the loss of TG storage in subcutaneous depots. Alternatively, a decrease in TG synthesis may trigger a domino affect of downstream metabolic impairments, which may vary by individual. Further investigations regarding changes in insulin-regulated genes and proteins would provide better insights into these responses.

*De novo* lipogenesis (DNL) is directly stimulated by insulin (26,27), thus quantifying DNL in the adipose provides a direct measurements of insulin action in the adipose. Our insulin sensitive subjects showed either a decrease or no change in DNL after overfeeding, similar to that observed with TG synthesis, indicating a lack of insulin's ability to stimulate lipogenesis in these subjects. Interestingly, IR subjects showed a slight increase in DNL, which contradicts our previous findings of greater TG and DNL is paired with greater insulin sensitivity. The IR subjects did not become significantly more insulin resistant after overfeeding, thus quantification of lipid synthesis in these subjects may reveal the differences between individuals that remain

insulin resistant for the remainder of their lives versus those that become diabetic. Further studies are required to determine this.
Overfeeding with a high carbohydrate diet increases hepatic and whole body DNL (29), however overfeeding with a mixed diet will not stimulate DNL to the same extent. This may explain the lack of change observed in our subjects. However, it's also been suggested that adipose may be the predominant site of DNL in the setting of overfeeding (28), but this is not the case in the steady state (1). Minehira and colleagues demonstrated that while DNL increases with just four days of overfeeding in both lean and obese subjects, but the increase in DNL was significantly lower in obese, overfed patients (30). Similar patterns of change were observed in lipogenic genes such as SREBP-1c, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), increasing in both groups, with a lesser increase in overweight subjects.

Adipocytes can expand up to 20 times their original size, and it has been hypothesized that once the threshold for lipid storage is met, lipids may spill over into visceral fat or ectopic depots, triggering insulin resistance (31,32). Data from our previous study (Chapter 1) suggests that insulin resistant patients have impaired complete differentiation of mature fat cells, consistent with this hypothesis. While we did not measure fat cell size after overfeeding in our patients, insulin sensitive subjects were unable to increase TG synthesis in their abdominal subcutaneous fat cells in response to increased caloric intake. This may indicate that the pre-existing adipocytes could not accommodate increases in lipid storage needs. Comparing baseline TG synthesis in the adipose to TG synthesis occurring after overfeeding, may suggest that TG synthesis in IS patients at baseline may have already been approaching a threshold and prior to overfeeding intervention, adipocytes were approaching their maximum storage capacity. A previous study in healthy lean patients showed abdominal subcutaneous adipocytes only increase in size, but not in number in response to overfeeding (33). Previous research shows that large subcutaneous adipocytes are associated with insulin resistance (34,35,36,31). In our study, a combination of decreased TG synthesis with no change in subcutaneous volume suggests maximum adipocyte capacity had been reached, which is further supported by an increase in visceral adiposity. Whether or not adipocytes located in the visceral depot have greater proliferative abilities than those in the subcutaneous depot it not known, but it provides a likely explanation as to why weight gain in the majority of our subjects lead to an increase in the ratio of visceral fat to subcutaneous fat.

An *in vitro* study, in which fat cells were isolated and digested from obese bariatric patients, showed that preadipocytes originating from the abdominal subcutaneous depots had greater differentiation capacity compared to those originating from visceral fat (37). This supports the notion that fat storage in the subcutaneous region is preferable over visceral fat storage, and may explain why visceral fat in lean subjects constitutes only 10% of total body fat in men (38). These findings, however contradict our results in that if differentiation were in fact greater in subcutaneous fat cells, we would have observed an increase in lipid synthesis and storage with weight gain. Since we did not, one possibility is that the pool of preadipocytes in our subjects lost the ability to fully differentiate into functional mature adipocytes, which would be indicated by an increase in TG storage as well as an increase in deuterium enrichment in mature adipocytes. In women, those with a higher BMI have fewer adipocytes and stromal vascular cell number compared to those with a lower BMI, providing a link between obesity and metabolic function (39). Whether or not this relation occurs in mean has not been shown, but it would

support our findings here. This hypothesis could be supported by observations of a decrease in stromal vascular cell proliferation, containing preadipocytes. Since we did not isolate preadipocytes from this fraction that contains many other cell types, we do not know if this change in proliferation is due to changes in preadipcoytes, specifically.

**Study Limitations**

The main limitation of this study was our small sample size that did not allow for significance in the majority of our outcome variables. This study has demonstrated that more subjects are required for future overfeeding studies evaluating the effects of adipose lipid metabolism in obese subjects, as metabolic responses may vary greatly amongst individuals. Furthermore, future studies should focus on the effects of obese insulin-sensitive patients only, making comparisons between changes in insulin sensitivity based on changes in fat deposition.

**References**


1. Hellerstein MK, Schwarz J, Neese RA. Regulation of hepatic de novo lipogenesis in humans. Annual Reviews 1996, 16:523-557.

2. Goldberg IJ, Eckel RH, Abumrad NA. Regulation of fatty acid uptake into tissues: lipoprotein lipase- and CD36-mediated pathways. Journal of Lipid Research 2008, 50:S86-S90.

3. McQuaid SE, Hodson L, Neville M, Dennis AL, Cheeseman J, Humphreys SM, Ruge T, Gilbert M, Fielding BA, Frayn KN, Karpe F. Down-regulation of adipose tissue fatty acid trafficking in obesity: A driver for ectopic fat deposition? Diabetes 2011, 60:47-55.

4. Lewis GF, Vranic M, Harley P, Giacca. Fatty acids mediate the acute extrahepatic effects of insulin on hepatic glucose production in humans. Diabetes 1997, 46:1111-1119.

5. Comert B, Mas MR, Erdem H, Dinc A, Saglamkaya U, Cigerim M, Kuzhan O, Unal T, Kocabalkan F 2001 Insulin resistance in non-alcoholic steatohepatitis. *Dig Liver Dis*33:353–358.

6. Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, McCullough AJ, Natale S, Forlani G, Melchionda N 2001 Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes* 50:1844–1850.

7. Aarsland A, Chinkes D, Wolfe RR. Hepatic and whole-body fat synthesis in humans during carbohydrate overfeeding. Am J Clin Nutr 1997, 65:1774-1782.

8. Minehira K, Betts V, Vidal H, Vega N, Di Vetta V, Rey V, Scheiter PH, Tappy L. Effect of carbohydrate overfeeding on whole body and adipose tissue metabolism in humans. Obes Res 2003, 11:1096-1103.

9. Diraison F, Dusserre E, Vidal H, Sothier M, Beylot M. Increasd hepatic lipogenesis but decreased expression of lipogenic gene in adipose tissue in human obesity. Am J Physiol Endocrinol Metab 2002, 282:E46-E51.

10. Minehira K, Vega N, Vidal H, Acheson K, Tappy L. Effect of carbohydrate overfeeding on whiole body macronutrient metabolism and expression of lipogenic enzymes in adipose tissue of lean and overweight humans. Int J Obes Relat Metab Disord 2004, 28:1291-1298.

11. Tan CY, Vidal-Puig A. Adipose tissue expandability: the metabolic problems of obesity may arise from the inability to become more obese. Biochem Soc Trans. 2008;36:935–940.

12. McLaughlin, T., Sherman A, Tsao P, Gonzalez O, Yee G, Lamdendola C, Reaven GM, Cushman SW. Enhanced proportion of small adipose cells in insulin-resistance vs insulin sensitive obese individuals implicates impaired adipogenesis. Diabetologia. 2007; 50(8): 1707-15.

13. Gray S and Vidal-Puig AJ. Adipose Tissue Expandability in the Maintenance of Metabolic Homeostasis. Nutrition Reviews 2007, 65: S7-S12.

14. Samocha-Bonet D, Campbell LV, Mori TA, Croft KD, Greenfield JR, Turner N, Heilbronn. Overfeeding reduces insulin sensitivity and increases oxidative stress without altering markers of mitochondrial content and function in humans. PLoS ONE 2012, 7:1-8.

15. Ogden CL, Carroll MD, Ki BK, Flegal KM. Prevalence of Obesity in the United States, 2009-2010. NCHS Data Brief 2012, 82:1-8.

16. Centers for Disease Control and Prevention (CDC). Childhood Overweight and Obesity. http://www.cdc.gov/obesity/childhood/index.html. Updated August 5, 2013. Viewed February 2014.

17. Manson JE, Colditz, Stamper MJ, Willet WC, Rosner B, Monson RR, Speizer FE, Hennekens CH. A prospective study of obesity and risk of coronary heart disease in women. N Eng J Med 1990, 322:882-889.

18. Garrison RJ, Higgins MW, Kannel WB. Obesity and coronary heart disease. Curr Opin Lipidol 1996, 7:199-202.

19. Danaei G, Ding EL, Mozaffarian D, Taylor B, Rehm J, Murray CJ, Ezzati M. The preventable causes of death in the United Staes: comparative risk assessment of dietary, lifestyle and metabolic risk factors. PLoS Med 2009, 6:1-23.

20. McLaughlin T, Allison G, Abbasi F, Lamendola C, Reaven G. Prevalence of insulin resistance and associated cardiovascular disease risk factors among normal weight, overweight, and obese individuals. Metabolism 2004, 53:495-499.

21. Johanson, EH, Jansson, PA, Lonn, L Matsuzawa Y, Funahashi T, Taskinen MR, Smith U, Axelsen M. Fat distribution, lipid accumulation in the liver, and exercise capacity do not explain the insulin resistance in healthy males with a family history for type 2 diabetes. *J Clin Endocrinol Metab*, 2003, 88, 4232-4238.

22. Banergi MA, Buckley MC, Chaiken RL, Gordon D, Lebovitz HE, Kral JG. Liver fat, serum triglycerides and visceral adipose tissue in insulin-sensitive and insulin-resistant black men in NIDDM. Int J Obes Relat Metab Disord 1995, 19:846-850.

23. Porter SA, Massaro JM, Hoffmann U, Vasan RS, O'Donnel CJ, Fox CS. Abdominal subcutaneous adipose tissue: a protective fat depot? Diabetes Care 2009, 32:1068-1075.

24. Klöting N, Fasshauer M, Dietrich A, Kovacs P, Schön MR, Kern M, Stumvoll M, Blüler M. Insulin-sensitive obesity. Am J Physiol Endocrinol Metab 2010, 299:E506-515.

25. Alligier M, Gabert L, Meugnier E, Lambert-Porcheron S, Chanseaume E, Pilleul F, Debard C, Sauvinet V, Morio B, Vidal-Puig A, Vidal H, Laville M. Visceral fat accumulation during lipid overfeeding related to subcutaneous adipose tissue characteristics in healthy men. J Clin Endocrinol Metab 2013, 98:802-810.

26. Wang, D and Sul HS. Insulin stimulation of the fatty acid synthase promoter is mediated by the phospatidylinositol 3-kinase pathway. J Biol Chem 1998, 273:25420-25426.

27. Sul HS, Latasa MJ, Moon Y, Kim KH. Regulation of the fatty acid synthase promoter by insulin. Ju Nutr 2000, 130:315S-320S.

28. Acheson K, Schutz Y, Bessard T, Anantharaman K, Flatt J, Jéquier E. Glycogen storage capacity and de novo lipogenesis during massive carbohydrate overfeeding in man. Am J Clin Nutr 1988, 48:240-247.

29. Marques-Lopes, I, Ansorena D, Astiasaran I, Forga L, Martinez JA. Postprandial de novo lipogenesis and metabolic changes induced by a high-carbohydrate, low-fat meal in lean and overweight men. Am J Clin Nutr 2001, 73:253-261.

30. Minehira K, Vega N, Vidal H, Achseon K, Tappy L. Effect of carbohydrate overfeeding on whole body macronutrient metabolism and expression of lipogenic enzymes in adipose tissue of lean and overweight humans. International Journal of Obesity 2004, 28: 1291-1298.

31. Weyer C, Foley JE, Bogardus C, Tataranni PA, Pratley RE. Enlarged subcutaneous abdominal fat cell size, but not obesity itself, predicts type II diabetes independent of insulin resistance. Diabetologia 2000, 43:1498-106.

32. Thomas EL, Hamilton G, Patel N, O'Dwyer R, Doré RD, Bell JD, Taylor-Robinson SD. Hepatic triglyceride content and its relation to body adiposity: a magnetic resonance imaging and protin magnetic resonance spectroscopy study. Gut 2005, 54:122-127.

33. Tchoukalova YD, Votruba SB, Tchkonia T, Giorgadze N, Kirkland JL, and Jensen MD. Regional differences in cellular mechanisms of adipose tissue gain with overfeeding. PNAS 2010, 107:18226-18231.

34. Krotkiewski M, Bjorntorp P, Sjostrom L, Smith U. Impact of obesity on metabolism in men and women. Importance of regional adipose tissue distribution. J Clin Invest 1983; 72:1150-1162.

35. Kissebeh AH, Vydelingum N, Murray R, Evans DJ, Hartz AJ, Kalkhoff RK, Adams PW. Relation of body fat distribution to metabolic complications of obesity. J Clin Endocrinol Metab 1982, 54:524-560.

36. Imbeault P, Lemieux S, Prud d'homme D, Trembaly A, Nadeau A, Després JP, Mauriège P. Relationship of visceral adipose tissue to metabolic risk factors for coronary heart disease: is there a contribution of subcutaneous fat cell hypertrophy? Metabolism 1999: 48:355-362.

37. Tchkonia T, Giorgadze N, Pirtskhalava T, Tchoukalova Y, Karagiannides I, Forse RA< DePonte M, Stevenson M, Guo W, Han J, Waloga G, Lash TL, Jensen MD, Kirkland JL. Fat depot origina affects adipogenesis in primary cultured and cloned human preadipocytes. Am J Physiol Regul Integr Comp Physiol 2002, 282:1286-1296.

38. Abate, N, Burns, D, Peshock, RM, Stray-Gundersen, J, Grundy, SM. Relationship of generalized and regional adiposity to insulin sensitivity in men. J. Clin. Invest. 1995. 96:88-98.

39. Van Harmelen V, Skurk T, Rohrig K, Lee YM, Apath-Husmann I, Hauner H. effect of BMI and age on adipose tissue cellularity and differentiation capacity in women. Int J Obese Relat Metab Disord 2003, 27:889-895.

**Table 1:** Subject characteristics before and after overfeeding.

| | IS | IR | p-value |
|---|---|---|---|
| Age | 54.9 ± 7.2 | 55.8 ± 5.3 | 0.84 |
| Race | 6/1/0 | 2/2 | 0.24 |
| Body weight (kg) | | | |
| Baseline | 89.2 ± 12 | 92.5 ± 13 | 0.52 |
| Post-Overfeeding | 94.5 ± 13 | 96.7 ± 14 | 0.63 |
| BMI ($kg/m^2$) | | | |
| Baseline | 28.1 ± 0.9 | 31.5 ± 1.8 | 0.10 |
| Post-Overfeeding | 29.1 ± 1.0 | 32.1 ± 1.8 | 0.16 |
| Fasting glucose | | | |
| Baseline | 96.3 ± 1.8 | 95.0 ± 1.8 | 0.66 |
| Post-Overfeeding | 97.0 ± 2.1 | 98.3 ± 2.5 | 0.72 |
| SSPG (mg/dL) | | | |
| Baseline | 84.6 ± 8.2 | 197.5 ± 17.9 | <0.0001 |
| Post-Overfeeding | 134.0 ± 14.6 | 212.8 ± 21.8 | 0.01 |

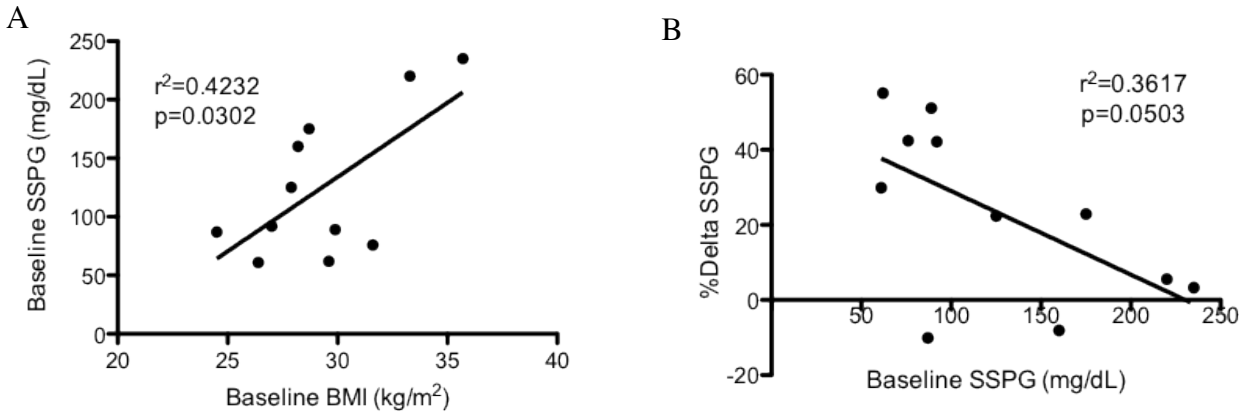



**Figure 1:** The relationship between baseline BMI and SSPG. A) Baseline BMI vs SSPG at baseline, $r^2$=0.42, p=0.03; B) Baseline SSPG vs percent change in SSPG after overfeeding, $r^2$=0.3617, p=0.05.

