Study Protocol

Project title: Protection against insulin resistance in obesity (PAIR-Study)
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BACKGROUND AND SIGNIFICANCE

Insulin resistance is a major contributor to the development of several obesity-related diseases. Despite the common link between obesity and insulin resistance, as many as one-third of obese adults appear to be "protected" against insulin resistance. Our preliminary findings indicate that insulin resistance can vary considerably even in a population of obese adults who are very similar in terms of the magnitude and distribution of their adiposity. **The overall goal of this project is to identify factors that "protect" some obese adults from becoming insulin resistant.** *Identifying mechanisms that help protect some obese adults from developing insulin resistance could lead to novel, targeted therapeutic and/or preventative strategies for obese adults who are insulin resistant*.

Excessive availability of fatty acids in the circulation (*which are largely derived from subcutaneous adipose tissue*) is one of the key factors underlying insulin resistance in obesity. Based on our preliminary findings, we contend that enhanced capacity for triglyceride storage in subcutaneous adipose tissue (thereby sequestering fatty acids - and attenuating their release) provides important protection against insulin resistance. This notion is not new, however, the specific metabolic events and/or cellular factors within subcutaneous adipose tissue that are responsible for more effective triglyceride storage are not known. *We hypothesize that obese adults who are protected against insulin resistance exhibit modifications in key factors that regulate adipose tissue structure and metabolic function (e.g., lipolytic regulation, adipogenic capacity (resulting in more, smaller fat cells), angiogenic capacity (enhancing capillarization), and fibrosis (altering extracellular matrix architecture))*

Although we contend that alterations in adipose tissue structure and metabolic function play a key role in determining insulin resistance in obesity, other factors obviously contribute as well. For example, because skeletal muscle is the predominant site of insulin-mediated glucose uptake, abnormalities in insulin signaling events within skeletal muscle also underlie systemic insulin resistance. Protein tyrosine phosphorylation (pTyr) plays critical role regulating key biological processes, including insulin signaling. Using state-of-the-art mass spectrometry-based proteomics, we can quantify hundreds of pTyr sites simultaneously, and by doing so we anticipate discovering novel pTyr targets that may differentiate insulin sensitive vs. insulin resistant obese adults. These findings will help identify new molecular mechanisms underlying insulin resistance, and will provide novel targets for preventing and treating type 2 diabetes and other obesity-related diseases.

In this project, we will measure insulin-mediated glucose uptake in a homogeneous cohort of abdominally obese adults, and then stratify them into three distinct groups based on their degree of insulin resistance. Our main comparisons will be made between the most- and least-insulin resistant groups.

SPECIFIC AIM #1: *Compare factors regulating triglyceride storage and adipogenesis in subcutaneous adipose tissue from insulin-sensitive obese adults compared with a well-matched cohort of insulin resistant subjects.*

Hypothesis 1: Compared with adipose tissue from obese adults who are insulin resistant, adipose tissue from those who remain relatively insulin sensitive will have: a) lower abundance and basal activity of lipolytic enzymes b) higher abundance and basal activity of esterification enzymes, c) greater anti-lipolytic response to insulin (measured *in vivo* and *in vitro*), d) greater expression of adipogenic markers (e.g., PPARγ and C/EBPα), e) elevated adipogenic capacity (measured *in vitro*), f) higher adipocyte cell density (cells/cm^2), g) greater angiogenic capacity (measured *ex vivo*), and h) less adipose tissue fibrosis.

SPECIFIC AIM #2: *Identify other important and novel factors that contribute to the protection against insulin resistance in some obese adults.*

Hypothesis 2A: Targeted analyses in adipose tissue and skeletal muscle will reveal that specific markers of: a) macrophage infiltration, b) pro-inflammatory cytokine/adipokine abundance, and c) skeletal muscle lipid accumulation, as well as d) hepatic lipid accumulation, will be lower in obese adults who are relatively insulin sensitive compared with a well-matched cohort of insulin resistant subjects.

