
eSupplement
eReferences
eTable. 5-day PROOF meal plans

This supplementary material has been provided by the authors to give readers additional information about their work.
I. Overview and statement of hypothesis

When body weight increases, the expenditure of energy increases as a mechanism to dissipate the excess calories. This occurs through several known mechanisms including increased spontaneous physical activity (SPA; fidgeting) (1) and probably increases in the activity of the sympathetic nervous system.

The role of diet composition in over-feeding - energy dissipation in humans is unknown. Animal studies, reviewed below, suggest that the amount of protein is important, with high protein diets and low protein diets less efficient in the deposition of fat and lean tissue than a modest protein diet. Our hypothesis is:

   a) that high and low protein diets will result in less weight gain as compared to a moderate protein diet during 56d high fat overfeeding.

   b) that increases in energy expenditure and SPA, adjusted for lean and fat mass will be greater in the high and low protein diets as compared to a moderate protein diet

   c) that the average size of the fat cells and the pattern of genes expressed in the adipose tissue, skeletal muscle and peripheral blood mononuclear cells will ‘predict’ which subjects will gain the most weight (and fat mass) independent of the level of the protein in the diet.

These hypotheses will be tested in the following aims:

   a) To determine the effect of overfeeding 40% above energy balance with a low (5%) or high (25%) vs. normal (15%) protein diet on body weight and body composition as well as energy expenditure and its components.

   b) To relate the baseline characteristics of the subjects [fat cell size, pattern of gene expression, body composition, family history of obesity, etc] to the degree of weight / fat gain during overfeeding.
II. Background and Significance

Epidemiological data have shown that the number of overweight and obese individuals in the United States is rapidly increasing. Because obesity is related to an increase of the risk of various conditions such as Type 2 diabetes, cardio-vascular disease, and some cancers it is important to understand, and possibly prevent weight gain as a means of preventing the complications of obesity. This current increase in the prevalence of obesity worldwide reflects the failure of mechanisms that regulate body weight to cope with environments that promote overeating and discourage physical activity. However, within any obesity-promoting environment there is a large variation among individuals regarding their susceptibility to weight gain. Some become obese despite a continuous struggle not to, whereas others are able to maintain body weight without effort. Such variations in the predisposition for weight gain may reflect varying susceptibilities to overeating or to a sedentary life-style. But we also know from overfeeding experiments that humans vary in their capacity to resist weight gain because of varying abilities to convert food directly into heat, a process called the thermic effect of food (TEF) (2). The magnitude of this 'waste' of energy is determined by the individual's genetic make up (3) as well as by the composition of their diet (4). In the described protocol we propose to explore the effect of both determinants on weight gain after an eight-week period of overfeeding with an emphasis on the effect of protein intake.

A. Overfeeding and energy balance

It has been suggested that total daily energy expenditure (TDEE) increases during periods of overfeeding. Studies show a lower weight gain than expected from the amount of calories given in excess. This was termed Luxusconsumption, and represents the loss of energy as heat or the work of physical activity. One explanation for the increase of energy expenditure is likely related to the costs of the gain in both lean and adipose tissue, as well as the higher costs of maintenance of the body mass gained. However, it has also been suggested, in addition to thermogenesis causes energy sympathetic nervous system illustrated by a study of of energy intake represented by presented in figure 1 and show the most common level of known for over 100 years that has been known as the specific thermogenesis rather than an association. All rights reserved.

Figure 1: Effects of varying dietary protein concentration (%ME) on the heat increment of feeding in rats. Adapted from Hamilton (4)
adaptive response. Diets high in protein have been shown to protect against weight regain after a weight loss program (6). However, that diets low in protein could influence metabolic efficiency has not been shown until the 1960s and in animals has been associated with an increase in thermogenesis response to norepinephrine and brown adipose tissue (BAT) thermogenesis, suggesting a role for the Sympathetic Nervous System (SNS) (7).

B. Overfeeding in humans

About a dozen overfeeding studies in humans have been conducted so far, and for most of them protein intake was in the normal range. Two of them used very low protein diets (4; 8). When comparing the costs of weight gain of the low protein studies with the normal protein studies was 2-2.5 times higher with the low protein studies. So far, no human overfeeding studies using a high-protein diet have been reported since all studies claiming a high-protein diet actually were in the normal intake range or 15-20%. Of the studies that used overfeeding as a model few actually measured TDEE under free-living conditions, which is likely a result of the difficulty in obtaining a reliable measurement. It should also be realized that for evaluating the response of energy metabolism, it is important to take the different components of TDEE into account, i.e. Resting Metabolic Rate (RMR), the Thermic Effect of Food (TEF), and the energy expenditure related to Physical activity (PA).

This study, will address these deficiencies in the literature and provide important information on the mechanisms by which dietary protein influences weight gain in a setting of high fat overfeeding, a setting not unlike our obesogenic environment.
III. Research Design and Methods

B. Inclusion/Exclusion Criteria

Inclusion Criteria

- Have a BMI of 19-30 kg/m²
  - A cutpoint of 26 kg/m² will be used to allocate treatment across the three diets. See the statistics section for more detail.
- Are willing to eat all of the study foods even when full
- Are willing to eat only foods provided by Pennington and all of the foods provided
- Are willing to live at Pennington for 10-12 weeks possibly without leaving the metabolic unit the entire time
- Are willing to avoid exercise while in the inpatient phase of the study
- Age 18 - 35

Exclusion Criteria

Participants are ineligible to participate in the study if they...

- Smoke
- Have Diabetes
- Have claustrophobia
- Have a Fasting Blood Sugar >110
- Have a history of cardiovascular disease
- Have an average screening blood pressure >140/90
- Have a history of a major psychiatric, addictive or eating disorder or any psychosocial or scheduling factors that could impede study outcomes
- Post obese (self-reported BMI) must never have had a BMI greater than 32
- Exercise more than 2 hours per week
- Unable to complete VO₂ max test.
- Weight gain or loss of >3 kg in the last 6 months
- Have significant renal, hepatic, endocrine, pulmonary or hematological disease, or a history of gout
• Unwilling or unable to abstain from alcohol, exercise and caffeine prior to visits and throughout the inpatient stay.
• Unwilling or unable to eat all study foods or to take study vitamins
• Have had previous GI surgery, Obstructive disease of the GI tract, Hypermotility disorder or a history of problems of impairment of the gag reflex.
• Corticosteroid use in the last 2 Months
• You are pregnant or breastfeeding
• Have Irregular menstrual cycles
• Use Depo-Provera, hormone implant or estrogen replacement therapy
• Have an allergy to PABA (a component of a B-vitamin often found in sunscreen).

You will also be excluded if you are unable to participate in the MRS portion of the study:

• Individuals who have a heart pacemaker, defibrillator, or non-removable hearing aid
• Individuals with any clips or metal plates in their head
• Individuals who have any artificial limbs or prosthetic devices
• Individuals who were ever injured by a metallic foreign body which was not removed
• Individuals, who wear braces on their teeth, have non-removable false teeth, or bridgework

Protocol Overview (train schedule)
The experimental protocol is graphically presented in the Appendix.