**BMI versus SSPG excluding subjects whose insulin sensitivity improved after overfeeding.**

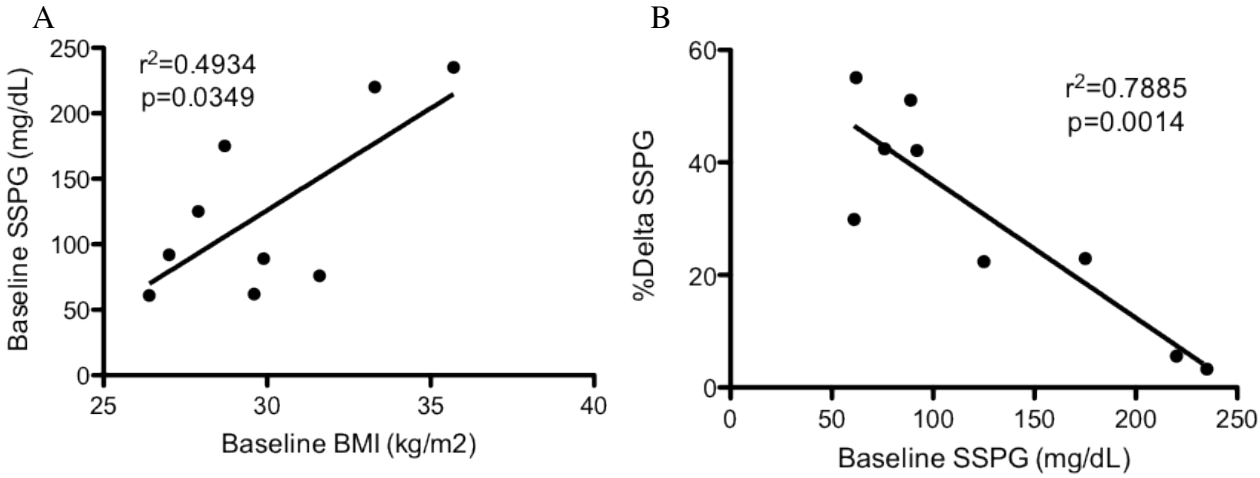



**Figure 2:** A re-analysis of the relationship between baseline BMI and SSPG, excluding the two subjects whose insulin sensitivity improved after overfeeding. A) Baseline BMI vs SSPG at baseline, $r^2$=0.0.49, p=0.0349; B) Baseline SSPG vs percent change in SSPG after overfeeding, $r^2$=0.79, p=0.0014.

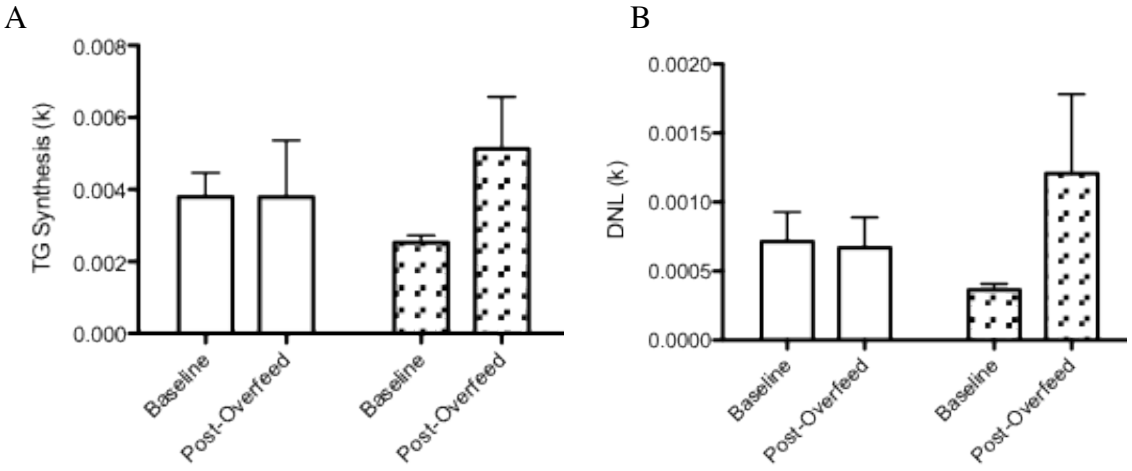
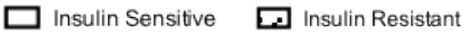



**Figure 3:** Adipose lipid synthesis before and after overfeeding. A) TG synthesis, p=0.98 based on insulin sensitivity; p=0.02991 based on affects of overfeeding; B) DNL, p=0.76 based on insulin sensitivity; p=0.1984 based on overfeeding.



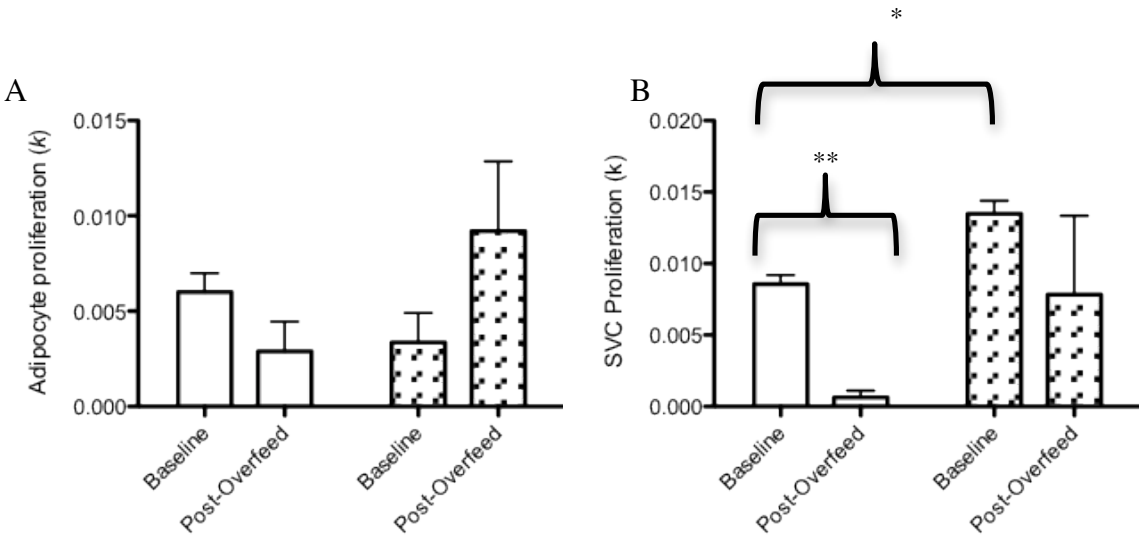




**Figure 4:** Cell proliferation before and after overfeeding in IS vs IR patients. A) Adipocyte proliferation, p=0.3615 based on insulin sensitivity; p=0.4929 based on overfeeding; B) SVC proliferation, **p=0.0099; *p=0.015.

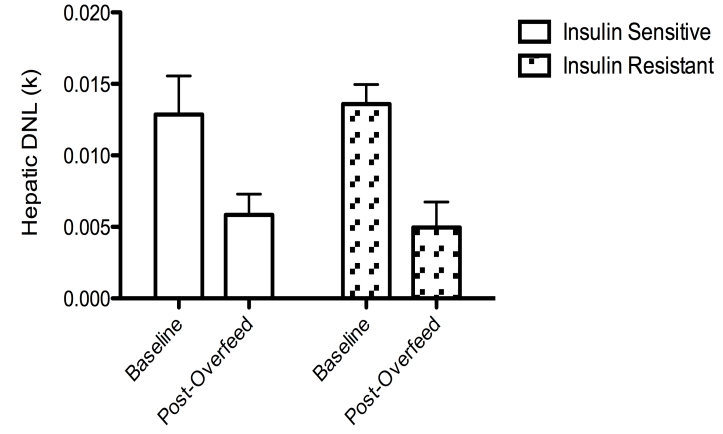



**Figure 5:** Hepatic DNL (k) per day before and after overfeeding in IS vs IR, p=0.0025 based on the affect of overfeeding; p=0.9785 based on differences between groups.

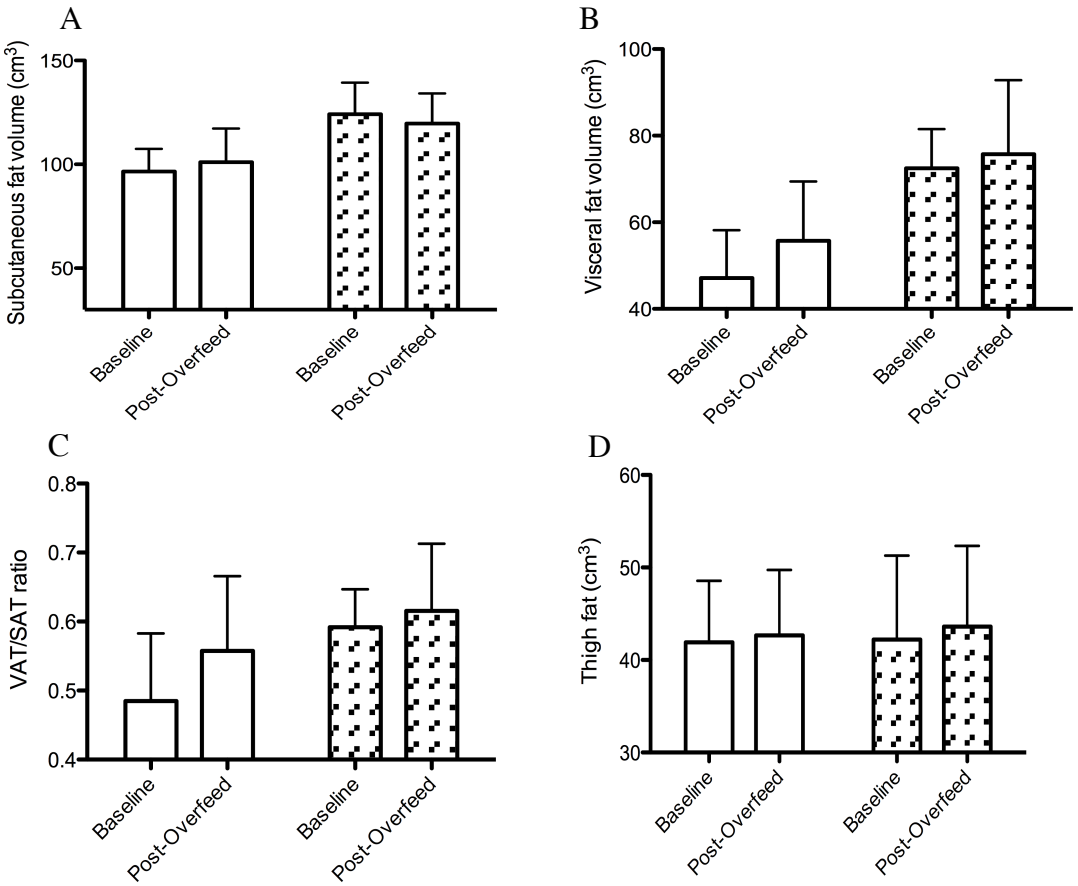



**Figure 6:** Abdominal subcutaneous vs visceral adipose tissue volume measured by CT scan before and after overfeeding in IS vs IR patients. A) Subcutaneous fat volume, p=0.1365 based on insulin sensitivity; p=0.9981 based on overfeeding; B) Visceral fat volume, p=0.1098 based on insulin sensitivity; p=0.6645 based on overfeeding; C) VAT/SAT ratio, p=0.4273 based on insulin sensitivity, p=0.6402, based on overfeeding; D) Thigh fat, p=0.9391 based on insulin sensitivity; p=0.8921 based on overfeeding.

Insulin Sensitive Insulin Resistant

| Subject | **Baseline Subcutaneous ($cm^3$)** | **Post-Overfeed Subcutaneous ($cm^3$)** | **Delta Subcutaneous ($cm^3$)** | **Baseline Visceral ($cm^3$)** | **Post-Overfeeding Visceral ($cm^3$)** | **Delta Visceral ($cm^3$)** |
|---|---|---|---|---|---|---|
| 1 | 94.58 | 94.29 | -0.29 | 56.46 | 69.63 | 13.17 |
| 2 | 118.64 | 150.66 | 32.02 | 37.41 | 38.45 | 1.04 |
| 3 | 91.94 | -- | | 32.31 | -- | |
| 4 | 62.56 | 52.14 | -10.42 | 18.56 | 18.44 | -0.12 |
| 5 | 129.94 | 133.73 | 3.79 | 43.36 | 61.05 | 17.69 |
| 6 | 104.72 | 115.37 | 10.65 | 96.09 | 112.73 | 16.64 |
| 7 | 68.64 | 60.13 | -8.51 | 30.74 | 33.97 | 3.23 |
| 8 | 125.27 | 97.91 | -27.36 | 58.05 | 31.8 | -26.25 |
| 9 | 164.9 | 156.99 | -7.91 | 98.84 | 112.78 | 13.94 |
| 10 | 113.87 | 128.19 | 14.32 | 65.42 | 88.88 | 23.46 |
| 11 | 92.59 | 95.67 | 3.08 | 67.63 | 69.44 | 1.81 |

**Table 2:** Fat distribution before and after overfeeding for each individual.

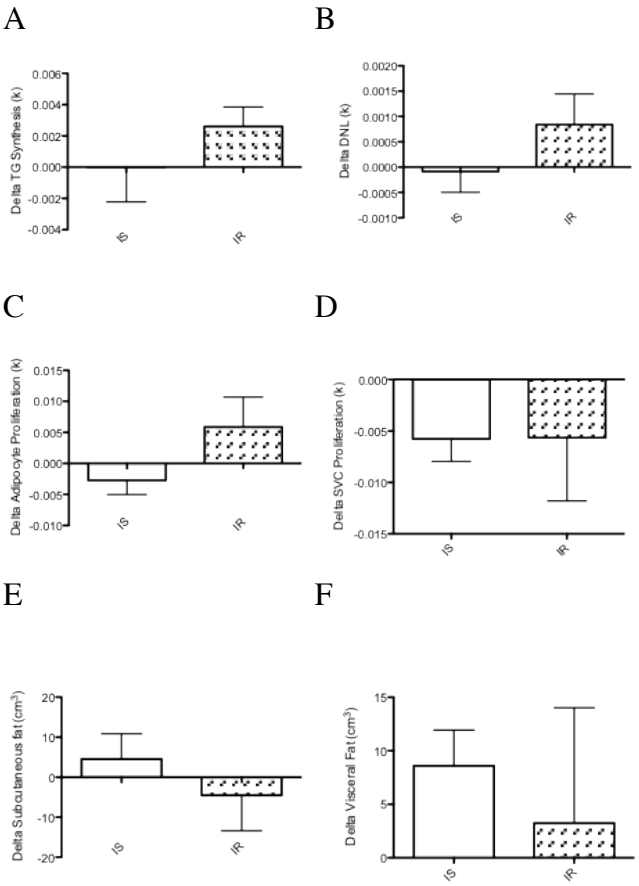



**Figure 7:** Delta values for adipose lipid kinetics and tissue volume after 4 weeks of overfeeding. A) Delta rate of TG synthesis, p=0.73; B) Delta rate of DNL, p=0.37; C) Delta rate of adipocyte proliferation, p=0.32; D) Delta rate of SVC proliferation, p=0.72; E) Delta subcutaneous fat volume, p=0.38; F) Delta visceral fat volume, p=0.66.

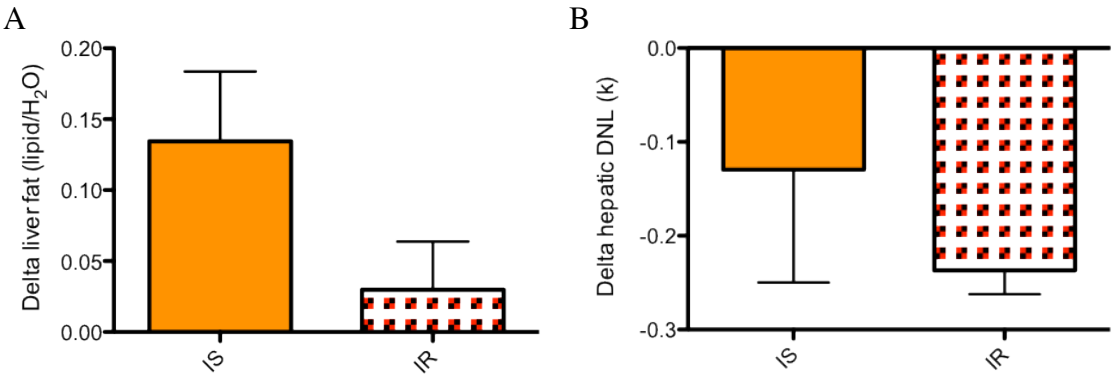



**Figure 8:** Changes in hepatic fat after overfeeding. A) Delta liver fat measured by MRI, p=0.63; B) Delta hepatic DNL measured by $^{2}H_2O$, p=0.26.