Hypothesis 2B: Unbiased proteomic analysis in skeletal muscle and adipose tissue will reveal novel differences between obese adults who are insulin sensitive compared with insulin resistant obese adults

RESEARCH DESIGN AND METHODS

Overall strategy

- We will recruit a cohort of abdominally obese adults who will be very similar in terms of body mass index (BMI), fat mass, fat free mass, waist circumference, and habitual physical activity behavior (sedentary).
- We will measure insulin sensitivity (hyperinsulinemic-euglycemic clamp) and then stratify the subjects into 3 groups based on their degree of insulin resistance. 1) "***Insulin resistant***" [**IR**], 2) "***Mildly insulin resistant***" [**MIR**], and 3) "***Non-insulin resistant***" [**NIR**]. The "cutoff points" used for stratifying our subjects into these 3 groups were defined by Stern et al., (25).
- We will also measure systemic fatty acid kinetics (stable-isotope dilution methods) and we will collect subcutaneous adipose tissue biopsy samples.
- In these adipose tissue samples, we will perform extensive, yet very relevant and integrated analyses of factors regulating fatty acid storage and mobilization, adipogenesis, fat cell size, capillarization, angiogenesis, and fibrosis.
- We will also collect skeletal muscle biopsy samples to assess factors in muscle that may also be different in insulin resistant [**IR**] and non-insulin resistant [**NIR**] obese adults.
- Several other important phenotypic measures will be performed to help characterize the subjects (e.g., subcutaneous and visceral adiposity [MRI analysis], , hepatic lipid accumulation accumulation and degree of hepatic fibrosis [MRI analysis], basal metabolic rate, blood lipid profile)
- Our primary comparisons to assess factors contributing to protection against insulin resistance will be performed on the most-insulin resistant [**IR**] and least-insulin resistant [**NIR**] groups, but we will also perform correlational analyses using the entire subject population.

Subjects

A total of 60 obese, pre-diabetic women [n=30] and men [n=30] will be recruited for this study. Eligible subjects will be homogeneous in terms of the magnitude and distribution of their adiposity (BMI: 30-40 kg/m^2; waist circumference: 88-98cm for women and 100-110cm for men), age (18-45 years old), and physical activity behavior (sedentary). All subjects must be weight stable (±3kg for ≥ 6 months) and they will be screened with a detailed health history survey. Subjects will not be taking medications known to affect lipid or glucose metabolism. Anyone with evidence and/or history of cardiovascular or metabolic diseases will be excluded. All women will be pre-menopausal and will have regularly occurring menses. To avoid potential confounding hormonal effects, all women will be studied in the early follicular phase of their menstrual cycle.

Based on past experience, we anticipate that approximately 50% of the subjects who sign an informed consent document before undergoing our more involved screening procedures will not participate in this study (largely due to being deemed ineligible after screening or because of personal reasons). Therefore, we anticipate a total of 120 subjects will sign our informed consent document.

Inclusion Criteria

- Body mass index [BMI]: 30-40 kg/m^2
- Age: 18-45 years
- All women must be pre-menopausal
- Non-exerciser: no regularly planned exercise/physical activity

Exclusion Criteria

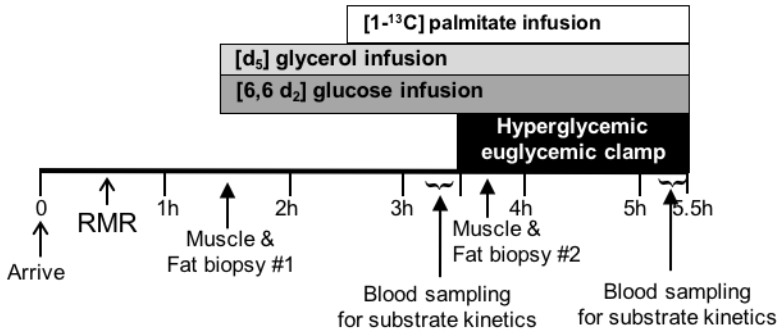
- Weight instability ≥ ±3kg ≥ 6 months
- Medications known to affect lipid and/or glucose metabolism
- Pregnancy or actively breast feeding

Additional preliminary testing will be performed on subjects deemed eligible after initial screening visit

- Physical activity questionnaire
- Urine pregnancy test for female subjects
- Body composition assessment (DEXA)