Participants must consume all foods provided and no additional foods will be allowed. All meals will be monitored with post meal checks of dishes and the eating area. The addition of DASH study acceptable spices (no salt or calories) will be permitted. A multivitamin will be given to each participant daily throughout the study beginning with the first run-in day. Participants will stay in the Metabolic Unit, or supervised on the Pennington grounds from admit to completion of overfeeding. The psychology staff will meet with the participants either in a group or individual basis throughout the study (weekly while inpatient and on one outpatient visit) to discuss their feelings about the study and evaluate potential problems. They will also monitor behavioral assessments that are collected monthly. Passes may be earned per primary investigator’s discretion based on strict adherence to protocol, in-patient guidelines, and good behavior, etc.
C: Run-In

Eligible participants will be admitted to the Metabolic Unit to consume a eucaloric diet of 15% protein, 25% fat & 60% carbohydrate. Weight maintenance will be established in a 13-25 day run-in phase during which continuous activity monitoring and daily fasting post-void weights in a gown will be recorded. For 2 days after admit participants will equilibrate NaCl and water and be fed a eucaloric diet of 1.3 to 1.4 RMR based on the average of 1) Rising’s equation

\[538+25(FFM)+5.5(FM)\]

and 2) the average of the Ravussin & Tataranni equation:

\[1294-7(age)+33(FFM)+3(FM)\]

for females, and

\[1294-7(age)+33(FFM)+3(FM)+92\]

for males. This is followed by 23 hours in the Metabolic Chamber including 24 hour urine collection for nitrogen, creatinine and protein. Core Temp will be measured while in the chamber. Discharge from the chamber begins the 1st of 5 days of the weight stabilization period. Doubly Labeled Water is given on the first stabilization day. This is followed by urine samples at 4.5 and 6 hours, the next day and every other day through 9 days post dosing regardless of the track taken. Untimed DLW urines are 2nd morning urines of at least 50ml. After completion of this 5-day period, progression is determined by weight stability (within 1 kg). If the participant meets these criteria participants begin 5 more days of energy balance. If they are not stable at the end of the stabilization period, calorie adjustments are made and they restart weight stabilization again. This cycle will not be completed more than 3 times. Volunteers that are not weight stable by completion of the third attempt are excluded.
Run-In Phase

**Day**  
1-2  2 days at 1.3-1.4 X RMR feeding based on ‘Rising’ equation and Ravussin & Tataranni equations (day 1-2) DLW baseline

3  23 hr Energy Expenditure in Metabolic Chamber

**DETERMINATION OF MAINTENANCE CALORIES (Stabilization)**

5 days stabilization @ TDEE (from the chamber) X 1.15 on 1st cycle (day 4-8)

Day 4 *(9,14)*  
*Doubly Labeled water baseline & dose with urines*  
*at 4.5 & 6 hours post on stabilization day 4*

5 *(10,15)*  
*DLW urine (2nd morning void) on stabilization day 5*

6 *(11,16)*

7 *(12,17)*  
*DLW urine (2nd morning void) on stabilization day 6*

8 *(13,18)*

*If Stable Wt.*  
*If Unstable Wt. → repeat 5 stabilization days (day 9-13)*  
*for second cycle*

**ENERGY BALANCE**

Day 9 *(or 14 or 19)*  
10 *(or 15 or 20)*  
11 *(or 16 or 21)*  
12 *(or 17 or 22)*  
13 *(or 18 or 23)*

*DLW urines days 9, 11 & 13 regardless of track*

Other parameters to be collected in or near the balance phase include:

- Behavior Assessment Battery including:
  - Three Factor Eating Questionnaire (TFEQ)
  - Body Shape Questionnaire (BSQ)
  - Body Morph Assessment (BMA)
Eating Disorder Diagnostic Scale (EDDS)
Multiaxial Assessment of Eating Disorder Symptoms (MAEDS)
Food-Craving Inventory (FQI)
Visual Analogue scales (VAS)
  - Weekly Assessment scales (X1)
  - Hunger & Satiety Scales (immediately before and after breakfast & dinner), and 2 hours and ½ hour before lunch and dinner.

- AIRG
- Two-step Clamp with microdialysis
- 24-hr urine for Catecholamines
- Three or more 24-hr urines for Creatinine, Urea, Na, K and P

D. Baseline 1: (Baselines may be reversed)
Chem. 15 (with lipids), CBC, U/A and urine HCG, glucose, insulin, free fatty acid, c-peptide, leptin, adipocytokines (sTNFRII, adiponectin with 1ml for HMW adiponectin, CRP, IL-6, resistin, sCD40L), archives (20ml), DNA, waist measurement, 30 min. RMR, TEF, urine for nitrogen & creatinine and DEXA. Physical Activity monitoring continues throughout baselines and overfeeding.

E. Baseline 2
Chem. 15 (with lipids), insulin, free fatty acid, c-peptide, leptin, sTNFRII, adiponectin with 1ml for HMW adiponectin, CRP, IL-6, resistin, sCD40L, PBML RNA for microarrays, TSH, T3 & RT3, archives (10ml) and DNA, 24h urine for nitrogen, creatinine, urea, Na, K & P, 30 minute RMR, DEXA, Visible Adipose Tissue (VAT) by Computerized Tomography (CT), and Magnetic Resonance Spectroscopy (MRS) of the muscle and liver and biopsies (muscle and fat) will also be performed for skeletal muscle culture and Fat Cell Size, and gene expression, etc.

F. Overfeeding
Beginning the next day, the overfeeding phase will begin. We will feed participants 40% more kcal per day than needed for weight maintenance. The diet will be randomly assigned to either high protein (25% protein, 34% fat, 41% carbohydrates) or low protein (5% protein, 54% fat, 41% carbohydrates) or standard protein (15% protein,
44% fat, 41% Carbohydrates) for the entire 8 weeks. As in the run-in phase, fasting weights and activity will be measured daily. Participants will be required to stay on the Metabolic Unit full time for their entire inpatient stay.

Diet compositions

Note: These examples are for baseline/run-in (2200 kcal) and overfeeding (3200 kcal), respectively, thus the 2200 kcal diets are identical in macronutrient content.

<table>
<thead>
<tr>
<th>Normal protein Diet (NP)</th>
<th>2200 kcal</th>
<th>3200 kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>330 g</td>
<td>330 g</td>
</tr>
<tr>
<td></td>
<td>60%</td>
<td>41%</td>
</tr>
<tr>
<td>Protein</td>
<td>82.5 g</td>
<td>120 g</td>
</tr>
<tr>
<td></td>
<td>15%</td>
<td>15%</td>
</tr>
<tr>
<td>Fat</td>
<td>61 g</td>
<td>155 g</td>
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<td></td>
<td>25%</td>
<td>44%</td>
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<th>High protein Diet (HP)</th>
<th>2200 kcal</th>
<th>3200 kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>330 g</td>
<td>330 g</td>
</tr>
<tr>
<td></td>
<td>60%</td>
<td>41%</td>
</tr>
<tr>
<td>Protein</td>
<td>82.5 g</td>
<td>200 g</td>
</tr>
<tr>
<td></td>
<td>15%</td>
<td>25%</td>
</tr>
<tr>
<td>Fat</td>
<td>61 g</td>
<td>120 g</td>
</tr>
<tr>
<td></td>
<td>25%</td>
<td>34%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Low protein Diet (LP)</th>
<th>2200 kcal</th>
<th>3200 kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>330 g</td>
<td>330 g</td>
</tr>
<tr>
<td></td>
<td>60%</td>
<td>41%</td>
</tr>
<tr>
<td>Protein</td>
<td>82.5 g</td>
<td>40 g</td>
</tr>
<tr>
<td></td>
<td>15%</td>
<td>5%</td>
</tr>
<tr>
<td>Fat</td>
<td>61 g</td>
<td>191 g</td>
</tr>
<tr>
<td></td>
<td>25%</td>
<td>54%</td>
</tr>
</tbody>
</table>