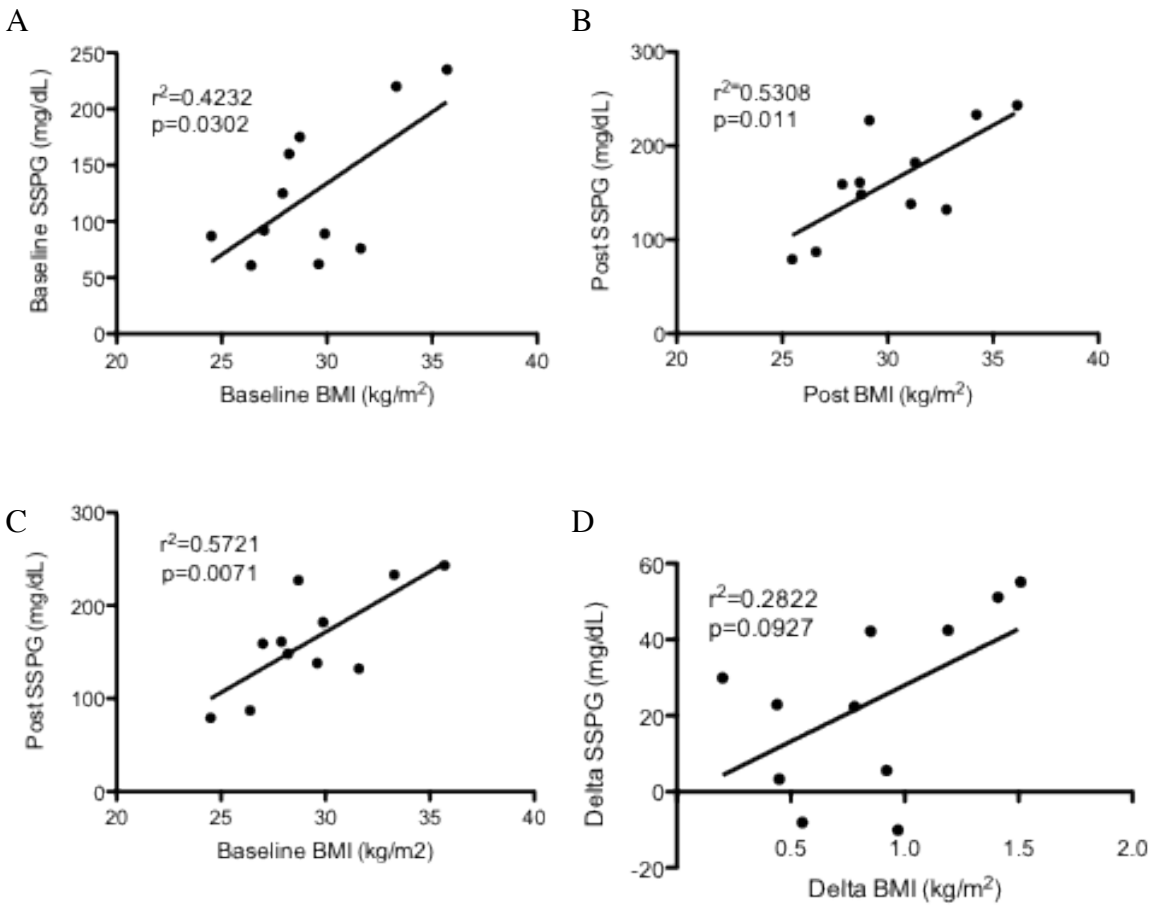



**Figure 9:** Relationship between BMI and insulin sensitivity measured by SSPG. A) Baseline BMI vs baseline SSPG; B) Post BMI vs Post SSPG; C) Baseline BMI vs Post SSPG; Delta BMI vs Delta SSPG.

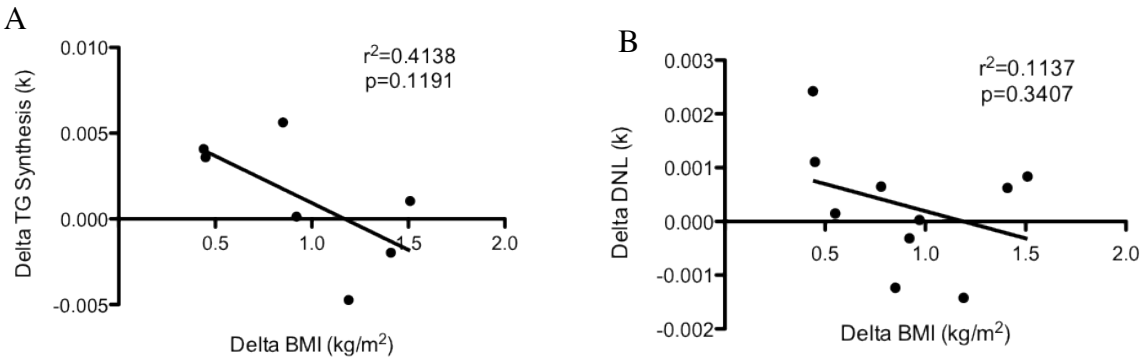



**Figure 10:** Relationship between change in BMI and change in adipose lipid kinetics A) Delta BMI versus delta TG synthesis after overfeeding; B) Delta BMI versus delta DNL after overfeeding.

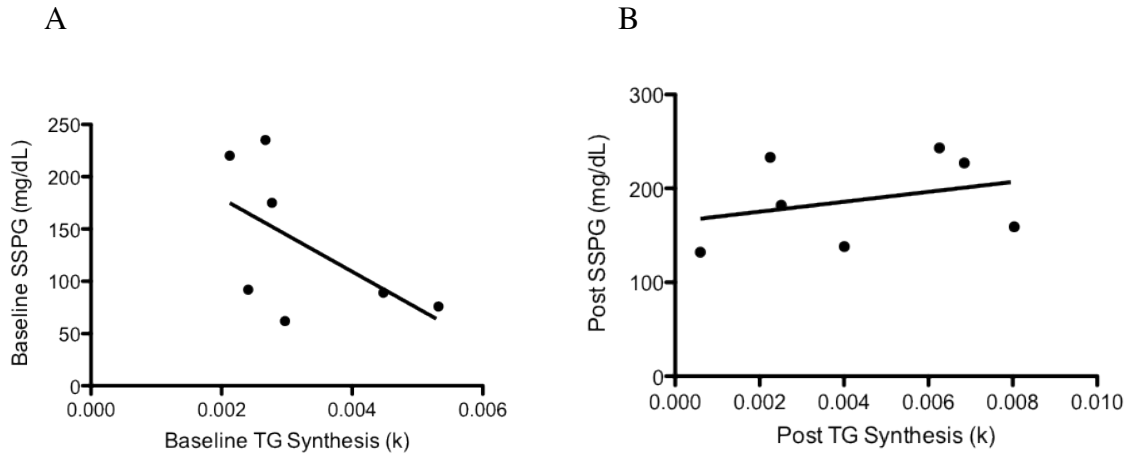



**Figure 11:** Relationship between TG synthesis and insulin sensitivity. A) Baseline TG synthesis vs baseline SSPG, $r^2$=0.33, p=0.18; B) Post-overfeeding TG synthesis vs post-overfeeding SSPG, $r^2$=0.097, p=0.50.

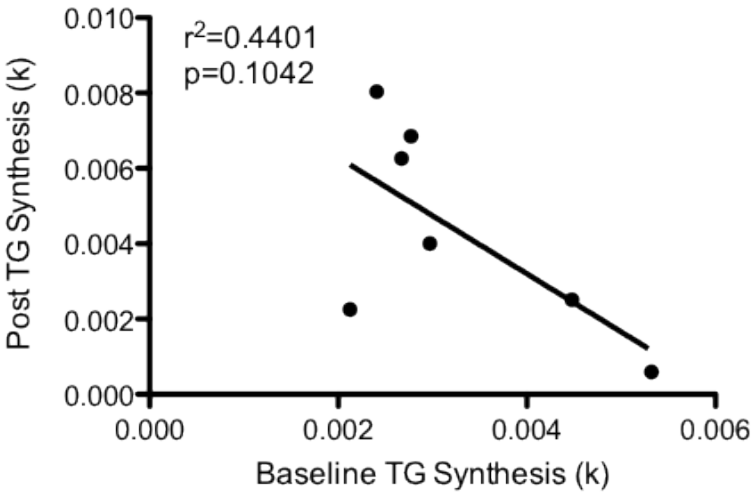



**Figure 12:** Baseline TG synthesis versus post-overfeeding TG synthesis.

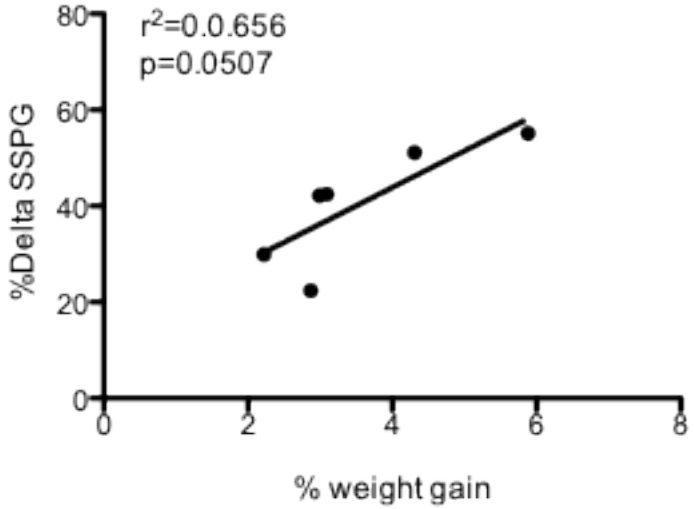



**Figure 13:** Relationship between percent weight gain and change in insulin sensitivity. A) Includes all subjects; B) Excludes

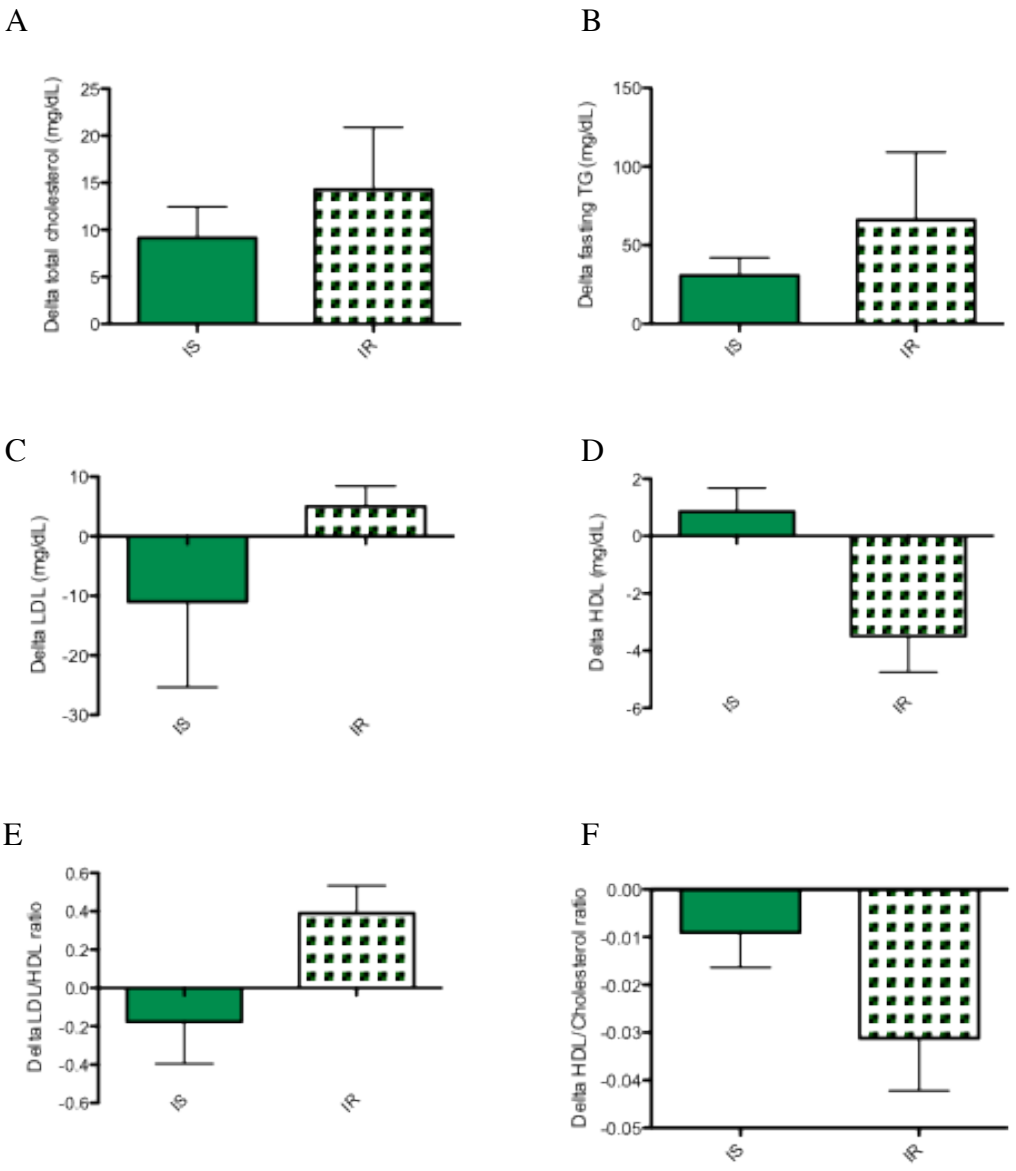



**Figure 14:** Changes in plasma lipids after overfeeding. A) Delta cholesterol, p=0.91; B) delta plasma TG, p=0.60; C) delta plasma LDL, p=0.63; D) delta HDL, p=0.19; E) delta LDL/HDL, p=0.11; F) delta HDL/cholesterol, p=0.59.

# Chapter 6

## Using $^{2}H_{2}O$ *in vivo* to determine the adipocyte proteome of insulin resistance using dynamic proteomics

**Abstract**

The etiology of obesity-induced insulin resistance is a complex network of conical pathways. Obesity is a major risk factor for insulin resistance due to the likelihood of elevated lipids in circulation not being properly stored in subcutaneous adipose tissue, which is thought to be metabolically protective. Lipids may interfere with insulin signaling in insulin-responsive tissues, and may also affect pancreatic beta cell release of insulin in efforts to maintain whole-body glucose homeostasis. Findings from our previous study demonstrate metabolic differences in the subcutaneous adipose tissue of insulin sensitive subjects that protects them from obesity-induced insulin resistance. Our observation of greater TG synthesis and DNL in this group lead us to further investigate the molecular mechanisms influencing the ability to maintain subcutaneous depot lipid synthesis in the obese state and other mechanisms driving insulin resistance. Using $^{2}H_2O$ labeling, we aimed to apply dynamic proteomics to quantify protein turnover rates of a large number of proteins from isolated subcutaneous mature adipocytes. In doing so, we hope to discover the underlying dynamics of the adipoycte proteome of insulin resistance. We investigated the adipocyte proteome dynamics in of 3 IS and 3 IR, equally obese subjects at steady state after 4 weeks of $^{2}H_2O$ consumption. Peptides were submitted through an LC/MS-MS instrument to separate and identify tryspinized peptides by size and charge, followed by Spectrum Mill analysis to organize peptides based on corresponding protein. A total of 620 proteins were identified amongst the six subjects, but after filtering for criteria (1<peptide and at least 1 duplicate per group), 438 proteins were identified and included in the final analysis. Proteins were then organized by pathway via KEGG analysis, resulting in a total 0f 218 pathways. We only focused on pathways known to directly affect or be affected by insulin, and of these, the majority of pathways showed greater protein fractional synthesis rates in IR subjects compared to IS subjects. Interestingly, the synthesis rates of proteins involved in lipid metabolism were greater in IR compared to IS, seemingly contrary to previous findings of reduced TG synthesis in this group. Similarly, proteins involved in cellular growth and proliferation had higher synthesis rates in IR vs IS, whereas adipocyte and stromal vascular cell proliferation were not different between groups. Our findings here reveal differences within tissue and pathways for resistance or sensitivity to insulin in states characterized as insulin resistant based on altered glucose metabolism.

## Introduction

Dynamic proteomics is the large-scale measurement of protein expression and turnover in a single specimen, biological tissue or bodily fluid. Protein turnover is the rate at which a protein is broken down and synthesized, and it is critical in determining circulating levels of proteins that control metabolic processes (1). Mitogenic hormones, like insulin, induce protein synthesis and cell growth via two different pathways: Ras and mTOR pathways (**Figure 1**). Ras pathway induces cell proliferation, while mTOR induces protein synthesis. When insulin is low, protein degradation will occur through the up-regulation of signals such as FoxO. In the insulin resistant state, hyperinsulinemia may up-regulate these pathways, however, given the lack of insulin response in this state, there may be a reduction, or perhaps no effects on these mitogenic signaling cascades. The consequence of systemic insulin resistance on cellular growth, maintenance and protein turnover has not been investigated. Our lab applies the use of $^{2}H_{2}O$ stable isotope technique to measure these dynamics *in vivo* to further investigate the role of hundreds of proteins in metabolic pathways. This technique can potentially identify biomarkers for disease states, such as insulin resistance, type 2 diabetes and cancer, eventually leading to advancements in therapeutic applications.

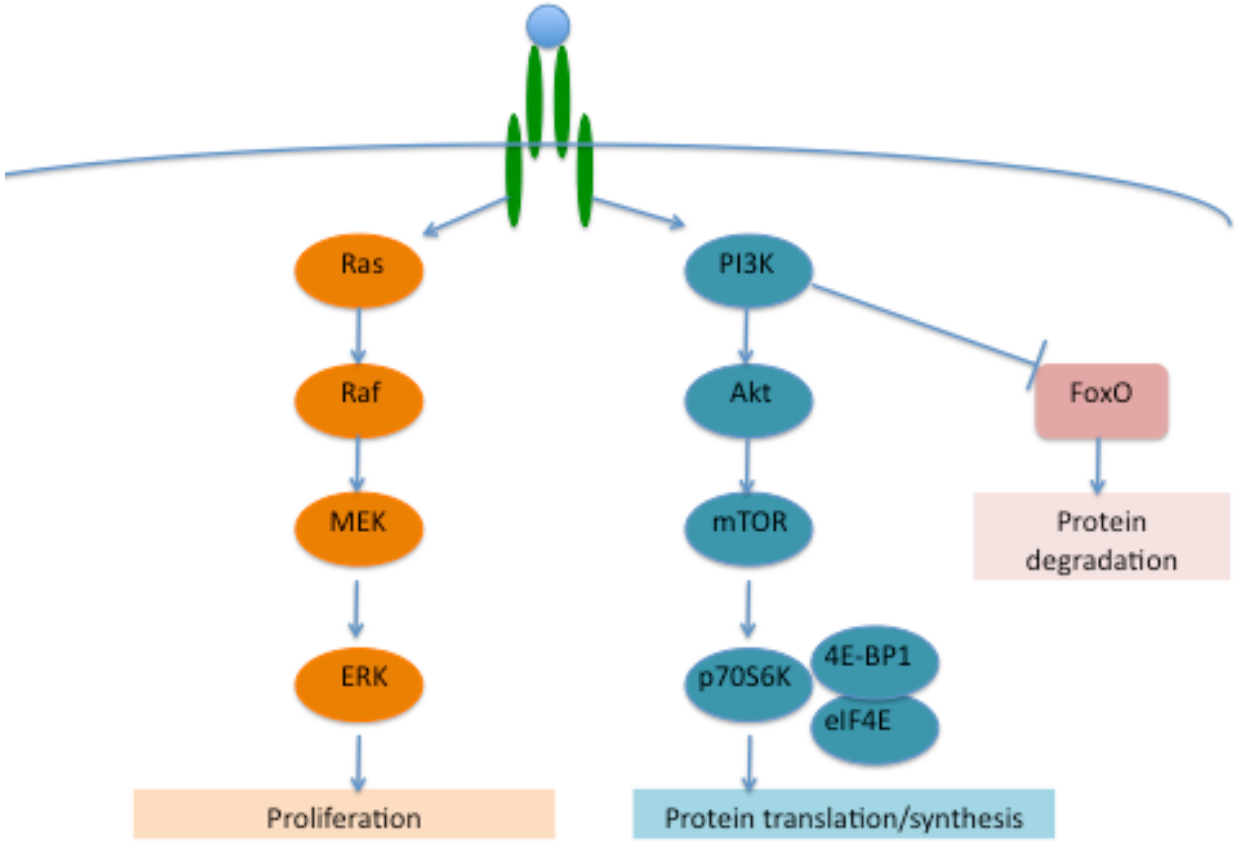



**Figure 1**: Following insulin binding to its receptor, tyrosine phosphorylation of IRS-1 leads to two downstream signaling cascades, Ras and mTOR, for cell growth. Ras stimulates Raf, which activates MEK, followed by ERK, leading to the transcription of proteins involved in cell proliferation. PI3K phosphorylation leads to both the activation of protein synthesis and the inhibition of protein degradation. Following activation of Akt by PI3K, mTOR stimulates p70S6K, 4E-BP1 and eIF4E initiating protein translation. PI3K blocks FoxO activity, preventing the degradation of proteins.

