Clinical Research Protocol

National Institute of Diabetes and Digestive and Kidney Diseases

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TITLE: Selective Reduction of Dietary Carbohydrate versus Fat: Effects on Metabolism, Endocrine Physiology, Brain Activity and Reward Circuitry

SHORT TITLE: Reduced Carbohydrate vs. Fat

IDENTIFYING WORDS: Obesity, Weight Loss, Carbohydrate, Fat, Macronutrient Balance, Brain Imaging

TYPE OF PROTOCOL: Natural History – Disease Progression/Physiology

ESTIMATED DURATION OF STUDY: 9 Years

START DATE: January, 2009 **END DATE:** January, 2018

NUMBER AND TYPE OF PATIENTS:

	Number	Sex	Age Range
Volunteers	**20 Control Subjects and 20 Obese Subjects**	**Male and female**	**18 to 45**

PRINCIPAL INVESTIGATOR:
Kevin Hall, Ph.D.
Laboratory of Biological Modeling (LBM)
National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)
Building 12A, Room 4007
12 South Drive, (MSC 5621)
Phone: 301 402-8248
Fax: 301 402-0535
E-mail: kevinh@niddk.nih.gov

MEDICAL ADVISORY INVESTIGATOR:

Ranganath Muniyappa, M.D.
NIDDK, DEOB
Building 10, CRC Room 5-3671

10 Center Drive
Bethesda, MD 20892-1613
Phone: 301-451-7702
Fax: 301-480-4517
E-mail: muniyapr@niddk.nih.gov

ASSOCIATE INVESTIGATORS:

Kong Y. Chen, Ph.D.
Clinical Endocrinology Branch (CEB)
NIDDK
Building 10, CRC, Room 5-3671
10 Center Drive, (MSC 1613)
Phone: 301 451-1636
E-mail: chenkong@niddk.nih.gov

Amber Courville, Ph.D., R.D.
Nutrition Department
Building 10, CRC, Room B2-2426
10 Center Drive, (MSC 1078)
Phone: 301 594-8051
E-mail: courvillea@mail.nih.gov

Juen Guo, Ph.D.
NIDDK
Building 12A, Room 4059
12 South Drive
Phone: 301 496-5436
E-mail: juen.guo@mail.nih.gov

Nora D. Volkow, M.D
National Institute on Drug Abuse (NIDA)
NSC - Neuro Science Ctr, 5274
6001 Exec Blvd
Rockville, MD
Phone: 301 443-6480
E-mail: nvolkow@nida.nih.gov

Peter Herscovitch, M.D.
Chief, PET Department
NIH Clinical Research Center
Building 10, Room 1C401
10 Center Drive, (MSC 1180)
Phone: 301 451-4248
E-mail: herscovitch@nih.gov

Lilian V. Howard, C.R.N.P.
Bldg 10 Rm 3-1604
10 Center Drive
Phone: 301-594-0298
E-mail: howardlv@mail.nih.gov

NON-INVESTIGATOR RESEARCH STAFF:

John E. Ingeholm, Ph.D.
Laboratory of Brain and Cognition, NIMH
Building 10, Room 4C-104
10 Center Drive, (MSC 1366)
Phone: 301 435-4940
E-mail: ingeholm@nih.gov

PROJECT USES IONIZING RADIATION:
No longer uses ionizing radiation since this study is data analysis only.
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OFF-SITE PROJECT: No

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Précis

Popular weight loss strategies often prescribe a targeted reduction of dietary carbohydrate or fat. But surprisingly, no controlled human feeding study has ever investigated the effects of a selective reduction of dietary carbohydrate versus fat while keeping the other dietary macronutrients at their baseline weight-maintenance values. The present study was designed to address this knowledge gap and improve our understanding of how selective reduction of dietary fat versus carbohydrate may differentially impact the many feedback control processes that act to resist weight loss.

After several days of eating a weight-maintenance diet, 20 obese adult volunteers (BMI above 30 kg/m^2) will be admitted to the metabolic clinical research unit (MCRU) and, after 5 additional days of the baseline diet, their diets will be modified to result in either 85% reduction of the baseline dietary fat or a 60% reduction of the baseline dietary carbohydrate for the next 6 days. These diet modifications produce an equivalent caloric reduction. The primary outcome measurements will be changes of metabolism, brain reward circuitry and regional brain activity in response to food stimuli measured during the baseline and reduced calorie diet phases. Immediately following each controlled diet, we will measure 3 days of ad-libitum food intake using a computerized vending machine system. The subjects will return to the MCRU after a 2-10 week washout period to receive the opposite reduced calorie diet. Twenty control subjects with normal body weight (BMI between 18.5 - 25 kg/m^2) will have measurements of brain reward circuitry and regional brain activity in response to food stimuli while on a balanced, weight-maintenance diet.

Immediately following the second in-patient visit, all of the obese subjects will be assigned to a 12 week out-patient weight loss program with the goal of achieving at least 5% weight loss. We will investigate the relationship between short-term fat imbalances measured during the in-patient phases, and the body weight and fat changes during the weight loss program. We will evaluate the effects of weight loss on metabolism, brain reward circuitry, and regional brain activity in response to food stimuli. Finally, if the subjects are available for long-term follow-up, then we will investigate their metabolic phenotype, brain reward circuitry, and regional brain activity in response to food stimuli yearly over the subsequent 5 years following the weight loss intervention. This study will result in an improved understanding of the physiological mechanisms that sense and respond to negative energy balance acutely, after several weeks, and after several years, and may eventually lead to increased long-term success of obesity treatment.

Introduction and Background

1. Dietary macronutrients and the treatment of obesity

The prevalence of obesity has reached epidemic proportions and the incidence and economic burden of obesity-related complications are rapidly increasing (1, 2). Any treatment of obesity must result in an imbalance between the energy expended by the body over the amount of energy derived from the intake of food. Popular weight loss strategies often achieve this negative energy balance by prescribing a targeted reduction of a dietary macronutrient such as carbohydrate or fat. Recent clinical trials in obese subjects have compared these strategies and determined that low-carbohydrate diets result in greater short-term weight loss compared with low-fat diets (3-5). The mechanisms underlying this result have not been determined. One hypothesis is that the macronutrient composition of a diet may significantly influence energy expenditure and fuel partitioning, and that low-carbohydrate diets may offer a "metabolic advantage" (6) in apparent violation of the long-lived principle that "a calorie is a calorie" (7). Nevertheless, proponents of the "metabolic advantage" concept base their arguments on sound biochemical mechanisms that may underlie the hypothesized alterations of metabolic efficiency and fuel partitioning with changes of diet composition (6). An alternative hypothesis is that the macronutrient content of the diet affects hunger and satiety possibly through altered hypothalamic signaling or brain reward pathways.

Given the importance of these topics and the intense public interest in how dietary macronutrients affect metabolism and weight loss, it is surprising that no human feeding study has ever investigated a controlled selective reduction of dietary carbohydrate versus fat while keeping the other dietary components at their balanced values. The present study was designed to address this knowledge gap and determine how a selective reduction of dietary fat versus carbohydrate affects whole-body metabolism, endocrine signals, hunger, satiety, regional brain activity and reward circuitry, and gene expression in skeletal muscle and adipose tissue. The general hypothesis of the present study is that a selective reduction of dietary carbohydrate, but not dietary fat, will result in significant changes of these variables with respect to the baseline diet.

2. Dietary macronutrients and their effect on fuel selection

Maintenance of a stable body weight requires a state of macronutrient balance where dietary carbohydrate, fat, and protein intake rates are balanced by their utilization rates. This fundamental concept of macronutrient balance and the relationship between metabolism and the chemical composition of the body (e.g., body fat and lean masses) is an extension of the energy balance concept (8, 9). While nothing is presently known about the effects of selective reduction of dietary carbohydrate versus fat on human metabolism, several *overfeeding* studies have investigated the effects of selective overfeeding of dietary carbohydrate versus fat (10-12). These studies found that carbohydrate overfeeding induced profound adaptations of metabolism by increasing carbohydrate utilization and decreasing fat utilization as indicated by an increase of the 24-hour respiratory quotient. However, fat overfeeding produced no significant changes of macronutrient utilization. We anticipate a similar dichotomy in the present study where we examine the effects of selective *underfeeding* of dietary carbohydrate versus fat for a 6-day

period in a controlled in-patient setting. In particular, we expect that a selective restriction of dietary carbohydrate will result in significant changes of 24-hour whole-body respiratory quotient and macronutrient utilization rates whereas selective restriction of dietary fat will cause no such changes.

3. Endocrine signals of dietary macronutrient intake

Diet composition has a profound effect on the concentration of a wide variety of circulating hormones involved in regulating metabolism, appetite and satiety. For example, dietary carbohydrates stimulate the secretion of insulin which orchestrates a complex sequence of events to promote the storage of fat as well as the uptake and oxidation of glucose (13). Dietary fat, on the other hand, does not directly impact insulin secretion but can stimulate the secretion of cholecystokinin (CCK) which is believed to play a role in satiety (14). Thus, dietary carbohydrate and fat alone are known to result in distinct endocrine signals. However, most previous studies have focused on the changes of circulating metabolites and hormones in response to single meals of varying macronutrient composition (15-20). Less is known about 24 hour changes of circulating metabolites and hormones after a period of adaptation to diets of differing macronutrient content, but it is clear that diet composition can significantly change the 24 hour circulating concentrations of key metabolites and hormones (21, 22).

For the first time, we will study the 24 hour circulating metabolite and hormone levels in response to selective reductions of dietary carbohydrate and fat. We expect that carbohydrate restriction, but not fat restriction, will significantly alter the 24 hour profiles of glucose, insulin, glucagon, ghrelin, PYY_{3-36}, and leptin. This will be assessed by collecting frequent blood samples over 24 hour periods before and on the 4^{th} day of selective reduction of dietary carbohydrate and fat. These expected changes of circulating metabolites and hormones mediate the anticipated changes of 24-hour fuel selection during the reduced carbohydrate diet. By measuring both the fuel selection and the metabolite and hormone profiles over the same 24-hour period, we can directly relate the measurements.

4. Influence of diet and weight loss on regional brain activity

Functional neuroimaging has revealed several areas of the human brain that play an important role in the neural response to feeding and have abnormal responses in obesity (23-25). Even short-term changes of diet can cause detectable changes of neural activity as was highlighted by a recent 3-day moderate overfeeding study where fMRI detected significant alterations of neural activity in the inferior temporal visual cortex, posterior parietal cortex, premotor cortex, hippocampus and hypothalamus in the response to visual food stimuli (26). We plan to measure the effects of diet and weight loss on regional brain activity through a series of four fMRI experiments. The first experiment will map the brain regions underlying gustatory perception of fundamental tastes. The second experiment will identify the brain regions responsible for perception of visual food stimuli. The third experiment will examine the neural basis of food preference and the final experiment will map functional connectivity among brain regions supporting gustation and perception of food stimuli. For a detailed description of the fMRI study design, see the Analytical Procedures section.

We hypothesize that obese subjects will show differences in their neural bases of food perception and preference versus normal weight control subjects. Furthermore, we hypothesize that after 4 days of selective restriction of dietary carbohydrate, but not fat, will induce changes of regional brain activity in the obese subjects and that these changes will be further enhanced following a 12 week out-patient weight loss program in the same subjects

5. Influence of diet and weight loss on brain reward circuitry

The response of the brain's reward system to the eating of food is believed to have played a fundamental role in evolution to ensure that an organism obtains sufficient energy reserves for reproduction (27). It has been hypothesized that the central reward pathways that evolved for this purpose can be hijacked and exploited to result in addiction to substances such as cocaine or methamphetamine (27). Similar to drug addiction, obesity is correlated with a decrease of dopamine D2 receptor availability in the striatum suggesting that increased food intake may be playing a compensatory role for the reduction of normal signaling through this reward pathway (28).

A recent study in mice with a functional knockout of the sweet taste receptor showed that post-ingestive signals from dietary carbohydrate can induce a central dopamine response that is independent of palatability (29). Thus, the metabolic and endocrine changes associated with altering dietary carbohydrate are expected to impact the dopamine reward system. Furthermore, food restriction in rats has been shown to increases the dopamine D2 receptor availability (30). The present study will investigate whether dopamine D2 receptor availability is altered during a reduced carbohydrate diet versus a reduced fat diet, and following weight lost.

Dopamine D2 receptor availability is measured by positron emission tomography (PET) using the positron emitting compound [^{18}F]fallypride which binds competitively with dopamine to the D2 receptor. We hypothesize that obese subjects will have increased [^{18}F]fallypride binding in the striatum following 4 days of a reduced carbohydrate diet, but not following a reduced fat diet. Furthermore, we expect that D2 receptor availability will be further enhanced following a 12 week out-patient weight loss program in the same subjects. In addition, a recent study has shown a negative relationship between the presence of a polymorphism associated with the dopamine D2 receptor gene and BMI, therefore genetic data from blood samples will be obtained for determination of the presence of the polymorphism in our study subjects (31). The presence of the polymorphism will be correlated with the measured dopamine D2 receptor availability, as well as other measured variables such as BMI.

6. Determinants of food intake following controlled diet restriction

A differential central response to the selective reduction of dietary carbohydrate versus fat may result in different degrees of hunger and satiety and may possibly lead to different food intake behaviors after the diet control is released. Certainly, the diets will result in different macronutrient oxidation rates which may independently affect food intake patterns and subsequent energy balance. For example, a high respiratory quotient has been correlated with subsequent gain of body weight (32, 33) and the degree of positive carbohydrate balance during

a high carbohydrate diet has been associated with protection from long-term gain of body fat (34).

Selective reduction of dietary carbohydrate versus fat may influence subsequent food intake patterns. Therefore, at the end of each dietary restriction period all subjects will be provided with ad libitum access to food for three days. We will investigate the correlations between the intake of food during the ad libitum diet period with a variety of measurements during the preceding diet-restriction period. In particular, 24-hour indirect calorimetry will be performed immediately prior to the ad libitum phase since previous studies have found correlations between carbohydrate oxidation rate and subsequent ad libitum food intake (35). Our study will further explore the relationships between macronutrient utilization and subsequent food intake since the two diet groups are expected to induce substantially different carbohydrate and fat oxidation rates prior to the ad libitum feeding period. Furthermore, we will relate the ad libitum feeding to the measured 24-hour metabolite and hormone levels, as well as hunger and satiety, measured during the reduced carbohydrate and reduced fat diets.

7. Macronutrient imbalance and body composition change

In the same obese subjects, we plan to assess the longitudinal dynamics of body composition change over the course of a 12 week out-patient weight loss program that will take place immediately following the final in-patient phase. We will investigate the relationship between short-term measurements of fat imbalance measured by indirect calorimetry during the in-patient diets and the long-term body weight and composition changes during the out-patient weight loss program. We have developed mathematical models that predict how short-term indirect calorimetry measurements obtained during the in-patient phase relate to the composition of weight loss during a more prolonged period of negative energy balance (8, 9), and these model predictions do not depend on the subjects adhering to a specific diet composition during the weight loss phase. We will test these mathematical models and investigate the factors that determine the relative loss of lean versus fat mass. Furthermore, we will investigate whether other measurements made during the in-patient phase can help explain the expected variability of weight loss and body composition change during the out-patient phase.

8. Long-term follow-up of obese subjects following the weight loss intervention

Because body weight change occurs over extended time scales it is important to assess changes of metabolism, body composition and brain activity and reward circuitry that occur over many years. Therefore, obese subjects that complete the out-patient weight loss phase of the study will be provided with the option to participate in follow-up assessments to investigate long-term changes of metabolism, body composition, brain reward circuitry, and regional brain activity. The follow-up assessments will occur on an approximately yearly basis for 5 years following the weight loss program and will repeat the set of procedures and timeline of the final 2 weeks of the weight loss phase. These data will allow for an improved understanding of body weight regulation on the characteristic time scale of natural weight change and will provide insights regarding factors that influence the relative success of weight loss and weight loss maintenance.

9. Summary

The importance of understanding the individual effects of selective dietary restriction of fat versus carbohydrate derives from the possibility that these macronutrients may differentially impact the many feedback control processes that sense whole-body energy imbalance and thereby act to resist weight loss. To further address the issue of adaptive responses to weight loss, we will evaluate changes of whole-body metabolism, body composition, regional brain activity and reward circuitry in the same obese subjects following a 12-week weight loss program. Therefore, this study will result in an improved understanding of the physiological mechanisms that sense and respond to negative energy balance acutely, after several weeks, and after several years, and may eventually lead to increased long-term success of obesity treatment.

Study Objectives

We will study obese adult volunteers in an in-patient setting to assess the changes of whole-body metabolism, endocrine physiology, psychology, and regional brain activity and reward circuitry in response to a 6-day isocaloric selective reduction of dietary fat versus carbohydrate by cross-over design. Following completion of both arms of the in-patient study, separated by a 2-10 week washout period, the same subjects will participate in a 12 week out-patient weight loss phase to assess changes of whole-body metabolism, body composition, and regional brain activity and reward circuitry following weight loss. To assess whether brain activity and reward pathways are altered in the obese subjects and, if so, whether the diet interventions normalize these variables, a group of normal weight control subjects will undergo the PET and fMRI procedures following three days of an out-patient balanced diet.

Primary Aims and Objectives:

1. To determine the effects of two isocaloric, reduced-energy diets for 6 days, selectively restrictive in either fat or carbohydrate calories, on 24-hour respiratory quotient, macronutrient utilization and energy balance in obese subjects.
2. To determine whether obese subjects have altered dopamine D2 receptor availability in comparison to lean subjects, and to measure changes of dopamine D2 receptor availability following 4 days of selective reduction of dietary fat versus carbohydrate, as well as after a 12 week out-patient weight loss program in the same obese subjects.
3. To determine whether obese subjects have altered regional brain activity in response to food stimuli and to measure changes of regional brain activity following 4 days of selective reduction of dietary fat versus carbohydrate, as well as after a 12 week out-patient weight loss program in the same obese subjects.
4. To measure the loss of fat and lean body mass in obese subjects during a 12 week out-patient weight loss program and use a mathematical model to relate the measured body composition changes to the indirect calorimetry measurements obtained during the in-patient diet perturbations.