**Cost of weight gain**

<table>
<thead>
<tr>
<th>DIET</th>
<th>Cost of weight gain (MJ/kg)</th>
<th>3-day overfeeding</th>
<th>4-day overfeeding</th>
<th>DIT MJ/d (kcal/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>38</td>
<td>0.33 kg</td>
<td>0.44 kg</td>
<td>-</td>
</tr>
<tr>
<td>HP</td>
<td>60</td>
<td>0.21 kg</td>
<td>0.28 kg</td>
<td>1.7 (400)</td>
</tr>
<tr>
<td>LP</td>
<td>83</td>
<td>0.15 kg</td>
<td>0.20 kg</td>
<td>2.3 (550)</td>
</tr>
</tbody>
</table>

Expected weight gain: If overfed 1000 kcal/day for 60 days
1000 kcal/day= 4189 kJ/day. Over 60 days total amount overfed = 251.3 MJ

<table>
<thead>
<tr>
<th>DIET</th>
<th>Cost of weight gain (MJ/kg)</th>
<th>Expected weight gain (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>38</td>
<td>6.614</td>
</tr>
<tr>
<td>HP</td>
<td>60</td>
<td>4.188</td>
</tr>
<tr>
<td>LP</td>
<td>83</td>
<td>3.02</td>
</tr>
</tbody>
</table>

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Participants will be given one plain multivitamin daily beginning with admit. Although the low protein diet will not meet the Adequate Intake requirements of calcium or magnesium, this will not be supplemented for this 8-week period.

Testing schedule: A physical activity monitor will be worn throughout the entire inpatient stay. Daily urines may be omitted if they conflict with other tests.

Week 1: Lipids, glucose, insulin, free fatty acid, 24h urine catecholamines (X1), daily 24-hour urine for urea, Na, K & P, 30 min. RMR, 23-hour metabolic chamber stay with 23-hour urine for creatinine and nitrogen, and VAS.

Week 2: Lipids, glucose, insulin, TSH, T3, reverse T3 free fatty acid, C-peptide, PBML RNA for microarrays, leptin, 24h urine for catecholamines, daily 24-hour urine for urea, Na, K & P, single label DLW with baselines X2, urines at 4.5 and 6 hours and followed by urines every other day X9 days, 30 min. RMR, DEXA, 23-hour metabolic chamber stay with 23-hour urine for nitrogen and creatinine, VAS, muscle/fat biopsy for skeletal muscle culture and fat cell size.

Week 3: Lipids, glucose, insulin, free fatty acid, daily 24-hour urine for urea, Na, K & P and a 30 min. RMR, and VAS.

Week 4: Chem. 15, CBC, TSH, T3, reverse T3, U/A glucose, insulin, free fatty acid, c-peptide, leptin, sTNFRII, adiponectin with 1ml for HMW adiponectin, CRP, IL-6, resistin, sCD40L, archives (10ml), 24h urine for cats, nitrogen and creatinine, daily 24-hour urine for urea, Na, K & P, DEXA, 30 min. RMR, and Behavioral Assessment Battery, and VAS.

Week 5: Lipids, glucose, insulin, free fatty acid, daily 24-hour urine for urea, Na, K & Ph, and 30 min. RMR, and VAS.

Week 6: Lipids, TSH, T3, reverse T3, glucose, insulin, free fatty acid, c-peptide, leptin, daily 24-hour urine for urea, Na, K & Ph and 30 min. RMR, DEXA and TEF with urine for nitrogen and creatinine, 24h urine for nitrogen and creatinine, and VAS.

Week 7: Lipids, glucose, insulin, free fatty acid, daily 24-hour urine for urea, Na, K & P, 30 min. RMR and doubly labeled water urine on day 44, dosing on 45 and urine collections at 4.5 and 6 hours, the next day and every other day through day 9 (ex. day 46, 48, 50, 52 & 54), and VAS.

Week 8: Chem. 15, CBC, U/A, TSH, T3, reverse T3, glucose, insulin, free fatty acid, C-peptide & leptin, sTNFRII, adiponectin with 1ml for HMW adiponectin, CRP, IL-6, resistin, sCD40L, PBML RNA, archives (20ml), 24h urine for cats, nitrogen and creatinine, daily 24-hour urine for urea, Na, K & P, DEXA (X2), CT, MRS, waist measurement, 30 min. RMR, Two 23 hr. metabolic chamber stays with urine nitrogen and creatinine, core temperature, muscle & fat biopsies with skeletal muscle culture and fat cell size, AIRG, a Two-step Clamp with microdialysis, Behavior Assessment Battery, and VAS.

G. F/U VISITS

After completion of all tests in week 8, participants will be discharged to return twice a week for follow-up measures on weeks 12, 16, 20 and 24.
Tests will include: Post-Void Weight in gown (X2), Chem. 15, CBC, U/A glucose, insulin, free fatty acid, and 30 min. RMR. On Week 16 the Behavior Assessment Battery will be administered excluding the VAS relating to meals. On Week 24 the DEXA, CT scan and Behavior Assessment Battery will also be repeated without the VAS relating to meals.

IV. Specific Methods
A. Anthropometry
Height, weight, waist circumference, thigh circumferences and waist to thigh ratio will be measured according to established PBRC clinical procedures

B. Electrocardiogram
The EKG will be measured according to established PBRC clinical procedures on Marquette’s MAC VU or MAC 5000.

C. Questionnaires
Questionnaires to be given on screening or during the study include:

- Demographic Questionnaire-This questionnaire asks general questions about marital status, education, employment, income and smoking status and family history
- Health Questionnaire- The PBRC screening Health Questionnaire will be used
- Medical History - The PBRC standard medical history form is completed by the screening physician at the time of the physical.
- TFEQ - TFEQ is a 56-item measure of 3 aspects of eating and hunger. The Restraint scale measures current dietary restriction. The Disinhibition scale measures the extent to which the participant eats in response to external (eg: emotional, social) triggers. The Perceived Hunger scale provides a global level of hunger and is correlated with the Disinhibition scale.
- \textbf{SCID-II (Structured Clinical Interview for DSM-IV Axis II Personality Disorders)}: This questionnaire measures factors often found in personality disorders. A Psychology staff member will review this questionnaire, and conduct 1 or 2 interviews to evaluate potential risks to the study.
- Body Shape Questionnaire (BSQ). The BSQ measures concern with body size and shape (9). The BSQ will be utilized to measure change in concern for body size and shape during the overfeeding and follow-up period of this study.
• **Body Morph Assessment 2.0.** The BMA is a computerized body image assessment procedure(10). The BMA has been found to be a valid measure of estimates of current, ideal, and reasonable body size. The BMA is computer-based, self-administered, and it can utilized with Caucasian and African-American men and women.

• **Eating Disorder Diagnostic Scale (EDDS).** The EDDS is a questionnaire that has been validated to quantify symptoms of eating disorders, consistent with the diagnostic nomenclature of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (11). In the current study, the EDDS will be used to detect the occurrence of eating disorder symptoms during the study.

• **Multiaxial Assessment of Eating Disorder Symptoms (MAEDS)(12).** The MAEDS is a self-report inventory that measures the six symptom domains related to eating disorders: binge eating, restrictive eating, purgative behavior, fear of fatness, avoidance of forbidden foods, and depression (12). Scores on all scales are standardized using t-scores.

• **Food-Craving Inventory (FCI).** The FCI is a validated self-report measure for general cravings and cravings for specific types of foods (13). Respondents rate the frequency of cravings over the past month for 33 food items using a 5-point Likert scale (Never, scored as 1; Rarely, 2; Sometimes, 3; Often, 4; Always/almost every day, 5).