Unlike other methodologies of measuring protein expression, our method of dynamic proteomics reveals changes in protein synthesis during the labeling period, but more importantly, we can obtain a snapshot of entire metabolic pathways from just one blood or tissue specimen. The western blot is a method commonly used to measure protein expression, but is significantly less efficient than dynamic proteomics. Western blots require the use of antibodies for the detection of one protein per antibody. Thus, not only does this method require more tissue, but it also is not time and cost efficient. Proteomics only requires anywhere from 8ul of plasma to 100mg of tissue, yet reveals more than any other technique could. Identification and quantification of peptides is detected by the use of an LC/MS-MS, which identified thousands of amino acid sequences based on their mass to charge ratio. Following MS-MS analysis, peptides are identified to their respective protein using Spectrum Mill computational analysis.

Currently, a few research groups have applied dynamic proteomics to understanding adipose tissue function, but none have performed proteomics experiments *in vivo*. After years of being neglected, the adipose has gained much attention as an endocrine gland, synthesizing and secreting hormones and other adipokines involved in various metabolic and immune responses. Because obesity is such a driving risk factor for insulin resistance and type 2 diabetes, measuring protein homeostasis in healthy versus insulin resistant patients may uncover target pathways for therapeutic interventions. Kratchmarova and colleagues were one of the first groups to identify proteins in the adipose using proteomics (2), followed by Chen and his group (3). This group demonstrated changes in the synthesis of over 1000 proteins with and without insulin treatment in cultured rat adipocytes, the majority of which were up-regulated by insulin. Adachi and colleagues reported to have the largest-scale adipose proteomics findings to date, however, they did not apply any stable isotope techniques to their *in vitro* experiment (4). Thus, their study identified proteins produced and secreted by the adipose, but they did not measure protein turnover rates in the adipose under any conditions. Further experiments should quantify changes in these proteins either between different adipose depots or disease states in which the adipose may play a role. Our previous findings of reduced triglyceride synthesis and *de novo* lipogenesis in IR subjects indicates adipocyte dysfunction as the potential driving force that links obesity to insulin resistance. Here, we provide the first study to reveal the synthesis and breakdown rates of the adipocyte proteome *in vivo* with the use of $^{2}H_2O$, to further investigate potential molecular mechanisms of the obesity-induced insulin resistance.

## Results

*Body water enrichments and protein yield*

Our human participants received doses of heavy water according to a previous study protocol (5) that does not enable them to achieve maximum body water $^{2}H_2O$ enrichments of about 1.5% until 14 to 28 days. A ramp up period in which maximum enrichment is achieved on day 1 is only feasible in animal models. Humans are at risk for side effects such as dizziness with rapid ramp-ups, thus such experiments require overnight in-patient hospital stay (6). Therefore, fractional protein synthesis rates were calculated based on the average body water enrichment for each individual subject at each labeling period (based on the trapezoidal equation)

Body water enrichments for some subjects were quite low at week 1, resulting in a low protein yield, while week 2 resulted in the greatest protein yield amongst all subjects. Week 4 resulted in fewer proteins than week 4, but more than week 1. Analyses discussed in this report include fractional synthesis data from week 2 only, given that this time point produced the greatest protein yield. Thousands of peptides were detected by LC/MS-MS based on the measurement of enriched amino acids. Based on peptide amino acid sequences, a total of 620 proteins were identified using Spectrum Mill software. Proteins for which only 1 peptide was identified were excluded from the analysis. With a total of 3 subjects per group, at least one duplicate was required for proteins with at least 1 peptide to be included in final analysis. After filtering for these criteria, our dataset yielded a total of 438 proteins.

The primary purpose of this analysis was to reveal potential biomarkers of insulin resistance that would correlate with our observation of reduced adipocyte lipid metabolism in IR compared to IS patients. Of the 438 proteins represented, 33 proteins involved in lipid metabolism were represented in at least 3 of the 6 subjects, and only 20 met filter criteria (1< peptide and at least one duplicate per group). These proteins were classified under at least one of the following KEGG pathways: fatty acid metabolism, fatty acid degradation, fatty acid elongation and biosynthesis of unsaturated fatty acids. **Figure 1** shows there were 30 proteins involved in glucose metabolism with 19 meeting filtering criteria. Pathways included in this group: glycolysis, gluconeogenesis, pyruvate metabolism, TCA cycle and pentose phosphate pathways. Glucose and lipid metabolism shared 9 proteins. Eighteen proteins (14 meeting filtering criteria) found in our dataset were found to be involved in protein metabolism, which included the TCA cycle, protein processing and protein digestion. Four of these 19 proteins were shared between protein and glucose metabolism between protein metabolism and lipid metabolism, with only 1 represented in both protein and lipid metabolism. Malate dehydrogenase was the only protein that was shared amongst all three metabolic categories. Other important metabolism-related pathways identified in this analysis are listed in **Table 1**. The number of proteins represented in each subject is listed. The top 5 pathways identified by KEGG (those with the most proteins identified amongst all subjects) include Ras signaling (18 proteins), Rap1 signaling (17 proteins), endocytosis (17 proteins), phagosome (17 proteins), and glycolysis/gluconeogenesis (16 proteins).

Of the 218 metabolic pathways identified by KEGG pathway analysis, we will only give attention to a few most pertinent to insulin action and/or obesity-induced metabolic dysfunction. **Figure 2** illustrates differences in the average fractional synthesis rates of proteins involved in

various pathways that may be potentially influenced by insulin. Such pathways included pancreatic secretion, insulin secretion, MAPK signaling, glycolysis, TCA cycle, and SNARE complex interaction in vesicular transport, just to name a few. Two-way ANOVA analysis shows significantly higher rates of synthesis of proteins involved in these pathways in IR versus IS, $p<0.0001$.

Based on our findings of a reduction in triglyceride synthesis and storage in the subcutaneous adipose tissue of IR patients versus IS, we hypothesized that a molecular basis for these differences could be found in the synthesis rates of proteins responsible for the up- and down-regulation of lipid metabolism in the adipose tissue. Since we observed reduced adipose function in IR, this may be a reflection of reduced synthesis of proteins stimulated by insulin to up-regulate TG synthesis. Instead, we found that proteins in lipid metabolic pathways had a greater rate of synthesis in IR compared to IS, with a p-value of 0.0350 (**Figure 3**).

One mechanism by which insulin stimulates lipid synthesis is through the up-regulation of PPAR gene transcription, which then stimulates protein downstream to uptake and synthesize lipids for storage. PPAR signaling consisted of 7 proteins found in our dataset, however whether KEGG analysis did not distinguish between the different PPAR isoforms (ie. PPAR-$\gamma$, PPAR-$\delta$ or PPAR-$\alpha$). These pathways are all involved in lipid metabolism and catabolism. Identified proteins had a higher fractional synthesis rates in IR compared to IS, $p=0.0469$ (**Figure 4**).

Taking a closer look, **Figure 5** shows at individual pathways, we found 9 proteins involved the insulin signaling cascade, including proteins directly regulated by insulin, such as fatty acid synthase and hormone-sensitive lipase. It appears that a few proteins had higher synthesis rates in the IR group, but overall, there was no significant difference between groups in this pathway, p-value=0.1401. Following tyrosine phosphorylation of the insulin receptor, several downstream proteins are activated, beginning with PI3K, stimulating several downstream effects. In order to determine if there existed any defects in proteins downstream of the insulin receptor, we examined the fractional synthesis rates of proteins constituted as being part of the PI3K signaling cascade. Compared to IS, IR patients showed higher fraction synthesis rates of proteins involved in this cascade, p-value= 0.0378 (**Figure 6**).

Further downstream of PI3K are MAPK signaling and Ras signaling, both of which are regulated by PI3K activation upon insulin receptor phosphorylation. In our IR patients, proteins involved in Ras signaling, which includes various isoforms of the Ras protein, had greater synthesis rates than those of IS patients, $p < 0.0001$ (**Figure 7**). Insulin stimulates cell proliferation and growth through the activation of the MAPK signaling cascade, which our analysis shows to also be significantly higher in IR compared to IS patients, p-value= 0.0062 (**Figure 8**).

We hypothesized that proteins involved in glycolysis would be downregulated and this would be reflected in reduced protein synthesis of glycolytic enzymes. In contrast, KEGG analysis revealed significantly greater synthesis of proteins involved in glycolysis and/or gluconeogenesis, p-value=0.0006 (**Figure 9**), as well as those proteins involved in pyruvate metabolism, p-value=0.0225 (**Figure 10**). Detailed examination of the glycolytic and gluconeogenic pathways do not reveal any clear patterns in terms of where in the pathway might serve as targets for further investigation in insulin resistance treatment. **Figure 11** displays the glycolytic/gluconeogenic pathway, highlighting those found in our dataset. Proteins highlighted

in red have greater synthesis rates in IR compared to IS; those in green were lower, and those in blue were unchanged. Most proteins identified in our analysis are part of the oxidative phase of glycolysis. Four proteins identified in the glycolytic pathway are enzymes responsible for ketogenesis or lactogenesis following the formation of pyrvuate.

Taken together, our results provide the first *in vivo* evidence of increase adipose protein synthesis rates in the insulin resistant state while eliminating obesity as the driving factor, since all subjects are BMI-matched. Using KEGG analysis, we have shown that the majority of proteins involved in major metabolic pathways, particularly those regulated by insulin, show greater lipid synthesis rates in IR patients compared to IS patients.

**Discussion**

Protein synthesis measured by $^2H_2O$ provides a quantitative measurement of protein translation, providing direct real-time *in vivo* quantification versus measuring protein expression by western blot of gene expression by qPCR. Insulin regulates protein synthesis via the mTOR and Ras MAPK pathways, thus in using $^2H_2O$ for dynamic proteomics, we can identify insulin's effects on the synthesis of proteins involved in several metabolic pathways in the insulin resistant state. This is the first study to demonstrate that the subcutaneous adipose tissue of obese insulin resistant individuals have an overall greater rate of protein turnover, compared to healthy obese individuals.

The rate at which proteins are synthesized is determined by transcriptional activity in the endoplasmic reticulum (ER) and autophagy activity in lysosomes. Autophagy maintains protein homeostasis by initiating cell death of damaged or harmful proteins (7), and is largely impacted by nutrition and energy requirements (8). Deficient autophagy may result in increases in cellular damage thus cell death may occur. Autophagy in the adipose tissue obese subjects is reported to be upregulated (9). They observed greater expression of autophagy-related proteins, such as Atg5, LC3A and LC3B in obese compared to lean subjects, and even more so in IR subjects. While we were unable to detect any autophagy proteins, the greater protein synthesis rates observed in IR subjects agree with previous findings and may reflect greater autophagy activity in that increases in cell death are pushing the system to replace proteins at a faster rate than that of a healthy state.

Insulin is a major inhibitor of autophagy, activating mammalian target of rapamycin (mTOR), which inhibits Atg1, the initiator of autophagy (10). The PI3K pathway plays an essential role in inhibiting the mTOR pathway, so one might predict an overall decrease in PI3K signaling. PI3K serves as link to many of insulin's downstream signaling cascades and relies on other proteins, such as p110 and p85 subunits to stimulate further downstream proteins. It is unclear whether or not PI3K is altered in insulin resistance, but scientists found that inhibition of the p85 subunit is required for PI3K activation and actually protects against insulin resistance. While seemingly contradicting, such results agree with our findings of greater synthesis rate for the majority of proteins involved in PI3K signaling, in which case inhibition of autophagy should be elevated as a result. However, PI3K stimulates Akt, controlling most of insulin's downstream signals, including protein synthesis. Thus greater protein synthesis of PI3K signaling may explain the greater protein synthesis rates observed in IR patients. However, there are two classes of PI3K components: Class I, which inhibits mTOR, while class II components up-regulate autophagy (11). We cannot distinguish between these two classes from which PI3K proteins identified in our data analysis are represented. We can however, examine two pathways that directly control cell proliferation, an important marker of longevity and metabolic function.

The Ras/MAPK pathway is predominately responsible for cell growth and is stimulated by PI3K in the presence of growth factors, such as insulin. Investigators find that inhibition of Ras signaling enhances insulin sensitivity (12), supporting our findings of slower synthesis of Ras signaling proteins in IS subjects. Ras proteins are predominately responsible for cellular proliferation and growth, counteracting the actions of insulin. However, Ras has also been shown to inhibit insulin's nutrient metabolic nutrients (12,13), mimicking insulin's ability to stimulate GLUT4 translocation to the cell membrane for glucose uptake. A study in obese diabetic rats

showed an increase in ERK and MAPK proteins, providing a potential link between obesity and cancer and insulin resistance (14). Other studies have suggested that increases in the MAPK pathway is linked to increases in adipogenesis in obesity, however we observed no differences in adipocyte proliferation between IR and IS, despite greater synthesis of proteins involved in the Ras and MAPK signaling. Pairing this outcome with our previous results of greater portions of small adipocytes and less TG synthesis in IR compared to IS supports our conclusion of impaired adipocyte function.

Adipocytes serve as storage reservoirs for excess energy stored in the form of lipids. Subcutaneous adipocyte function has been hypothesized to be protective against insulin resistance, by reducing the amount of fat stored in the harmful visceral fat depot. We previously demonstrated an impaired response to insulin's effects on lipid synthesis in the adipose tissue of IR patients, leading us to hypothesize that the synthesis of proteins responsible for regulating lipid metabolism would also be impaired. Here, we found 33 proteins to play a role lipid metabolism, but unexpectedly, the majority of proteins in IR individuals had higher turnover rates compared to IS individuals. Of these 33, key proteins known to be regulated by insulin, hormone sensitive lipase (HSL) and fatty acid synthase (FAS), showed similar turnover rates between groups. In the adipose tissue, insulin stimulates synthesis and uptake of fatty acids, triglycerides, while suppressing lipolysis in the effort to maintain lipids in the adipose and out of plasma and ectopic storage. HSL is the rate-limiting step for lipolysis and is a direct target of insulin. FAS is upregulated by insulin to stimulate fatty acid synthesis, and this will be discussed later. Its mRNA and protein expression in subcutaneous adipose tissue are decreased in insulin resistance (15,16), while levels in the visceral depot are increased (15). HSL is reduced in subcutaneous tissue of IR patients, based on qpcr and western blot analysis (17). Yet in our analysis, HSL is no different between IR and IS. Its interaction with perilipin determines the rate at which lipolysis occurs. In the postprandial state, perilipin resides on lipid droplets to trap triglycerides inside the cell. In the fasting state, HSL is phosphorylated by activation of PKA, and invades the lipid droplet, causing dissociation between perilipin and TG (18). Alternatively, if perilipin is first phosphorylated, it will change conformation, exposing TG to the actions of HSL. Once in contact with TG, HSL hydrolyzes TG, releasing free fatty acids (FFA) into circulation (17). Studies in mice have found that perilipin is reduced in obesity and over-expression of perilipin protects mice against diet-induced obesity (19,20). However, these results were challenged by findings of elevated perilipin expression in obese humans *in vivo*, with authors suggesting that this may indicate compensation to reduce elevated basal lipolysis (21,22). We observed impaired insulin-inhibition of lipolysis in IR, yet dynamic proteomic analysis did not show any differences in perilipin synthesis between IS and IR. In comparing the few subjects between groups, HSL synthesis was only about 7% higher in IR subjects. This difference was not significant, likely due to the small sample size. Insulin inhibits lipolysis by inhibiting HSL activity, yet HSL turnover was not different between groups. This can be explained by previous studies demonstrating that insulin has no effect on the transcription of translation of HSL, but rather only has the ability to de-phosphorylate HSL, inhibiting its actions (23,24). When HSL is phosphorylated, it becomes active and stimulates the release of fatty acids from lipid droplets. HSL activity is suppressed by insulin's activation of a dephosphorylase. Thus, in our IR subjects, insulin cannot promote the synthesis of new TG, the uptake of free fatty acids nor suppress lipolysis, but these impairments cannot be explained by a lack of synthesis of proteins involved in these processes. However, it is important to note that

we cannot determine the action of these proteins using the dynamic proteomic method. HSL activity can be represented by the amount of phosphorylated HSL through the actions of insulin, as can perilipin. Unfortunately, we cannot measure the portion of newly synthesize HSL or perilipin that were phosphorylated. Without this pertinent outcome, it cannot be determined whether or not HSL or perilipin activity was greater in IR subjects. We also cannot determine from which subcellular location HSL measured here represents. Inactivated HSL remains in the cytosol before being translocated to the lipid droplet (25,26). In our study, adipocytes were thoroughly sonicated, releasing all proteins into a soluble lysate, while the majority of lipids are removed. Thus, it is likely that HSL from either location was pooled together, so without either specifically measuring phosphorylated HSL or separating subcellular fractions by ultra centrifugation, we cannot make any conclusions about these results. Nonetheless, elevated plasma free fatty acids is a common phenotype of insulin resistance, and along with elevated glucose levels, contributes to negative metabolic reactions in diabetics. Lipolysis releases fatty acids and glycerols into circulation, which then travel to the liver for metabolism via gluconeogenesis or glycolysis.