Secondary Aims and Objectives:

1. In obese subjects, to assess the impact of two reduced-energy diets selectively restrictive in either fat or carbohydrate on:
    a. Nitrogen balance (i.e. urinary nitrogen excretion)
    b. 24-hour plasma concentration of hormones and peptides related to feeding and energy balance
    c. Whole-body lipolysis, proteolysis, and glucose turnover rates.
    d. Expression of pro-inflammatory markers and of genes involved in macronutrient utilization in skeletal muscle and adipose tissue
    e. Changes in psychological measurements such as affective state, mood, hunger, satiety, as well as implicit and explicit measures of liking and wanting of food
2. In obese subjects, to evaluate 3-day ad libitum food intake following 6 days of selective restriction of dietary carbohydrate versus fat and investigate correlations between the intake of food during the ad libitum diet day with a variety of measurements during the previous controlled diet restriction period.

Study Design and Methods

This is a randomized, cross-over study of adult, obese, non-diabetic individuals to determine the metabolic, endocrine, psychological, and brain reward circuitry responses to controlled, selective reductions of dietary carbohydrate versus fat. To assess whether brain activity and reward pathways are altered in the obese subjects, a group of control subjects with normal BMIs will undergo the PET and fMRI procedures following three days of an out-patient balanced diet.

Subjects

Human subjects (18-45 years of age) will be recruited to the study via inquires received from interested study subjects through the NIH Patient Recruitment and Public Liaison Office. Pre-screening by this office will exclude subjects with a BMI less than 30 k/m^2 for obese subjects, and greater than 25 kg/m^2 or less than 18.5 k/m^2 for lean control subjects, those with diabetes or any other metabolic disorders, and those who require assistance to complete activities of daily living. All others will be contacted by the protocol team to review exclusion criteria. All subjects will be fully informed of the aims, nature, risks, and potential benefits of the study prior to giving written consent.

Inclusion and Exclusion Criteria

Obese Subjects

Inclusion criteria:

- Age 18-45 years, male or female
- Body mass < 350 lbs. (max. weight dictated by table limit for fMRI scanner)
  - when acquisition of large bore fMRI is complete, max. wt. limit will increase to 400 lbs.
- Weight stable (< ± 5 kg over past 6 months)
- Body mass index > 30.0 kg/m^2
- Premenopausal (women only)
- Healthy, as determined by medical history and laboratory tests
- Able to complete daily bouts of walking at a moderate rate
- Written informed consent

Exclusion criteria:

- Body mass > 350 lbs. (max. weight dictated by table limit for fMRI scanner)
  - when acquisition of large bore fMRI is complete, max. wt. limit will increase to 400 lbs.
- BMI < 30.0 kg/m^2
- Evidence of metabolic or cardiovascular disease, or disease that may influence metabolism (e.g. cancer, diabetes, thyroid disease)
- Taking any prescription medication (except birth control) or other drug that may influence metabolism (e.g. diet/weight-loss medication)
- Hematocrit < 34% (women only)
- Hematocrit < 40% (men only)
- Pregnancy, lactation (women only)
- Allergy to lidocaine or ethanol
- Participating in a regular exercise program (> 2h/week of vigorous activity)
- Caffeine consumption > 150 mg/day (will be clamped at baseline intake during study)

- Regular use of alcohol (> 2 drinks per day), tobacco (smoking or chewing) amphetamines, cocaine, heroin, or marijuana over past 6 months
- Past or present history of eating disorder (including binge eating) or psychiatric disease, including claustrophobia since part of the protocol will involve being confined to a small room for whole-body indirect calorimetry
- Volunteers with strict dietary concerns (e.g. vegetarian or kosher diet, multiple food allergies)
- Are claustrophobic to a degree that they would feel uncomfortable in the MRI machine.
- Having any metal in their body (for example, pacemakers, metallic prostheses such as cochlear implants or heart valves, shrapnel fragments, etc.).
- Left-handedness
- Non-native English speakers
- Volunteers unwilling or unable to give informed consent

Control Subjects
Inclusion criteria:

- Age 18-45 years, male or female
- 18.5 kg/m^2 < BMI < 25.0 kg/m^2
- Weight stable (< ± 5 kg over past 6 months)
- Premenopausal (women only)
- Healthy, as determined by medical history and laboratory tests
- Written informed consent

Exclusion criteria:

- BMI < 18.5 or > 25.0 kg/m^2
- Evidence of metabolic or cardiovascular disease, or disease that may influence metabolism (e.g. cancer or diabetes)
- Taking any prescription medication (except birth control) or other drug that may influence metabolism (e.g. diet/weight-loss medication)
- Hyperlipidemia (fasting plasma triglyceride concentration > 150 mg/dl)
- Hematocrit < 34% (women only)
- Hematocrit < 40% (men only)
- Pregnancy, lactation (women only)
- Participating in a regular exercise program (> 2h/week of vigorous activity)
- Caffeine consumption > 150 mg/day
- Regular use of alcohol (> 2 drinks per day), tobacco (smoking or chewing), amphetamines, cocaine, heroin, or marijuana over the past 6 months
- Past or present history of eating disorder (including binge eating) or psychiatric disease
- Volunteers with strict dietary concerns (e.g. vegetarian or kosher diet, multiple food allergies)
- Are claustrophobic to a degree that they would feel uncomfortable in the MRI machine.
- Having any metal in their body (for example, pacemakers, metallic prostheses such as cochlear implants or heart valves, shrapnel fragments, etc.).
- Left-handedness

- Non-native English speakers
- Volunteers unwilling or unable to give informed consent

Screening Procedures

Eligible volunteers will be invited to the Clinical Research Center for a screening visit, and after signing the informed consent, will undergo a series of screening tests, including a history and physical examination. Subjects will be required to fast for at least 12 hours before the screening tests begin, which will include a blood draw for assessment of blood lipid profile, liver panel, electrolytes, and blood count. A urine pregnancy test will be performed on all female subjects. Body weight and height will be measured and body mass index will be determined. All subjects will undergo a resting electrocardiogram (EKG), and resting metabolic rate will be measured by indirect calorimetry. Obese subjects, but not control subjects, will then walk on a treadmill at a self-selected pace for 15 minutes. This walking speed will be repeated on days of scheduled physical activity during the obese subjects' in-patient visit. All subjects will also undergo psychiatric evaluation by Structured Clinical Interview for DSM-IV (SCID) during the screening process. The results of the interview will be evaluated during the screening phase by a clinical psychologist and any clinically significant findings will be communicated to the subject by a qualified member of the research team and appropriate follow-up with their primary care physician will be planned. During the screening procedures, subjects will also briefly visit the MRI mock scanner. The mock scanner simulates the noise and dimensions of the MRI scanner that will be used for this study and will allow us to screen for subjects that may be unwilling to participate in the scanning procedures due to noise level, claustrophobia, or overall fit within the scanner. Following the screening visit, volunteers will be asked to continuously wear physical activity monitors and keep food diary and activity records for 3 days. The monitors and records will be returned and reviewed after completion of the three days to ensure adequate macronutrient and energy intake in the habitual diet, as well as to estimate the average daily caloric requirements for use during the baseline period. Along with the 3-day dietary record, volunteers will also be asked to provide a list of any dietary restrictions or allergies.

Study Overview (see Appendix for a detailed description of the study procedures)

Control Subjects

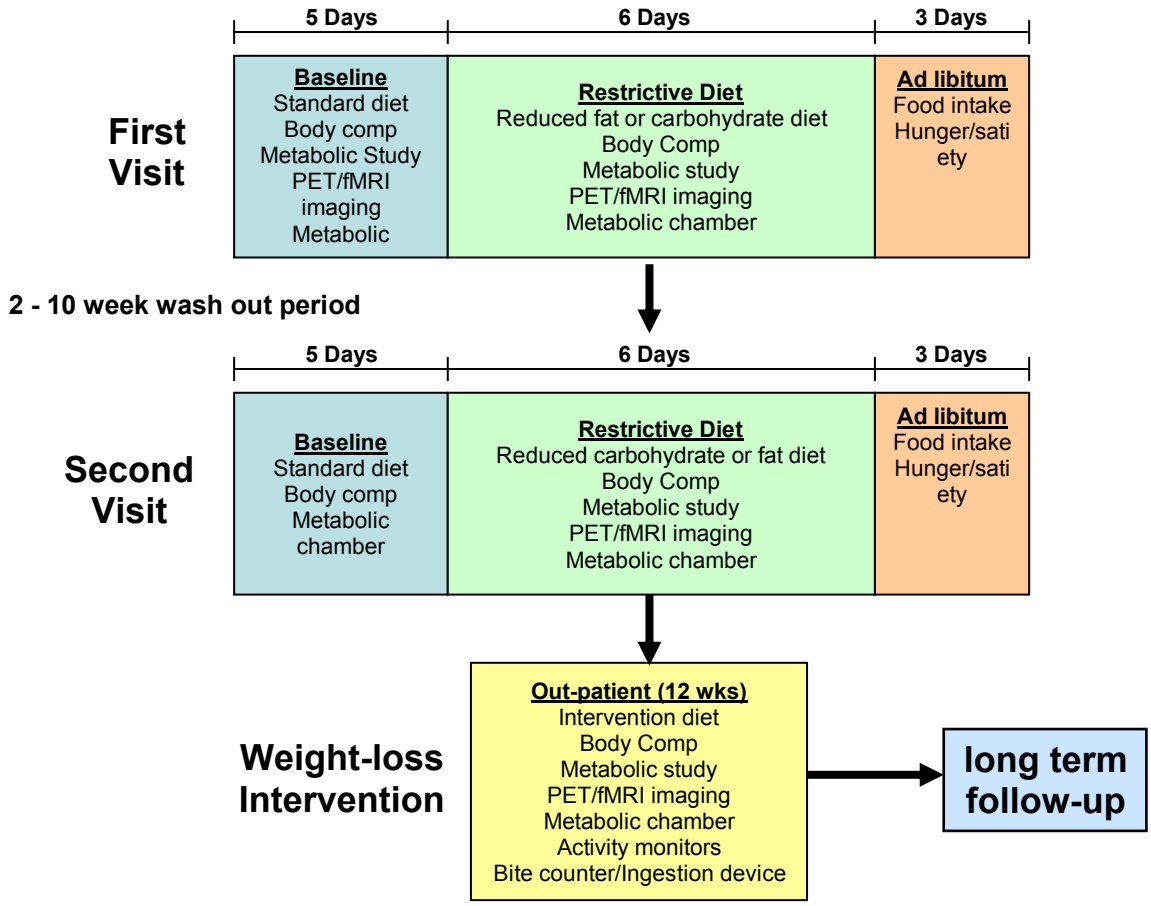
A total of 20 normal control subjects will undergo PET and fMRI procedures. For 2 days before admission to the NIH Metabolic Clinical Research Unit (MCRU), each subject will be provided with a weight-maintenance diet using a standard diet composition of 50% carbohydrate, 35% fat, and 15% protein on an out-patient basis. The food will be supplied by the Clinical Center Metabolic Kitchen and subjects will eat one meal per day on the metabolic unit. The daily caloric content of the diet during the out-patient segment will be based on the 3-day diet and activity records, as well as resting metabolic rate and body size measurements made during screening. On the morning after the second day of the out-patient diet, subjects will arrive at the MCRU at 0800 h and be admitted to the MCRU. During the first day of their in-patient visit, subjects will undergo a series of procedures including measurement of body composition by dual energy x-ray absorptiometry (DEXA) and air-displacement plethysmography (BodPod), measurement of total body water by bioelectrical impedance spectroscopy (BIA), assessment of taste sensitivity (including PROP taste test), three-factor eating questionnaire and socioeconomic questionnaire, assessment of liking and wanting of food, and a gustatory, blood draw for measurement of plasma hormones and substrate, and food perception fMRI scan session. During

the second day of their in-patient visit, subjects will undergo a baseline blood draw for measurement of plasma hormones and substrate, an PET [^{18}F]fallypride imaging session, assessment of delay discounting of monetary versus food rewards, an additional blood draw for measurement of plasma hormones and substrate, and a separate food rating fMRI scan session. Hunger will be assessed by visual analog scale periodically throughout the two days. The subjects will be fed the standard baseline diet during this in-patient period. These measurements on the control subjects will be used for comparison with similar measurements on obese subjects as described below.

Obese Subjects

A total of 20 obese subjects will undergo and complete this cross-over study. The outline of the study is displayed in Figure 1. Subjects will undergo two separate in-patient visits to the research unit followed by a 12 week out-patient weight loss program. For all female subjects, both in-patient visits will be carried out during the follicular phase of the menstruation cycle. On the first visit, subjects will be required to stay 14 consecutive days in the MCRU, beginning with a 5-day weight maintenance diet phase, followed by a 6-day reduced calorie diet phase, and ending with a 3-day ad libitum diet phase. For 2 days before admission to the NIH Metabolic Clinical Research Unit (MCRU), each subject will be provided with a weight-maintenance diet using a standard diet composition of 50% carbohydrate, 35% fat, and 15% protein on an out-patient basis. The food will be supplied by the Clinical Center Metabolic Kitchen and subjects will eat one meal per day on the metabolic unit.

Figure 1. General time line of in-patient and out-patient weight loss phases followed by yearly follow-ups for 5 years.



The daily caloric content of the diet during the initial out-patient segment will be based on the 3-day diet and activity records, as well as resting metabolic rate and body size measurements made during screening. Upon admission, the subjects will continue to be fed the standard diet. The subjects will spend the second day in the metabolic chamber where the standard diet will be fine-tuned to achieve energy balance. The standard diet will then be continued for the next 3 days during the first in-patient stay.

Over the first 5 days following admission to the MCRU, subjects will undergo baseline metabolic studies consisting of a daily blood draw, a 24-hour blood sampling, indirect calorimetry, measurement of body composition (twice) by dual energy x-ray absorptiometry (DEXA) and air-displacement plethysmography (BodPod), measurement of total body water by bioelectrical impedance spectroscopy (BIA) (twice) and by administration of sodium bromide, administration of doubly labeled water (DLW) for measurement of average total energy expenditure, isotopically-labeled substrate infusions (optional), muscle and adipose tissue biopsies (optional), frequently sampled intravenous glucose tolerance test (FSIVGTT) for measurement of insulin sensitivity, positron emission tomography (PET) imaging, magnetic resonance imaging (MRI) scanning sessions of food perception and food rating, Profile of Mood States (POMS) questionnaire, three-factor eating questionnaire socioeconomic questionnaire,

hunger and satiety assessment, assessment of taste sensitivity (including PROP taste test), assessment of delay discounting of monetary versus food rewards, assessment of liking and wanting of food, as well as two 24h stays in a metabolic chamber. Physical activity monitors will be worn continuously, and urine will be collected daily for measurement of 24h urinary nitrogen excretion.

The purpose of these measurements is to provide a comprehensive assessment of baseline whole-body metabolism and body composition in the obese subjects as well as baseline measures of brain reward circuitry and regional brain activity in response to food stimuli. The baseline measurements will then be compared with similar measurements during the in-patient diet interventions and after the outpatient weight loss program as described below.

Directly following the baseline period on the standard diet, subjects will continue their stay at the MCRU for the next 6 days and will be randomly assigned to one of the following reduced calorie diets:

1. **Red-FAT** (selective reduction of 85% of baseline fat calories per day)
2. **Red-CHO** (selective reduction of 60% of baseline carbohydrate calories per day)

Both diet perturbations were designed to produce identical caloric deficits when compared to the standard diet (see Table 1). The two reduced calorie periods will have dietary protein, sodium, and water intakes maintained corresponding to the baseline diet and all subjects will receive a daily multivitamin.

To address primary aim #1 of the study, the subjects will spend the second and final baseline diet days and the first, fourth, and sixth reduced-calorie diet days in the metabolic chamber to measure 24-hour respiratory quotient, substrate utilization rates, as well as macronutrient and energy balances. Primary aims #2 and #3 will be addressed by repeat PET and fMRI studies after 4 days of the reduced-calorie diets.

Table 1. Diet composition for hypothetical subject with estimated total daily energy intake of 2000 kcal/day and estimated total daily energy intake of 3000 kcal/day.

	Macronutrient Ingestion, g/day (% Δ from std diet)			**Total Energy Intake** kcal/day (% Δ from std diet)
	Carbohydrate	**Fat**	**Protein**	
Baseline diet	250g	78g	75g	2000
Red-Fat	250g (0%)	12g (-85%)	75g (0%)	1405 (-30%)
Red-Carb	101g (-60%)	78g (0%)	75g (0%)	1405 (-30%)
Baseline diet	375g	117g	113g	3000
Red-Fat	375g (0%)	18g (-85%)	113g (0%)	2100 (-30%)
Red-Carb	152g (-60%)	117g (0%)	113g (0%)	2100 (-30%)

To address the secondary aims listed above, after several days on the reduced-calorie diets we will repeat the daily blood draw, the 24-hour blood sampling, indirect calorimetry, measurement

of body composition by dual energy x-ray absorptiometry (DEXA) and air-displacement plethysmography (BodPod), measurement of total body water by bioelectrical impedance spectroscopy (BIA), isotopically-labeled substrate infusions (optional), muscle and adipose tissue biopsies (optional), positron emission tomography (PET) imaging, magnetic resonance imaging (MRI) scanning session of food rating, Profile of Mood States (POMS) questionnaire, hunger and satiety assessment, assessment of delay discounting of monetary versus food rewards, and assessment of liking and wanting of food. Immediately after the conclusion of the 6-day calorie reduction period, the subjects will remain at the MCRU for an additional 3 days during which they will have ad libitum food access and the food intake will be measured.

After discharge from the MCRU, a wash-out period of at least 2 weeks will be required before subjects return for the second portion of the study (i.e., the alternate reduced calorie diet than what was consumed during their first visit). This period of time will allow for sufficient wash-out of any effects the diet manipulation may have on subsequent measurements during the second stay at the MCRU. Subjects will be required to begin completion of the second reduced calorie portion of the study within 10 weeks after discharge in order to avoid large changes in physiological state. During the wash-out period, subjects will be encouraged to return to normal living patterns and consume a diet as similar to the standard diet as possible and maintain weight stability (within 2 kg of their baseline weight).