Detailed clinical study procedures

Subjects will complete a physical activity questionnaire and a three day food journal before their hospital visit. On the morning of the study, subjects will be admitted to the Michigan Clinical Research Unit (MCRU) at 0700h. Subjects will arrive at the Michigan Clinical Research Unit (MCRU) in the University of Michigan Medical Center in the morning after an overnight fast. After arrival, subjects will rest quietly in their room for 30 min. We will then measure their resting metabolic rate (RMR) using a canopy-equipped metabolic cart (Vmax metabolic cart; Viaysis, Inc.) for 20min. After RMR measurement, intravenous catheters (IVs) will then be placed in a forearm or hand vein of both arms. Approximately 1.5h after admission to the MCRU, we will begin primed, stable isotope infusions of [6,6 d_2]glucose (to assess the rates of hepatic glucose production and systemic glucose uptake) and [d_5]glycerol (to assess whole body lipolytic rate). At the same time we begin the tracer infusions, we will collect skeletal muscle (vastus lateralis) and subcutaneous abdominal adipose tissue biopsy samples. Approximately 2.5h after admission we will begin an infusion of [^{13}C]palmitate (to assess the rate of systemic fatty acid mobilization). Three blood samples will be collected in five minute intervals starting ~50min after beginning the [^{13}C]palmitate infusion. Immediately, after collection of these blood samples we will begin a hyperinsulinemic-euglycemic clamp (insulin infusion rate = 40 mU/m^2/min) to assess insulin resistance, as described previously by Defronzo, et al. (6). During the clamp procedure we will collect small blood samples every 5 minutes to assess blood glucose concentration and the glucose infusion rate will be adjusted as necessary to maintain euglycermia, as described previously (6). Thirty minutes after starting the hyperinsulinemic-euglycemic clamp procedure we will collect another skeletal muscle and adipose tissue biopsy samples to acquire a sample in the insulin stimulated condition. Finally, we will collect 5 blood samples during the final 20 minutes of the clamp. Detailed descriptions of these procedures can be found in our previous publications (8, 9, 16, 23), and blood sampling timeline is provided in Appendix 1 of this document.



(**Hours on the timeline refer to time after admission**)

We will also measure percent body fat using a Dual Energy X-ray Absorptiometry [DEXA: Lunar DPX, DEXA Scanner] and We also plan to measure hepatic fibrosis, the amount of fat stored in the liver, intra-abdominal adiposity (i.e., visceral adiposity), and visceral fat (or intra-abdominal fat) using magnetic resonance imager (MRI) with elastography – but depending on scheduling, these tests may be performed on a separate occasion.

The blood collection during the clinical trial will be conducted in the Michigan Clinical Research Unit (MCRU), which is located in room 1111 of the Cardiovascular Center (CVC). The clinical suite is a research unit, specifically designed for performing clinical procedures in human subjects. The suite contains a padded patient treatment table for blood draws, IV placements and biopsies. A television is mounted on the wall for participant's entertainment during the testing procedures.

The intravenous (IV) catheter placement and blood draws will be performed by Jeff Wysocki, RN, Dr. Horowitz, or a qualified member of Dr. Horowitz's research team. Horowitz research staff members, Alison Ludzki, Suzette Howton, Benjamin Ryan, and Michael Schleh have completed Phlebotomy training offered through MLearning (supervisor: Christine Conrad, MSN, RN, CNS-BC, OCN), and they are trained and qualified to perform venipuncture blood draws.

Dr. Burant will provide clinical oversight/responsibility for this study. In general, Dr. Burant will not be present in the MCRU during the testing, but he will be alerted in advance to when all testing will be occurring, he will be available for immediate consultation by phone, and available to come to the MCRU within ~15 min to attend to any concerns that may need his direct attention. In the event Dr. Burant is unavailable/out of town during a testing day, arrangements will be made with either Drs. Kraftson, Rothberg, Oral, or Carr to provide direct clinical oversight during that particular experiment.

SOP for Skeletal Muscle and Subcutaneous Adipose Biopsies

Screening and pre-visit procedures

• Anti-coagulant medication (e.g., Coumadin, Rivaroxaban) and Lidocaine allergy/sensitivity are exclusion criteria for the biopsy procedure.

• Aspirin (ASA) and/or other nonsteroidal anti-inflammatory medications (NSAIDS; e.g., Ibuprofen, Naproxen) must be avoided for 3 days before the biopsy procedure (Tylenol can be used)

Before performing the tissue biopsies, Dr. Horowitz (or his qualified co-investigator who is performing the biopsies) will:

1) confirm the participant's identification
2) thoroughly explain the biopsy procedures to the participant, describing what they may experience during the procedures.
3) confirm the location of the procedures (e.g., left or right thigh for muscle biopsy - and left or right of the umbilicus for the adipose tissue biopsy/aspiration

Skeletal Muscle Biopsy Procedure

• Confirm that the participant does not have any known allergies/sensitivity to local anesthetic (e.g., Lidocaine/Bupivacaine) – this should also have been addressed before/during informed consent process.