The ability to continue TG synthesis in subcutaneous adipose tissue has reverses insulin resistance in patients taking the anti-diabetes drug, Metformin. Metformin, which inhibits lipolysis, causes a down-regulation of perilipin, preventing an increase in plasma free fatty acids (27,28). Metformin also decreases gluconeogenesis, increases peripheral glucose uptake, and helps prevent dyslipidemia (29). Increasing glucose uptake in the adipose will result in an increase in newly synthesized glycerol, allowing for greater uptake and storage of fatty acids in the adipose. IR subjects have reduced peripheral glucose uptake, because insulin action is impaired. Thus, the reduced TG synthesis observed in our IR subjects may be in part caused by reduced esterification in addition to an impaired ability to synthesize new glycerol in the adipose.

One of the most important roles of insulin is to regulate glucose homeostasis, by pushing glucose into cells for metabolism or storage. Glycolysis is the process by which glucose is broken down into macromolecules that will later become glycogen, fatty acids, glycogen or purines for DNA synthesis. Under conditions of intense exercise or diabetes, ketogenesis can also occur, in which by products of glycolysis are used to make ketones. Observations of higher synthesis of proteins involved in ketogenesis suggest an up-regulation of ketogenic products, potentially reflecting impaired pyruvate oxidation in the mitochondria. Interestingly, however, this would normally occur in the liver, and thus the impact of ketogenesis occurring in the adipose may not be of importance (30).

The last step of glycolysis is the conversion of phosphoenolpyruvate into pyruvate via pyruvate kinase. Pyruvate links together lipid metabolism, TCA cycle and glycolysis, as it gets converted to acetyl-CoA via pyruvate dehydrogenase E (PDE) in the mitochondria. PDE1 component beta was found in two IS (fractional synthesis was 25 and 20%) and two IR subjects (fractional synthesized equaled 63% and greater than 100%), although because fractional synthesis values greater than 100% cannot be interpreted, PDE was not included in our results. However, these values still reveal that PDE synthesis was greater in IR than IS. PDE is increased in the fed state, but is inactivated by pyruvate dehydrogenase kinase (PDK) in the fasted state (31).

Insulin is known to regulate PDE activity, causing an increase in PDE in the adipose as well as other tissues (32,33). In the IR state, insulin is higher than normal in the fed states, thus observations of high synthesis of PDE in IR subjects may be a reflection of hyperinsulinemia. This increase in PDE is also reflected in other molecular reactions, such as ketogenesis, lactogenesis and TCA cycle proteins in our IR patients. PDE leads to the synthesis of acetyl-CoA linking together the major metabolic pathways. Tying in with the main goal of our studies, acetyl-CoA reacts with malonyl-CoA for the synthesis of fatty acids. This reaction is controlled by the rate-limiting enzyme acetyl-CoA carboxylase (ACC) (34). ACC is regulated by insulin, thus to observed the synthesis of this protein would have revealed the relationship between protein synthesis and insulin resistance. Unfortunately, we did not observed ACC in our dataset. Other proteins of fatty acid metabolism, however, were identified in our dataset, mostly those involved in fatty acid oxidation. For example, four-*N*-trimethylaminobutyaldehyde dehydrogenase was one of the proteins identified in pyruvate metabolism by KEGG analysis with the highest synthesis rate in IR subjects. This enzyme is involved in the synthesis of carnitine, a known transporter of long-chain fatty acids into the mitochondria for a fatty acid oxidation. Carnitine's role in fatty acid transport is facilitated by carnitine palmitoyltransferase 1 (CPT1), which promotes fatty acid oxidation, while inhibiting malonyl-CoA. Low insulin causes activation of CPT-1, thus inversely, insulin blocks CPT-1, indirectly by increasing glucose metabolism and oxidation, stimulating the production of malonyl-CoA for TG synthesis to occur (35,36). Hyperinsulinemia caused by insulin resistance elevates CPT-1, and paired with elevated free fatty acids, lipids are pushed into the cytoplasm for lipid synthesis and into the mitochondria for fat oxidation. Long-chain fatty acids (LFCA) are transported into the mitochondria for oxidation, which is well-documented to be decreased in diabetic patients (37,38). Interestingly, however, proteins involved in oxidative phosphorylation show higher fractional synthesis rates in IR subjects compared to IS. This is contrary to previous observations of reduced oxidative phosphorylation in insulin resistance, as well as our findings of reduced TG synthesis and DNL in our IR patients. Long-chain fatty acid Co-A (LCFA) ligase showed elevated fractional synthesis in IR compared to IS, perhaps reflecting an influx of FFAs into the adipocyte. Since our IR subjects showed high plasma FFAs paired with impaired TG synthesis, the demand for LFCA ligase may be high, but the resources (ie. glycerol-α-phosphate) are low. Alternatively, LFCA ligase may be responding to either hyperglycemia and/or hyperlipidemia, as has been shown previously, *in vitro* (39). In order for fatty acid oxidation to be greater in addition to lipid synthesis being reduced, our subjects would have to be either lipodystrophic or under going weight loss. Instead, our subjects are in steady state, so increases in oxidative proteins may simply be a result of nutrient-sensing as free fatty acids remain high, but may not reflect fat oxidation activity in the adipose.

Our observation of higher synthesis in proteins isolated from adipose of IR compared to IS may reflect cellular compensation in that protein synthesis must be increased due to a decline in adipocyte function. This would analogous to β-cell compensation in the islets of the pancreas. When there isn't enough insulin to meet the demands of elevated glucose, β-cells proliferate in order to pump out more insulin (40). Thus, perhaps a decrease in adipocyte function causes an increase in protein synthesis to meet increasing demands of excess energy storage.

Previous studies investigating the effects of caloric restriction on protein turnover contradict our findings. Caloric restriction is known to improve insulin sensitivity, decrease cell proliferation

and enhance longevity (41,42). Part of calorie restriction's ability to increase longevity may be due to its ability to lessen the decline in protein degradation and synthesis. Studies found that protein synthesis rates decline during aging, thus preserving protein synthesis will maintain cellular function and prevent aging (43,44). In fact, one study found an increase in protein turnover rates in response to calorie restriction (45). Based on this, plus findings of reduced protein synthesis in T2D patients (46), we would have hypothesized that IR subjects would show reduced protein turnover in their adipose tissue, reflecting adipocyte dysfunction. Instead we observed greater protein synthesis in the majority of proteins extracted from IR subjects compared to IS. Interestingly, hormones in our dataset, like FAS, that are involved in lipid synthesis were not different between groups, while those involved in fatty acid oxidation were higher in IR.

Our findings here suggest that hyperinsulinemia in insulin resistance enhances the mitogenic actions of insulin, resulting in increases in protein synthesis in adipocytes. However, despite enhances synthesis of proteins in key metabolic pathways, such as glycolysis, lipid metabolism and suppression of lipolysis, the outcomes of these signaling cascades are not reflected in the IR phenotype. This divergence in pathways, demonstrates differential insulin resistance at the cellular level. Furthermore, the IR phenotype is not reflected in the turnover of proteins seemingly responsible for these phenotypes. For example, despite glycolytic enzymes being greater in IR, IR have reduced glucose uptake and metabolism shown by the steady state plasma glucose test. IR subjects in our previous study showed reduced TG synthesis and DNL, but this was not reflected in the synthesis of proteins involved in these pathways. Therefore, if protein synthesis is upregulated in the hyperinsulinemic state, while the corresponding metabolic processes are downregulated, defects in metabolism must occur at the posttranslational level. This can be supported by studies performed in the adipose of *ob*/*ob* mice, in which normal levels of mRNA expression of lipogenic genes was not reflected in significantly elevated rates of TG synthesis and DNL quantified by $^{2}H_{2}O$ (47). Thus, there are regulators beyond transcription and translational which regulate the actions of newly synthesized proteins in metabolic processes.

Our results demonstrate that obese IR individuals have an increase in turnover of proteins involved in metabolism to meet the increased demands of maintaining homeostasis. The subjects in our study are not diabetic, thus, future studies comparing adipose protein turnover in IR versus diabetic may provide further insight into the enhanced protein turnover observed IR. It may be that in the IR state, all metabolic pathways are working to compensate for reduced insulin response, but over time, cells can no longer increase protein production and thus the whole body metabolic homeostasis fails and type 2 diabetes develops. Future studies investigating this hypothesis would reveal the proteome of the development of diabetes and important targets for therapeutic interventions may be discovered.

**Study Limitations**

This study provided the first *in vivo* evidence of an adipocyte proteome in the insulin resistant state. Due to our small sample size, we were unable to detect significant differences amongst individual proteins within a particular pathway, although observation of protein synthesis globally, we were able to detect significant differences amongst all pathways of interest. One advantage of our study was that dynamic proteomic analysis was performed in isolated mature adipocytes, rather than whole adipose tissue, providing a cell-specific proteome profile.

Therefore, future efforts investigating the adipocyte proteome of the insulin resistant state should include a larger sample size to detect significant differences in protein turnover rates of proteins involved in insulin-regulated pathways.

**References**


1. Eagle H, Piez KA, Fleischman R, Oyama VI. Protein turnover in mammaliar cell cultures. *J. Biol. Chem* 1959, 234:592–597.

2. Kratchmarova I, Kalume DE, Blagoev B, Scherer PE, Podtelejnikov AV, Molina H, Bickel PE, Andersen JS, Fernandez MM, Bunkenborg J, Roepstorff P, Kristiansen K, Lodish HF, Mann M, Pandey A. A proteomic approach for identification of secreted protein during the differentiation of 3T3-L1 Preadipocytes to Adipocytes. Molecular and Cellular Proteomics 1.3 2002, 1:213-222.

3. Chen X, Cushman S, Pannel LK, Hess S. Quantitative proteomic analysis of the secretory proteins from rat adipose cells using a 2D liquid chromatography—MS/MS Approach. Journal of Proteome Research 2005, 4:570-577.

4. Adachi J, Kumar C, Zhang Y, Mann M. In-depth analysis of the adipocyte proteome by mass spectrometry and bioinformatics. Molecular and Cellular Proteomics 6.7 2007, 6:1257-1273.

5. Strawford A, Antelo F, Christiansen M, Hellerstein MK. Adipose tissue triglyceride turnover, de novo lipogenesis, and cell proliferation in humans measured with $^{2}H_{2}O$. Am J Physiol Endocrinol Metab. 2004, 286:E577-88.

6. Money KE, Myles WS. Heavy water nystagmus and effects of alcohol. Nature 1974, 247: 404-405.

7. White, D. A.; Bennett, A. J.; Billett, M. A.; Salter, A. M. The assembly of triacylglycerol-rich lipoproteins: an essential role for the microsomal triacylglycerol transfer protein. British Journal of Nutrition1998, 80: 219–229.

8. Todde V, Veenhuis M, van der Klei IJ. Autophagy: principle and significance in health and disease. Biochim Biophys Acta 2009, 1792:3-13.

9. Jansen HJ, van Essen P, Koenen T, Joosten LAB, Netea G, Tack CJ, Stienstra R. Autophay activity is up-regulated in adipose tissue of obese individuals and modulates proinflammatory cytokine expression.

10. Codogno P. and Meijer AJ. Autophagy and signaling: their role in cell survival and cell death. Autophagy and signaling: their role in cell survival and cell death. Cell Death Differ 2005, 12:1509-1518.

11. Meijer AJ, Codogno P, 2004 Regulation and role of autophagy in mammalian cells. Int J Biochem Cell Biol 36: 2445-2462.

12. Mor A, Aizman E, George J, Kloog Y. Ras inhibition induces insulin sensitivity and glucose uptake. PLoS ONE 2011, 6:1-11.

13. Kozma L, Baltensperger K, Klarlund J, Santos E, Czech MP. The ras signaling pathway mimics insulin action on glucose transporter translcoation. PNAS 1993, 90:4460-4464.

14. Jiang ZY, Lin Y, Clemont A, Feener EP, Hein KD, Igarashi M, Yamauchi T, White MF, King GL. Characterization of selective resistance to insulin signaling in the vasculature of obese Zucker (*fa*/*fa*) rats. J Clin Invest 1999, 104:447-457.

15. Berndt J, Kralisch S, Klöting, Ruschke K, Kern M, Fasshauer M, Schön MR, Stumvoll M, Blüler M. Adipose triglyceride lipase gene expression in human visceral obesity. Exp Clin Endocrinol Diabetes 2008, 116:203-210.

16. Jocken JW, Langin D, Smit E, Saris WH, Valle C, Hul GB, Holm C, Arner P, Blaak EE. Adipose triglyceride lipase and hormones-sensitive lipase protein expression in is decreased in the obese insulin resistant state.

17. Brasaemle, DL. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and controls of lipolysis. Journal of Lipid Research 2007, 48:2547-2559.

18. Souza SC, Muliro KV, Liscum L, Lien P, Yamamoto MT, Schaffer JE, Dallal GE, Wang X, Kraemer FB, Obin M, Greenberg. Modulation of hormone-sensitive lipase and protein kinase A-mediated lipolysis by perilipin A in an adenoviral reconsistuted system. J Biol Chem 2002, 277:8267-8272.

19. Wang Y, Sullivan S, Trujillo M, Lee MJ, Schneider SH, Brolin RE, Kang YH, Weber Y, Greenber AS, Fried SK. Evidence for an important role of perilipin in the regulation of human adipocyte lipolysis. Obes Res 2003, 11:930-936.

20. Miyoshi H, Souza S, Endo M, Sawada T, Perfield JW, Shimizu C, Stancheva Z, Nagai, Strissel KJ, Yoshioka N, Obin MS, Koike T, and Greenberg. Perilipin overexpression in mice protects against diet-induced obesity. J Lipid Res 2010, 51:975-982.

21. Kern, PA, Gregorio GD, Lu T, Rassouli N, Ranganathan G. Perilipin expression in human adipose tissue is elevated with obesity. J Clin Endocrinol 2004, 89:1352-1358.

22. Mottagui-Tabar S, Ryden M, Lofgren P, Faulds G, Hoffstedt J, Brookes AJ, Andersson I, Arner P. Evidence for an important role of perilipin in the regulation of human adipocyte lipolysis. Diabetologia 2003; 46:789–797.

23. Smith F, Rouet P, Lucas S, Mairal A, Sengenes C, Lafontan M, Vaulont S, Casado M, Langin D. Transcriptional regulation of adipocyte hormone-sensitive lipase by glucose. Diabetes 2002, 51:293-300.

24. Nishino N, Tamori Y, Kasuga M. Insulin efficiently stores triglycerides in adipocytes by inhibiting lipolysis and repressing PGC-1alpha induction. Kobe J Med Sci 2007, 53:99-106.

25. Eagan JJ, Greenberg AS, Chang MK, Wek SA, Moos MC Jr, Londos C. Mechanism of hormone-stimulated lipolysis in adipocytes: translocation of hormone-sensitive lipoase to the lipid storage droplet. Proc Natl Acad Sci 1992, 89:8537-8541.

26. Clifford GM, Londos C, Kraemer FB, Vernon RG, Sj Yeaman. Translocation of hormone-sensitive lipase and perilipid upon lipolytic stimulate of rat adipocytes. Biol Chem 2000, 275:5011-5015.

27. Ren T, He J, Jiang H, Zu L, Pu S, Guo X, Xu G. Metformin reduces lipolysis in primary rat adipocytes stimulated by tumor necrosis factor-alpha or isoproterenol. J Mol Endocrinol 2006; 37:175–183.

28. Abbasi F, Carantoni M, Chen YD & Reaven GM. Further evidence for a central role of adipose tissue in the antihyperglycemic effect of metformin. Diabetes Care 1998, 21:1301–1305.

29. Bailey CJ. Biguanides and NIDDM. Diabetes Care 1992, 15:755–772.

30. Noda G, Ischihara A. Control of ketogenesis from amino acids. IV. Tissue specificity in oxidation of leucine, tyrosine and lysine. J Biochem 1976, 80:1159-1164.

31. Gudi R, Bowker-Kinley MM, Kedishvili NY, Zhao Y, Popov KM. Diversity of the pyruvate dehydrogenase kinase gene family in humans. *J Biol Chem* 1995, 270: 28989-28994.

32. Coore HG, Denton RM, Martin BR, Randle PJ. Regulation of adipose tissue pyruvate dehydrogenase by insulin and other hormones. Biochem J 1971:115-127.

33. Hutson, NJ, Kerbey AL, Randle PJ, Sugden PH. Regulation of pyruvate dehydrogenase by insulin action. Prog Clin Biol Res 1979, 31:707-719.

34. Volpe JJ, Marasa JC. Hormonal regulation of fatty acid synthetase, acetyl-CoA carboxylase and fatty acid synthesis in mammalian adipose tissue and liver. Biochimica et Biophysica Act- Lipids and Lipid Metabolism 1975, 380:454-472.

35. Dayanand CD, Krishnamurthy N, Ashakiran S, Shashidhar KN. Carnitine: A novel health factor-An overview. Int J Pharm Biomed Res 2011, 2:79-89.

36. Bremer J. The biochemistry of hypo-hyperlipidemic fatty acid derivatives: metaboism and metabolic effects. Prog Lipid Res 2001, 40:231-268.

37. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, MunE, DeFronzo R, Finlayson J, Kahn CR Mandarino LJ. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC-1 and NRF1. Proc Natl Acad Sci 2003, 100:8466-8471.

38. Morino K, Petersen KK, Shulman GI. Molecular mechanism of insulin resistance in humans and their potential links with mitochondrial dysfunction. Diabetes 2006, S9-S15.