After the wash-out period, subjects will return to the MCRU for the second segment of selective reduced calorie intake. Before being admitted to the MCRU, subjects will again consume the standard diet for 2 days on an out-patient basis, however, after subjects are admitted to the MCRU, they will undergo a reduced number of baseline metabolic studies in comparison with their first in-patient stay. Of the baseline metabolic studies that were conducted during the first in-patient stay, only the following measurements will be repeated: body composition by dual energy x-ray absorptiometry (DEXA) and air-displacement plethysmography (BodPod), total body water by bioelectrical impedance spectroscopy (BIA), average total energy expenditure by administration of doubly labeled water (DLW), hunger and satiety assessment, and two 24h stays in a metabolic chamber. Physical activity monitors will be worn continuously, and urine will be collected daily for measurement of 24h urinary nitrogen excretion. Once the 6-day reduced calorie diet has begun, the timeline and measurements will be the same as during the reduced calorie portion of the first visit.

We have designed this protocol to avoid the confounding influence of the menstrual cycle phase on measurements of energy balance in our female subjects (36). The metabolic studies of the baseline period and calorie reduction period of the first in-patient visit, as well as the second in-patient visit, will be carried out during the follicular phase for all female subjects.

At the end of the second visit, subjects will be discharged from the MCRU and begin a 12 week out-patient weight loss program to address our final primary aims. During this period, all subjects will be prescribed a lifestyle modification consisting of a reduced-calorie diet and increased physical activity with a goal to lose at least 5% of body weight by the end of 12 weeks. Subject's will be given a scale to take home with them and their body weight will be monitored daily (by phone or email) and subjects will meet with registered dietitians biweekly to receive nutrition education and goal assessment. Subjects will also return periodically (at 2, 6, 10, and

12 weeks after discharge) to the MCRU for metabolic studies consisting of body composition, indirect calorimetry, and 24h stay in the metabolic chamber. At the end of the 12 week period, subjects will undergo similar metabolic studies as those performed during the calorie reduction periods.

The obese subjects that complete the out-patient weight loss phase will be provided with the option to participate in follow-up assessments to investigate long-term changes of metabolism, body composition, brain reward circuitry, and regional brain activity. The follow-up assessments will occur on an approximately yearly basis for 5 years following the weight loss program and will repeat the procedures and timeline of the final 2 weeks of the weight loss phase.

Analytical Procedures

Resting energy expenditure

The measurement will be performed in the morning after a 12 h fast and overnight rest with the subject in a supine position. An indirect calorimeter with the ventilated hood technique will be used for the 30 min measurement (the first 5 – 10 mins will be excluded from analysis). The respiratory quotient will be calculated as the ratio of carbon dioxide production to oxygen consumption. Resting metabolic rate will be calculated from VO_2 and VCO_2 measurements using the Weir equation (37) and whole body fat oxidation will be calculated using the equations of Frayn (38).

MedGem Handheld Indirect Calorimeter

This measurement will be performed at home every morning after an overnight fast during the first two weeks and the last two weeks of the 12-week weight-loss program. The device is light-weight and equipped with a disposable mouth piece that the subject breathes through for 5 to 10 minutes while seated and resting comfortably. Subjects will also be wearing a disposable nose clip. This device reports 24hour resting energy expenditure in kcals and VO_2 in mL/min.

Respiratory chamber: 24h energy expenditure

The respiratory chamber is a specially constructed room to assess the metabolism of subjects for a period of 24 hours. Designed as a walk-in "pull" calorimeter, it is an open circuit unit that draws conditioned room air into the chamber at the same flow rate as it is extracted into the gas analysis system. Each of the three rooms is equipped with a toilet and sink with privacy screen, treadmill, bed, desk, and computer with access to television and other forms of entertainment. General physical activity level is measured continuously through a wall mounted monitoring device (microwave sensor). Food and fresh water is passed through an air-lock drawer system. Telemetry and a nurse call are available to enhance subject safety. In the chamber, 24h energy expenditure (EE), sleeping EE, respiratory quotient (RQ), and dietary induced thermogenesis (DIT) will be assessed.

Plasma substrate and hormone concentrations

Blood samples will be collected into chilled test tubes containing EDTA and aprotinin as preservatives. Those sample for which analysis of gastrointestinal peptides will take place will also contain dipeptidyl peptidase IV inhibitor (10 $\mu L \cdot mL^{-1}$ blood) as preservative and be collected into chilled glass tubes. All samples will be kept on ice and then centrifuged (1600 *g* for 15 min at 4°C) within 30 min of collection for isolation of plasma. After centrifugation, the

plasma will immediately be frozen and stored at -80°C for later analysis. Plasma substrate and hormone concentrations will be assessed using the procedures outlined in Table 2. Genomic DNA will be isolated from peripheral mononuclear cells using the QIAmp® system (QIAGEN).

Table 2. List of procedures for measuring plasma substrates and hormones

	Metabolite/hormone	Method
Metabolites	triglycerides	colorimetric assay
	fatty acids	colorimetric assay
	β-hydroxybuterate	fluorometric assay
	glucose	colorimetric assay
Hormones	insulin	RIA
	glucagon	RIA
	leptin	RIA
	c-peptide	RIA
	cortisol	chemiluminescence
	epinephrine	HPLC
	growth hormone	chemiluminescence
	leptin	RIA
	adiponectin	RIA
	GLP-1 (active form)	RIA
	PYY_{3-36}	RIA
	ghrelin	RIA
	CCK	RIA
	GIP	RIA
Markers of Inflammation	CRP	ELISA
	IL-6	ELISA
	TNF-alpha	ELISA

RIA = radioimmunoassay; HPLC = high performance liquid chromatography, ELISA = enzyme-linked immunosorbent assay

Isotope tracer enrichment *(optional)*

The tracer:tracee ratio (TTR) of plasma phenylalanine, glucose, and glycerol will be measured by electron impact ionization gas chromatography/mass spectroscopy (GC/MS). For plasma phenylalanine, GC/MS analysis will be performed after conversion to the tertiary-butyldimethylsilyl derivative of phenylalanine in deproteinized plasma (39). Heptafluorobutyric (HFB) anhydride will be used to form a heptafluorobutyric derivative of glycerol and glucose for GC/MS analysis (40). The TTR in plasma will be used to calculate phenylalanine, glycerol, and glucose rate of appearance (R_a) into plasma. Whole-body plasma phenylalanine R_a, glycerol R_a, and glucose R_a will be determined from the steady-state isotope dilution equation $R_a = I/TTR_A$ (41), where I is the tracer infusion rate ($\mu mol \bullet kg^{-1} \bullet min^{-1}$) and TTR_A is the TTR of [*ring*-$^2H_5$]-phenylalanine, [6,6-$^2H_2$]-glucose, or [$^2H_5$]-glycerol in the arterialized sample.

Muscle biopsy *(optional)*

About 100 – 150 mg of muscle will be taken from the thigh during each biopsy. The muscle biopsy procedure involves numbing a nickel-sized portion of the skin of the thigh with a local anesthetic (lidocaine), making a small incision (1/4 inch), and removing a small piece of muscle.

The muscle will quickly be partitioned into small pieces (~25-50 mg), rinsed with saline, and frozen in liquid nitrogen. The incision will then be closed with a piece of sterile tape. Intramuscular triglyceride (IMTG) concentration will be measured from the liberation of free glycerol (42). Muscle glycogen will be determined from the measurement of glucose after acid hydrolysis of muscle glycogen with hydrochloric acid (43). Gene expression micorarrays will be conducted on muscle biopsy samples for measurement of skeletal muscle gene expression of proteins involved in substrate utilization within the muscle (e.g. GLUT4, PDK-4, PDH, ACC, IRS-1, PI3-K, PDK-1, MAPK, CPT1, FATP, LPL, CD36/FBP).

Subcutaneous Adipose Tissue Biopsy *(optional)*

Subcutaneous adipose tissue (5-10 g) will be removed from the abdominal region by aspiration with a 16-gauge needle under local anesthesia (2% xylocaine). The relatively large sample of fat from each subject will ensure extraction of sufficient RNA for microarrays, follow up, and quantitative real time-PCR validation. A representative portion of the tissues will be formalin-fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin using standard histological methods. The remaining tissue will be separated into aliquots of approximately 500 mg for RNA isolation and protein extraction, flash frozen in liquid nitrogen, and stored at -80 ºC. Gene expression micorarrays will be conducted on adipose tissue biopsy samples for measurement of adipose tissue gene expression of proteins involved in substrate utilization within the adipose tissue (e.g. leptin, adiponectin, HSL, LPL, CD36/FBP, Il-6, PAI-1,TNF-α).

Frequently-sampled intravenous glucose tolerance test (IVGTT)

We will use the IVGTT for measurement of insulin sensitivity using the well-established sensitivity index (SI) derived from Bergman's minimal model (44). Two intravenous catheters will be placed: one will be inserted in an antecubital vein for infusion of glucose and insulin and a second retrograde catheter will be inserted into a dorsal hand of wrist vein (the hand will be enclosed in a warming chamber (65°C) to "arterialize" the blood obtained from all samples). At time 0, an intravenous bolus of glucose (300 mg/kg body weight) will be administered over 1 – 2 minutes. At 20 min, a bolus of insulin (0.03 U/kg body weight) will be given. Blood samples (~2 mL) for glucose and insulin will be taken at -10, -5, 0, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, and 120 minutes. Plasma glucose and insulin will be measured by the methods indicated in Table 1.

Dual energy x-ray absorptiometry (DEXA)

This technique has found extensive clinical and research applications in addition to the assessment of bone mineralization status. With this technique, one can determine total and regional body fat and fat-free masses and can estimate appendicular muscle. This procedure will be performed in the MCRU.

Air-displacement plethysmography

Assessment of body composition will be derived from an estimate of body density obtained using an air displacement plethsymography chamber (BOD POD). Subjects will be weighed and then evaluated in the chamber while clothed in a tight-fitting bathing suit or underwear and acrylic bathing cap. Final body volume will be calculated based on initial body volume corrected for the subject's thoracic gas volume as measured by the breathing circuit housed in

the rear of the chamber through a disposable tube. The two compartment model of Siri will be used to estimate body fatness from the body density measurements obtained using the BOD POD.

Physical activity monitors

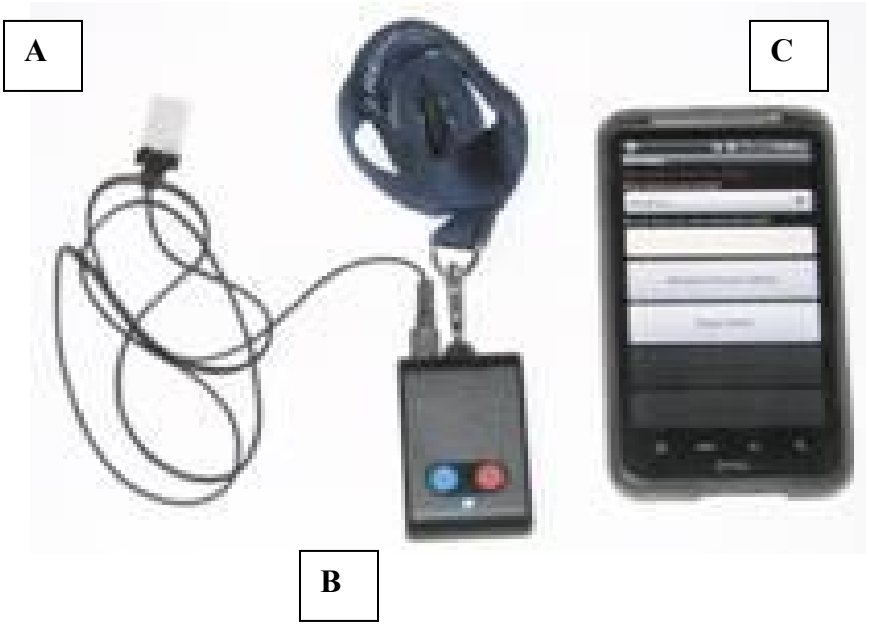
Free-living physical activity will be quantified with activity monitors using high sampling frequencies (32 samples per second in the chamber – minute-to-minute sampling other times) during all waking periods using small, portable pager-type and watch accelerometers (Mini Mitter / Respironics Co, Bend OR) at the subjects' hip, dominant wrist, and non-dominant ankle. Overall physical activity levels, daily changes, amount of time spent in sedentary, moderate, vigorous intensity categories and activity-associated energy expenditures will be extracted (45). In addition, a portable accelerometer + heart rate combination monitor (Actiheart, also by Mini Mitter) will be used. These monitors will be worn throughout both in-patient studies and periodically (i.e. from weeks 2 – 4, weeks 6 – 8, and weeks 10 – 12) during the 12-week out-patient period.

Bite Counter

This method detects eating behavior and provides real-time information concerning bites taken during a meal by counting the total number of bites the user has taken, and providing the bites-taken rate (bites per minute) with a 91% sensitivity (46). This method use an orientation sensor placed on the wrist of a user, and analyze the rolling motion of the wrist in order to detect a pattern related to biting behavior.

Automatic Ingestion Monitor

The automatic ingestion monitor is a device capable of automatically detecting food intake episodes; estimating the number of episodes in a period of time and the frequency, duration, rate and mass of ingestion. The principle of operation is based on non-invasive sensing of jaw motion characteristic of food intake, hand-to-mouth gestures during food intake, and (optionally) swallowing events. Automatic computer algorithms process the sensor signals to recognize and characterize food intake episodes. AIM consists of a jaw motion sensor connected by a wire to a wireless module worn as a pendant around the neck, a small radio transmitter worn on a wrist (used for detection of hand-to-mouth gestures), and a smart phone used for logging of the sensor data. The jaw motion sensor is attached to the jaw. This sensor is connected to the pendant (worn around the neck) by a wire. The pendant is wireless and will send data about the jaw sensor to the smart phone. A picture of an AIM is shown below.



A) Strain sensor (jaw sensor) that will attach by medical tape to the area immediately below the outer ear. B) Wireless module worn as a pendant around the neck. It is connected to the jaw sensor via wire. C) Smart phone that will log the sensor data via a wireless transmission.

There are no electrical risks associated with the automatic ingestion monitor, but subjects may occasionally find wearing the jaw motion sensor inconvenient. The automatic ingestion monitor also involves a neck band which utilizes a breakaway clasp that uses either a magnetic clasp or a breakaway buckle that pops apart under tension and prevents suffocation if a pulling force is applied to the band.

Earlier models of this device have been approved by the Institutional Review Board at Clarkson University for Dr. Sazonov. This device has previously been studied and is included in the following references:

Detection of food intake from swallowing sequences by supervised and unsupervised methods. Lopez-Meyer P, Makeyev O, Schuckers S, Melanson EL, Neuman MR, Sazonov E. Ann Biomed Eng. 2010 Aug;38(8):2766-74. Epub 2010 Mar 30.

Automatic detection of swallowing events by acoustical means for applications of monitoring of ingestive behavior. Sazonov ES, Makeyev O, Schuckers S, Lopez-Meyer P, Melanson EL, Neuman MR. IEEE Trans Biomed Eng. 2010 Mar;57(3):626-33. Epub 2009 Sep 29.

Non-invasive monitoring of chewing and swallowing for objective quantification of ingestive behavior. Sazonov E, Schuckers S, Lopez-Meyer P, Makeyev O, Sazonova N, Melanson EL, Neuman M. Physiol Meas. 2008 May;29(5):525-41. Epub 2008 Apr 22.

Toward objective monitoring of ingestive behavior in free-living population. Sazonov ES, Schuckers SA, Lopez-Meyer P, Makeyev O, Melanson EL, Neuman MR, Hill JO. Obesity (Silver Spring). 2009 Oct;17(10):1971-5. Epub 2009 May 14.

In addition, Dr. Sazonov has been awarded the following NIDDK grant to investigate the use of this device with the following information:

Project Number: 5R21DK085462

Contact PI / Project Leader: SAZONOV, EDWARD S

Title: OBJECTIVE MONITORING OF ENERGY INTAKE AND INGESTIVE BEHAVIOR IN A FREE LIVING POP

Awardee Organization: UNIVERSITY OF ALABAMA IN TUSCALOOSA

Doubly Labeled Water

In the morning on the first day of both in-patient periods, as well as at the beginning and end of the 12-week out-patient period (i.e. the beginning of week 1 and week 10) we will make a collection of a baseline (predose) urine sample. Subjects will then drink from a stock solution of a mixture of 1.5 g of 10% enriched $H_2{}^{18}O$ and 0.08 g of 99% enriched ^2H_2O per kg of body weight followed by 100-200 mL tap water to rinse the dose container. Throughout both in-patient periods, urine samples will be collected each morning in the fasted states. During the out-patient period, after giving the initial dose, urine samples will be collected on three occasions each week for two weeks (i.e. from weeks 1 – 2and 10 – 12). Isotopic enrichments of urine samples will be measured by isotope ratio mass spectrometry. CO_2 production rate will be estimated from the differential disappearance of the 2 isotopes with use of the equation developed by Schoeller et al (47).

Bioelectric Impedance

We will use bioelectrical impedance spectroscopy (BIS) to measure the impedance of the body. BIS spectra will be obtained with a HYDRA bio-impedance spectrum analyzer (model 4200, Xitron Technologies, San Diego, CA) using a spectrum of 50 programmed frequencies spaced throughout a 5KHz to 1MHz range. From this we can estimate total body water (TBW) and extracellular water (ECW), as tissue impedance at low frequencies is controlled by the electrical properties of ECW and at high frequencies by the electrical properties of TBW. Subjects are measured by two sets of electrodes placed on the hand/wrist and foot/ankle while lying supine on a nonconductive surface. This is a non-invasive test.

Bromide dilution

Bromide dilution method will be used to measure ECW. Blood samples will be taken before and 10 h and 11 h after a dose of sodium bromide (60 mg bromide/liter of estimated TBW) (48). The bromide concentration in serum ultrafiltrate will be determined by high performance liquid chromatography (49).