• If necessary, shave ~5" x ~5" square above the vastus lateralis

• Put on surgical gown, mask and bonnet (assistants in procedure room also must wear mask and bonnet)

• Disinfect skin above the vastus lateralis with betadine

• Put on sterile gloves and all the following procedures must be performed using aseptic technique

• Create sterile field on tray top and around the betadine covered area of the participant's thigh

• Infiltrate skin and underlying tissue with 2% lidocaine using a 25g x 1.5" needle (~6-10 ml of lidocaine: total dose of lidocaine not to exceed 4.5mg/kg)

• Make an incision above the vastus lateralis with a #10 scalpel (cut skin and try to cut fascia if possible)

• Apply pressure to incision site with sterile gauze until bleeding stops (or is very light)

• While pressure is being applied to the thigh, the biopsy assistant attaches a 3-way stopcock to a 60cc syringe

• One end of sterile pressure tubing is attached to the sterilized biopsy needle (4-5g Bergstrom or UCH biopsy needle), and the other end of the tubing is handed to the assistant to attach to their 3-way stopcock/60cc syringe set-up for suction.

• The biopsy needle is inserted into the incision with firm pressure. The biopsy needle is inserted past the fascia, in the location/direction of the applied anesthetic

• Once the biopsy needle is fully inserted, the biopsy assistant pulls back firmly on the plunger of the 60cc syringe to apply steady suction. The sample is collected by closure of the biopsy needle – To increase sample yield, the biopsy needle can be quickly rotated then opened and closed 3-5 times to obtain a few "snips" of sample before removing the biopsy needle from the thigh.

• The sample is placed on a sterile absorbent pad, and pressure is applied to the biopsy site with sterile gauze

• The biopsy assistant quickly rinses/cleans the sample with saline and gently dabs the sample on the absorbent pad to remove excess liquid. The sample is placed in collection vial - and placed in liquid N2.

• While assistant is cleaning and storing the sample, manual pressure is applied to the incision site

• The biopsy procedure can be repeated in the same incision site (or separate preprepared site) if more tissue is needed.

• When the entire biopsy procedure is finished, firm direct pressure is applied to the incision site until bleeding stops (~10 minutes)

• Incision site is cleaned with 70% ethanol and dried with sterile gauze

• The incision is closed with steri-strips and an overlaying Tegaderm bandage (with absorbent pad)

• A pressure dressing is applied to the thigh until bedtime the night of the biopsy procedure

• Participant is instructed to avoid water submersion for 2 days and to keep the bandages on for 5 days.

• Upon discharge, provide participant with pre-printed post-procedure care instructions with emergency contact information.

Subcutaneous Adipose Biopsy Procedure

• Confirm that the participant does not have any known allergies/sensitivity to local anesthetic (e.g., Lidocaine/Bupivacaine) – this should also have been addressed before/during informed consent process.

• Clean the anterior abdominal wall in the periumbilical area with betadine
• Put on sterile gloves and all the following procedures must be performed using aseptic technique
• Create sterile field on tray top and around the betadine covered area of the participant's abdomen
• The location of the incision will be 5-10cm lateral to the umbilicus – infiltrate the skin and underlying tissue in this location with ~1-2ml of 2% lidocaine using a 25g x 1.5" needle. Then, with the lidocaine needle inserted at the intended incision site direct the needle at ~20 degree angle with the abdomen and anesthetize a quadrant of the abdomen with a 1.5" radius between the intended incision site and the umbilicus (this will anesthetize the sampling region). Total lidocaine dose for abdomen = 5-8ml of 2% lidocaine; total dose of lidocaine must not exceed 4.5mg/kg – this includes any lidocaine administered for any other procedures during the test day).
• Make an incision in this anesthetized site (5-10cm lateral to the umbilicus) with #10 or #11 scalpel
• Apply pressure to incision site with sterile gauze until bleeding stops (or very light)
• While pressure is being applied to the abdomen, the biopsy assistant attaches a 3- way stopcock to a 60cc syringe
• One end of sterile pressure tubing is attached to the sterilized biopsy needle (4-5g Bergstrom or UCH biopsy needle), and the other end of the tubing is handed to the assistant to attach to their 3-way stopcock/60cc syringe set-up for suction.
• For collection of the core tissue sample, insert the biopsy needle (4-5g Bergstrom or UCH biopsy needle) into the incision, directed at ~20 degree angle with abdomen. The biopsy needle is inserted ~2cm in the direction of the anesthetized area (toward the umbilicus).
• Once the biopsy needle is inserted ~2cm, suction is applied to the needle (by an assistant) via an attached extension tubing and 60cc syringe. The sample is collected by closure of the biopsy needle – and extracted.
• The sample is placed on a sterile absorbent pad. The biopsy assistant rinses and cleans the sample with saline and gently dabs the sample on a on absorbent pad to remove excess liquid
• The sample is placed in collection vial (typically containing formalin).
• The biopsy procedure can be repeated in the same incision site if a larger core sample is needed.
• After completing the core sample collection, more tissue can extracted using aspiration.
• For the subcutaneous adipose tissue aspiration method, attach a sterilized aspiration needle (e.g., Spirotri cannula) to sterile pressure tubing, attached to sterile 60cc syringe with stopcock attached.
• Insert aspiration needle into incision site (~3cm), retract plunger, and apply constant negative pressure with the syringe during the aspiration process.
• With needle inserted into this incision site, one hand lightly pinches the surface of the biopsy site, while the other hand gentle maneuvers the biopsy needle under the skin for 30-60 seconds to extract tissue (tissue will accumulate in the syringe)
• Remove aspiration needle from incision site –
• The extracted tissue can be removed from the syringe either by ejecting through the tip of the syringe – or by removing the syringe plunger and pouring the tissue out. In both cases the tissue should be placed on a sterile absorbent pad for the biopsy assistant to clean and process the sample.
• Aspiration procedure can then be repeated in the same incision, but try to direct the needle in a slightly different "track" within the anesthetized area in order to avoid tissue collection within exactly the same area.
• After completion of all tissue extraction, pressure should be applied to the biopsy site for 5-10 min. • Clean biopsy site with alcohol, place steri-strip on site to close the small incision, and tegaderm bandage is then placed over the steri-strips.
• Apply ice to biopsy site for 10-20 min
• Participant is instructed to keep the bandages on for 5 days.
• Upon discharge, provide participant with pre-printed post-procedure care instructions with emergency contact information.