39. Prentk M, Vischer S, Glennon MC, Regazzi R, Deeney JT, Coreky BE. Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling in nutrient-induced insulin secretion. Biological Chemistry 1992, 257:5802-5810.

40. DeFronzo RA, Bonadonna RC, Ferrannini E. Pathogenesis of NIDDM. Diabetes Care 1992, 15:318-368.

41. Larson-Meyer DE, Heilbronn LK, Redman LM, Newcomer BR, Frisard MI, Anton S, Smith SR, Mapistat AA, Ravussin E, Pennington CALORIE Team. Effect of calorie restriction with or without exercise on insulin sensitivity, β-cell function, fat cell size, and ectopic lipid in overweight subjects. Diabetes Care 2006, 29:1337-1344.

42. Bruss MD, Thompson AC, Aggarwal I, Khambatta CF, Hellerstein MK. The effects of physiological adaptations to calorie restriction on global cell proliferation rates. Am J Physiol Endocrinol Metab 2011, 300:E735-45.

43. Ward WF. The relentless effects of the aging process on protein turnover. Biogerontology 2000, 1:195-199.

44. Rattan SI. Synthesis, modifications, and turnover of proteins during aging. Exp Gerontol 1996, 31:33-47.

45. Lewis SE, Goldpink DF, Phillips JG, Merry BJ, Holehan AM. The effects of agining and chronic dietary restriction on whole body growth and protein turnover. Exp Gerontol 1985, 20:253-263.

46. Pereira S, Marliss EB, Morais JA, Chevalier S, Gougeon R. Insulin resistance of protein metabolism in type 2 diabetes. Diabetes 2007, 57:56-63.

47. Turner SM, Roy S, Sul HS, Neese RA, Murphy EJ, Samandi W, Roohk DJ, Hellerstein MK. Dissociation between adipose tissue fluxes and lipogenic gene expression in ob/ob mice. Am J Physiol Endocrinol Metab 2007, 292:E1101-1109.

**Figures**

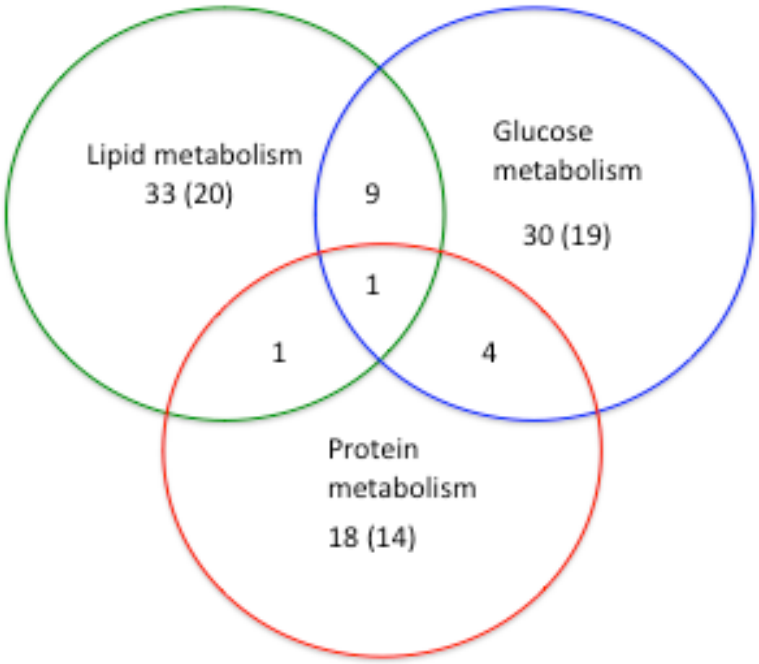



**Figure 1:** Comparison of the number of proteins found in 3 macronutrient metabolic categories: lipid, glucose, proteins. Lipid metabolism includes proteins involved in fatty acid metabolism, degradation, elongation, and biosynthesis of unsaturated fatty acids; Glucose metabolism includes

| | # of Proteins Represented at Week 2 | | | | | |
|---|---|---|---|---|---|---|
| Pathway | 1520 | 1448 | 1584 | 1007 | 1535 | 1752 |
| Insulin signaling pathway | 9 | 10 | 8 | 6 | 9 | 7 |
| Ras signaling pathway | 17 | 21 | 19 | 14 | 20 | 19 |
| Biosynthesis of unsaturated fatty acids | 5 | 5 | 3 | 0 | 6 | 4 |
| Wnt signaling pathway | 5 | 5 | 4 | 4 | 5 | 5 |
| Carbon metabolism | 27 | 32 | 24 | 24 | 30 | 27 |
| Phagosome | 17 | 19 | 15 | 11 | 19 | 19 |
| Fatty acid metabolism | 13 | 14 | 10 | 7 | 16 | 10 |
| Oxidative phosphorylation | 15 | 19 | 8 | 6 | 14 | 14 |
| Insulin secretion | 5 | 6 | 4 | 3 | 5 | 5 |
| Gastric acid secretion | 9 | 9 | 8 | 6 | 9 | 8 |
| PI3K-Akt signaling pathway | 16 | 16 | 15 | 11 | 13 | 13 |
| Thyroid hormone synthesis | 4 | 6 | 4 | 5 | 6 | 4 |
| Fructose and mannose metabolism | 3 | 3 | 3 | 4 | 3 | 3 |
| Rap1 signaling pathway | 19 | 20 | 16 | 13 | 19 | 18 |
| HIF-1 signaling pathway | 6 | 9 | 5 | 6 | 7 | 6 |
| Peroxisome | 12 | 13 | 9 | 6 | 12 | 12 |
| mTOR signaling pathway | 3 | 3 | 3 | 3 | 3 | 3 |
| Steroid hormone biosynthesis | 5 | 5 | 4 | 3 | 5 | 5 |
| Thyroid hormone signaling pathway | 3 | 3 | 3 | 3 | 5 | 3 |
| Non-alcoholic fatty liver disease (NAFLD) | 9 | 12 | 7 | 3 | 9 | 9 |
| Endocytosis | 14 | 20 | 16 | 11 | 17 | 14 |
| MAPK signaling pathway | 11 | 14 | 11 | 10 | 12 | 13 |
| PPAR signaling pathway | 11 | 14 | 10 | 9 | 14 | 10 |
| Pyruvate metabolism | 8 | 11 | 9 | 9 | 11 | 9 |
| Pancreatic secretion | 7 | 8 | 7 | 4 | 7 | 8 |
| Protein processing in endoplasmic reticulum | 9 | 14 | 5 | 9 | 12 | 8 |
| Fatty acid degradation | 14 | 17 | 11 | 10 | 18 | 14 |
| Glycolysis / Gluconeogenesis | 16 | 19 | 13 | 16 | 16 | 18 |
| Vasopressin-regulated water reabsorption | 7 | 9 | 10 | 7 | 9 | 9 |
| Fat digestion and absorption | 2 | 3 | 1 | 1 | 2 | 1 |
| SNARE interactions in vesicular transport | 3 | 3 | 3 | 1 | 3 | 3 |
| FoxO signaling pathway | 2 | 2 | 1 | 2 | 2 | 2 |
| Pentose phosphate pathway | 7 | 8 | 5 | 6 | 7 | 8 |
| Citrate cycle (TCA cycle) | 8 | 12 | 2 | 5 | 10 | 8 |

**Table 1:** A sample of important metabolic pathways found in this study, listing the number of proteins per pathways found for each subject..