Profile of Mood States (POMS) Questionnaire

The Profile of Mood States (POMS) is a widely used questionnaire for measuring distinct mood states. The POMS has 65 items, each with a 5-point adjective rating scale, that measures six identifiable moods or feelings: Tension-Anxiety (T), Depression-Dejection (D), Anger-Hostility (A), Vigor-Activity (V), Fatigue-Inertia (F), and Confusion-Bewilderment (C). Completion of the POMS takes 5-10 minutes.

Structured Clinical Interview for DSM-IV (SCID)

The SCID is a widely used semi-structured interview for making most of the major psychiatric diagnoses. The interview will be conducted by a trained mental health professional.

Three-Factor Eating Questionnaire

The three-factor eating questionnaire (TEFQ) is a self-assessment questionnaire developed to measure dietary restraint, disinhibition and hunger (50). The questionnaire contains 36 items with a yes/no response, 14 items with a 1-4 response scale, and 1 item with a 1-5 response scale.

MacArthur Socioeconomic Status (SES) Questionnaire

The MacArthur SES questionnaire is a widely used self-assessment questionnaire. It begins with subjective social status questions, followed by questions assessing educational attainment, occupational status, income and assets.

Hunger & Satiety Assessment

At multiple times during their in-patient visit, subjects will be asked to complete a survey to identify their perception of hunger (i.e., visual analog scale [VAS]) (51). More specifically, the VAS survey will consist of four questions: 1) "How hungry do you feel?" 2) "How much do you think you can eat?" 3) "How satisfied do you feel?" and 4) "How full do you feel?" Below each question on the survey there is a horizontal 100mm line with qualifying statements on the extreme left and right side of this line. In response to each question, subjects will be asked to draw a vertical mark on the horizontal line to represent the magnitude of their response to the question. A value for each response is quantified by measuring the distance of their mark (in mm) relative to the left end of the line. Therefore, the values (or "scores") for each question range from 0 to 100. To ensure consistency, all VAS ratings will be measured by the same member of the research team.

Taste Intensity Assessment

The perception of taste intensity in response to standard taste stimuli will be assessed. The standard stimuli will be sweet, sour, salty, bitter, savory (umami), and fat. The subjects will be asked to take a sip of each tastant solution, hold it in their mouth for ~5 sec, and then swallow. A minimum 60 sec interval will be required between each taste stimuli during which subjects will rate their perceived intensity and rinse their mouth with a tasteless rinsing solution. The rating of perceived intensity of each tastant will be recorded by VAS (anchored with “not at all” and “extremely”).

In addition, the gustatory response to the bitter tasting agent, 6-n-propylthiouracil (PROP) will be assessed. A difference in the perception of 6-n-propylthiouracil has been linked to greater perceived bitterness of some bitter compounds and divides the population into nontasters

and tasters (52). Subjects will place a PROP filter paper on their tongue, allow it to moisten, and then rate the bitterness intensity using VAS.

Liking and Wanting Computer Procedure

The liking and wanting procedure comprises two tasks designed to assess (1) explicit liking and wanting, followed immediately by (2) implicit wanting for the same target food stimuli (53, 54). The separate task elements will be integrated to fully randomize explicit and implicit trials. Experiment generator software (E-prime v1.2) is used to integrate the single stimulus trials for the liking task with the paired stimuli trials for the wanting task. The integrated software is also programmed to center the cursor between each trial to produce more consistent response times, and different question prompts will be presented in contrasting colors to encourage discrimination.

Food stimuli presented in the procedure are selected based on two key dimensions associated with loss of appetite control and over-consumption: the fat/carbohydrate content and taste/sensory properties of foods. Stimuli will be presented on a 17-in flat-screen monitor. The food items are selected from a database of photographic stimuli and sorted according to their fat content and taste properties into one of four separate categories: high-fat savory (HFSA); low-fat savory (LFSA); high-fat sweet (HFSW); and low-fat sweet (LFSW). Each category is represented by four different foods; hence a total of 16 different food stimuli will be presented in the procedure (see Table 3).

The aim of the explicit task is to obtain introspective hedonic measures for the same stimuli used in the implicit wanting task. Therefore, each food stimulus is assessed independently using visual analogue scales (VAS). The explicit computer task trials consist of 16 food stimuli presented one at a time and rated according to a 100-mm VAS anchored at each end by the statements "not at all" and "extremely". Subjects will be prompted with the statements "How pleasant would it be to taste some of this food now?" and "How much do you want some of this food now?". In particular, the liking question is constructed to reflect the pleasure of the experience of tasting some of the given food to avoid eliciting a more general evaluation to do with properties inherent to the food itself (53).

Table 3. Photographic food stimuli used in liking and wanting computer task (grouped by food category).

HFSA	LFSA	HFSW	LFSW
Salted peanuts	Savory biscuits	Blueberry muffin	Jelly (jello)
Salted crisps	Pilau rice	Milk chocolate	Popcorn
Swiss cheese	Boiled potatoes	Jam doughnut	Jelly sweets (candies)
French fries	Bread roll	Cream cake	Fruit salad

HFSA: high-fat savory; LFSA: low-fat savory; HFSW: high-fat sweet; LFSW: low-fat sweet

The VAS will be presented on-screen beneath each food stimulus and subjects use the computer mouse to move a centered cursor along the line to indicate their response. When a rating is made, the procedure automatically cycles to the next stimulus trial. Responses on the software will be recorded online and mean ratings for each food category (HFSA, LFSA, etc.) are automatically computed.

Implicit wanting is measured by a behavioral "forced choice" methodology. In this task, a food stimulus from one of the four food categories is paired with one stimulus from the

remaining categories to form a series of 96 trials in which the subjects will be given the standardized instruction to select the food they "most want to eat now". In addition to recording the frequency of selections made in each category (with a possible range of 0–48) which may reveal a relative preference, reaction time (in milliseconds) of each choice is also measured. By covertly recording reaction time, subjects will be unaware of implicit changes in their behavior on the task, while remaining free to determine the direction of their choices. In this measure, the motivated behavioral response independent of the explicit awareness of its incentive value is the key variable. Data from the forced-choice task—including frequency of choice for each food category and reaction time of choice—will be recorded online for later calculation of the means.

Implicit Liking and Wanting Procedure using the ISCAN® ETL-300 Eye Tracker

We will perform pilot testing of this commercially available device to measure eye gaze duration, direction, and pupil dilation during the assessment of liking and wanting of food procedure. The ISCAN® ETL-300 Eye Tracking device shown in the figure below provides detailed measurements about what people look at when they are presented with a visual stimulus such as a picture of food. We will use the eye tracker to measure various quantities like pupil dilation and gaze time when people are presented with various food images. These measurements will help us better understand the psychological factors underlying food choices.

The ISCAN® ETL-300 Eye Tracker is a lightweight (< 150 g or 1/3 of a pound) head-mounted device with an adjustable headband, thus allowing freedom of head movement (See Figure). This device can be used with contact lens or glasses. The subject's eyes are viewed using a specialized miniature infrared video camera system embedded in the headgear. A second video imaging system captures the scene that the subject is viewing. A 25 foot tether will connect the head-mounted device to a computer for data storage and analysis.

The measurements obtained from the device will be used in conjunction with food liking and wanting measures to provide an implicit measure of food wanting. The liking and wanting procedure lasts about 45 min. The eye tracker device will only be worn during the final liking and wanting procedure of each in-patient visit for pilot testing.

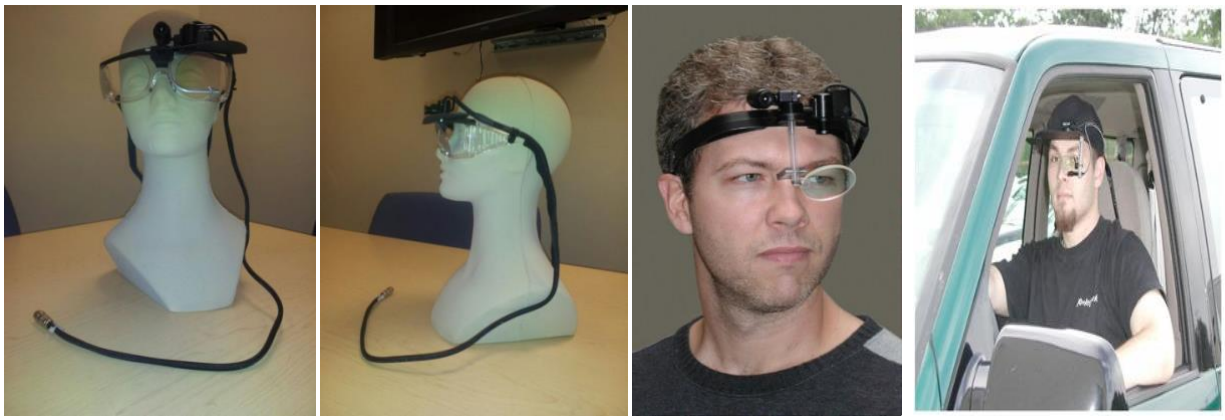


Figure - ISCAN® ETL-300 Eye Tracker

Delay Discounting Computer Procedure

People generally prefer immediate rewards in comparison to delayed rewards, even when the delayed reward has a higher value. The degree to which delayed rewards are discounted in comparison to immediate rewards may provide an index of impulsive decision making. It has been hypothesized that obese individuals may have difficulty adhering to diets because the long-term rewards of weight loss are strongly discounted in comparison to the immediate rewards of palatable food (55).

We will implement a delay discounting computer procedure using an image of a food that scored highly on each individual's liking scale. This food image will be presented along with a forced choice statement such as "Would you rather eat this food now or receive $10 tomorrow?" The subject must select either the "food" or "money" option. We will also measure how long it takes to make each selection. The same food image will always be used as the hypothetical immediate reward, but hypothetical monetary choices will vary in the amounts of: $1, $5, $10, $20, $50, $100 and the associated delay times will vary from: now, 4 hours, tomorrow, 1 week, 2 weeks, and 1 month. Experiment generator software (E-prime v1.2) will be used to present the paired stimuli trials and will be programmed to center the cursor between each trial to produce more consistent response times. Stimuli will be presented on a 17-in flat-screen monitor.

PET imaging

PET scans will be performed on a dedicated head scanner, the High Resolution Research Tomograph (HRRT) with ~2.5 mm resolution. Prior to the scan, a swimming cap with small light reflectors will be put on the subject's head. It is used to monitor the position of the head during the scan. Information about head movement is used in the image reconstruction process to reduce the blurring effect of head movement on the PET images. Scanning will be carried out for 3.5 hours, with short breaks to allow the subject to get up and to void. A transmission scan will be obtained with a ^{137}Cs rotating pin source before radiotracer injection and before the emission scan to correct for attenuation. Scans will start immediately after intravenous injection of 5 mCi of [^{18}F]fallypride using a Harvard® pump and will be carried out for a total of 3.5 hours. After completion of the scan, subjects will be asked to void to minimize radiation exposure to the bladder. We will control the environmental stimulation to the subjects to ensure that it is standardized for all subjects (noise kept to a minimum and room dimly light).

To estimate D2 receptor availability we will obtain the time-activity curves for tissue concentration in striatum (ST) and in cerebellum (CB) along with the time activity curves for [^{18}F]fallypride, which we will use to calculate the distribution volume (DV). The DV corresponds to the equilibrium measurement of the ratio of tissue concentration to plasma concentration and is estimated using a graphical analysis technique for reversible systems (56). The ratio of the DV in ST to CB, which corresponds to (Bmax/Kd) +1 and is insensitive to changes in cerebral blood flow, will be used as model parameter for determination of specific binding (56).

Functional Neuroimaging

All MRI studies will be performed at the High-Field MRI Laboratory on a 3.0T whole body instrument. A 27 cm tube resonator will be used for radio frequency transmission and detection. Structural images will be obtained with a T1-weighted axial 3D modified driven equilibrium Fourier transform (57), and a T2-weighted coronal hyper-echo pulse sequences (58). fMRI will

be performed using a coronal single-shot gradient-echo echo-planar imaging (EPI) sequence. Padding will be used to minimize motion.

Neural bases of gustatory perception. As part of a larger battery of fMRI scans examining the neural representation of food perception in obese and lean subjects, we will localize in each subject the brain regions underlying perception of gustatory stimuli. The gustatory mapping task will involve three types of events: tastant delivery events, cue-catch events, and wash/swallow events.

During tastant delivery events, the subject will see a word-cue for five seconds indicating the imminent delivery of either a sweet (pleasant) tastant, or a tasteless solution. Next the cue word will be replaced with the word "TASTE" for 5 seconds, during which time the subject will receive 0.4 ml of either a sweet tastant, or a tasteless (neutral) solution. The sweet tastant will be composed of apple juice sweetened with sucrose, and the tasteless solution will be composed of 12.5 mM KCL + 1.24 mM NaHCO3. The neutral solution closely matches the properties of human saliva, and thus serves as a good control stimulus because its delivery closely mimics all properties of the sweet solution, yet has no taste. After delivery of the tastant, the subject will next see a fixation mark appear on the screen for between 2.5 and 12.5 seconds, after which the word "WASH" will appear on the screen for 2.5 seconds and the subject will receive .8ml of the tasteless control solution to wash away the preceding tastant. Immediately after the wash period, the word "SWALLOW" will appear, at which time the subject will swallow the fluid in the mouth. Tastant delivery events will occur eighteen times in each scanning run, 9 for the sweet tastant, and 9 times for the neutral tastant.

During cue-catch events, a subject will see a word-cue for five seconds. Importantly, these events only involve the presentation of the cue-words, and not the delivery of fluid. Cue-catch events will be followed by a variable duration fixation period. These free-standing "catch-trial" events, which will occur 12 times in each scanning run (6 for the sweet cue, 6 for the neutral cue), and will be modeled in the design matrix along with the cues in the tastant delivery events, thus allowing us mathematically deconvolve the response to the cues from the tastants.

Finally, during wash/swallow events, a subject will see the word "WASH" appear on the screen for 2.5 seconds and the subject will receive .8ml of the tasteless control solution. Immediately after the wash period, the word "SWALLOW" will appear, at which time the subject will swallow the tasteless solution. Wash/swallow events will be followed by a variable duration fixation period. Freestanding wash/swallow events will occur 9 times in each scanning run, and will be modeled in the design matrix along with the 18 wash/swallow events during the tastant delivery events, again allowing us to mathematically deconvolve the response associated with receiving a tastant from receiving a wash solution and swallowing.

By comparing activations following administration of tastant and neutral control stimuli, we will be able to functionally localize in each subject the brain regions underlying gustatory perception. Additionally, by comparing patterns of activity in response to sweet and neutral cues independent of the delivery of tastants into the mouth, we will be able to map regions engaged during anticipation of rewarding tastes. The brain regions identified in this study will be used as functional regions of interest (ROIs) in the fMRI studies of food perception performed with these

same subjects. In addition, the dataset will allow us to examine whether there are group differences in the neural bases of gustatory perception for obese and lean subjects. Finally, we will also be able to examine whether there are group differences between obese and lean subjects in their anticipatory responses for rewarding tastes.

Neural bases of food stimulus perception. To evaluate the neural bases of food stimulus perception, subjects will be presented with photographs of four different types of foods: high-fat savory foods, low-fat savory foods, high-fat sweet foods, and low-fat sweet foods. Presentation of the food stimuli will be blocked by food category. Each block will contain 5 photographs of food stimuli from a given category, each presented for 2.5 seconds with a 500 ms interstimulus interval. In addition to food picture blocks, subjects will also be shown blocks of non-food object photographs that will serve as an object perception control condition. The participants' task while viewing the stimuli will be simply to press a button on a hand-held response box anytime they see the same picture presented twice in a row. Picture presentation blocks will be separated by 10-second 'null-stimulus' blocks in which only a small fixation mark will be presented on the screen. These null periods will be included to allow the blood oxygenation-related fMRI signal to return to baseline after each stimulus block. By comparing the activity associated with viewing food- and non-food stimuli, we will localize in each subject the brain regions supporting food perception in general. In addition, by comparing activity among the four food stimulus categories, we will localize ROIs supporting perception of each food type. Finally, by comparing activity between obese and lean individuals, this research design will also allow us to identify group differences in the perception of food stimuli in general, as well as among the four food stimulus categories

Relationship between diet and activation in brain regions supporting food preference judgments. To investigate the effects of dietary changes on neural responses to food stimuli and food preference, obese subjects will undergo multiple fMRI scan sessions in which they will provide preference ratings in response to photographs of high-fat savory foods, low-fat savory foods, high-fat sweet foods, and low-fat sweet foods. Lean subjects will also undergo this procedure, though only in a single scan. Food stimuli will be presented in a fast event-related design, with individual stimuli appearing in a pseudo-random order. Each stimulus will be presented for 5-seconds during which subjects will provide one of two different ratings:
"if given the opportunity right now, how pleasant it would be to eat this food?"
AND
"if given the opportunity right now, how much self-control would it take to NOT eat this food?"
by manipulating a handheld MR-compatible scroll wheel to select a location on a number line.
To allow mathematical deconvolution of the responses to stimulus presentations, each picture presentation will be followed by the appearance of a simple 'null-stimulus' fixation mark for between 2 and 12 seconds. Analyses will proceed in the following manner. First, subjects' ratings on the "liking" question will be used as covariates on the imaging data to identify brain regions supporting food preference judgments for each of the four classes of food stimuli. Second, activity during the liking task will be subtracted from the activity during the food impulse control question to identify regions involved in behavioral inhibition in response to food stimuli. A corollary prediction is that these regions should be modulated by subjects' responses on the food impulse control question. To explore whether diet affects brain regions supporting food preference judgments and impulse control, we will compare activity measured in the first

scan session collected during the baseline diet phase, with activity measured in subsequent scan sessions when obese subjects are in either the dietary carbohydrate- or fat-reduction phase of the study. In addition to addressing these two questions using whole-brain imaging analyses, we will also explore how diet affects activity during food-preference judgments and impuse control within the functionally-defined ROIs identified in the gustatory stimulation and food perception functional localizer experiments described above.