• **Visual Analogue Scales (VAS).** Computerized Visual Analogue Scales (VAS) are used to measure subjective states such as hunger or satiety. VAS will be used to measure satiety and hunger before and after breakfast and dinner on the days that participants complete the psychological questionnaires. VAS will also be collected 2 hours and ½ hour before lunch and dinner on a weekly basis. In addition, a “weekly” VAS will be collected on the same day as the psychological questionnaires. The “weekly” VAS assessed average ratings of satiety and hunger for the past week. Participants rate the variable being assessed on a 100 mm line, anchored from “not at all” to “extremely.” The reliability and validity of the VAS used at the Pennington Center have been established (14).
- VO2 max screen – This form includes a list of symptoms as well as a CHD risk factors to be considered in deciding if a physician is required for the VO2 max test.
- Core Temp Inclusion / Exclusion Checklist - This form is a checklist of exclusions for the core temperature pill. If any of these factors are present the participant will not be given this large radio transmitter pill. However, this is not a reason for exclusion from the protocol.

D. Fasting RQ (Resting Metabolic Rate)
The fasting respiratory quotient will be measured using a Deltatrac II Metabolic cart (Sensor Medics Corporation, Anaheim, CA or Datex-Ohmeda, Helsinki, Finland). This is done for 30 minutes at the beginning. It is preceded by a 15-minute rest period.

E. TEF (The thermic effect of food)
TEF and the RQ response to a standardized meal will be measured on two occasions, at baseline and at week 6 of overfeeding. After an overnight fast RMR and RQ will be measured for 30 minutes after which a standard breakfast containing 40% of calories as measured by RMR on the baseline day will be given. The meal will consist of 50% CHO, 15% protein and 35% fat. Thirty minutes after the start of the meal metabolic rate and RQ will again be measured for 30 minutes each hour for a total of 6 hours.

Metabolic rate will be measured by indirect Calorimetry using a Deltatrac II metabolic Monitor (DATEX-Ohmeda, Helsinki, Finland). TEF will be calculated as increase above RMR over 6 hours and will be expressed as absolute increase in kcal/6 hours, % of energy intake and as % of RMR. In addition, postprandial substrate oxidation will be measured from npRQ and expressed as gram/6hours.

F. Activity monitor (Accelerometer)
A RT3 activity monitor (or equivalent) will be worn for 7 days during screening. It may be repeated as necessary to complete this collection. The RT3 will also be worn continuously during run-in and the entire inpatient stay.

G. Laboratory Samples
Screening labs include Chem. 15, CBC, U/A and urine HCG. Safety labs will include: Weekly chemistries and lipids are collected in the overfeeding phase. CBC, TSH, T3 & reverse T3, urinalysis and urine HCG are also collected. Metabolic labs include (glucose, insulin and free fatty acid. Adipocytokines include sTNFRII, adiponectin with 1ml for HMN adiponectin, CRP, IL-6, resistin and sCD40L. C-peptides and Leptin will be collected at B1, B2, and every other week. Peripheral

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blood and mononuclear cell RNA for microarrays are scheduled for baseline, week 2 and week 8. 24 Hour urines are collected for catecholamines, protein and nitrogen. Inpatient archives will be obtained at baseline and every 4 weeks. Urine drug, alcohol and tobacco tests may be performed at any time during the study.

Blood volumes needed for the study include: 22ml for screening plus 515 ml for the protocol totaling 537ml for the entire study. This is a little over 1 unit (480ml) of blood spread over the 7-month (3 blood donation periods) study.

H. Doubly-Labeled Water and Deuterium (heavy water)

Following 2 baseline urines at –2 and the morning of dosing, subjects will drink from a stock solution with 0.2g/kg total body water (TBW) of H218O (Cambridge Isotopes, Cambridge, MA) and 0.115g/kg TBW of 2H2O (Isoteric Inc., Miamisburg, OH or Cambridge Isotopes, Cambridge, MA), then they will drink the tap water used to rinse the dose container. Consecutive urine samples are collected at 4.5 and 6 hours post dosing. For the next 9 days the 2nd morning urine samples will be collected every other morning. The 2H and 18O isotope elimination rates (kO and kH) will be calculated using linear regression using the isotopic enrichment relative to pre-dose enrichment of the urine samples from the beginning and end of the metabolic study. TBW will be determined from the enrichment at time zero, obtained from the zero intercept from the regression analysis. The rate of CO2 production is calculated using the equations of Schoeller et al. (15) and later modified as follows:

\[
\text{rCO2} = \text{rCO2} (\text{moles/d}) = (N/2.078) (1.007 kO - 1.041 kH) - 0.0246 r_{GF}
\]

where rCO2 is the rate of carbon dioxide production; N is total body water calculated from N0/1.007 (we will also examine individually measured dilution spaces); kO and kH represent the elimination rates of oxygen-18 and deuterium, respectively, from body water; and r_{GF} is the rate of fractionated gaseous (evaporative) water loss, which is estimated to be 1.05N (1.007kO - 1.041kH) (16). Total energy expenditure will be calculated by multiplying rCO2 by the energy equivalent of CO2 based on the estimated food quotient of the diet and estimated changes in body energy stores:

\[
\text{TEE (kcal/d)} = 22.4 \text{rCO2} (3.9/RQ + 1.10)
\]

Where TEE is total daily energy expenditure and RQ is the estimated respiratory quotient.

Additionally, Deuterated (non-radioactive heavy water) water will be used to measure total body water at day +10 of overfeeding. This procedure, including sample collection, is identical to the doubly labeled water (DLW except that the water is singly labeled with only deuterium.
I. PABA Absorption Test:
Para-aminobenzoic acid (PABA), a B-vitamin component, is rapidly metabolized and excreted by the kidneys and can be used as a marker to validate food consumption or as a measure of intestinal absorption. During run-in and throughout overfeeding, divided daily doses totaling 240mg of free PABA (in 4 divided doses) may be added to the food of participants in order to confirm subjects are not purging. The doses will be given in divided doses over 3 meals and a snack. 24 Hour urine collections will be examined for the supplement and its metabolites. Sulfanilamides and Tylenol will be avoided during PABA administration to avoid drug interactions or alteration of the assay.

J. DEXA
DEXA scans will be performed using a new Hologic QDR 4500A whole-body scanner. The subject will lie on a table wearing a hospital gown and no metal containing objects, while the scanner emitting low energy X-rays, and a detector passes along the body. The scan takes less than 4 min. and the radiation dose is less than 1 mrem, equal to about 12h of background radiation. The scans are analyzed with the latest software QDR for Windows V11.1. DEXA will be obtained post-void. Repeat analyses in 40 subjects gave coefficients of variation (CV) for body composition measurements of lean mass, fat mass and percentage of body fat were 0.6%, 1.1% and 1.1% respectively. We will also use the DEXA data to estimate muscle mass using a method developed by Dr. Steven Heymsfield’s group.

K. Computed Tomography
  • CT measurement of adipose tissue volume
    Volunteers will change into a gown and lie on the CT scanner table with the arms over their head. A “scout” image will be acquired to plan the image acquisition. Based on the scout, 8 contiguous images will be obtained every 5cm; 5 above and 2 below a slice centered on L4-L5 inter-vertebral disc. The volunteer will be removed from the table and allowed to clothe. The images will be stored on a digital tape and backed up on an optical disk.
  • CT measurement of skeletal muscle lipid
    Single slice CT will be used to measure intramuscular fat using the method of Kelley et al. A 10mm slice through the vastus lateralis muscle and calf will be obtained at 14kV, 240mA.
  • Image analysis
    The digital tape will be transported to the PBRC for quantitation of the adipose tissue distribution as follows. Images will be imported into the AnalyzePCT™ software package on a computer workstation. Adipose tissue will be anatomically defined and quantified as intra-abdominal, subcutaneous-deep and subcutaneous-superficial. Areas will be calculated using a triangulation method.
Women who are of childbearing potential will have a pregnancy test within twenty-four hours of the CT scan. A negative result will be documented prior to CT scanning.