As an alternative approach, a 1.5in x 16g needle or a "Coleman needle" may be used for tissue aspiration instead of the Spirotri cannula, under the same conditions as described above. However, when using these alternative needles, more aspiration "passes" may be required to extract the necessary amount of adipose tissue.

Diets for clinical study: Subjects will be advised what they should be eating in the 3 days leading up to their hospital visit to maintain energy balance (total daily energy intake will be approximated to be 370 + (21.6* fat free mass)] x 1.5), with a macronutrient content of approximately 55% carbohydrate, 30% fat, and 15% protein, which represents the macronutrient content of a "typical" western diet (2). On the experimental day, subjects will arrive in the overnight fasted state and will not eat anything until completion of the clamp procedure.

Subject stratification

As noted above, subjects will be stratified into 3 groups based on their degree of insulin resistance, as defined by their glucose disposal rate (GDR) measured via hyperinsulinemic/euglycemic clamp during the experiment.

1) "***Insulin resistant***" [**IR**] (GDR < 28μmol/kg fat free mass [FFM]/min),
2) "***Mildly insulin resistant***" [**MIR**] (GDR > 28μmol/kg FFM/min and < 40μmol/kg FFM/min)
3) "***Non-insulin resistant***" [**NIR**] (GDR > 40μmol/kg FFM/min).

The "cutoff points" used to stratify our subjects into these 3 groups were defined by Stern et al., (25). Stern, et al., used a statistical approach using hyperinsulinemic-euglycemic clamp data from 2,138 ethnically diverse non-diabetic subjects (insulin infusion rate = 40 mU/m^2/min, which is the same as we propose here). They defined the "optimal insulin resistance cutoff" as being < 28 μmol/kg FFM/min. We will make every effort to closely balance subject characteristics across the three study groups (including sex).

Primary phenotype measurements for Specific Aim #1

During the clinical study outlined above, we will perform several clinical and sub-clinical assessments to help characterize our subject population.

Insulin resistance will be assessed during the hyperinsulinemic-euglycemic clamp. The primary measure of insulin resistance will be calculated as the steady-state rate of glucose infusion (GDR) during the 20 min period at the end of the clamp. We will also assess hepatic insulin resistance (i.e., magnitude of reduction in hepatic glucose production (HGP) in response to insulin) using stable isotope tracer methods, and peripheral tissue insulin resistance (i.e., difference between whole-body GDR and HGP). Detailed methods for this procedure, including methods used to quantify the tracer-tracee ratio (TTR) for d_2-glucose using gas chromatography/mass spectrometry (GC/MS) can be found in our recent publication (16).

In vivo rates of lipolysis and fatty acid mobilization in the systemic circulation We will measure TTR for d_5-glycerol and ^{13}C-palmitate using the same GC/MS analysis methods we have used previously in many of our studies (4, 17, 21, 22, 26).