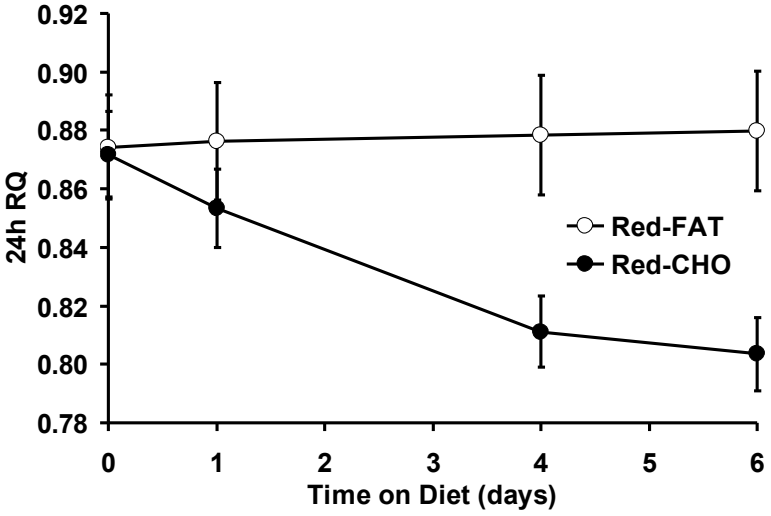
Functional connectivity among brain regions supporting gustation and perception of food stimuli. Resting-state functional connectivity analyses will be used to examine group differences in connectivity between gustatory and food perception areas, as well as possible changes in the connectivity among these regions as a function of diet. During the initial baseline scan session, both obese and lean subjects will undergo an 8-minute "resting state" fMRI scan. During this scan subjects will be asked to stare at a fixation mark located in the center of the display, and to do their best to clear their minds and not think about anything in particular. In the subsequent scan sessions, obese subjects will again undergo this 8-minute resting state scan. Seed voxels for the resting-state connectivity analyses will be selected using data from gustatory perception ROIs and food perception ROIs. Group differences in functional connectivity will be explored by comparing connectivity profiles in the data collected during the baseline scan sessions for obese and non-obese individuals. Diet-related changes in obese subjects' functional connectivity profiles will be explored by comparing data collected during the first session (baseline diet) with the data collected in the subsequent scan sessions (low-fat or low-carbohydrate diets).

Statistical Analysis

We performed our power/sample size calculations based on predicted measurements described in our primary aims. No direct preliminary data is available for our power/sample size calculations for this study. However, we have performed computer simulations of the proposed study design using a mathematical model of human macronutrient metabolism developed by our group (9) and the predicted changes and standard deviations are consistent with the literature as will be described.

Sample size for Primary Aim #1. Figure 2 shows the simulated 24-hour respiratory quotient (RQ) values for the two in-patient phases using a mathematical model of human macronutrient metabolism (9). The computer simulations were obtained by generating a population of 100 "virtual study subjects" (50 male and 50 female) with a range of initial body weights matching our inclusion criteria (e.g., body weight = 110 ± 20 kg). The two in-patient phases of the protocol were simulated with an

Figure 2. Simulated RQ Changes



intervening 2 week washout period when the standard baseline diet was reinstated. We also simulated realistic variations of physical activity, body water, as well as random errors in our assessment of the baseline diet such that the simulated average energy balance before the reduced calorie diets had a realistically wide uncertainty range of ± 340 kcal/d. The simulated standard deviations of the 24-hour RQ values were 0.02 which are similar to those observed in other feeding studies (59, 60). As shown in Figure 2, the 24-hour RQ was predicted to change from 0.87 ± 0.02 to 0.85 ± 0.02 after the first day of the reduced carbohydrate diet which is consistent with data where dietary carbohydrate was reduced but replaced isocalorically with dietary fat (59, 60).

From these estimates, we calculated that detection of a significant decrease of 24-hour RQ on day 1 of the reduced carbohydrate diet requires sample size of N = 4 for one sided test at 80% power and α = 0.05. We expect an attrition rate of roughly 50%, therefore achievement of adequate statistical power for this outcome measurement will require a recruitment sample size of N = 8.

Sample size for Primary Aims #2 and #3.

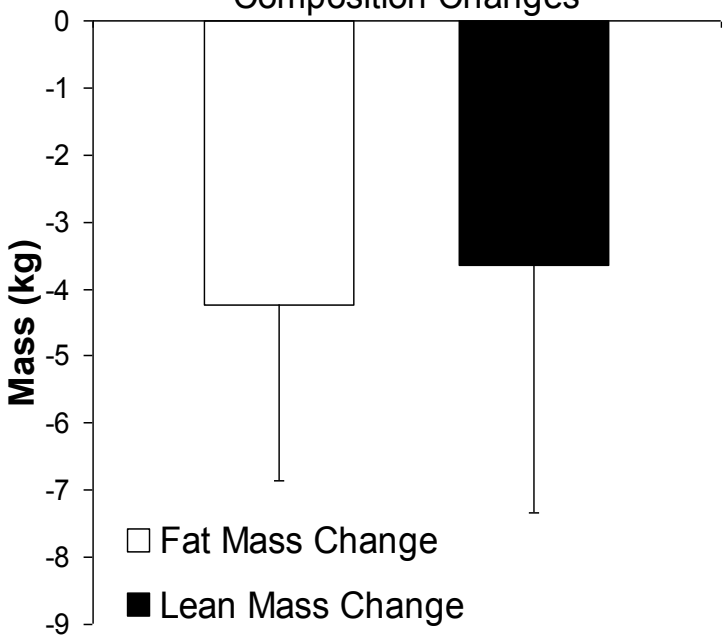
Our power analysis is based on the primary analysis of the main hypothesis, that is, to assess the effects of diet on D2 receptor availability and on reactivity of brain limbic regions when exposed to food stimuli. We will compare the D2 receptor availability before and after diet using the paired samples t-test or its nonparametric counterpart. In order to detect a meaningful difference of effect size 1 (the effect size is the ratio of the expected mean and the standard deviation of the paired differences), with 20 subjects measured, we will achieve a power of 98% via the paired samples t-test ($\alpha = 0.05$, 2-sided). The normality assumption will be examined via the Shapiro-Wilk test. In the event of non-normality, the paired samples t-test will be replaced by the Wilcoxon signed-rank test, which will result in an estimated 5% loss in test power and thus we estimate a power of 93% for the Wilcoxon signed-rank test (61). For fMRI scanning, we base our power analysis on the number of subjects needed to detect significant differences in fMRI activation between two randomized conditions in normal control subjects (62). As a result of the preliminary data we know that: for control subjects the inter-individual variability of fMRI signals in cognitive activation tasks is be approximately 0.7%. We aim to detect larger signal changes than 0.5%.

Assuming a Type I error of 0.05 and 80% power, we need a minimum of 20 obese subjects and 20 control subjects. We expect an attrition rate of roughly 50% with the obese subjects. Therefore, achievement of adequate statistical power for these outcome measurements will require a recruitment sample size of N = 40 obese subjects.

Sample size for Primary Aim #4.

The goal for primary aim #4 is to measure the change of body fat mass and fat-free mass during the 12 week out-patient weight loss period and relate those measured body composition changes to the macronutrient imbalances measured during the in-patient diet perturbations. Using our computational model, we simulated a prescribed decrease of 750 kcal/d from the standard baseline diet. The predicted changes in body composition are depicted in Figure 3. The overall body weight decreased by an average of 7.9 ± 4.6 kg with a fat mass loss of 4.2 ± 2.6 kg and a lean mass loss of 3.6 ± 3.7 kg. These estimates are similar to data from previous studies reporting changes in body composition after 12 weeks of a prescribed 750 kcal/d reduction in dietary intake leading to a reduction of body weight by 9.5 ± 5 kg, a fat mass decrease of 6.6 ± 3 kg, and a fat-free mass decrease of 2.8 ± 2.5 kg (63).

Figure 3. Simulated Body Composition Changes



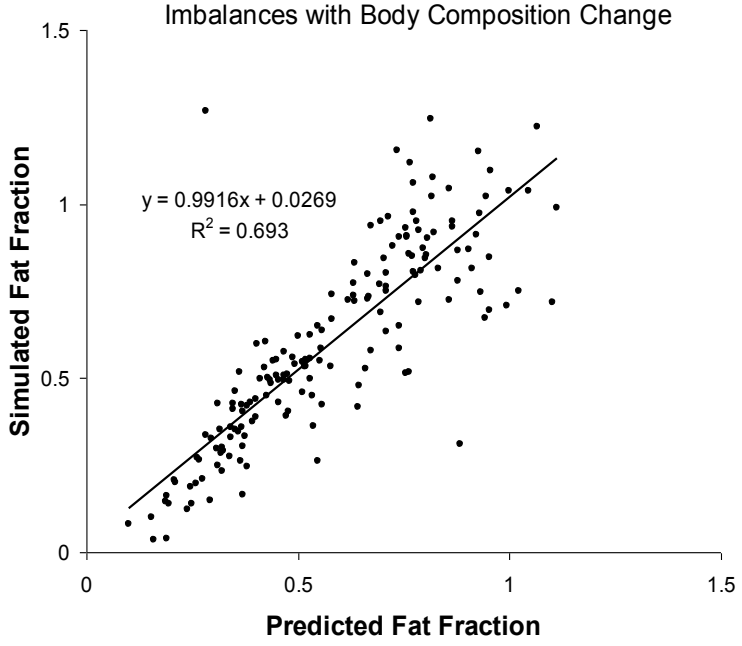
From these estimates, we calculated that detection of a significant difference in fat mass requires sample size of N = 4 for two sided test at 80% power and α = 0.05. We expect an attrition rate of roughly 50%, therefore achievement of adequate statistical power for this outcome measurement will require a recruitment sample size of N = 8.

We have previously described a simple equation for using short-term measurements of macronutrient imbalance to predict the fraction of body weight loss resulting from loss of body fat (8). The predicted fat fraction of weight loss is calculated as follows:

$$\frac{\Delta FM}{\Delta BW} = \frac{\rho_L\left(\text{24h Fat Imbalance}\right)}{\rho_L\left(\text{24h Fat Imbalance}\right) + \rho_F\left(\text{24h Energy Imbalance - 24h Fat Imbalance}\right)}$$

where ρ_F = 9.4 kcal/g and ρ_L = 1.8 kcal/g are the energy densities of body fat and lean mass changes, respectively. Using the average imbalances measured during the last days of the two reduced calorie diet periods, this simple equation was used to predict the fat fraction of weight loss in our simulated weight loss intervention and the results are shown in Figure 4. These simulations suggest that the predictions from the simple equation can relate short-term energy imbalances to long-term body composition changes. We estimate that an N = 20 will be sufficient to detect significant correlations between the predicted fat fraction of weight loss and the body composition changes following the 12 week weight loss period. With an expected attrition rate of 50%, we estimate that we will need to recruit 40 subjects to detect significant correlations between the predicted fat fraction of weight loss and the body composition changes.

Figure 4. Simulated Correlations of Short-term Imbalances with Body Composition Change



Safety Considerations

Possible Risks and Hazards

Research-related risks in this study include those associated with study procedures, namely blood drawing, indirect calorimetry, measurement of body composition by dual energy x-ray absorptiometry (DEXA) and air-displacement plethysmography (BodPod), measurement of total body water by bioelectrical impedance spectroscopy (BIA) and by administration of sodium bromide, administration of doubly labeled water (DLW) for measurement of average total energy expenditure, isotopically-labeled substrate infusions (optional), muscle and adipose tissue biopsies (optional), frequently samples intravenous glucose tolerance test (FSIVGTT) for measurement of insulin sensitivity, positron emission tomography (PET) imaging, magnetic resonance imaging (MRI) scanning sessions of food perception and food rating, Profile of Mood States (POMS) questionnaire, hunger and satiety assessment, assessment of liking and wanting of food, wearing physical activity monitors, handheld indirect calorimeters, as well as 24h stays in the metabolic chamber. There are no study medications and we do not expect any diet intervention-related risks since the study will be under close follow-up with trained dietitians/nutrition experts throughout the study.

Blood drawing. The placement of intravenous needles may cause transient pain, and may also result in bruising, bleeding, and/or clotting at the site of needle insertion. The application of

direct pressure at the catheterization site will be used to help prevent these symptoms. There is a possibility that a catheter placement would be unsuccessful or need to be removed. If this should occur, another catheter would be placed. It is possible that this may occur more than once during the subject's participation in the protocol. The daily blood draw schedule for the obese subjects is as follows:

BLOOD DRAWN – 1ST IN-PATIENT VISIT

Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**	**Day 15**	**Total**
0 mL	12 mL	10 mL	85 mL	60 mL	15 mL	4 mL	19 mL	57 mL	15 mL	8 mL	3 mL	3 mL	3 mL	3 mL	**297 mL**

BLOOD DRAWN – 2ND IN-PATIENT VISIT

Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 13**	**Day 15**	**Total**
0 mL	12 mL	0 mL	0 mL	5 mL	11 mL	4 mL	19 mL	57 mL	15 mL	8 mL	3 mL	3 mL	3 mL	3 mL	**143 mL**

Buccal mucosa sampling. During the in-patient visit, an oral (buccal) mucosa sample will be obtained for DNA. This is a non-invasive procedure and there are no known risks with performing this procedure.

Indirect calorimetry. The use of the ventilation hood may cause some minimal discomfort in claustrophobic subjects.

DEXA. The amount of radiation during the DEXA scan is less than one mRem to the whole body. This amount is less than the radiation exposure received by anyone living in the United States in one day. The use of the DEXA scan apparatus may cause some minimal discomfort in claustrophobic subjects and may cause some minimal back pain in a small minority of the individuals.

Air-displacement plethysmography. This procedure may cause discomfort in claustrophobic subjects.

Bioelectrical impedance spectroscopy. There are no risks involved with this measurement and subjects will not feel the electric current.

Sodium bromide dilution. At the dose administered, there are no known toxicities, although the salty taste may be unpleasant.

Doubly labeled water. The doubly labeled water procedure has no known risks.

Intravenous glucose tolerance test. Minor discomforts include arm tenderness and transient flushing and nausea resulting from the glucose infusion. The major risk of this procedure is hypoglycemia due to the administration of insulin. To allow for early detection of this potential,

though infrequent complication, study participants will be closely monitored throughout the test. Glucose levels will be assessed at the beside throughout the test. Should chemical (plasma glucose < 60 mg/dL) or symptomatic hypoglycemia (hunger, sweating, palpitations, or mental status change) occur, the test will be stopped immediately and the study volunteer will be treated with oral glucose or with 50% glucose IV, if medically indicated.

Profile of mood states questionnaire. There is no known risk associated with the POMS questionnaire, however, there is the potential of discovering clinically relevant information requiring further follow-up. If this is the case, subjects will be notified by a qualified member of the research team and appropriate follow-up with the primary care physician will be planned.

Structured clinical interview for DSM-IV. There is no known risk associated with the SCID interview, however, there is the potential of discovering clinically relevant information requiring further follow-up. If this is the case, subjects will be notified by a qualified member of the research team and appropriate follow-up with the primary care physician will be planned.

Three-factor eating questionnaire. There is no known risk associated with the TFEQ.

MacArthur Socioeconomic Status (SES) Questionnaire. There are no known risks associated with the MacArthur SES questionnaire although some subjects may find completing the task tedious or some may find the questions probing and too personal in nature to comfortably answer. Subjects will be informed that they do not have to respond to all the questions if they have reservations about sharing such personal information.

Hunger and satiety assessment. There are no known risks associated with hunger and satiety assessment

Taste intensity assessment. There is no known risk associated with assessment of taste intensity, however an unpleasant sensation from tasting the stimuli may result in some individuals, specifically in response to propylthiouracil (PROP).

Delay discounting computer procedure. There is no known risk associated with the assessment of delay discounting by computer.

Assessment of liking and wanting of food. There is no known risk associated with assessment of liking and wanting of food.

Assessment of implicit liking and wanting of food using the ISCAN® ETL-300 Eye Tracker. There are no known risks associated with assessment of liking and wanting of food or the eye tracking device. It may be temporarily inconvenient to wear the head-mounted eye tracking device. The device will not be used to diagnose, treat or prevent disease during the procedure and does not significantly or permanently affect any function or structure of the body. The device can be used with contact lens or glasses.

Metabolic chamber. Besides inconveniences that can reasonably be expected as a result of spending an extensive time (24h) in the live-in room calorimeter, the serious risk to subjects' health is minimum.

Physical activity monitors. There are no risks associated with the monitors, but subjects may find them to occasionally be inconvenient.

MedGem handheld indirect calorimeters. The test is non-invasive, but subjects may find the mouth piece and nose clip to be slightly uncomfortable.

Tracer infusion (optional). The tracer-labeled solutions infused are not commercially prepared and will be prepared by the pharmacy using proper compounding procedures. Although unlikely, the infusions may cause discomfort at the catheter site, fever, and/or infection.

Subcutaneous fat and skeletal muscle biopsy (optional). The muscle biopsy may cause transient pain, and may also result in bruising, bleeding, clotting, infection, and/or scaring at the site of needle insertion. The application of direct pressure at the biopsy site will be used to help prevent these symptoms. This procedure may also result in dizziness or fainting. To reduce the chance of feeling faint or dizzy, this procedure will be performed while the subject is in bed. The procedure will be performed under sterile technique to minimize the chances of infection. Pain will be reduced during the muscle biopsy by using a local anesthetic (lidocaine). Injection of lidocaine itself can result in brief discomfort (a "stinging" sensation). A very low dose of lidocaine will be used, as doses several times greater that what will be administered in this protocol can affect the central nervous system and the cardiovascular system. Post-biopsy, the needle site will be cleaned and covered with gauze and translucent dressing tape. Study participants will be instructed to report to the clinical staff any changes at the biopsy site including bleeding, secretion, erythema, pain, and signs and symptoms of infection. Study participants will be instructed to self-monitor the biopsy site after discharge from the Clinical Center.

Positron emission tomography. This research study involves exposure to radiation from the radioactive tracer [^{18}F]fallypride and the transmission scans. The total amount of radiation obese subjects will receive in this study is from four injection of 5 millicuries of [^{18}F]fallypride (control subjects will receive just one injection). The NIH Radiation Safety Committee has reviewed the use of radiation in this research study and has approved this use as necessary to obtain the research information desired.