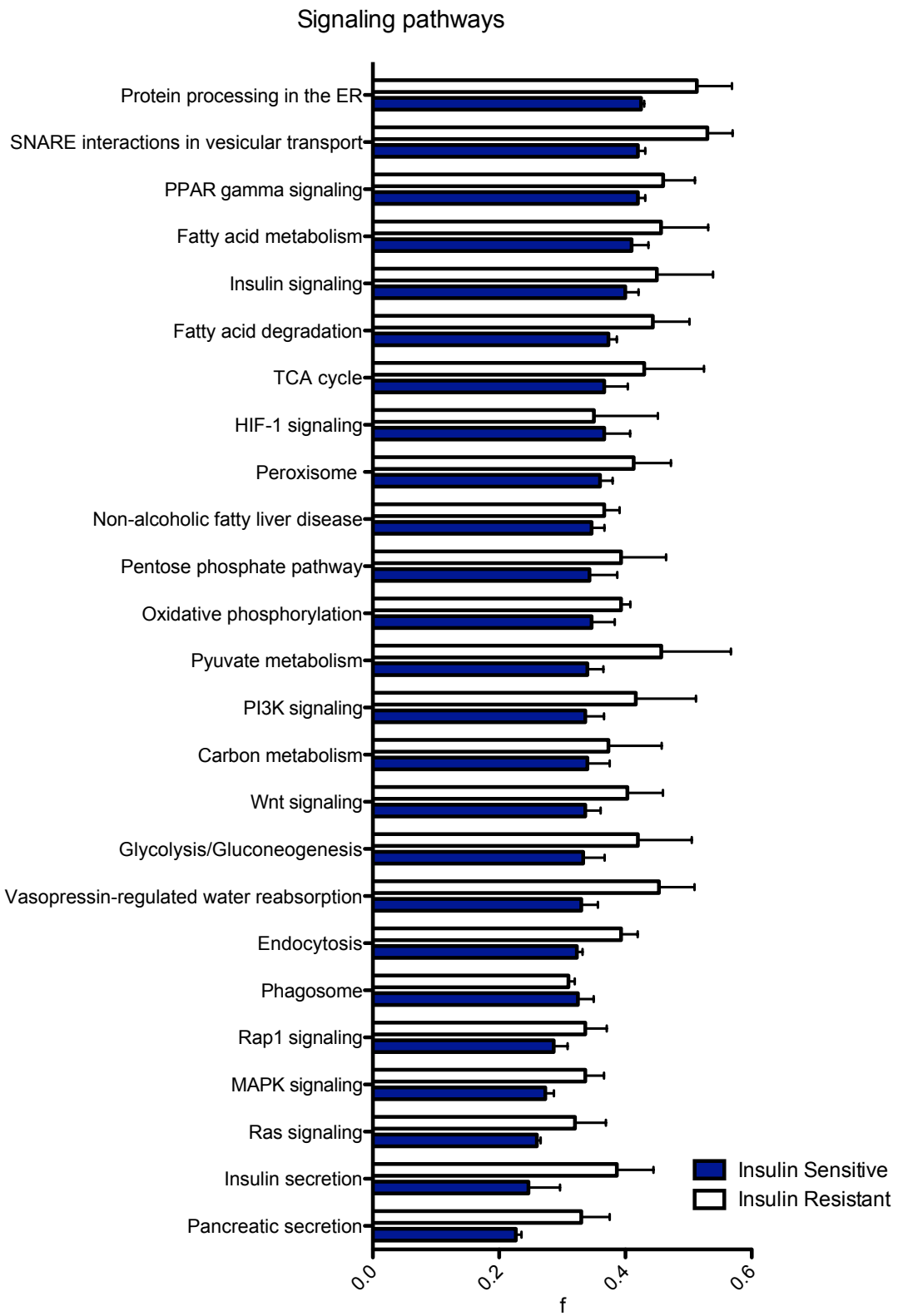



**Figure 2 :** Mean fractional synthesis rates in various pathways related to insulin action and metabolism, $p<0.0001$.

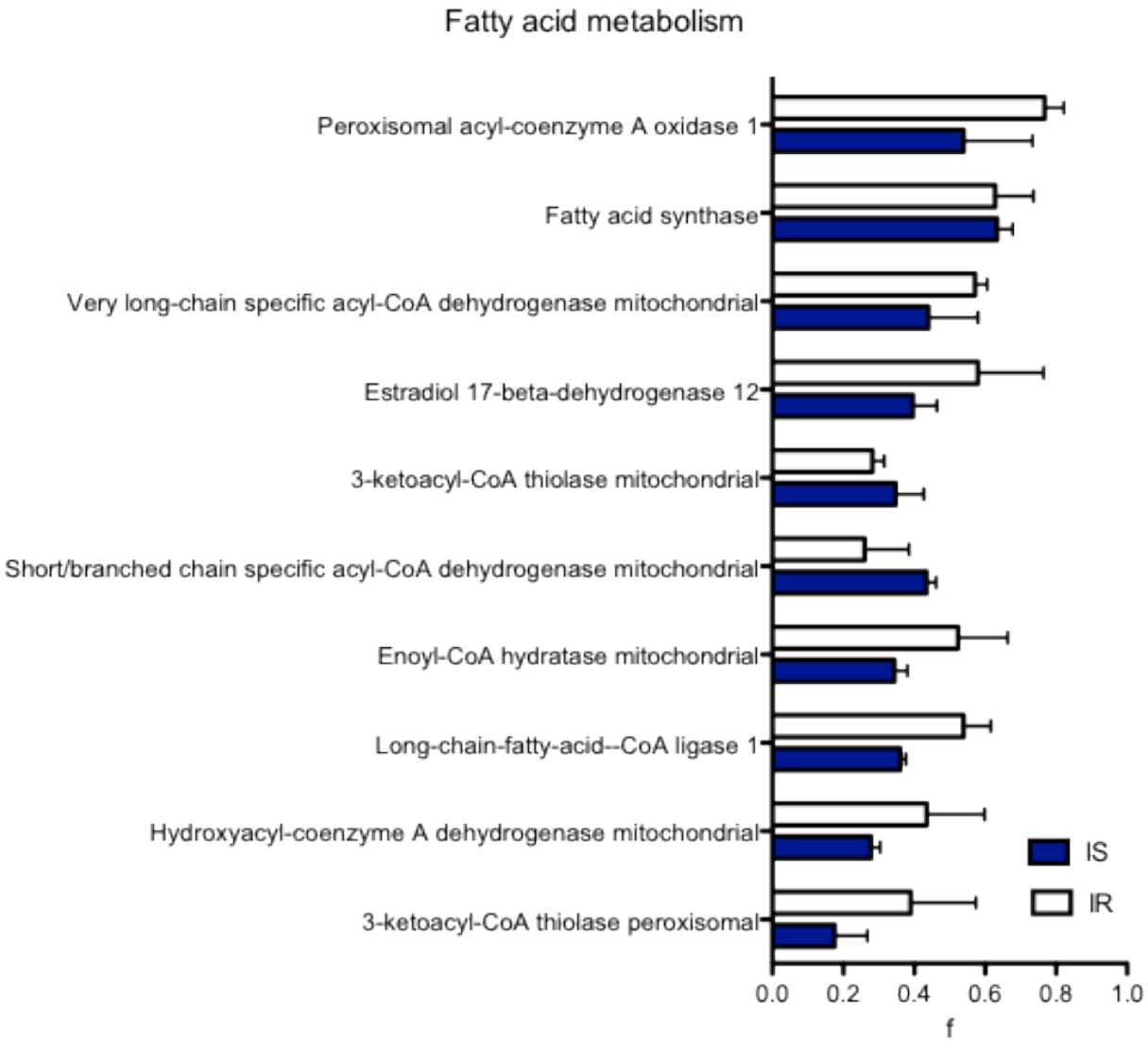



**Figure 3:** Lipid metabolism, p=0.0350.

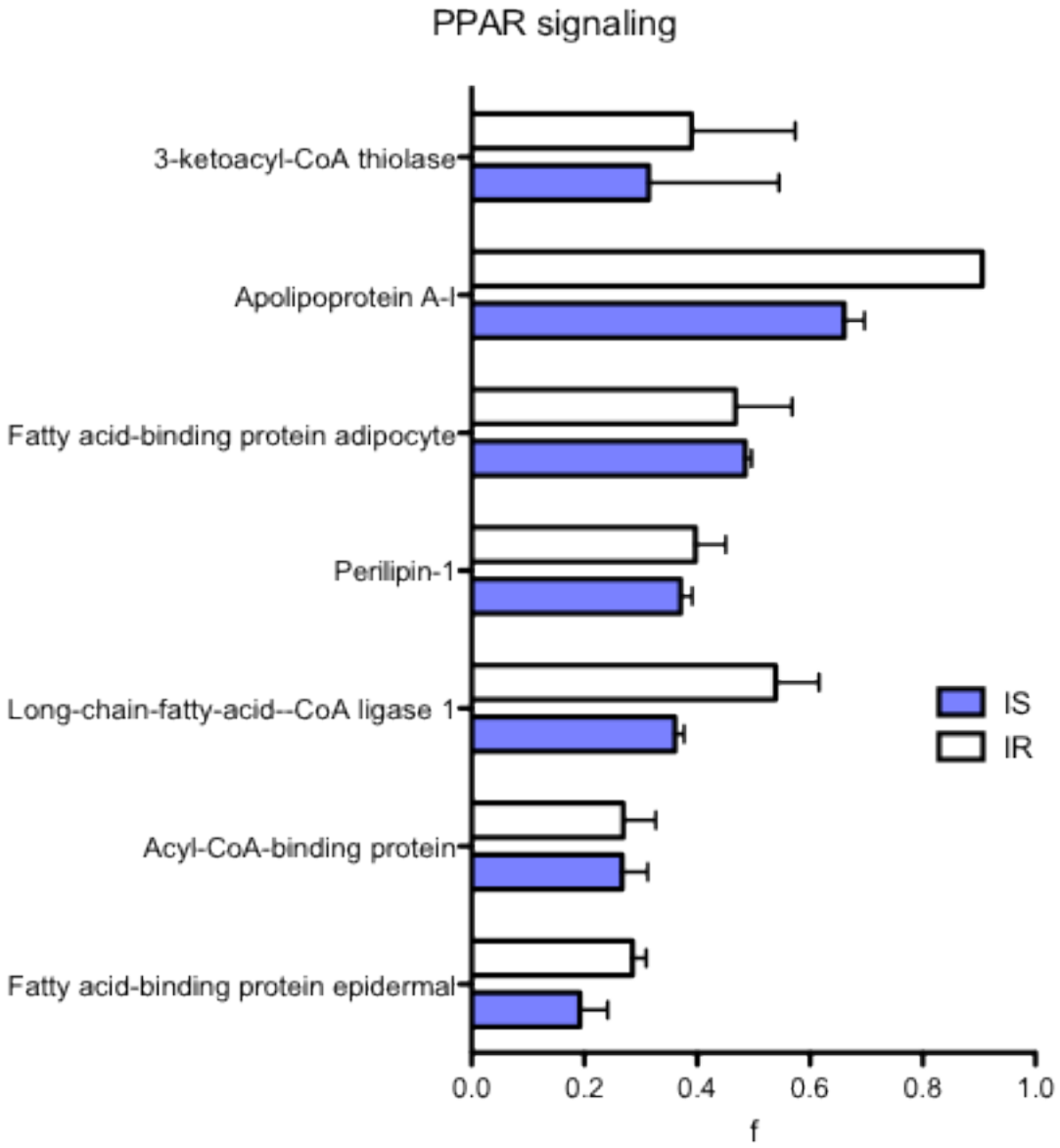



**Figure 4:** PPAR signaling pathway, p=0.0469.

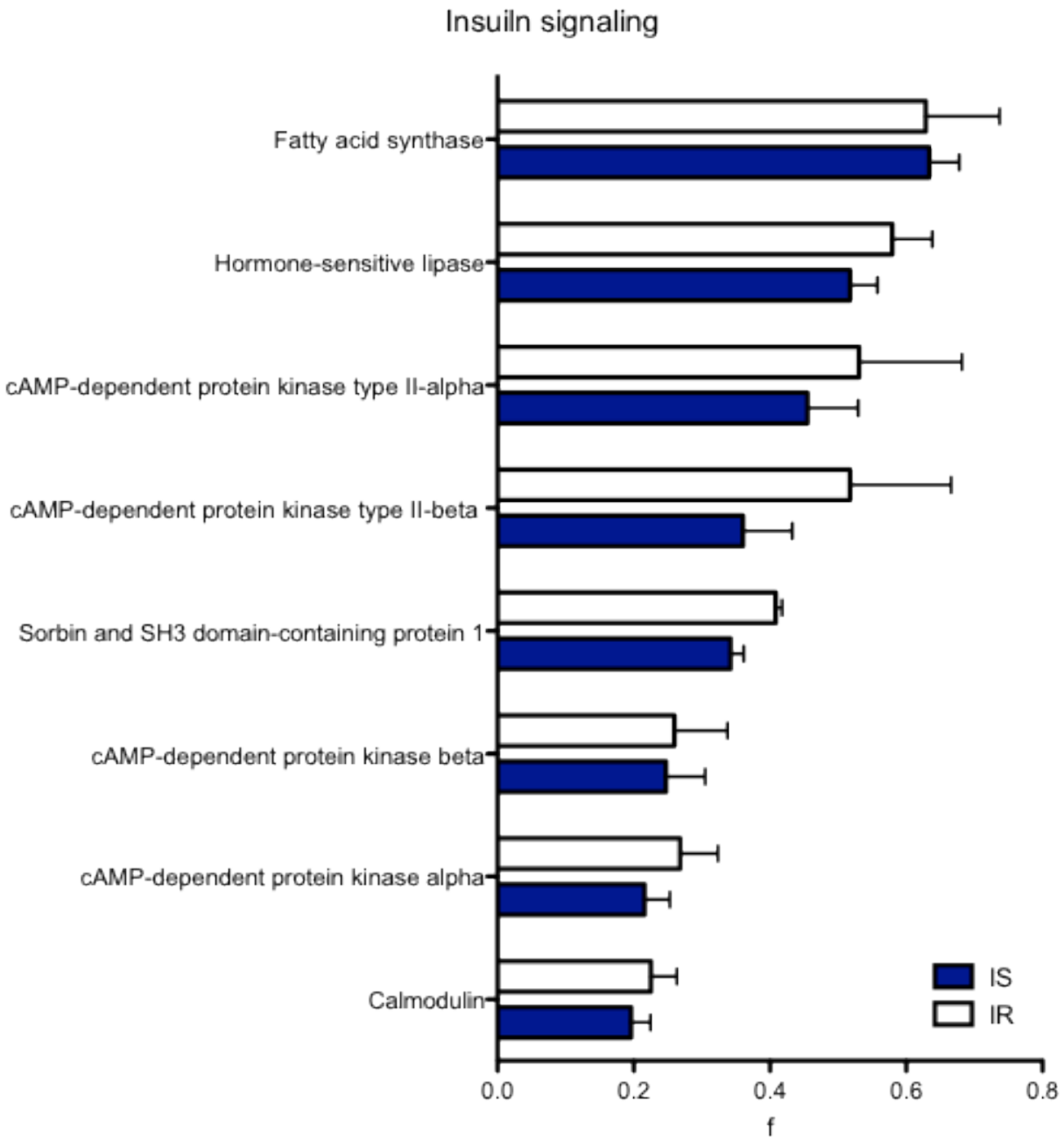



**Figure 5:** Insulin signaling pathway, p=0.1401.

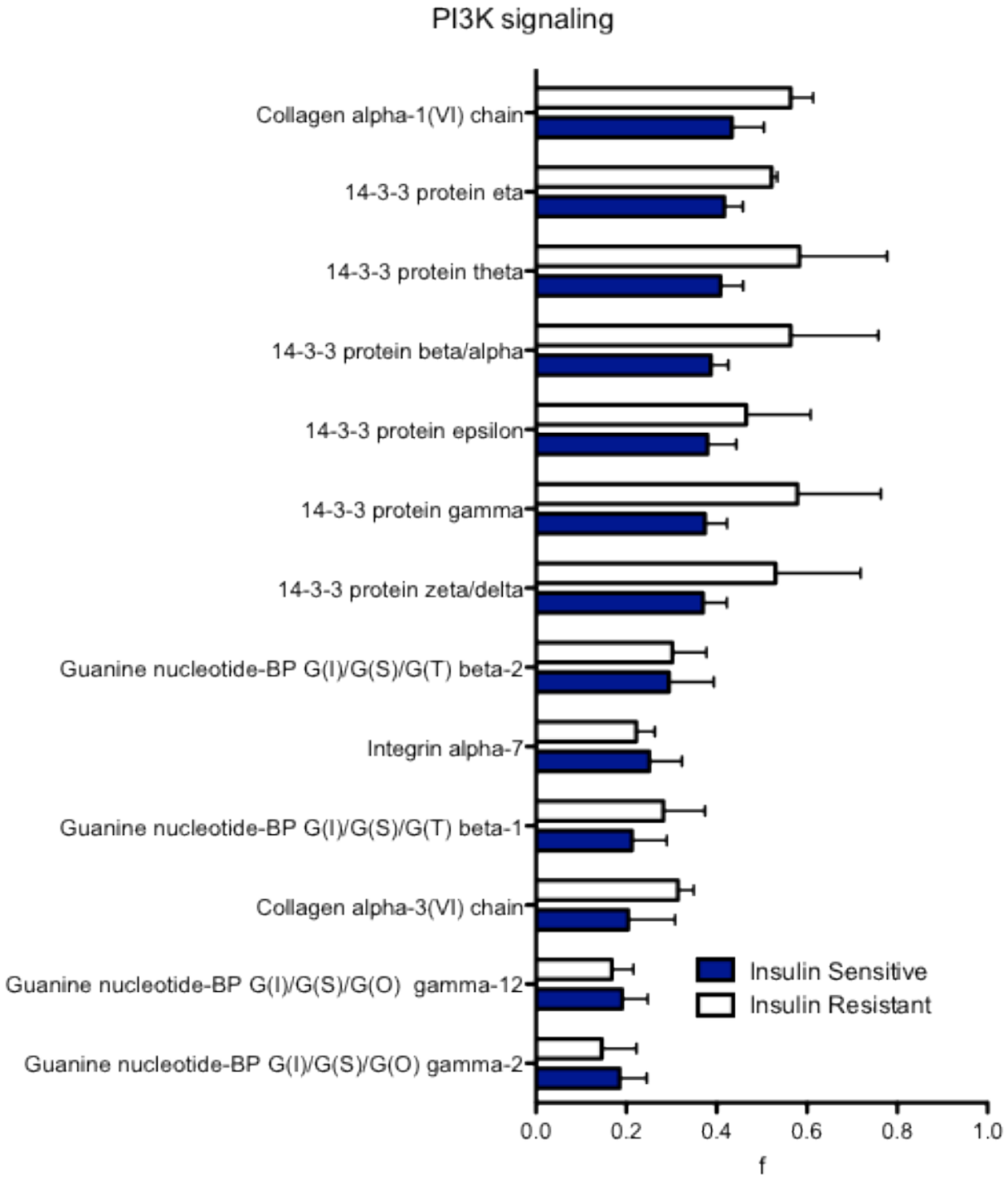



**Figure 6:** PI3K signaling pathway, p=0.0378.

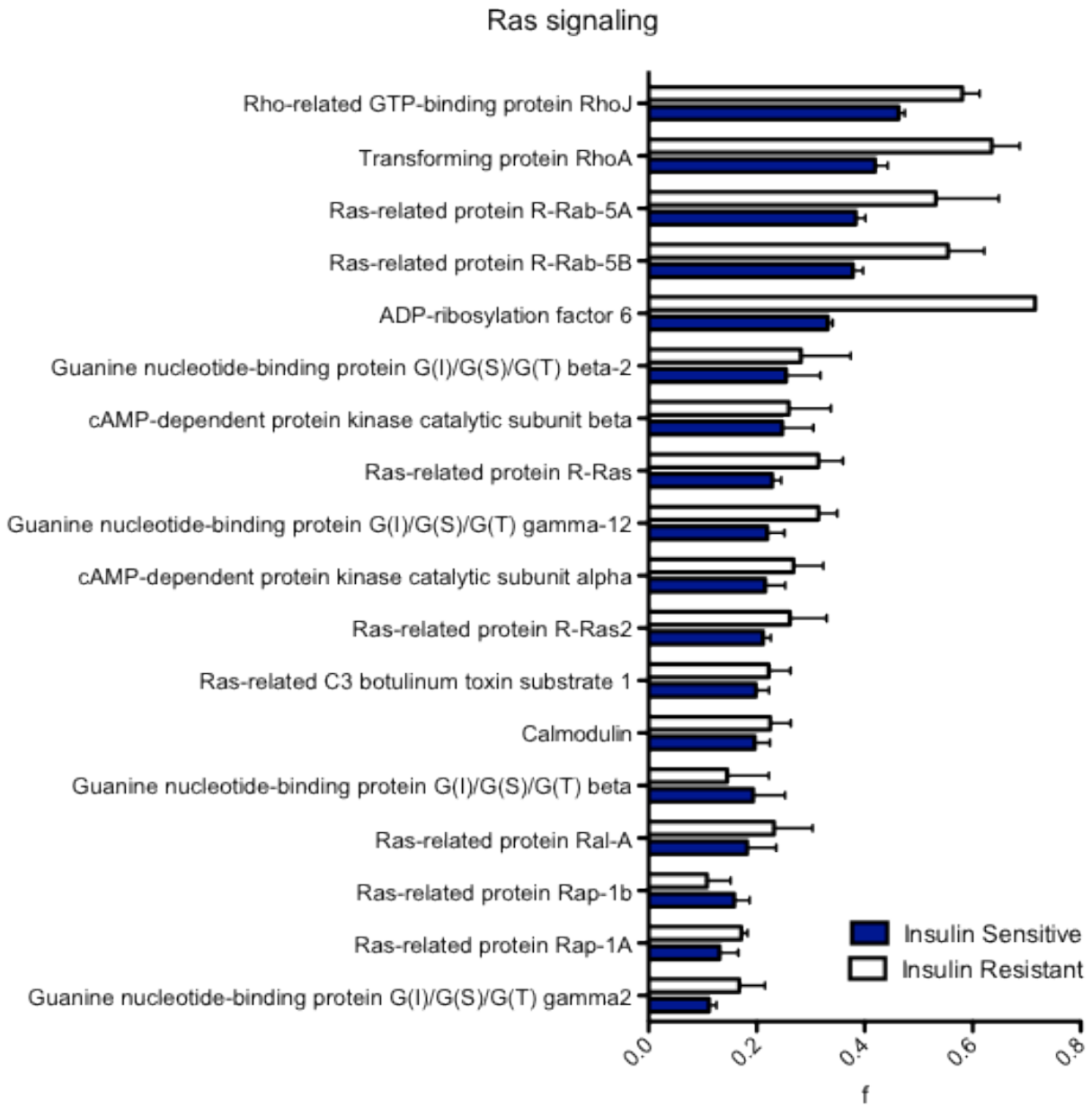



**Figure 7:** Ras signaling pathway, p< 0.0001.

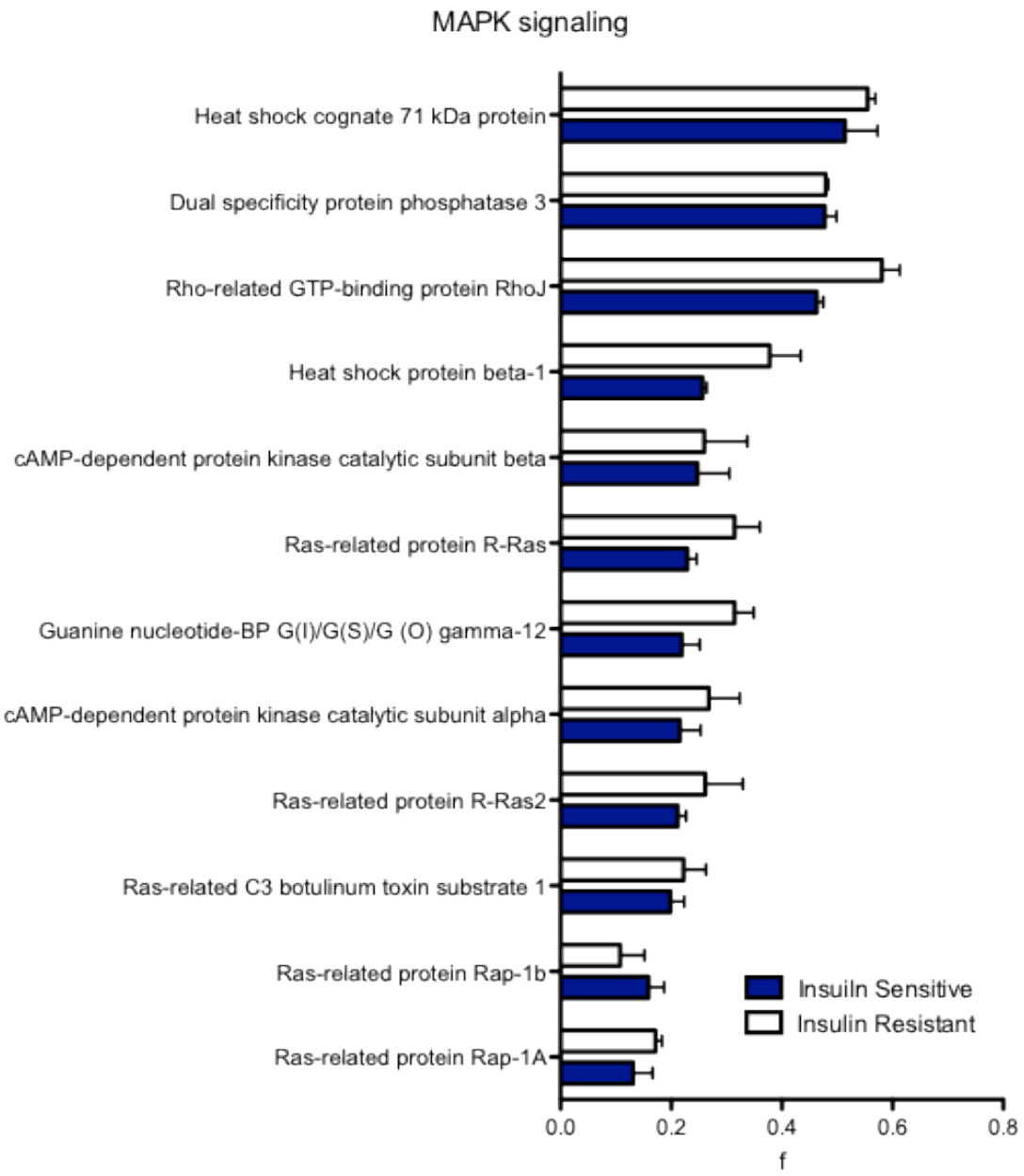



**Figure 8:** MAPK signaling pathway, p=0.0062.

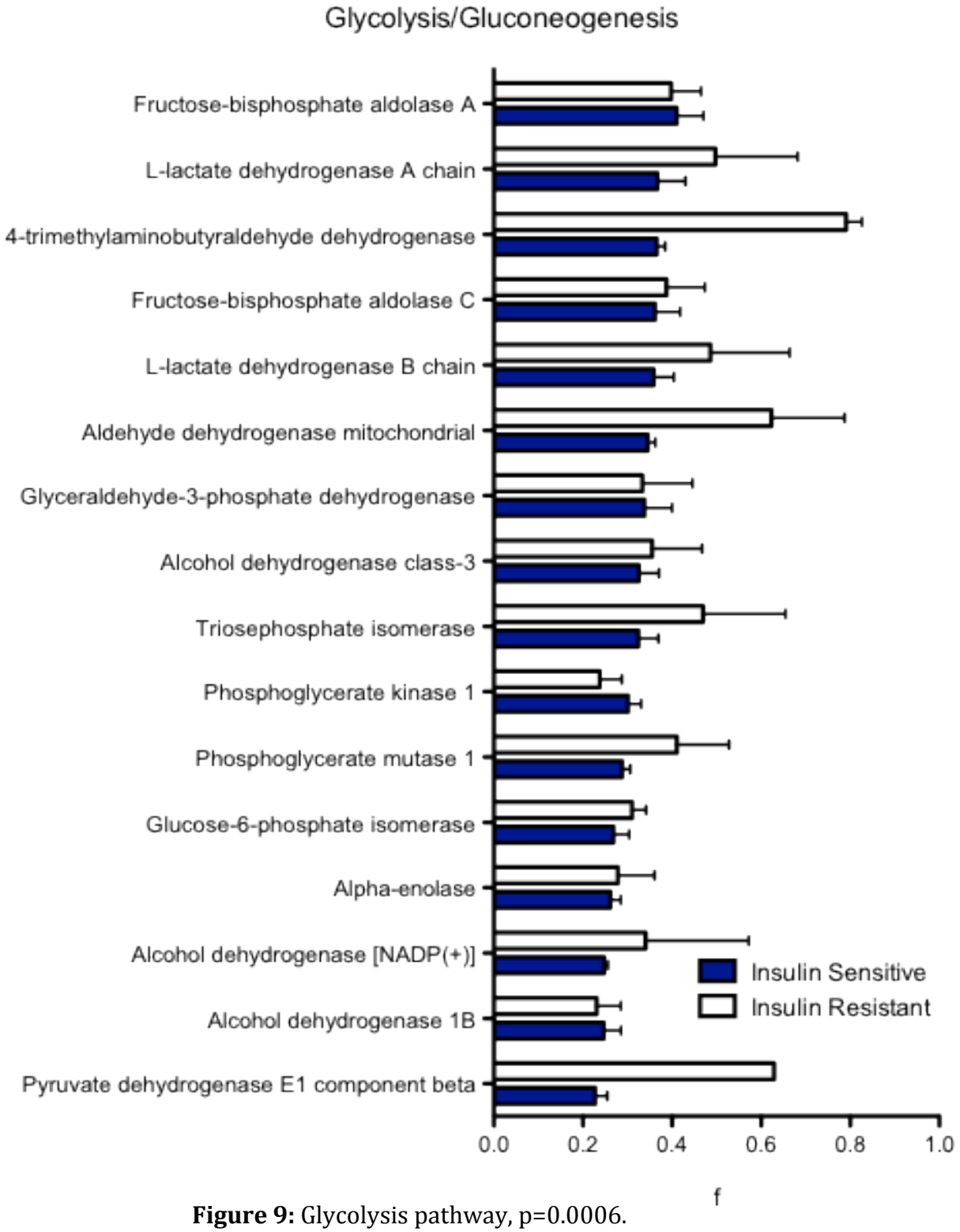



**Figure 9:** Glycolysis pathway, p=0.0006.

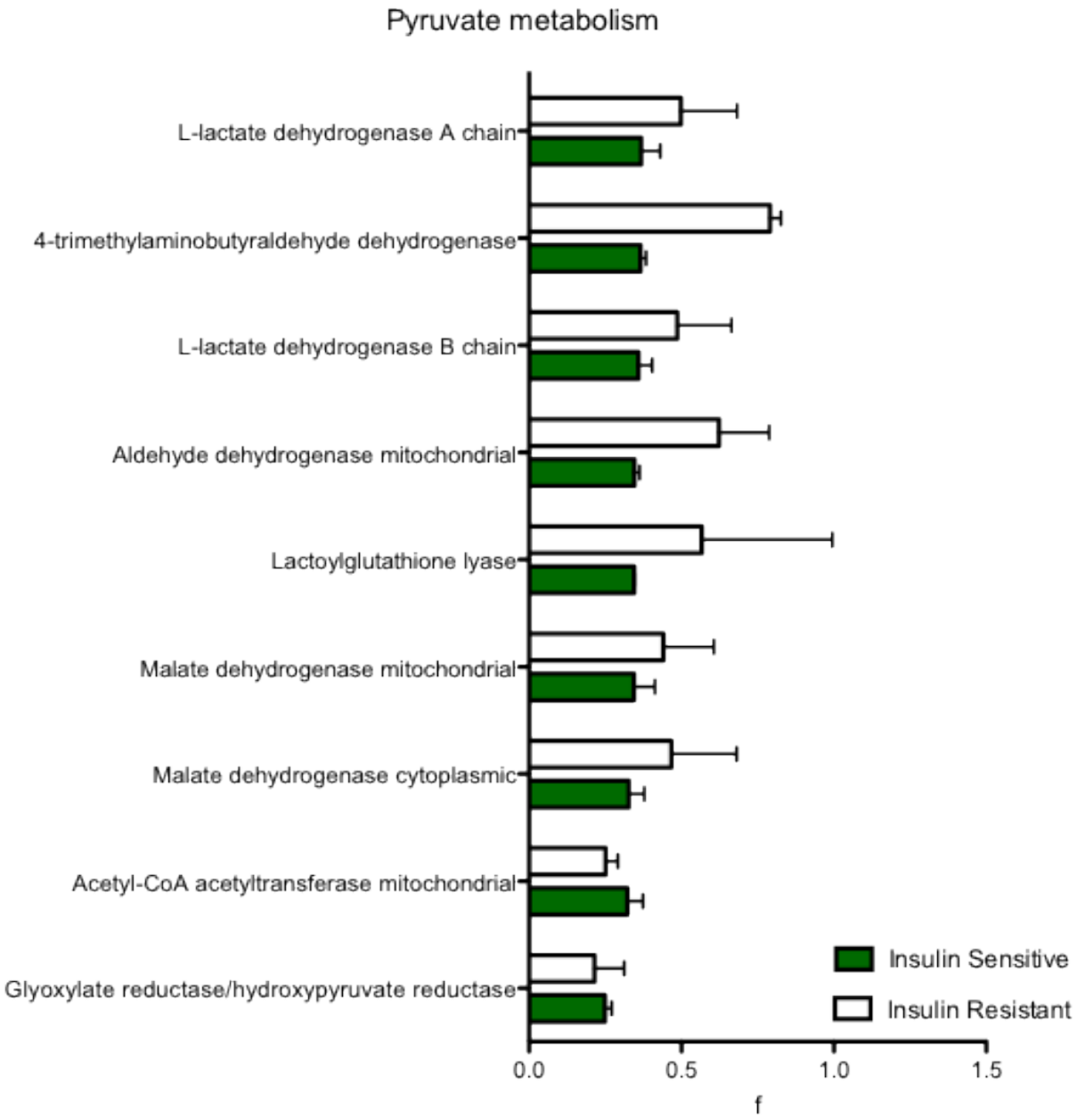



**Figure 9:** Glycolysis pathway, p=0.0006.

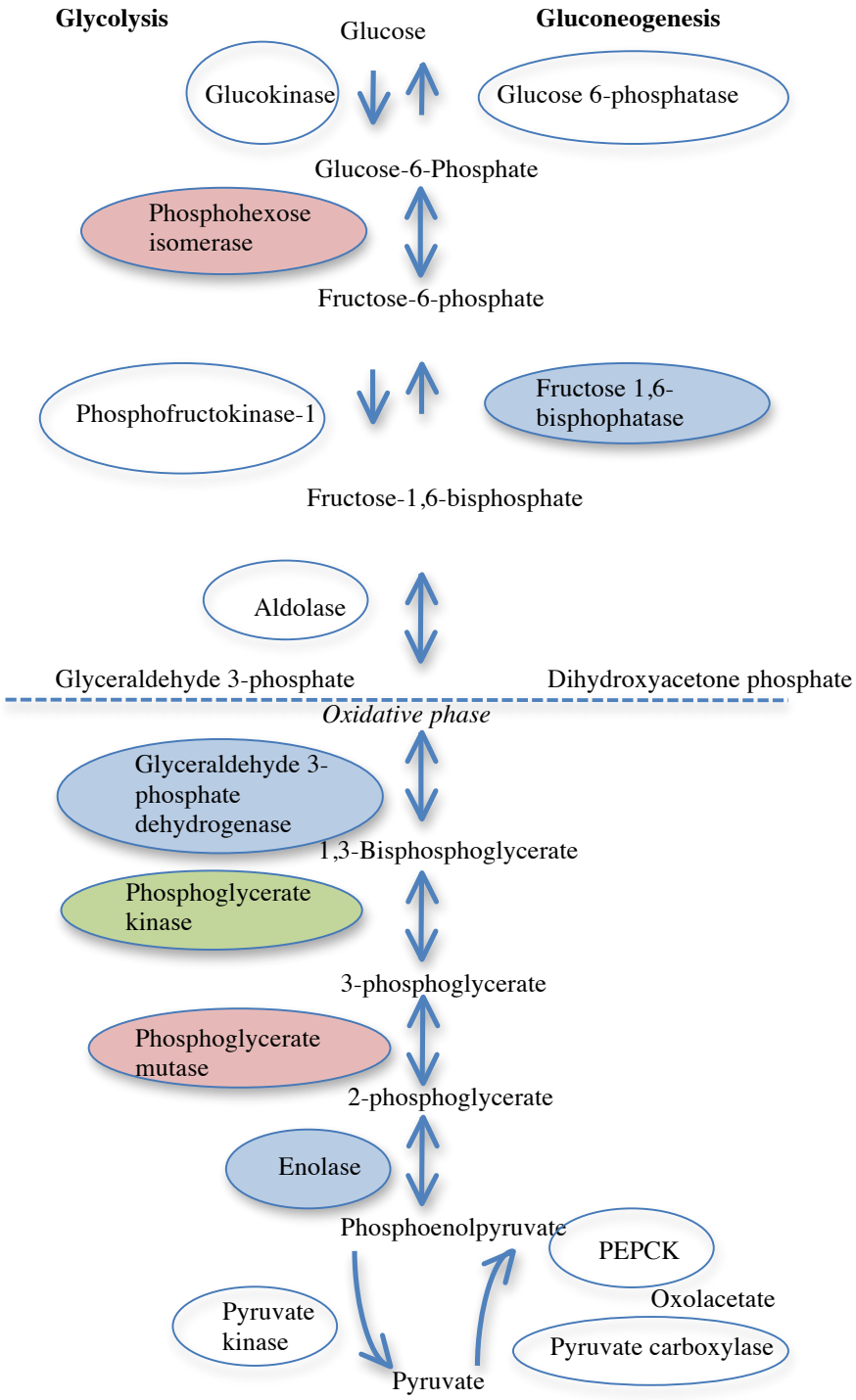



**Figure 11:** Glycolysis/Gluconeogenesis metabolic pathways schematic. Only proteins found in our dataset are highlighted in color. Proteins in red are those with greater fractional synthesis rates in IR compared to IS; green are those that are lower fractional synthesis rates in IR vs IS; blue represents no differences in

## Conclusions

Obesity continues to plague Americans, and if its prevalence increases, it will continue to remain the leading cause of death in the United States. Obesity is a major risk factor for the diabetes, cardiovascular disease and even cancer, with costs of such obesity-induced illnesses reaching well over 100 billion dollars a year. Therefore, treating, but more importantly, preventing obesity-induced type 2 diabetes and other diseases, will save our nation billions of dollars.

Type 2 diabetes is triggered by the loss of beta-compensation in the insulin resistant state, causing reduced synthesis and secretion of insulin and thus, hyperglycemia. Extensive research as shown that obesity may impair beta-cells, liver, muscle and as we show here, adipose function in response to insulin. Elevated free fatty acids that invade these tissues impair insulin signaling, leading to an overall decrease in efficient metabolism. Research has shown that different adipose storage depots may be responsible for lipotoxicity in insulin-responsive tissues, and our research here demonstrates that not only is it the type of depot, but also the function of cells within the depot that may trigger the onset of insulin resistance.

The four studies performed here aimed to investigate the molecular mechanisms of adipocyte function in the etiology of insulin resistance using $^{2}H_{2}O$ *in vivo*. Unlike most studies, we examined obese insulin sensitive and obese insulin resistant patients in order to remove obesity as the driving factor of potentially differing outcomes. The first study consisted of a predominately Caucasian cohort and provides one of the first studies providing *in vivo* evidence of adipocyte dysfunction in insulin resistant humans. Deuterated water directly quantified the rate of lipid synthesis in obese individuals, and demonstrated that those who were able to remain healthy, despite being obese, had greater TG synthesis and DNL in their subcutaneous abdominal adipose depot compared to obese IR. While there were differences in lipid synthesis, there were no differences in adipocyte or stromal vascular cell proliferation, indicating the inability to synthesize and store lipids may be largely due to an impaired insulin-This may also suggest that somehow increasing adipocyte proliferation in IR patients may reverse insulin resistance, allowing for the uptake and storage of free fatty acids into the subcutaneous adipose depot and away from the lipolytic visceral depot and ectopic sites. This is supported by observations of greater visceral fat, hepatic DNL, and fasting TG, in IR subjects. Evidence provided in this experiment suggests that adipocyte dysfunction triggers the onset of insulin resistance, and thus future preventative and treatment interventions should work towards preserving and enhancing abdominal subcutaneous adipose tissue function.

The second study was developed to put forth more attention towards investigating the etiological differences of insulin resistance in African-Americans as it compares to Caucasians. Current research has shown that despite being more insulin resistance than their Caucasian counterparts, African-Americans have less visceral and hepatic. This paradoxical phenotype has puzzled investigators as we work towards a deeper understanding of insulin resistance and type 2 diabetes on a global scale. It is now clear that obesity-induced insulin resistance may not be driven by the same molecular pathways in all individuals. Our use of $^{2}H_{2}O$ *in vivo* revealed great insight into the subcutaneous adipose of African-Americans, as it compares to Caucasians. Interestingly, African-Americans in our cohort had a greater volume of abdominal subcutaneous fat, yet less TG synthesis in this depot and no differences in visceral fat. Thus, it is possible that