Resting energy expenditure and fat oxidation rate will be measured using indirect calorimetry (7).

Plasma substrates and hormone concentrations. Plasma concentrations of triglyceride, fatty acid, glucose and insulin will be assessed using commercially available assays kits (Sigma, Wako, and ThermoDMA, Millipore, respectively). Plasma epinephrine and norepinephrine will be measured by HPLC with electrochemical detection by the pathology lab at the University of Michigan Medical Center.

Blood lipid profile. Plasma concentrations of total- and high-density lipoprotein cholesterol (Total-C and HDL-C) will be measured using commercially available colorimetric assay kits; Cholesterol E and HDL-Cholesterol E; WAKO Life Sciences, Inc.) and plasma low-density lipoprotein (LDL) cholesterol will be calculated as: [LDL-C] = [Total-C] – [HDL-C] – [Triglyceride])/5.

Body composition and visceral adiposity. Percent body fat will be assessed using a Dual Energy X-ray Absorptiometry [DEXA: Lunar DPX, DEXA Scanner]. We also plan to measure hepatic fibrosis, the amount of fat stored in the liver and visceral fat (or intra-abdominal fat) using magnetic resonance imager (MRI) with elastography as described previously (1).

Liver function. Circulating markers of liver function will be assessed in serum.

Quantifying factors regulating triglyceride storage and adipogenic markers.

Isolation of adipocytes and stromal vascular fraction (SVF) will be performed by collagenase digestion and centrifugation methods on fresh adipose tissue biopsy samples as previously described (3, 24). Immunoblot analysis to quantify protein abundance of enzymes and other co-factors regulating lipolysis and fatty acid esterification will be performed on the purified adipocyte fraction (13, 17). We will quantify the abundance of key adipogenic markers in the SVF. Table 1 provides a list of some of the proteins we plan to measure. We also plan to measure mRNA expression of many of these factors using the StepOnePlus real time PCR system (Life Technologies) with fluorescent probe-based assays (IDT).

Table 1. Adipose proteins to measure

	LIPOLYSIS	TRIGLYCERIDE SYNTHESIS	ADIPOGENIC MARKERS
Proteins of interest	ATGL, CGI-58, HSL, caveolin, p-HSL-s565, 563, 660, Perilipin-1	DGAT, mGPAT, AGPAT-1, PAP (LIPIN-1), SCD-1, SREBP-1c	PPARγ, C/EBPa, b, g, FABP4, Chemerin, Adiponectin, FAS

Lipolytic activity in adipose tissue explants and cultured cells (basal, stimulated and insulin inhibited). Lipolytic experiments will be performed on fresh adipose tissue samples *ex vivo* (~50mg; cultured at 5% CO_2 in Krebs buffer + 2.5% BSA (pH 7.4)), as well as in primary cells differentiated from adipose stromal vascular cells (aSVC) derived from these adipose tissue biopsy samples. Lipolysis will be assessed in optimally differentiated aSVC (12). The differentiated cells will be washed, serum-starved for 4 hours, and incubated in basal [1 U/mL adenosine deaminase + 20 n*M* N6-(L-2-phenylisopropyl)-adenosine], stimulated (1 m*M* 8-bromo-cAMP), or stimulated + insulin (30 p*M*, 120 p*M*, or 600 p*M* insulin) conditions for 2 hours. Importantly, by performing these experiments on fresh tissue and aSVC, we will examine the lipolytic action of existing mature adipocytes, as well as the innate lipolytic action of derived pre-adipocytes.

Esterification activity in adipose tissue samples. We will measure basal GPAT and DGAT activity in adipose tissue lysate samples using ^{14}C-G3P and ^{14}C-palmitoyl-CoA, as we have reported previously (17, 23).

Adipogenic capacity assays (in vitro). Adipose stromal vascular cells (aSVC) will be derived from fresh adipose tissue samples as described above. Subcultured aSVC from individual subjects will be plated on 24-well plates. When confluent, cells will be incubated for 7 days in either: 1) optimal differentiation media (+IBMX and +TZD; (12)), 2) suboptimal differentiation media (optimal media without IBMX and TZD), or 3) control media (containing 15% FBS). Incubation in the suboptimal media will provide information regarding whether the aSVCs from the different subject groups are already primed for differentiation, while the optimal media will allow us to compare the maximum capacity of the aSVCs to differentiate. After 7 days of incubation in these different media, cells will incubate in a maintenance medium (12). Ten days after the induction of differentiation, cells will be harvested for qPCR analysis of *PPARG* and *FABP4* gene expression levels.