Using the standard way of describing radiation dose, from participating in this study, obese subjects will receive a total of 8.80 rem to their gallbladder wall, 6.60 rem to their urinary bladder wall, and 5.20 rem to their liver. All other organs will receive smaller amounts of radiation. Control subjects will receive a fourth of this radiation to their respective organs. Although each organ will receive a different dose, the amount of radiation exposure obese subjects will receive from these procedures is equal to a uniform whole-body exposure of 1.56 rem (0.39 rem for control subjects). The amount of radiation received in this study is within the dose guideline established by the NIH Radiation Safety Committee for research subjects. The guideline is an effective dose of 5 rem (or 5,000 mrem) received per year.

One possible effect that could occur at these doses is a slight increase in the risk of cancer. The natural chance of a person getting a fatal cancer during his/her lifetime is about 1 out of 4 (or 25 percent). The increase in the chance of getting a fatal cancer, as a result of the radiation exposure received from this research study, is 0.06 percent for obese subjects (0.02 percent for control subjects). Therefore, the total risk of fatal cancer may be estimated to increase from 25 percent to 25.06 percent in obese subjects (25.02 for control subject). This change in risk is small and cannot be measured directly. Compared with other everyday risks, such as flying in an airplane or driving a car, this increase is considered slight.

If subjects are pregnant or breast feeding, they may not participate in this research study. It is best to avoid radiation exposure to unborn or nursing children since they are more sensitive to radiation than adults.

Another unlikely risk associated with this procedure is intravenous infiltration and radioactive ligand extravasation into the tissues in which case standard care will include warm compresses to the area of infiltration, administration of intravenous fluids, and use of a survey meter to take recordings over the infiltration site. The appearance of radioactivity increase in the brain will be closely monitored to enable early detection of intravenous infiltration in which case the scan will be terminated. In order to minimize risks of intravenous line infiltration, attempts may be made to use wrist or hand rather than antecubital veins for intravenous placement.

Magnetic resonance imaging. People are at risk for injury from the MRI magnet if they have pacemakers or other implanted electrical devices, brain stimulators, some types of dental implants, aneurysm clips (metal clips on the wall of a large artery), metallic prostheses (including metal pins and rods, heart valves, and cochlear implants), permanent eyeliner, implanted delivery pump, or shrapnel fragments. Welders and metal workers are also at risk for injury because of possible small metal fragments in the eye of which they may be unaware. Subjects will be screened for these conditions before having the scan, and if they have any, they will not receive an MRI scan. In addition, all magnetic objects (for example, watches, coins, jewelry, and credit cards) will be removed before entering the MRI scan room.

It is not known if MRI is completely safe for a developing fetus. Therefore, all women of childbearing potential will have a pregnancy test performed on the morning of the scanning visit before any scans are performed. The scan will not be done if the pregnancy test is positive. People with fear of confined spaces may become anxious during an MRI. Those with back problems may have back pain or discomfort from lying in the scanner. The noise from the scanner is loud enough to damage hearing. Subjects should not have a research MRI scan if they know that they have hearing loss. Everyone having a research MRI scan will be fitted with hearing protection. If it is difficult for subjects to tolerate the confinement or the noise within the scanner they will be taken out immediately if they so request. There are no known long-term risks of MRI scans.

DNA and tissues containing genetic material. Some of the blood drawn for this study and some of the adipose and muscle tissue collected at the biopsy will be used to analyze genes that may be important in obesity and its related traits. Family studies are not conducted under this protocol. The results of the testing are likely not to have direct clinical relevance and thus, we will not

provide participants with the results of such testing. By agreeing to participate in this study, participants do not waive any rights regarding access to and disclosure of medical records. If our testing discovers information important to the health of the participant or the health of the participant's offspring, we will share it with the participant and recommend appropriate follow-up which may include genetic counseling. Possible risks of knowing such results include: anxiety or other psychological distress; and the possibility of insurance and job discrimination. A possible risk of not knowing includes being unaware of the need for treatment. These risks can change depending on the results of the research and whether there is a treatment or cure for a particular disease. Sometimes patients have been required to furnish information from genetic testing for health insurance, life insurance, and/or a job. A Federal law, called the Genetic Information Nondiscrimination Act (GINA), generally makes it illegal for health insurance companies, group health plans, and most employers to discriminate against employees based on genetic information. This law generally will protect participants in the following ways:

- Health insurance companies and group health plans may not request genetic information that we get from this research.
- Health insurance companies and group health plans may not use genetic information when making decisions regarding patient eligibility or premiums.
- Employers with 15 or more employees may not use genetic information that we get from this research when making a decision to hire, promote, or fire employees or when setting the terms of employment.

All health insurance companies and group health plans must follow this law by May 21, 2010. All employers with 15 or more employees must follow this law as of November 21, 2009. This Federal law does not protect against genetic discrimination by companies that sell life insurance, disability insurance, or long-term care insurance. Donation of tissues (blood, fat, and muscle) for these research purposes will not be included the participant's medical records.

Risks Related to Clinical Relevance of Test Results

If any lab tests, questionnaires, PET or MRI scans, or any other measurements made during the screening or procedures of this protocol show clinically significant abnormalities that may impact on the health and well-being of the subjects, they will be notified by a qualified member of the research team and appropriate follow-up with their primary care physician will be planned.

Safety and Event Reporting and Data Monitoring

Adverse events, protocol deviations, unanticipated problems (UP), serious adverse events, sponsor and serious, are defined in NIH HRPP SOP 16 ("Reporting Requirements for Unanticipated Problems, Adverse Events and Protocol Deviations."). All adverse events occurring during the study either observed by the physicians, nurses, or dietitians or reported by the subject will be recorded.

Serious unanticipated problems and serious protocol deviations will be reported to the IRB and CD (Clinical Director) as soon as possible, but not more than 7 days after the PI first learns of the event. Unanticipated problems that are not serious will be reported to the IRB and CD as soon as possible, but not more than 14 days after the PI first learns of the event. Protocol deviations that are not serious will be reported to the IRB as soon as possible, but not more than 14 days after the PI first learns of the event.

SAE's and AE's that are at least possibly related to the research interventions and are not UPs will be reported only as aggregated summaries at Continuing Review (CR). If the rate and/or severity of these events exceeds those of comparable events previously observed in this or similar studies, the events will be classified and reported as though they are Unanticipated Problems.

All deaths will be reported to the Clinical Director and the IRB within 7 days after the PI first learns of the event.

Data and Safety Monitoring Plan

No DSMB will be instituted to record adverse events given the modest level of risk involved in the research and the involvement of a multidisciplinary team in the study. Adverse events will be recorded and monitored by the Principal Investigator and Medical Advisory Investigator: Dr. Kevin Hall and Dr. Ranganath Muniyappa, respectively.

Human Subject Consideration

Recruitment Strategies

Subjects will be recruited from the community through advertisements in clinicaltrials.gov and possibly, local flyers, newspapers, or magazines. We will utilize the Patient Recruitment and Public Liaison Office in the Clinical Research Center in establishing and implementing a recruitment strategy.

The age range for inclusion in this protocol is 18-45 years. The rational for excluding subjects older than 45 years is multi-fold. First, obese and lean subjects will not be matched for age; therefore, in order to diminish the chance of a type 1 error in our results we chose to limit the age range to provide us with a more homogenous subject population. Second, a number of the major metabolic outcome variables of this protocol have been shown to change significantly with aging (e.g., metabolic rate and body composition). Third, much of the current information on our primary outcome measurements was gathered from subjects within the age range specified by this protocol; therefore, we believe that this age range is appropriate as it allows us to put our results into direct context with the current knowledge base. Finally, a more homogeneous population of obese subjects with respect to age will allow us to randomize the first in-patient diet intervention to match for gender and BMI without large variations of age. This is important because we anticipate a relatively high attrition rate following the first in-patient stay and the crossover study design may therefore be compromised. In that case, our backup plan is to compare the diet interventions across subjects and such a comparison is facilitated when the variance of age is not too great.

Recruitment of Women, Children, and Minority Individuals

We will actively encourage the participation of women and minorities. Considering the prevalence of obesity in the general population, we expect that women will represent approximately half of the study population. Moreover, given the increased incidence and morbidity of obesity among minorities and the social demographics of the greater Washington, DC area, we anticipate that African-Americans and Hispanics will constitute at least 20% of enrollees.

On the other hand, we have deliberately chosen not to study children and adolescents at this time pending the outcome of the findings of this protocol. At present, there is not information suggesting that the metabolic responses to selective reductions of dietary macronutrients are likely different in adults and children and so we see no compelling reason to expose children to the rigors and inconvenience of this type of study. We fully recognize, however, that the insights derived from the data analysis in this study may very well direct our attention to other potentially fruitful areas of clinical research involving childhood obesity, given the early onset and alarming risks associated with obesity and other major public health issues in this population.

Subject Withdrawal

Participants may voluntarily withdraw from this study at any given time. Also, there are several conditions that require the Principal/Co-Investigator to drop a study volunteer from this protocol, but not limited to the following:

- Development of any new medical condition or start of medications that would have prevented enrollment in this study as it pertains to the exclusion criteria.
- Inability or unwillingness to comply with study requirements
- The Principal/Co-Investigator, Dr. Kevin Hall or Dr. Nick Knuth, deems it unsafe to remain in the study
- Pregnancy
- The study is terminated

Research Use, Storage, and Disposition of Human Subjects' Samples and Data

As with all clinical data, the findings will be kept confidential. Volunteer clinical data will be protected and tracked using standard operating procedures in the medical record department. All research charts and records will be kept in a secure place in a locked file cabinet in the office of the Principal Investigator. All research samples and data will be identified by a study code linked to the subject's name and the code and the results of all analyses will be kept strictly confidential. All research samples (blood, fluids, tissue, DNA, mRNA) will be coded for storage in refrigerators and freezers in a locked laboratory. The plan is to store the samples until they are analyzed and a sample of serum, tissue, and genetic material will be safeguarded for future analysis of other factors related to obesity and its phenotype. These samples will be stored indefinitely. The IRB will be notified in the event these samples are accidentally destroyed, lost or are anonymized. Studies on these samples will always be related to the scope of this proposal.

Data from the assessment of liking and wanting of food will be sent to Graham Finlayson, PhD, a collaborator on this study, at the University of Leeds. These data will be coded and will include the measured rating, selection, and reaction time to visual food stimuli. No identifying information will be sent. The Investigators will retain the link to all coded samples. It is necessary to retain the link to the volunteer's identity to correlate study data with the individual's

clinical information for this collaboration and all other collaborations where data will be shared in a coded manner.

Dr. Edward Sazonov from the University of Alabama will be a collaborator on this study but will not have any direct contact with the study subjects. Data files from the automated ingestion monitor will be coded by subject ID and the coded data will be sent to Dr. Sazonov for further processing to identify periods of food intake. The data will remain in a protected storage in Dr. Sazonov's lab until the study is complete. The data collected from the device will include the following:

A. Data stream from the chewing sensor, which is a single channel sampled at 1000Hz.
B. Data stream from the hand gesture sensor, single channel sampled at 10Hz.
C. Data stream from the motion sensor, three channels sampled at 100Hz
D. Data stream from self report buttons, two channels samples at 10Hz

All of these sensor streams carry no personal data that could be used to identify subjects. The smartphone portion of the device serves as the data logger storing sensor information transmitted in real time via Bluetooth on its SD card. The wireless transmission is protected by the encryption mechanism of Bluetooth standard. The data on the smartphone is deprived of any personal information on the subject. The data is stored in a binary format which is impossible to decipher without knowledge of its internal structure. The subjects will not have access to the data stored on the cell phone as initiation, completion of data collection and downloading on the data will performed by the investigators with phone being password locked at other time. The data from the phone will be retrieved after every 24-hr period.

Dr. Eric Muth from Clemson University will be a collaborator on the study but will not have any direct contact with the study subjects. Data files from the bite counter will be coded by subject ID and the coded data will be sent to Dr. Muth for further processing to quantify food intake.

Dr. Carla Prado from Florida State will be a collaborator on the study, but will not have any further direct contact with the study subjects. Data files from diet, hunger and satiety will be coded by subject ID and the coded data will be sent to Dr. Prado for further processing.

Dr. Siervo from Newcastle University will be a collaborator on the study, but will not have any further direct contact with the study subjects. Data files from the indirect calorimetry, urinary nitrogen, diet, and body composition data will be coded by subject ID and the coded data will be sent to Dr. Siervo for further processing.

Some of the blood drawn for this study and some of the muscle and fat tissue collected at the biopsy (for obese subjects only) will be used to analyze certain genes that are important in the development of obesity. We will be looking for changes in the expression of different genes comparing obese subjects with control subjects and studying the obese subjects before and after weight loss. We will also search for changes (mutations) in genes directly associated with

obesity, but not test for mutations that cause known genetic diseases or conduct a study on family history of health or illness.

Some clinically relevant research data will be stored indefinitely in the medical record and will be accessible to the subject for review by others of their choosing (doctors, insurance companies etc.) after executing a release of information. This and other data will be maintained in databases in the Clinical Endocrine Section of the CEB, password protected and secure.

Collaborations on Stored Tissue and Blood Samples

Collaborations requiring transfer of stored tissue and blood samples will be done in a coded fashion after informing the IRB and obtaining necessary assurances from the outside institution. The protocol will be amended and IRB approval will be sought when such collaborations are established.

Informed Consent

The investigational nature of the study and its risks will be carefully explained to the subject and a signed consent will be obtained prior to performing any protocol described testing. The informed consent process will be documented on a progress note and a copy of the note and the original informed consent will be filed in the subject's record.

As we have amended our protocol to include the MacArthur Socioeconomic Status (SES) Questionnaire recently, we are lacking data for those subjects recruited prior to amendment approval. These patients will be contacted by the principal investigator or one of the study co-investigators. The investigational nature of the questionnaire and its attendant risks will be carefully explained to the subject and a signed consent will be obtained prior to collecting the data. For those subjects who are unable or unwilling to travel to the NIH the consent will be executed by telephone. The subject will receive a copy of the protocol consent in the mail prior to being consented. After they have had an opportunity to review the consent the investigator will contact the subject by telephone. The investigator will review the investigational nature of the protocol with the subject and answer questions in the presence of a witness. If the subject chooses to participate, the subject and the witness will sign and date the consent. The informed consent document will be mailed to the principal or associate investigator who led the discussion, who will sign and date and mail back a fully executed copy for the subject's records. The informed consent process will be documented on a progress note and a copy of the note and the original informed consent will be filed in the subject's record. We will ask the subject to also mail a copy of the completed SES questionnaire to the study team.

We have also amended our protocol to include buccal cell DNA collection. We are lacking data for those subjects recruited prior to amendment approval. These patients will be contacted by the principal investigator or one of the study co-investigators. The investigational nature of this DNA sample and its attendant risks will be carefully explained to the subject and a signed consent will be obtained prior to collecting the data. For those subjects who are unable or unwilling to travel to the NIH the consent will be executed by telephone. The subject will receive a copy of this protocol telephone consent in the mail prior to being consented. After they have had an opportunity to review the consent the investigator will contact the subject by telephone. The investigator will review the investigational nature of the protocol with the subject and answer questions in the presence of a witness. If the subject chooses to participate, the subject and the witness will sign and date the consent. The informed consent document will be mailed to the principal or associate investigator who led the discussion, who will sign, date and mail back a

fully executed copy for the subject's records. The informed consent process will be documented on a progress note and a copy of the note and the original informed consent will be filed in the subject's record. We will ask the subject to also mail the DNA sample to the study team.

Benefits to Study Participants

This study will enroll both lean (control) and obese individuals. All volunteers will receive a full history and physical and information about their health prior to beginning their individual study. For the obese subjects, this study has a goal of a achieving a 5% weight loss. As noted in the introduction, as weight loss is difficult, this study will offer a supervised outpatient program designed to achieve weight loss. This may lead to favorable future metabolic outcomes in individuals who are successful in losing and maintaining weight loss (such as decreased risk of diabetes or hypertension). There will be no other direct benefits from participation in this study aside from the knowledge that they are contributing to advancing our understanding of the brain regions involved with food intake and our understanding of obesity, and that these insights may lead to new treatment options in the future. Abnormal values will be discussed with the study volunteers and forwarded to their primary care physicians.