K. Magnetic Resonance Spectroscopy (MRS)
The magnetic resonance scans to measure lipid stores in muscle and liver will be performed in the MRS lab of Pennington Biomedical Research Center on the new GE Signa 3T Excite HD system. For the muscle scan, subjects will be asked to lie supine on the patient table of the 1.5 magnet (picker Edge Eclipse) for approximately 45 min. Measurements will be obtained using the PRESS box technique. The subject’s right leg will be positioned inside a commercially made radio frequent (RF) $^1$H knee coil with the knee in extension and the ankle in a neutral position. Four to six separate water suppressed PRESS boxes (7.5 x 7.5 x 10 mm3 voxels) will be collected from the largest volume of the calf muscle (echo time (TE) =35msec and a resonance time (TR)=2 seconds). Boxes will be positioned to avoid facia, vascular structures and gross marbling as determined using the cross-sectional and axial scout images. For the liver scan, the subject will first exit the magnet and then be repositioned on their belly. A $^1$H body coil (which is not visible and sits beneath the patient bed) will be used to measure intrahepatic fat stores. A single PRESS box will be collected in an area of the liver that is free from heavy vascularization as determined from the scout images. This measurement takes less than 30 minutes. Foam pads and pillows will be used to ensure the subject will rest comfortably during the testing. The subject will be informed that the imaging scans will produce loud “knocking” noises and a headset with music will be provided to mask and minimize this noise. As needed, the subjects will also be allowed to stretch ad walk around briefly between scans.

M. Aerobic fitness exercise test (a.k.a. VO2 max)
Aerobic fitness will be determined by measuring maximal O$_2$ consumption during (a graded) treadmill exercise testing. A doctor will be present during treadmill testing for volunteers who are at risk according to ACSM guidelines.

N. Insulin sensitivity and insulin secretion
   1) Acute insulin response to glucose
   We will conduct a abbreviated FSIGTT protocol to determine the insulin Acute insulin response to glucose (AIRG). Subjects will be studied after an overnight fast while residing on the metabolic ward. 2 IV lines will be placed and 3 baselines drawn. Briefly, 300mg/kg glucose is injected at TIME 0, followed by collection of blood samples (4cc) at 1, 3, 4, 5 6,7,8, 10, 12, 14, 16, and 20 minutes. Each blood sample is analyzed for glucose and insulin. These data are then submitted for calculation AIRG using the Minimal Model method of Bergman. The minimal model analysis is accomplished using a program developed for a PPC (MINMOD-Millenium, © R. Bergman).
2) Insulin sensitivity and metabolic flexibility

2-Step hyperinsulinemic euglycemic clamp at 90 mg/dl glucose. The glucose clamp is the gold standard for measurement of insulin sensitivity and will be performed as previously described (Defronzo 1979) during the baseline testing of participants. After an overnight 12-hour fast, an intravenous catheter will be placed in an antecubital vein for infusion of insulin and glucose. A second catheter will be placed retrograde in a dorsal vein of the contralateral hand for blood withdrawal. The hand will be placed in a heating box or pad at 70°C for arterIALIZation of venous blood. A primed low-dose infusion of regular insulin (5 mU/min/m2) will be initiated and continued for 60 min, followed by a high-dose rate of 80 mU/min/m2 for 90 min, where the dose of the insulin infusion has been calculated prior to the study for each participant. Plasma glucose will be allowed to either decrease to 90 mg/dl or will be increased to 90 mg/dl in subjects depending on the fasting blood glucose levels. Arterialized plasma glucose will be measured at 5 min intervals and a variable infusion of exogenous glucose (20% solution) will be given to maintain plasma glucose concentration at ~90 mg/dl. The resting metabolic rate (RMR) will be measured three times during the clamp procedure, baseline, first and second steps of the clamp. Each RMR will last 30 minutes and will include first 10 minutes as an adjustment period and next 20 minutes as a steady period. Two blood samples will be collected during each RMR period for determination of plasma insulin, glucose and FFA concentration at –15 and –5 before the insulin infusion during baseline, and end-15 and 5 for the second and third RMR periods. The clamp will last 2.5 hours from the time at which the insulin infusion is initiated. IL-6 and TNF-a will be determined in the 2 baseline samples. Patients will void before the test, and urine will be collected during and at the end of the clamp to determine urinary nitrogen and glucose loss.

Calculations: An insulin infusion of 80 mU/min/m2 is expected to result in plasma insulin concentration of ~200 mU/ml. Hepatic glucose production is expected to be completely suppressed at this level of insulin, even in diabetic subjects. In this case, peripheral glucose uptake (Rd) should equal the glucose infusion rate (GINF) during steady state (the last 20 minutes of the clamp) after correction for urinary glucose loss.

That is: - Rd = Steady State GINF - urinary glucose loss

Insulin sensitivity (SI) will be calculated using the formula: SI = Rd / (steady state insulin level - basal insulin level) where steady state insulin equals the average insulin concentration during the last 20 minutes of the clamp and basal insulin equals the average insulin level in the 20 minutes before starting the insulin infusion.

We are aware that a hyperinsulinemic euglycemic two-step clamp may impose a significant burden on potential volunteers. However, we feel that a glucose clamp is the most reliable and sensitive method to assess insulin sensitivity.

Microdialysate sample collection and analysis
A microdialysis probe (CMA 60: Stockholm, Sweden) with a 30 mm-long membrane will be inserted into subcutaneous abdominal tissue under the local anesthesia and 60 minutes will be allowed for recovery of the tissue from insertion trauma and for the baseline calibration of the perfusion system before activated of the dialysis protocol. The Ringer solution will be infused through the probe at 0.3 ul/min flow rate. The microvials, collected about every hour, will be placed in the microvial rack and stored in the ice box until analyzed. Interstitial glucose, glycerol and lactate levels in the microdialysis will be measured in duplicate by an automated spectrophotometric kinetic enzymatic analyzer (CMA 600, CMA, Solna, Sweden). A dose response curve will be generated from the concentration of insulin vs. the glycerol concentrations in the dialysate [baseline/5mlU/m^2/80mlU/m^2]

O. Metabolic chamber

24 hour Energy Expenditure and RQ
The room indirect calorimeter measures 10 feet x 12 feet x 8 feet, with a total volume of 27,000 liters. The chamber is ventilated with fresh air at a rate of ~60L/min, and operates at an ambient temperature of 22.2 ± 0.5 °C, and a relative humidity of 50-65%. The room has two windows, and is furnished with a futon bed, desk and chair, television, radio/tape player, telephone, microwave, sink and toilet with privacy curtain, a treadmill, a small refrigerator for the storage of urine or fecal samples, and an air-locking food passage through which the meals are given. Video cameras and microwave motion detectors continuously monitor the subject’s movement. Oxygen and carbon dioxide levels in the chambers are measured using a Magnos 4G magneto-pneumatic oxygen analyzer, and a Uras 3G infrared CO2 analyzer (Hartmann and Braun, Frankfurt am Main, Germany), both of which sample O2 and CO2 concentrations 60 times per second. Every ten seconds a computer program averages these values, calculates the volumes of O2 consumption and CO2 production, and plots the average values at 10-minute intervals.

On every test day the chambers are calibrated using pure gas mixtures, and for determination of the accuracy and precision of the calorimeters, 23-hour propane combustion tests are performed on a weekly basis. The accuracy of our chambers is 98.25% and 96.55 %, for O2 and CO2, respectively. The raw data for each chamber day are corrected by using the recovery rates for the propane test closest to the test day. Energy expenditure and substrate oxidations (including RQ) are calculated from oxygen consumption, CO2 production and 24-hour urinary N excretion by using the equations established by Acheson et al. (17).