African-American individuals included in our study either have greater deep subcutaneous fat storage, which may contribute to impaired insulin response in tissues. It is also possible that synthesis is down but re-esterification of already existing lipids is occurring. Another alternative is the consumption of a more lipid-rich diet, however food logs did not reveal any significant differences in macronutrient content amongst subjects. Because African-Americans in our study showed a trend towards greater thigh fat compared to Caucasians, it is possible that any newly synthesized fat may be occurring in the thigh. Given that the thigh is a subcutaneous depot, one might expect that a re-distribution to the thigh would be protective against insulin resistance. In fact, the majority of African-Americans in our study were actually insulin sensitive. Thus further studies examining adipose function and fat distribution in IR-only African-Americans should be explored. In summary, this pilot study is the first to provide *in vivo* quantification of lipid synthesis in the subcutaneous adipose tissue of a small cohort of African-Americans and potentially reveals a very different physiological mechanism of the development of insulin resistance in this minority, yet most at-risk population. It is important to note that our study does not provide a representation for adipose physiology of the African-American population as a whole, but rather includes a select few, and thus future studies would require a larger sample size to evaluate potential racial differences. Greater efforts should be made to perform *in vivo* studies to improve efforts in preventing and treating obesity in African-Americans.

The first two studies provided insight into mechanisms of insulin resistance in the steady state. However determining the physiological adaptations of obesity provide even greater understanding of metabolic switches in responses to excess caloric intake. Unlike most studies that measuring before and after effects of an intervention, our use of $^2H_2O$ allows to not only quantify cellular changes before an intervention but also changes that occurred during the intervention. By placing subjects on an overfeeding diet for 4 weeks with a weight gain goal of at least 4kg, we were able to quantify changes in adipocyte function in response to excess energy consumption. Unexpectedly, all of our IS patients became IR in response to overfeeding and this was matched by a lack of increased TG synthesis, DNL, adipocyte proliferation or SVC proliferation. These surprising results indicate that adipocytes in these IS patients were unable to respond to the demands of surplus energy storage needs, and thus showed a reduction in adipose-insulin action. Even more surprising was the decrease in hepatic DNL observed in both groups, an unusual finding based on evidence of enhanced hepatic lipid infiltration with weight gain and overfeeding. There was great variability amongst subjects in response to overfeeding, especially in the IS groups, suggesting adipose adaptations in response to overfeeding may vary amongst individuals. Nonetheless, our results demonstrate that metabolically healthy obese individuals can become insulin resistant under conditions of short-term weight gain. Further studies involving a larger sample would be of value to understand the physiological mechanisms responsible for the development of insulin resistance and understanding the metabolic adaptations of overfeeding.

Lastly, we used $^2H_2O$ to apply to dynamic proteomics, the large-scale measurement of protein synthesis, in adipocytes of insulin sensitive versus insulin resistant patients. A few studies have begun the innovative task of unveiling the proteome of adipocytes, yet none have measured the synthesis and breakdown rates of identified proteins. Furthermore, none have been able to quantify *in vivo* the dynamic proteome of adipocytes in obese IS and IR patients. Our study here, does just that and reveals unexpected differences in cellular pathways potentially involved

in insulin resistance. Our first study showed IS subjects had greater TG synthesis and DNL in their subcutaneous adipose tissue, compared to IR. Therefore, one might hypothesize that proteins involved in lipid metabolism would mimic this pattern, showing higher synthesis rates in IS versus IR. The opposite was observed in the majority of pathways chosen for analyses. Pathways such as Ras signaling, MAPK signaling, endocytosis, insulin secretion, glycolysis/gluconeogenesis, and pancreatic secretion showed a lower overall average rate of proteins involved in these pathways of IS subjects. While surprising, these findings may suggest cellular stress and compensation. Like the pancreatic β-cells in response to insulin resistance, dysfunctional adipocytes may upregulate protein synthesis in efforts to maintain metabolic homeostasis and normal adipocyte function. Similar to prolonging lifespan, a reduction in protein synthesis may preserve adipocyte function and thus protect against obesity-induced diseases. This is the first study revealing the dynamic proteome of adipose insulin resistance *in vivo* in humans. Further studies, involving larger sample sizes and different tissues would reveal tremendous molecular mechanisms responsible for the onset of insulin resistance.

**Summary**

In summary, these studies here are one of the first, if not the first, to provide *in vivo* physiological and molecular mechanisms of adipose insulin resistance. Our findings and future studies will lead scientists to creating innovative therapeutic options for insulin resistant and diabetic patients, allowing for a future of individually tailored treatment options. More importantly, our research will assist in the prevention of obesity-induced diseases, by informing individuals, physicians and even policy-makers of lifestyle changes that promote positive fat storage and healthy metabolic adaptations to protect against the ill fate of obesity.