Remuneration

Subjects will receive payments for time and effort connected with the outpatient visits, hospital stays, and procedures according to the following schedule:

Control Subjects

EVENT	COMPENSATION	OCCURANCE	TOTAL
out-patient food record	$20	1	$20
in-patient per diem	$40	2	$80
body composition (DEXA, Bod-Pod, BIA) (2 units)	$50	1	$50
Hunger and satiety assessments	$25	1	$25
POMS questionnaires (1 unit)	$25	1	$25
SES and TFE questionnaires (1 unit)	$25	1	$25
taste sensitivity assessment (1 unit)	$25	1	$25
delay discounting procedure (1 unit)	$25	1	$25
food preference assessment (1 unit)	$25	1	$25
PET (4 units)	$100	1	$100
fMRI (gustatory and food perception scans) (4 units)	$100	1	$100
fMRI (resting-state and food rating scans) (4 units)	$100	1	$100
		TOTAL:	**$600**

Obese Subjects, Main Study

EVENT	COMPENSATION	OCCURANCE	TOTAL
out-patient food record	$20	1	$20
in-patient per diem	$40	32	$1280
body composition (DEXA, Bod-Pod, BIA) (2 units)	$50	10	$500
sodium bromide dose	$25	2	$50
doubly labeled water (DLW) (1 unit)	$25	4	$100
24-h blood sampling (2 units)	$50	3	$150
tracer labeld substrate infusions (2 units) ***(optional)***	***$50***	***4***	***$200***
muscle & fat biopsy (4 units) ***(optional)***	***$100***	***4***	***$400***
indirect calorimetry (1unit)	$25	7	$175
IVGTT (1 unit)	$25	1	$25
PET (4 units)	$100	4	$400
fMRI (gustatory and food perception scans) (4 units)	$100	1	$100
fMRI (resting-state and food rating scans) (4 units)	$100	4	$400
24-hour respiratory chamber (4 units)	$100	12	$1200
physical activity monitors (1 unit)	$25	5	$125
bite counter (1 unit)	$25	3	$75
automatic ingestion monitor (1 unit)	$25	3	$75
hunger questionnaires (1 unit)	$25	17	$425
POMS questionnaire (1 unit)	$25	4	$100
SES & TFE questionnaires (1 unit)	$25	1	$25
taste sensitivity assessment (1 unit)	$25	1	$25
delay discounting procedure (1 unit)	$25	4	$100
food preference assessment (1 unit)	$25	4	$100
Attendance at bi-weekly counseling	$20	5	$100
At-home weights & indirect calorimetry	$10	28	$280
out-patient meal days	$10	4	$40
bonus for completing study	$200	1	$200
	TOTAL REMUNERATION:		**$6,670**

Obese Subjects, Each Yearly Follow-up

EVENT	COMPENSATION	OCCURANCE	TOTAL
out-patient food record	$20	1	$20
in-patient per diem	$40	2	$80
body composition (DEXA, Bod-Pod, BIA) (2 units)	$50	2	$100
sodium bromide dose	$25	1	$25
doubly labeled water (DLW) (1 unit)	$25	1	$25
tracer labeld substrate infusions (2 units) ***(optional)***	***$50***	***1***	***$50***
muscle & fat biopsy (4 units) ***(optional)***	***$100***	***1***	***$100***
indirect calorimetry (1unit)	$25	1	$25
IVGTT (1 unit)	$25	1	$25
PET (4 units)	$100	1	$100
fMRI (resting-state and food rating scans) (4 units)	$100	1	$100
24-hour respiratory chamber (4 units)	$100	1	$100
physical activity monitors (1 unit)	$25	1	$25
bite counter (1 unit)	$25	1	$25
automatic ingestion monitor (1 unit)	$25	1	$25
hunger questionnaires (1 unit)	$25	1	$25
POMS questionnaire (1 unit)	$25	1	$25
SES & TFE questionnaires (1 unit)	$25	1	$25
delay discounting procedure (1 unit)	$25	1	$25
food preference assessment (1 unit)	$25	1	$25
At-home weight	$10	1	$10
		REMUNERATION:	**$960**

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Appendix

Detailed Study Design and Methods

Screening Visit

At the initial screening visit, study volunteers will be asked questions about their general health status and undergo a routine blood test to measure basic metabolic functions in an effort to address the various inclusion/exclusion criteria. All subjects who are interested in participating in the full study protocol will have the following procedures and test performed after an overnight fast and after signing the standard informed consent document:

- History and physical examination
- Fasting blood tests [complete blood count (CBC), lipid profile, glucose, insulin]
- Body weight and body mass index (BMI) determination
- Resting electrocardiogram (EKG)
- Resting metabolic rate (measured by indirect calorimetry)
- 15 minute treadmill walking at self-selected speed – (obese subjects only)
- Psychiatric evaluation by Structured Clinical Interview for DSM-IV (SCID)
- Visit to the MRI mock scanner
- Pregnancy test (women only)

Volunteers will be asked to continuously wear physical activity monitors and keep food diary and activity records for the subsequent 3 days. All subjects will be given directions to record their dietary intake for three days. The physical activity monitors and food records will be returned and reviewed after 3 days to ensure adequate macronutrient and energy intake in the habitual diet, as well as to gage the average daily caloric requirements for use during the baseline period. Food records will be analyzed using Nutrition Data Systems for Research (NDS-R) software, developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN. If qualified for entry into the study on the basis of the determinations listed above, discussions will take place as to the scheduling of the beginning of active participation in the study (i.e. beginning of the baseline period for obese subjects, or scanning day for control subjects).

Control Subjects

If the results from the screening visit permit their continued participation in this research study control subjects will be invited to the Clinical Research Center for a second visit. During the 2 days before their second visit, control subjects will receive a standard diet with a relative macronutrient content of 50% carbohydrate, 35% fat, and 15% protein on an out-patient basis. Subjects will be required to consume one meal per day of the standard diet at the hospital, and will take away with them the remainder of the day's meals. The daily caloric content of the diet during the out-patient segment will be based on the 3-day dietary record provided during screening. On the morning after the second day of the out-patient diet, subjects will arrive at the MCRU at 0800 h and be admitted to the MCRU. At 0815h, body weight and body composition (by DEXA and air-displacement plethysmography) will be measured, as well as measurement of total body water content by bioelectrical impedance. At 1100 h subjects will undergo assessment of taste sensitivity (including PROP) and complete the three-factor eating, profile of mood state,

and MacArthur socioeconomic questionnaires. At 1345 h, a blood sample will be taken and hunger will be assessed by VAS, and then at 1400 h, subjects will undergo an fMRI scanning session comprised of gustatory functional localizer scans, food perception functional localizer scans, and an anatomical scan (see Analytical Procedures). Finally, at 1700 h hunger will again be assessed by VAS before subjects undergo assessment of liking and wanting of food by computer presentation of four separate food categories: high-fat savory (HFSA), low-fat savory (LFSA), high-fat sweet (HFSW), and low-fat sweet (LFSW). Subjects will be fed the standardized diet at 0900h, 1200 h, and 1900 h during their first in-patient day.

The following morning, a blood sample will be taken at 0715 h. Between 0930 and 1130h (dependent on available scan time), subjects will undergo a positron emission tomography (PET) scan. Briefly, after placement of an intravenous catheter and positioning, subjects will be given an intravenous injection of ~5 mCi of [^{18}F]fallypride. For the next 3.5 hours, scanning will be carried out in sets with periodic breaks to allow the subjects to get up and to void. At 5.5h after the scheduled PET scan subjects will undergo a delay discounting computer procedure where they will be required to make decisions between hypothetical immediate food rewards in comparison to various delayed monetary rewards. Finally, at 1745 h subjects will have a final blood draw followed by, at 1800 h, a second fMRI scanning session, comprised of the required clinical scans, a resting-state scan, food rating scans, and a second anatomical scan (see Analytical Procedures). Hunger will be assessed by VAS before the PET scan, before the delayed discounting procedure, and before the final fMRI scan. Subjects will be fed the standardized diet during their second in-patient day at 1200 h, 1500 h, and 2000 h. The subjects will then be discharged following completion of their evening meal.

Obese Subjects

First In-Patient Visit

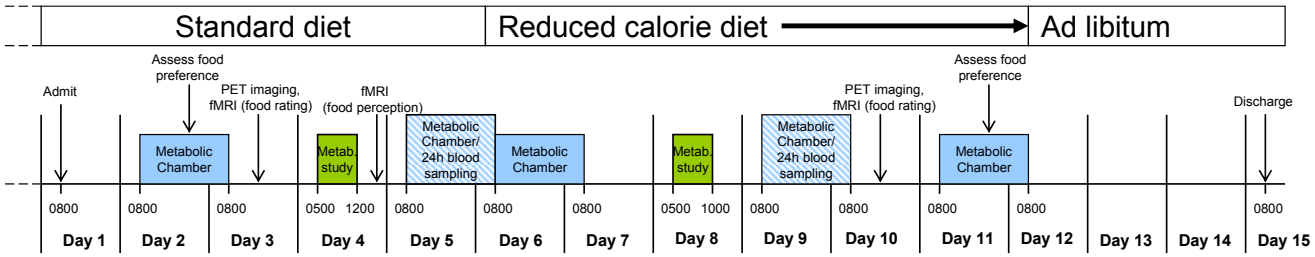
Baseline dietary procedures

If the results from the screening visit permit their continued participation in this research study obese subjects will be invited to the Clinical Research Center for their first in-patient visit, which will last 14 days. During the 2 days before admission to the MCRU, subjects will receive a standard diet with a relative macronutrient content of 50% carbohydrate, 35% fat, and 15% protein on an out-patient basis. Subjects will be required to consume one meal per day of the standard diet at the hospital, and will take away with them the remainder of the day's meals. The daily caloric content of the diet during the out-patient segment will be based on the 3-day dietary record provided during screening. After these 2 days, subjects will be admitted to the hospital and will continue to consume the standard diet for an additional 5 days. The initial daily caloric content of the standard diet will be equal to 1.5 x resting metabolic rate (RMR), which will be measured during their initial screening visit. This caloric content has been found to approximate the total energy daily energy expenditure when sedentary (64). Body weight will be monitored daily and the amount of food given to the subjects will be adjusted to maintain a stable body weight.

Specific procedures during baseline period

A schematic timeline of events during the first in-patient visit is depicted in Figure A1. The first 5 days of this visit will serve as the baseline period, and during this baseline period, subjects will consume a standard diet supplied by the clinical center metabolic kitchen (as described above). Beginning two days before admission, all food to be consumed by the subjects will also

Figure A1. Schematic timeline of first in-patient visit.

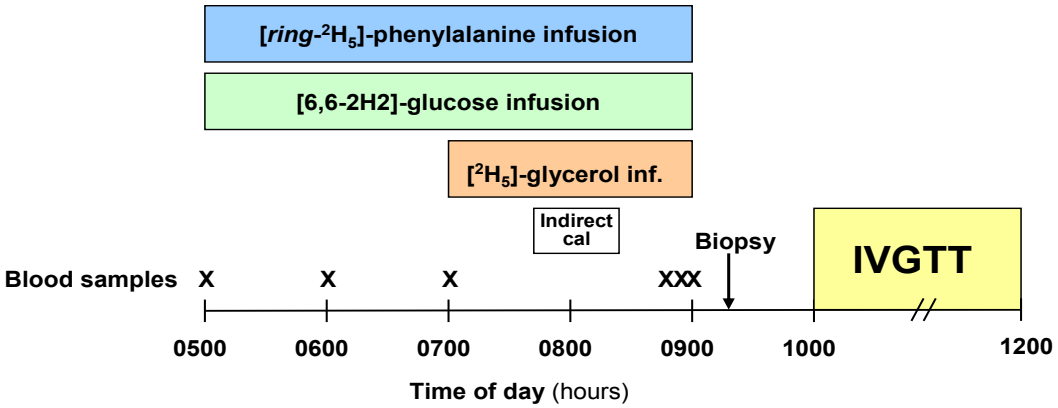


be supplied by the clinical center metabolic kitchen. Subjects will arrive each morning at the clinical center at 0800 h for measurement of body weight, after which they will eat breakfast, and then take the remainder of the day's meal with them to consume later during the day. Subjects will be instructed to consume only and all the food provided to them and to return those portions that they were unable to eat the following day when they return for the next day's meals.

On the morning following the second day of the out-patient standard diet, subjects will arrive in the fasted state at the MCRU at 0800 h for admission to the MCRU (Day 1, Figure A1). Following admission, subjects will be fitted with accelerometers, a bite counter and an automatic ingestion monitor for continuous measurement of activity, which will be worn throughout their entire in-patient visit. At 0845 h a baseline urine sample will be collected (24h urine specimens will be collected throughout the duration of the in-patient period for measurement of nitrogen excretion). At 0855 h subjects will drink from a stock solution of 0.15 g/kg body weight of $H_2{}^{18}O$ and 0.08 g/kg body weight of ^2H_2O followed by 100-200 mL tap water to rinse the dose container (measurement of tracer decay will be made in urine samples collected every morning for the remainder of the study). At 1100 h subjects will undergo assessment of taste sensitivity (including PROP) and complete the three-factor eating,profile of mood state, and MacArthur socioeconomic questionnaires. Subjects will be fed the standardized diet at three meals during the day on Day 1 at 0900 h, 1300 h, and 1800 h. At 1500 h, subjects will begin a regular scheduled (i.e. daily) physical activity session of treadmill walking for a total of 60 minutes (6 x 10-minute walking period with 10-minute rest period between each walking period) at the self selected speed determined during the screening process. This session of walking will be repeated each day during the remainder of the in-patient period, including while in the metabolic chamber. The daily caloric intake will account for the additional calories expended by the added physical activity.

At 0700 h on the morning of Day 2 body weight and body composition (by DEXA and air-displacement plethysmography) will be measured, as well as measurement of total body water content by bioelectrical impedance. At 0745 h a blood sample will be taken, and at 0800 h, subjects will enter the metabolic chamber for measurement of 24h energy expenditure. Subjects will be fed the standardized diet at three meals during the day on Day 1 at 0900 h, 1300 h, and 2000 h while in the metabolic chamber, and hunger and satiety will be assessed by visual analog scale and questionnaire before each meal, and every 30 min for 2h and then every hour after each meal. At 1500 h subjects will undergo their scheduled session of treadmill walking, and at 1800 h, subjects will also undergo assessment of liking and wanting of food by computer presentation of four separate food categories: high-fat savory (HFSA), low-fat savory (LFSA), high-fat sweet (HFSW), and low-fat sweet (LFSW). At 2200 h, overall energy balance will be assessed and a standard shake will be administered to maintain energy balance for the remainder of the stay in

Figure A2. Schematic timeline of metabolic study.



the metabolic chamber. The amount of shake required to maintain energy balance will be given with the dinner for the remainder of the baseline days (Day 3-5).

On the morning of Day 3, subjects will be allowed to leave the chamber. At that time a urine sample will be collected, body weight will be measured, a blood sample will be taken, and subjects will be allowed to shower but not to engage in any other activities. Between 0930 and 1130h (dependent on available scan time), subjects will undergo a positron emission tomography (PET) scan. Briefly, after placement of an intravenous catheter and positioning of the subjects, subjects will be given an intravenous injection of ~5 mCi of [^{18}F]fallypride. For the next 3.5 hours, scanning will be carried out in sets with periodic breaks to allow the subjects to get up and to void. At 5.5h after the scheduled PET scan subjects will undergo a delay discounting computer procedure where they will be required to make decisions between hypothetical immediate food rewards in comparison to various delayed monetary rewards. At 1500 h, subjects will begin the regular scheduled session of treadmill walking for a total of 60 minutes (6 x 10-minute walking period with 10-minute rest period between each walking period). At 1730 h, a blood sample will be taken, and then at 1800 h subjects will undergo fMRI scanning, comprised of the required clinical scans, a resting-state scan, food rating scans, and an anatomical scan (see Analytical Procedures). On day 3, subjects will be fed their standardized diet at 1200 h and 1500 h and 2000 h. At 2200 h on Day 3, two intravenous catheters will be inserted for infusions during the "metabolic study" the next day, during which we will assess protein synthesis, lipolysis, glucose uptake, and insulin sensitivity (Figure A2). The tracer infusion procedures and the muscle and adipose tissue biopsies are optional procedures; however the indirect calorimetry and measurement of insulin sensitivity (beginning at 1000 h on Day 4) will not be optional.

At 0500 h on the morning of Day 4, a blood sample will be obtained from the hand vein in order to measure baseline isotope enrichment of phenylalanine, glycerol, and glucose. After the blood draw, a primed-constant infusion of [*ring*-$^{2}H_5$]-phenylalanine and [6,6-$^{2}H_2$]-glucose will be started ([*ring*-$^{2}H_5$]-phenylalanine: 2 μmol/kg priming dose, 0.05 μmol/kg/min continuous infusion; [6,6-$^{2}H_2$]-glucose: 35 μmol/kg priming dose, 0.41 μmol/kg/min continuous infusion). Two hours after starting the [*ring*-$^{2}H_5$]-phenylalanine and [6,6-$^{2}H_2$]-glucose infusions, we will begin a 2h constant infusion of [$^{2}H_5$]-glycerol (1.5 μmol/kg priming dose, 0.1 μmol/kg/min continuous infusion). After 3h 50min of the phenylalanine and glucose infusions and 1h 50min

of the glycerol infusion (the time necessary to achieve isotopic equilibrium of phenylalanine, glucose, and glycerol within the body), three arterialized blood samples will be obtained from the heated hand vein (to obtain "arterialized" blood samples (65)) in 5 min intervals for determination of phenylalanine, glucose, and glycerol rate of appearance (R_a) into the circulation. Substrate oxidation will be measured using indirect calorimetry from 0745-0815 h and all the infusions will be stopped at 0900 h. At 0915 h, we will obtain a small muscle sample (~100 mg) from the muscle of the thigh for measurement of intramuscular substrates (e.g. glycogen, lipids), and a subcutaneous adipose tissue sample (5-10 g) will be obtained from the abdominal region for measurement of adipose tissue proteins. At 1000 h, subjects will undergo an intravenous glucose tolerance test (IVGTT) to assess insulin sensitivity using the minimal model method (66). Briefly, subjects will be injected with a bolus of glucose (300 mg/kg body weight) at 1000 h and a bolus of insulin (0.03 U/kg) at 1020 h and blood samples will be taken at frequent time points for 2h after the glucose bolus. Subjects will be fed immediately following the conclusion of the IVGTT at 1200 h, as well as at 1600 h and 1800 h on Day 4. At 1345 h, a blood sample will be taken, and then at 1400 h subjects will undergo an fMRI scanning session comprised of gustatory functional localizer scans, food perception functional localizer scans, and an anatomical scan (see Analytical Procedures). At 1600 h, subjects will begin the regular scheduled session of treadmill walking for a total of 30 minutes (3 x 10-minute walking period with 10-minute rest period between each walking period). The second 30 minutes of scheduled walking will occur at 1900 h (3 x 10-minute walking period with 10-minute rest period between each walking period). Finally, at 2200 h, subjects will be administered a dose of a sodium bromide solution for measurement of extracellular water content. Subjects will be weighed, and a urine and blood sample will be collected before the sodium bromide dose is given.

On Day 5, at 0700 h, a urine sample will be collected, and subject's body weight and body composition (by DEXA and air-displacement plethysmography) will be measured, as well as measurement of total body water content by bioelectrical impedance. At 0730 h, an intravenous catheter will be inserted and a blood sample will be taken. Throughout the following 24h period, additional blood samples will be frequently taken to allow measurement of plasma hormone concentrations (i.e every 30 min for 4h after breakfast and lunch, and for 2h after dinner, and then at 2100 h and 2200 h on Day 5 and 0300 h and 0700 h on Day 6). While in the metabolic chamber, hunger and satiety will be assessed by visual analog scale and questionnaire before each meal, and every 30 min for 2h and then every hour after each meal. Subjects will be fed their standardized diet at 0900 h, 1300 h, and 1800 h, while in the metabolic chamber and undergo their scheduled session of treadmill walking at 1500 h.