Measurement of adipocyte cell size and cell density, and capillary density
Adipocyte size and number in adipose tissue biopsy samples will be quantified after staining with hematoxylin and eosin (H&E) using MetaMorph Image Analysis software (Molecular Devices) (18, 19, 24). Similarly, We will quantify the capillary:adipocyte ratio (#capillaries/cell) and capillary density (capillaries per adipocyte cell area) using Rhodamine-labeled GSL to stain for capillaries with standard immunohistochemistry techniques (14).

Angiogenic potential (ex vivo): Quantification of angiogenic potential will be examined *ex vivo* by incubating subcutaneous adipose tissue explants in extracellular matrix components in the presence of pro-angiogenic factors as described in detail by Rojas-Rodriguez et al. (20). Under these conditions, the adipose tissue explants sprout capillaries into the extracellular matrix environment (i.e., Matrigel). We will examine capillary "sprouting" over 7 days. Quantification of capillary growth (i.e., number of capillary sprouts, growth area, and percent of tissue with growth) will be performed using ImageJ software.

Extracellular matrix remodeling in adipose tissue. We will perform immunohistochemistry techniques along with qPCR analysis on adipose tissue samples (24) to quantify expression of some key factors involved in extracellular matrix remodeling in adipose tissue. Weight gain has been reported to increase expression of Collagen I, III, and VI, as well as Secreted Protein, Acidic and Rich in Cysteine (SPARC) (11), and we will compare the expression of these factors in adipose tissue samples from our insulin resistant and insulin sensitive obese subjects.

Unbiased proteomic analysis of the phosphoproteome in skeletal muscle. The untargeted phosphoproteomic

analyses will be performed in Dr. Zhengping Yi's laboratory at Wayne State University (~1h drive from Dr. Horowitz's lab at the University of Michigan). Dr. Yi will be performing the proteomic analysis on skeletal muscle samples collected in this study. We will bring de-identified muscle samples to Dr. Yi's laboratory at Wayne state for this analysis. Dr. Yi is an expert in proteomic analysis of skeletal muscle samples. Protein isolation, and in-solution trypsin digestion and phosphopeptide enrichment using titanium dioxide (TiO_2) will be performed as described previously by Dr. Yi (10, 28). Mass spectrometry, data analysis and bioinformatics will all also be performed as detailed previously by Dr. Yi (10, 28). We will aliquot muscle protein lysates and use some aliquots to perform western blot analysis on the proteins with significant differences between our insulin resistant and insulin sensitive subjects. We will assess the proteomic findings in context of the subjects' muscle fiber composition, which we will assess using standard immunohistochemistry methods.

Statistical analysis

Student's t-test will be used to assess statistical significance of our major endpoints between **NIR** and **IR**. In addition, we will perform linear regression analyses using our entire cohort of subjects (**NIR, MIR**, and **IR**) to assess relationships between *each* of our major endpoints and insulin resistance. Finally, we will also conduct multiple regression analysis using our entire cohort to assess the relative contribution of each of our major endpoints to the variance of insulin resistance. A *P* value of ≤ 0.05 will be considered statistically significant.

For our untargeted analyses, the false discovery rate (FDR) for both proteins and peptides will be set to 0.01. In addition, only phosphosites with a localization probability greater than 0.75 will be considered as "identified", a threshold used commonly in phosphoproteome studies. To be used in comparisons between groups, phosphorylation sites need to satisfy the following criteria: 1) identified in more than half of the samples; and 2) with a fold change (or difference) greater than 1.5 between comparisons. For comparisons between groups for both our untargeted and target endpoints, a Student's t-test will be used to assess statistical significance of our major endpoints for our primary comparison between the **NIR** and **IR** groups. In addition, we will perform simple linear regression analyses to assess relationships between *each* of our major endpoints and insulin resistance. Finally, we will also conduct multiple regression analysis to assess the relative contribution of each of our major endpoint measures to the variance of insulin resistance. A *P* value of ≤ 0.05 will be considered statistically significant.