The first and last chamber measurements will be obtained under energy balance conditions. Energy balance is maintained via two procedures. First, the energy requirements are calculated based on the RMR and the average free-living energy expenditure measured by a triaxial accelerometer. The middle three

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chambers will be obtained under overfeeding conditions. As the chamber has limited area, physical activity typically decreases by approximately 15% compared to free living values. The total daily energy expenditure is then calculated based on 1.4 X RMR, and the expected spontaneous physical activity. Fecal energy is assumed to be 10% of dietary energy for purpose of maintaining energy balance. Meals are prepared in the Metabolic Kitchen using menus that have been previously validated with regards to energy content and % fat.

The middle three metabolic chambers will be obtained under overfeeding conditions

P. Core temperature

The core body temperature will be measured using a swallowed radio transmitter pill. The signal will be received by a belt worn monitor/recorder (MTI, Inc., FL, CorTemp recorder and pills).

Q. Skeletal muscle biopsy

- Muscle biopsy

Vastus Lateralis muscle biopsy will be performed using the Bergstrom technique. Volunteers will be placed in the supine position and the skin cleansed with povidone-iodine solution. After a sterile drape is placed, the skin, adipose tissue and skeletal muscle fascia anethetized using approximately 5ml of a 50% / 50% mixture of bupivacaine and lidocaine (final concentrations 1.0% and 0.125%). The skin is incised (approximately 0.75cm) with a #11 scalpel. The fascial fibers are separated with the blunt edge of the scalpel or the Bergstrom needle (4-6mm) is inserted into the vastus lateralis. After suction is applied from the proximal port, the sample (approximately 50mg) is cut and the needle removed. Pressure is applied and the skin is closed with sterile tape. Samples for RNA extraction (75mg) are placed in saline, quickly washed and trimmed, and stored in liquid nitrogen. Samples for skeletal muscle culture (100-150mg) are washed in Ham’s F10 media @ 4°C and immediately transported to the laboratory for culture.

- RNA extraction –skeletal muscle

Tissue will be pulverized in a Bessman tissue homogenizer under liquid nitrogen, homogenized in guanidium thiocyanate, extracted with phenol and chloroform, and alcohol precipitated by the method of Chomczynski and Sacchi, (18). Pelleted RNA will be quantified by spectrophotometry, quality documented by PAGE (gel electrophoresis) / Sybr Green II staining, and stored in ultrapure water at -70°C.

- Skeletal muscle culture

Initial skeletal muscle culture techniques were developed in the molecular endocrinology lab using primary skeletal muscle cultures obtained from Clonetics, Inc. We have been growing human skeletal muscle cells for almost 3 years using the methods of Blau and Webster (19). Culture conditions

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were followed as described by Henry (20; 21) and Ciaraldi (22) using commercially available proliferation and differentiation medias (SkGM media, Clonetics, Inc., catalog CC-3160). These cultures will be used to finalize the protocols for proliferation, differentiation, glucose and fat oxidation, etc.

Skeletal muscle (75-100mg) is placed in Ham’s F-10 media @ 4oC, strained, minced and washed three times in Ham’s F-10 media @ 4oC and once @ 37oC. The cells are then dissociated with trypsin 0.05%/EDTA at room temperature. Dissociated cells are then centrifuged at 600 X g for 4 minutes at 37oC and resuspended in SkGM bullet media (Clonetics, Inc.) with 2%FBS. Cells are plated on 100-mm dishes and placed in an incubator with 95% air/5%CO2. Media is changed every 3 - 4 days until < 70% confluent. Plates are then split into 12 X 24 well plates and grown to 70% confluence. At this stage, cells are differentiated into myotubes using α-MEM with 2% FBS. Myotube formation will be confirmed by microscopy and induction of α-actin and myosin heavy chain by Western immunoblotting, and creatine phosphokinase isoenzyme analysis (Clinical Chemistry laboratory).

- **RNA extraction – cultured cells**

  Total cellular RNA will be extracted using the method of Chomczynski and Sacchi, (23). Briefly, media will be removed from cultured cells using suction and 1.0ml TriReagent™ /35mm dish added while swirling on a rotary shaker. After 15 minutes, the cells and guanidinium mixture are pipetted up and down 20X to shear the DNA. Mixture is processed in Kontes glass homogenizers at low speed on ice and phenol / chloroform extracted as described above.

- **In-vitro Substrate Oxidation and Storage**

  After proliferation and differentiation into myotubes, skeletal muscle cultures will be assayed for fat oxidation, triglyceride synthesis, glucose oxidation, and glycogen synthesis. The methods of Muio (as adapted by Ukropkova (24)) (25; 26) will be used. Prior to assay, the media will be changed to serum free DMEM buffered with 10mM HEPES, containing 5mM glucose, 1.0mM oleate, 1.0 % dialyzed BSA. 1.0mM carnitine, and the cells incubated overnight for assay the next morning.

- **Fatty Acid Oxidation and Triglyceride Synthesis**

  After incubation overnight in serum free media, the media is exchanged to fresh serum free DMEM buffered with 10mM HEPES, containing 5mM glucose, 1.0mM oleate, 1.0 % dialyzed BSA, and 1.0mM carnitine with 1uCi/mL [1-14C] oleate added. Insulin, 10mU/ml, leptin, 10ugm/ml or both will be added to quadruplicate wells. The cells will be incubated for 90 minutes and [14CO2] captured by the addition of 100uL of perchlorate using a NaOH filter paper capture technique as described by Muio (25-27). After incubation at 37°C for 90 minutes, the filter paper is cut, washed in 2ml of ddH2O and dual channel counted by liquid scintillation (channel 1 = [14CO2], channel 2 = [3H-thymidine] incorporation). The cells are lysed, scraped from the plate, and

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lipid is extracted using chloroform-methanol. The lipid fraction is then dried under nitrogen and counted as described above (channel 1 = [14CO2], channel 2 = [3H-thymidine] incorporation). FA oxidized or TAG synthesized will be expressed as mmol/hour and normalized to the cell number.

- **Glucose transport, Oxidation and Glycogen Synthesis**

  After incubation overnight in serum free media, the media is exchanged to fresh serum free DMEM buffered with 10mM HEPES, containing 5mM glucose, 1.0mM oleate, 1.0 % dialyzed BSA. 1.0mM carnitine with 1uCi/ml [UL-14C] glucose added. Insulin, 10mU/ml, leptin, 10ugm/ml or both will be added to additional wells. The cells will be incubated for 90 minutes and [14CO2] captured by the addition of 100uL of perchlorate using a NaOH filter paper capture technique as described above. After incubation at 37°C for 90 minutes, the filter paper is cut, washed in 2ml of ddH2O and dual channel counted by liquid scintillation (channel 1 = [14CO2], channel 2 = [3H-thymidine] incorporation). The cells are lysed, scraped from the plate, and 4mg of carrier glycogen is added. The samples are incubated for 1 hour at 80°C and glycogen is precipitated by adding ice-cold ethanol to a final concentration of 70%. The samples are incubated overnight at -20°C and the sample centrifuged at 8000g for 20 minutes. The resulting pellet is washed x 3 with 70% ethanol, resuspended in H2O, and dual channel counted by liquid scintillation (channel 1 = [14C-glycogen], channel 2 = [3H-thymidine] incorporation). Glucose oxidized or glycogen synthesized will be expressed as mmol/hour.

These techniques are now a routine in the Smith laboratory.