Sample size calculations

Specific Aim #1: The number of subjects necessary to find statistical differences between our insulin resistant (**IR**) and our non-insulin resistant (**NIR**) groups in major endpoints of this study was calculated based on mean and standard deviation values derived from our preliminary studies and our previous publications (18, 24). The sample size calculations (Table 2) indicate that at least 17 subjects will be required for each group in this study. Our primary comparisons will be between the **IR** and **NIR** groups, but subjects will also be stratified into the **MIR** ("mildly insulin resistant") group. Based on previous work (27), we anticipate that our subjects will be stratified relatively evenly into our three study groups (i.e., **NIR**, **MIR**, and **IR**), but it is quite possible this may not be the case. Therefore, we plan to enroll a total of 60 subjects into this study in order to end with at least 17 subjects in both the **IR** and **NIR** groups.

Table 2. Sample size calculations for Specific Aim #1

	Power	α	Expected Δ (%)	Estimated SD (%)	# of subjects
Fatty acid Ra	0.8	0.05	35%	30%	**13**
Adipogenic capacity assays	0.8	0.05	20%	20%	**17**
Adipocyte cell size	0.8	0.05	25%	25%	**17**

Specific Aim #2: The number of subjects necessary to find statistical differences between **IR** and **NIR** groups in key endpoints of the studies designed to address Specific Aim #2 was calculated based on mean and standard deviation values derived from our preliminary studies, work published in our previous publications (15, 16) and from previous work (14). For Specific Aim #2, we will only perform the analyses on samples from subjects in the **IR** and **NIR** groups. The sample size calculations (Table 3) indicate that we will need at least 17 subjects in both groups. For our proteomic analyses, we will use the samples collected from the number of subjects required for our targeted analyses to be statistically significant.

Table 3. Sample size calculations for Specific Aim #2

	Power	α	Expected Δ (%)	Estimated SD (%)	# of subjects
Macophage infiltration	0.8	0.05	20%	20%	**17**
Cytokine abundance	0.8	0.05	50%	40%	**12**
Muscle ceramide concentration	0.8	0.05	20%	20%	**17**

APPENDIX 1 - Blood sampling timeline

The following table provides the blood sampling timeline for the infusion studies performed in the MCRU. The blood volume for the trial is 199.5 mL.

Time	Sample	Blood Volume (mL)
8:25am	EDTA tube, heparin tube, SST tubes	45
8:30am	EDTA tube, heparin tube	12
10:20am	EDTA tube, heparin tube, glucometer	9.5
10:25am	EDTA tube, heparin tube, glucometer	9.5
10:30am	EDTA tube, heparin tube, glucometer	9.5
10:30am	**BEGIN CLAMP**	
10:35am	Glucometer	0.5
10:40am	Glucometer	0.5
10:45am	Glucometer	0.5
10:50am	EDTA tube, heparin tube, glucometer	9.5
10:55am	Glucometer	0.5
11:00am	Glucometer	0.5
11:05am	Glucometer	0.5
11:10am	EDTA tube, heparin tube, glucometer	9.5
11:15am	Glucometer	0.5
11:20am	Glucometer	0.5
11:25am	Glucometer	0.5
11:30am	EDTA tube, heparin tube, glucometer	9.5
11:35am	Glucometer	0.5
11:40am	Glucometer	0.5
11:45am	Glucometer	0.5
11:50am	EDTA tube, heparin tube, glucometer	9.5
11:55am	Glucometer	0.5
12:00pm	Glucometer	0.5
12:05pm	Glucometer	0.5
12:10pm	EDTA tube, heparin tube, glucometer	12.5
12:15pm	EDTA tube, heparin tube, glucometer	12.5
12:20pm	EDTA tube, heparin tube, glucometer	12.5
12:25pm	EDTA tube, heparin tube, glucometer	12.5
12:30pm	EDTA tube, heparin tube, glucometer	12.5
*	Additional glucometer measures if clamp takes longer	6
	Total Volume (mL)	199.5

Appendix 2 – Infusion rates

TRACER TYPE	
[6,6 d_2]-Glucose	35 umol/kg bolus priming dose 0.41 umol/kg/min infusion for 4 hours
[d_5]-Glycerol	1.5 umol/kg bolus priming dose 0.10 umol/kg/min infusion for 4 hours
^{13}C-Palmitate	No priming dose 0.04 umol/kg/min infusion for 3 hours

u = “micro”, kg = kg body weight

The tracer glucose and glycerol infusions begin with a bolus “priming” dose (injected over 1-2 min) – followed immediately by the steady state infusion at the rates and durations indicated above. The palmitate has no bolus/priming dose.

INSULIN AND KCl INFUSION RATE

Time of clamp procedure	
0-5 min	160 mU/m^2/min
5-10min	80 mU/m^2/min
10-120min	40 mU/m^2/min

KCl INFUSION RATES = *20Meq at 30ml/hr for all protocols*

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