R. Adipose tissue biopsy

After vital signs are obtained, the skin over the lateral abdomen will be cleansed with povidone-iodine solution, and a sterile drape placed over the site. Topical anesthesia will be obtained by the use of a 50% - 50% mixture of lidocaine and bupivacaine (approximately 5 mL). A 1.0-cm incision will be made in the skin and a 4-6mm Bergstrom needle inserted to collect under aspiration approximately 500 mg of adipose tissue. The sample will be washed in sterile PBS and snap frozen in liquid nitrogen, with 50mg placed in osmium tetraoxide for the determination of fat cell size. After the biopsy is complete, the incision will be closed with a sterile bandage (butterfly), and antibiotic ointment / sterile dressing applied.

- **Fat cell size and number using the Coulter principle**

  All the fat cell analyses (morphology and gene expression) will be performed at the Pennington. The method used for fat cell size and number has been previously described (28). At each center, adipose tissue will be fixed in a solution containing collidine HCl (0.2M) and osmium tetraoxide (31mg/mL-collidine HCL buffer). After fixation, samples are stable for prolonged periods. Samples are diluted with 154mM NaCl, filtered over a 10-micron nylon screen, recollected in 10mL 154mM NaCl, and dissociated over 1 week by the addition of 10mL of 8M urea in 154mM NaCl. The sample is filtered through a 250-micron nylon filter into a weighed beaker. The volume is increased to 300mL with 154mM NaCl containing 0.1% TritonX-100. The cells are then counted during on a Multisizer-3 (Beckman Coulter, Fullerton, CA, USA) using a 400 micron aperture (dynamic linear range =12-320 micron). Adipocyte

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cell number (cells per mg wet weight of tissue) is then determined from the amount of sample (ml) the quantity of cells (per ml), and the tissue weight (mg). Cell size distribution is displayed as cells per 10-micron bin, mean cell size, median cell size, and % of cells greater than 100 micron. Additional sample characteristics can be determined from the table of individual cell sizes. This technique is routine in the Smith laboratory.

- **Adipose tissue gene expression**
  
  Total RNA will be extracted using the technique of Harris (29). Adipose tissue gene expression will be performed using the Taqman RT-PCR technique. Expression profiling will be performed as described, below. This technique is routine in the Smith laboratory.

- **Quantitative RT-PCR**
  
  Quantitative RT-PCR will be performed on the PE-7700 instrument. This instrument combines PCR with real time fluorescent detection to measure gene expression in samples as small as 2ng of total RNA. This allows for the measurement of multiple signals from small samples (< 10mg skeletal muscle, for example.) As described by the manufacturer, during PCR, a fluorogenic probe, consisting of an oligonucleotide with both a reporter and a quencher dye attached, anneals specifically between the forward and reverse primers. When the probe is cleaved by the 5’ nuclease activity of the DNA polymerase, the reporter dye is separated from the quencher dye and a sequence-specific signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored during the PCR. This technique has been used in the laboratory. For a detailed description see Bustin (30).

- **Expression profiling**
  
  In addition to testing hypotheses utilizing the candidate gene approach, expression profiling will be conducted using the core resources of the molecular genetics laboratory. A cDNA library, constructed from the human genome project contains approximately 18, (16) 861 elements spotted on glass slides.

  These cDNA microarrays will be used to compare
  
  a) The change in gene expression before vs. after the overfeeding period (skeletal muscle, adipose tissue and peripheral blood mononuclear cells – [PBML])
  
  b) Across individuals at baseline to if there is a pattern of genes that determines the differences between individuals in the amount of weight/fat gain.

    o The following slide arrangement will be used:

      - adipose tissue → skeletal muscle
      - Skeletal muscle → PBML

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– PBML → adipose tissue

This head-to-tail has been recommended by Churchill, et al. (31).

– These samples will then be labeled with Cy3 and Cy5 dyes and applied to the glass microarrays.
– Differential gene expression will be confirmed using RT-PCR (Taqman - PE 7700) and Western immunoblotting where appropriate. RT-PCR confirmation will be performed on the entire population.
– See Sparks, et al. (32) (in-press and appended) for a summary of the statistical approaches to the clustering for experimental design a) above.

Sparks, Smith, et al – the paper in review at diabetes
– See (33; 34) for a summary of the statistical approaches to the clustering for experimental design b) above.

• DNA for single nucleotide polymorphism (SNP) analysis

Blood will be collected (buffy coats) to extract DNA.

S. Statistical Considerations

• Randomization

Randomization will be performed on or about study day R10 by either the study statistician or the study dietitian and will be based upon the Minimization Allocation method (35; 36). The randomization will employ stratification for sex and BMI, and is designed to yield samples that are comparable with regard to this set of baseline prognostic factors. For this randomization, BMI will be dichotomized as follows: 19 ≤ BMI < 26 and 26 ≤ BMI ≤ 30.

• Power Analysis

A power analysis was conducted for weight gain as the response of primary interest. It is anticipated that, at the end of the overfeeding period, the standard protein population will exhibit a mean increase in body weight of 6.6 kg, the high protein group 4.2 kg, and the low protein group 3.0 kg. A common dispersion estimate for weight gain, σ_d=3.01 kg, was employed. The power analysis was conducted for the test of the null hypothesis of equality of the means of the standard protein and high protein populations.

A type I error rate (alpha) of 5% was used; a one-tailed alternative hypothesis was employed for the research hypothesis that weight gain for the standard protein population exceeds that of the high protein population by 2.4 kg. A single interim analysis was taken into consideration; O’Brien-Fleming
significance boundaries were used to maintain the overall false positive rate at the nominal (5%) level. Results of the power analysis indicate that twenty (20) completing subjects in each treatment arm (total 60 subjects) would be required to have 80% power to reject the null hypothesis.

- **Statistical Methods**

A single interim analysis is planned after the 30th randomized subject has completed the protocol. This analysis will test the null hypothesis of equality of the means for weight gain of the standard protein and high protein populations versus a one-tailed alternative hypothesis that weight gain for the standard protein population exceeds that of the high protein population. To control the false positive rate, one-sided O'Brien-Fleming spending function significance boundaries will be used in the interim and final analyses of weight change for this hypothesis.

Effects of treatment on change in weekly average body weight from baseline, change in body composition measures from baseline, and change in energy expenditure from baseline will be evaluated employing repeated measures models. These models will include sex as a fixed effect and baseline BMI as a covariate (i.e., the prognostic variables used in the randomization), along with the baseline value of the response measure as an additional covariate.

Tests related to pre-planned hypotheses (treatment effect comparisons) will be rendered unadjusted for multiple comparisons. Statistical significance will be defined relative to a nominal 5% type 1 error rate. Final analysis and reporting will be conducted by the PBRC Biostatistics unit subsequent to the study termination.

V. Risks
<table>
<thead>
<tr>
<th>Procedure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overfeeding by 40% for 8 weeks (10% weight gain)</td>
<td>Nausea, bloating, approximately 10-15% weight gain</td>
</tr>
<tr>
<td>High Fat diet for 8 weeks</td>
<td>Nausea, increase in cholesterol levels</td>
</tr>
<tr>
<td>Weight gain</td>
<td>Being overweight increases your risk of diabetes, hypertension and high cholesterol</td>
</tr>
<tr>
<td>Confinement</td>
<td>RESTLESSNESS, IRRITABILITY, AND LONELINESS</td>
</tr>
<tr>
<td>Questionnaires/Interviews</td>
<td>NONE</td>
</tr>
<tr>
<td>Behavior Assessments</td>
<td></td>
</tr>
<tr>
<td>EKG</td>
<td>Skin irritation if allergic to electrodes</td>
</tr>
<tr>
<td>Fasting blood samples</td>
<td>Bruising, bleeding, pain, Infection. These will be minimized by our trained phlebotomists using sterile technique.</td>
</tr>
<tr>
<td>Urine &amp; stool collections</td>
<td>None</td>
</tr>
<tr>
<td>Measurement of resting energy expenditure</td>
<td>Claustrophobia because of the transparent hood around your head</td>
</tr>
<tr>
<td>Physical activity monitor</td>
<td>NONE</td>
</tr>
<tr>
<td>Exercise testing</td>
<td>Fatigue. Muscle, ligament, tendon or bone soreness/injury. Abnormal blood pressure. Fainting.</td>
</tr>
<tr>
<td></td>
<td>Heart irregularities and in rare instances heart attack, stroke or death are also possible.</td>
</tr>
<tr>
<td></td>
<td>The increase in this risk is very low (&lt;0.01%) and similar to when you exercise during your daily life. You may experience 'shortness of breath or become 'dizzy' or 'lightheaded' during the test. These feelings are normal and transient in nature. If these feelings are prolonged and increase in intensity after the end of exercise, notify the exercise physiologist or Principal Investigator immediately. A doctor will be present during treadmill testing for volunteers who are at risk according to ACSM guidelines.</td>
</tr>
<tr>
<td>DEXA</td>
<td>Minimal x-ray exposure. Example: 12 hours background radiation from the sun. Exposure to radiation can harm an unborn child and pregnant women are not allowed to undergo this procedure.</td>
</tr>
<tr>
<td>CT scan</td>
<td>Involves x-ray exposure equivalent to 2 chest x-ray series. Exposure to radiation can harm an unborn child and pregnant women are not allowed to undergo this procedure.</td>
</tr>
<tr>
<td>Magnetic Resonance Spectroscopy</td>
<td>There are no significant risks associated with MR spectroscopy and imaging. There is a small chance of claustrophobia or muscle-skeletal discomfort from lying partially in the magnet for up to 45 minutes. During the imaging measurement, you may hear loud banging that may be somewhat unpleasant. Earplugs and/or a head set, however, are provided to mute this banging. Although the long-term risk of exposure to a magnetic field is not known, the possibility of any long-term risk is extremely low in view of the information accumulated over the past 10 years.</td>
</tr>
<tr>
<td>Metabolic Chamber</td>
<td>Claustrophobia</td>
</tr>
<tr>
<td>Muscle &amp; Fat Biopsies</td>
<td>Pain, infection, scar, bleeding, loss of sensation of skin around biopsy site. This loss of skin sensation may be temporary (3 months) or in some cases permanent.</td>
</tr>
<tr>
<td>Insulin Sensitivity by FSIGTT</td>
<td>Bruising at the site of needle insertion in veins. The major risk of this test is that your blood sugar may fall due to the insulin. Low blood sugar can make you feel sick to your stomach, sweaty, irritable and sometimes confused. We will check your blood sugar after we inject insulin every 10 minutes. After the study is finished, you will be given high carbohydrate meal and fruit juice..</td>
</tr>
<tr>
<td>Microdialysis</td>
<td>Pain, infection, bruising and bleeding, similar to those seen with an IV</td>
</tr>
<tr>
<td>Core Temperature</td>
<td>Previous GI surgery increases the risk of intestinal blockage. The pill will not be used if you have had previous GI surgery.</td>
</tr>
<tr>
<td>PABA absorption test</td>
<td>Skin rash or other allergy symptoms</td>
</tr>
</tbody>
</table>

In addition to the risks listed above, a previously unknown risk or side effect may occur.
VI. REFERENCES

1. Levine JA, Eberhardt NL, Jensen MD: Role of nonexercise activity thermogenesis in resistance to fat gain in humans [see comments]. Science 283:212-214, 1999

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eTable: 5-Day PROOF Meal Plans
<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lemon Bread</td>
<td>Blueberry Crisp</td>
<td>Biscuit or English Muffin</td>
<td>Banana nut muffin or Special K Cereal w/ banana</td>
<td>Pancakes</td>
</tr>
<tr>
<td>Butter</td>
<td>Fruit Cocktail</td>
<td>*Butter</td>
<td>Breakfast pie</td>
<td>Maple Syrup</td>
</tr>
<tr>
<td>Cranberry Juice</td>
<td>Cheesy Breakfast Pie</td>
<td>Cheddar Cheese</td>
<td>Pineapple Chunks</td>
<td>*Butter</td>
</tr>
<tr>
<td>Fruit Cocktail</td>
<td>Canadian Bacon</td>
<td>Hard Boiled Egg</td>
<td>or Banana</td>
<td>Blackberries</td>
</tr>
<tr>
<td></td>
<td>*Apples &amp; Cinnamon</td>
<td>Canadian Bacon</td>
<td>Apple Juice</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oatmeal</td>
<td>Grits</td>
<td>Butter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Milk</td>
<td>Pears</td>
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<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
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</thead>
<tbody>
<tr>
<td>Turkey Mozzarella Cheese</td>
<td>Grilled Chicken Breast or Breaded Chicken Breast</td>
<td><strong>Tuna Salad</strong>&lt;br&gt;Wheat Bread or Croissant&lt;br&gt;Trail mix or Balance Bar or Potato Chips</td>
<td>Ham</td>
<td>Chicken Salad&lt;br&gt;Croissant or Whole Wheat Pita Bread&lt;br&gt;<strong>Cucumber Slices</strong>&lt;br&gt;Italian Salad Dressing&lt;br&gt;Potato Chips&lt;br&gt;*Chocolate Pudding</td>
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<tr>
<td>Lettuce&lt;br&gt;Tomato&lt;br&gt;Mustard or Mayonnaise</td>
<td>Lettuce&lt;br&gt;Tomato Bun&lt;br&gt;Mustard or Mayonnaise&lt;br&gt;Sun Chips or Potato Chips&lt;br&gt;*Shortbread Cookie&lt;br&gt;*Jelly Beans</td>
<td>Baby Carrots, raw</td>
<td>Lettuce&lt;br&gt;Tomato</td>
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<tr>
<td>Chocolate Chip Cookie</td>
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<tr>
<td>Snack</td>
<td>Day 1</td>
<td>Day 2</td>
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<td>Balance Nutrition Bar</td>
<td>Balance Nutrition Bar</td>
<td>Wheat Thins &amp; Cheese or Nutrigrain Bar or Shortbread Cookie + Starbursts</td>
<td>Fruits for Salad or Peaches String cheese or Fruit Chews or Balance Bar</td>
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<td></td>
<td>Mozzarella String Cheese or Carrots w/ Ranch Dressing</td>
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<td></td>
<td>Mustard or Mayonnaise Yogurt Granola Butterscotch or Chocolate Chip Cookie BBQ Potato Chips</td>
<td>Mozzarella String Cheese or Jelly Beans or Balance Bar or Butterscotch Cookies</td>
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<th>Day</th>
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<tbody>
<tr>
<td>Day 1</td>
<td>Meatballs &amp; Spaghetti or Vegetarian Spaghetti Parmesan Cheese Garden Salad Italian Dressing Dinner Roll *Butter Peaches Butterscotch Cookies Milk</td>
<td>Red Beans &amp; Rice w/ Sausage and or Ham Lettuce &amp; Cucumber Salad Italian Dressing Cornbread *Butter</td>
<td>Mexican Casserole Lettuce &amp; Tomato Salad Ranch Dressing Pineapple Chunks Dinner Roll Milk</td>
<td>Chicken Alfredo</td>
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**Bold-** Depends on diet. **Italics-** Depends on calorie level. ***Italics/ Bold-** Depends on both diet and calorie level.