Calorie reduction dietary procedures

After the 5-day baseline period, subjects will be randomized into either the *Red-Fat* or *Red-Carb* segments of the protocol:

Red-Fat: Total caloric content of the "reduced-fat" diet will be 30% per day lower than the calories required to maintain energy balance during the baseline period, and will be achieved strictly by the removal of 85% of the baseline fat calories from the diet (the absolute amount of fat [in grams] removed will be dependent on the initial caloric intake during the baseline period, see Table 1). The absolute amount of carbohydrate and protein (in grams) will be identical to

that provided in the baseline diet. The relative macronutrient content for this diet will be approximately 71% carbohydrate, 8% fat, and 21% protein.

Red-Carb: Total caloric content of the "reduced-carbohydrate" diet will also be 30% per day lower than the calories required to maintain energy balance during the baseline period, and will be achieved strictly by the removal of 60% of baseline carbohydrate calories from the diet (the absolute amount of carbohydrate [in grams] removed will be dependent on the initial caloric intake during the baseline period). The absolute amount of fat and protein (in grams) will be identical to that provided in the baseline diet. The relative macronutrient content for this diet will be approximately 29% carbohydrate, 50% fat, and 21% protein. An example of real foods for the two calorie-restricted diets, as well as the standard diet, can be found in Appendix Table A1.

Specific procedures for calorie reduction period

A schematic timeline of events during the calorie reduction period (Days 6 – 11) is depicted in Figure A1. On the morning of Day 6 (after the 24h metabolic chamber stay that will begin on Day 5) subjects will be allowed to leave the chamber. At that time a urine sample will be collected, body weight will be measured, and subjects will be allowed to shower but not to engage in any other activities. After collection of a basal blood sample, subjects will then re-enter the metabolic chamber for measurement of 24h energy expenditure. The first meal of the reduced calorie diet will be given in the chamber as breakfast on Day 6 at 0900 h, and the remainder of the meals will be fed at 1300 h and 1800 h while in the chamber. While in the metabolic chamber, hunger and satiety will again be assessed by visual analog scale and questionnaire before each meal, and every 30 min for 2h and then every hour after each meal, and subjects will undergo their scheduled session of treadmill walking at 1500 h. Subjects will leave the chamber on the morning of Day 7 and a basal blood sample will be collected. Subjects will be fed their reduced calorie diet at 0900 h, 1300 h, and 1800 h, on Day 7 and undergo their scheduled session of treadmill walking at 1500 h. The procedures during the last four days of the reduced calorie period (Days 8 – 11) will be similar to those during the last four days of the baseline period as described above with the exceptions noted below. Briefly, at the end of Day 7 at 2200 h two intravenous catheters will be inserted for infusions during the "metabolic study" (i.e. tracer infusion, indirect calorimetry, muscle and adipose tissue biopsy) that will occur on Day 8. The "metabolic study" procedures will again be optional. On Day 8, at 0500h, the metabolic study will begin, however the IVGTT procedure, the fMRI (gustatory and food perception) scanning session, and the sodium bromide dose that will all performed following the metabolic study on Day 4 will be not be repeated on Day 8. Therefore, the subjects will be fed at 1000 h, 1300 h, and 1800 h, and at 1500 h they will undergo their regular scheduled session of treadmill walking for a total of 60 minutes (6 x 10-minute walking period with 10-minute rest period between each walking period). On Day 9, at 0730 h, after three days on the reduced calorie diet, an intravenous catheter will be inserted and a blood sample will be taken. At 0800h subjects will enter the metabolic chamber and undergo a 24h stay in order to measure changes in energy expenditure as a result of the diet. Hunger and satiety will be assessed by visual analog scale and questionnaire while in the chamber. Throughout the 24h period in the chamber, blood samples will be frequently taken to allow measurement of plasma hormone concentration. On Day 10, after leaving the metabolic chamber, a blood sample will be taken before subjects undergo PET imaging, followed by the delay discounting computer procedure at 1400 h, a blood

sample at 1730 h, and at 1800 h subjects will undergo the same fMRI session as preformed on Day 3 of the baseline diet period. On Day 11, at 0715 h, subject's body weight, body composition (by DEXA and air-displacement plethysmography), and total body water content (by bioelectrical impedance) will be measured, and then at 0800 h, subjects will enter the metabolic chamber for measurement of 24h energy expenditure. While in the metabolic chamber, hunger and satiety again will be assessed by visual analog scale, the POMS questionnaire will be administered, and subjects will undergo assessment of liking and wanting of food by computer presentation of four separate food categories.

Ad libitum dietary procedures

Subjects will begin an ad libitum period on the morning of Day 12 after their 24h stay in the metabolic chamber, and continue this period for 3 days until 0800 h on Day 15 (Figure A1). An automated food-selection system made up of a refrigerated vending machine that contains multiple trays of food will be used. Multiple food items will be available to the subjects during the ad libitum period and will accommodate the appropriateness of certain foods for breakfast, lunch, dinner, and evening snacks. The subjects will have unrestricted access to the vending machines and will be instructed to follow their typical eating pattern as closely as possible. The subjects will be instructed to eat whatever and whenever they desire and to return the food wrappers and unconsumed food portions to the vending machines. Physical activity during this period will be unregulated, but monitored by the accelerometers that the subjects will be wearing. Daily energy intake and protein, fat, and carbohydrate intake will be calculated by weighing of the food consumed and eating behavior will also be assessed with the bite counter and the automatic ingestion monitor.

Specific procedures during ad libitum period

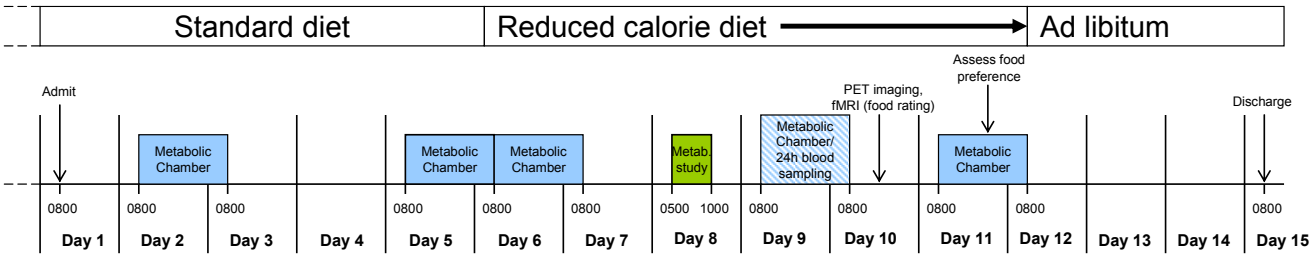
On the morning of Day 12, subjects will leave the metabolic chamber. At 0800 h, a urine sample will be collected, body weight will be measured, and a blood sample taken. Hunger and satiety will be assessed by visual analog scale and questionnaire hourly from 0800 h to 2200 h. These procedures will be repeated during each day of the 3-day ad libitum diet period. On the morning of Day 15, after collection of a urine sample and body weight, at 0730 h, a blood sample will be taken for measurement of baseline plasma substrate and hormone levels. At 0800 h, subjects will be discharged.

<u>Second In-Patient Visit</u>

After discharge from the MCRU, a wash-out period of at least 2 weeks will be required before subjects return for the second portion of the study (i.e. the alternate reduced calorie diet than what was consumed during their first visit). This period of time will allow for sufficient wash-out of any effects the diet manipulation may have on subsequent measurements made during the second stay at the MCRU. Subjects will be required to begin completion of the second reduced calorie portion of the study within 10 weeks after discharge in order to avoid large changes in physiological state. During the wash-out period, subjects will be encouraged to return to normal activity patterns, to consume a diet as similar to the standard diet as possible (50% carbohydrate, 35% fat, and 15% protein), and to maintain weight stability (within 2kg of the starting weight).

Specific procedures during baseline period

Figure A3. Schematic timeline of second in-patient visit.



A schematic timeline of events during the second in-patient visit is depicted in Figure A3. Beginning two days before admission, all food to be consumed by the subjects will again be supplied by the MCRU. During this time, subjects will arrive each morning at the MCRU at 0800 h for measurement of body weight, after which they will eat breakfast, and then take the remainder of the day's meal with them to consume later during the day. Subjects will be instructed to consume only and all the food provided to them and to return those portions that they were unable to eat the following day when they return for the next day's meals.

On the morning following the second day of the out-patient standard diet, subjects will arrive in the fasted state at the MCRU at 0800 h for admission to the MCRU (Day 1, see Figure A3). Following admission, subjects will be fitted with accelerometers (activity monitors), a bite counter to detect a pattern related to biting behavior and an automatic ingestion monitor to estimate frequency, duration, rate and mass of ingestion. Both will be worn continuously during the in-patient period. At 0845 h a baseline urine sample will be collected (24h urine specimens will be collected throughout the duration of the in-patient period for measurement of nitrogen excretion). At 0855 h subjects will drink from a stock solution of 0.15 g/kg body weight of $H_2{}^{18}O$ and 0.08 g/kg body weight of ^2H_2O followed by 100-200 mL tap water to rinse the dose container (measurement of tracer decay will be made in urine samples collected every morning for the remainder of the study). Subjects will be fed the standardized diet at three meals during the day on Day 1 at 0900 h, 1300 h, and 1800 h. At 1500 h, subjects will undergo the same treadmill walking session as described above.

At 0700 h on the morning of Day 2 body weight and body composition (by DEXA and air-displacement plethysmography) will be measured, as well as measurement of total body water content by bioelectrical impedance. At 0745 h a blood sample will be taken, and at 0800 h, subjects will enter the metabolic chamber for measurement of 24h energy expenditure. While in the metabolic chamber, hunger and satiety will be assessed by visual analog scale and questionnaire before each meal, and every 30 min for 2h and then every hour after each meal. At 1500 h, subjects will undergo the same treadmill walking session as described above. This physical activity session will be repeated every day during the second in-patient period, and daily caloric intake will be adjusted to reflect the increase in caloric expenditure as a result of the added physical activity in order to maintain energy balance. Subjects will be fed the standardized diet at 0900 h, 1300 h, and 1800 h while in the metabolic chamber. At 2200 h, overall energy balance will be assessed and a standard shake will be administered to maintain energy balance for the remainder of the stay in the metabolic chamber. The amount of shake required to maintain energy balance will be given with the dinner for the remainder of the baseline days (Day 3-5).

On the morning of Day 3, subjects will be allowed to leave the chamber. At that time a urine sample will be collected and body weight will be measured. Subjects will be fed the standardized diet at 0900 h, 1300 h, and 1800 h, and undergo their scheduled session of treadmill walking at 1500 h. The timeline of events on Day 4 will be identical to those on day 3.

At 0700 h on the morning of Day 5 body weight and body composition (by DEXA and air-displacement plethysmography) will be measured, as well as measurement of total body water content by bioelectrical impedance. At 0745 h a blood sample will be taken, and at 0800 h, subjects will enter the metabolic chamber for measurement of 24h energy expenditure. While in the metabolic chamber, hunger and satiety will be assessed by visual analog scale and questionnaire before each meal, and every 30 min for 2h and then every hour after each meal. At 1500 h, subjects will undergo the same treadmill walking session as described above. Subjects will be fed the standardized diet at 0900 h, 1300 h, and 1800 h while in the metabolic chamber.

Specific procedures for calorie reduction period

A schematic timeline of events during the calorie reduction period of the second in-patient visit (Days 6 – 11) is depicted in Figure A3. The procedures during this calorie reduction period will be identical to those during the calorie reduction period of the first in-patient visit described above. Briefly, on the morning of Day 6 (after the 24h metabolic chamber stay that will begin on Day 5) subjects will leave the chamber, a urine sample will be collected, body weight measured, and a basal blood sample take. Subjects will then be allowed to shower before re-entering the metabolic chamber at 0800 h for measurement of 24h energy expenditure. The first meal of the reduced calorie diet will be given in the chamber as breakfast on Day 6 at 0900 h, and the remainder of the meals will be fed at 1300 h and 1800 h while in the chamber, and hunger and satiety will again be assessed throughout the day. At 2200 h on Day 7, an intravenous catheters will be inserted for infusions during the "metabolic study" (i.e. tracer infusion, indirect calorimetry, muscle and adipose tissue biopsy) that will occur on Day 8. The "metabolic study" procedures will again be optional. At 0730 h on Day 9, after three days on the reduced calorie diet, an intravenous catheter will be inserted and a blood sample will be taken. Throughout the following 24h period, additional blood samples will be frequently taken to allow measurement of plasma hormone concentrations. At 0800h, subjects will then enter the metabolic chamber and undergo a 24h stay in the metabolic chamber in order to measure changes in energy expenditure as a result of the diet. Hunger and satiety again will be assessed by visual analog scale and questionnaire while in the chamber. On Day 10, after leaving the metabolic chamber, subjects will have a blood sample taken, undergo PET imaging, followed by the delay discounting computer procedure, a blood sample at 1730 h, and at 1800 h subjects will undergo the same fMRI session as preformed on Day 2 of the initial baseline diet period (first in-patient visit). On Day 11, at 0715 h, subject's body weight, body composition (by DEXA and air-displacement plethysmography), and total body water content (by bioelectrical impedance) will be measured, and then at 0800 h, subjects will enter the metabolic chamber for measurement of 24h energy expenditure. While in the metabolic chamber, hunger and satiety again will be assessed by visual analog scale, the POMS questionnaire will be administered, and subjects will undergo assessment of liking and wanting of food by computer presentation of four separate food categories.

Specific procedures during ad libitum period

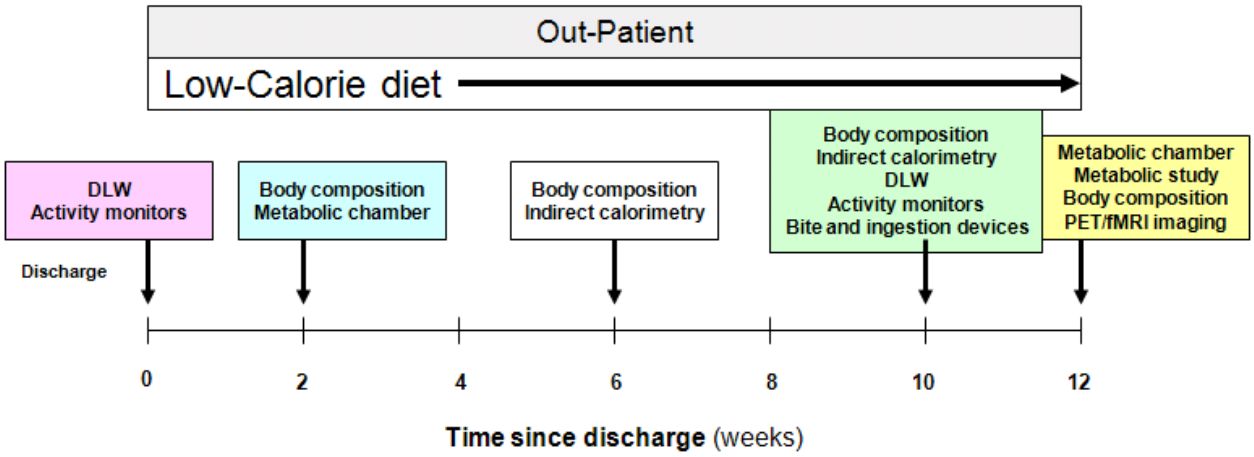
Procedures during the ad libitum period will be identical to those during the first in-patient visit. Briefly, on Day 12, subjects will leave the metabolic chamber. At 0800 h, a urine sample will be collected, body weight will be measured, and a blood sample taken. Hunger and satiety will be assessed by visual analog scale and questionnaire hourly from 0800 h to 2200 h. These procedures will be repeated during each day of the 3-day ad libitum diet period. On the morning of Day 15, after collection of a urine sample and body weight, at 0730 h, a blood sample will be taken for measurement of baseline plasma substrate and hormone levels. At 0800 h, subjects will be discharged.

Out-patient lifestyle intervention period

Specific procedures during out-patient period

At the end of the ad libitum period of the second in-patient visit, subjects will be discharged from the MCRU and begin a 12 week out-patient period. During this period, all subjects will be encouraged to undergo lifestyle modification consisting of diet modification and increased physical activity with a goal to reduce body weight by at least 5% by the end of 12 weeks. At the onset of the out-patient period, all volunteers will be provided with a weight-loss counseling session with registered dietitians to set their weight-loss goals and explain their prescribed diet. Based on the measurements conducted during the in-patient stay, we will calibrate a mathematical model of the individual subject's metabolism and body composition and this mathematical model will be used to project the expected weight loss and set appropriate goals (9). All subjects will be asked to report their body weights on a daily basis (by phone or email) using bathroom scales provided to them. On a biweekly basis subjects will meet with registered

Figure A4. Schematic timeline of out-patient period.



dietitians and receive reminders about their diet and weight loss goals, along with general nutrition and lifestyle information, as well as additional feedback using the mathematical model on their average degree of energy imbalance, how this conforms to their weight loss goals, and their projected total weight loss. If the subjects are not on track to meet their weight loss goals, then specific corrective adjustments of diet or physical activity will be advised. In between their biweekly meetings, subjects will receive guidance from the registered dietitians by phone or email.

A number of metabolic studies will take place periodically during the 12 week out-patient period, with the first studies beginning at the conclusion of the subject's second in-patient visit (Figure A4). Specifically, before subjects are discharged, a collection of a baseline urine sample will occur, after which subjects will drink from a stock solution of 0.15 g/kg body weight of $H_2{}^{18}O$ and 0.08 g/kg body weight of ^2H_2O followed by 100-200 mL tap water to rinse the dose container (urine samples will be collected twice more during the week and three times during the following week for measurement of tracer decay). Subjects will be given a handheld indirect calorimeter (MedGem, Dunedin, FL) to measure and record resting metabolic rate at home each morning for the next two weeks and will also be given physical activity monitors, a bite counter and an automatic ingestion monitor to wear continuously during these two weeks. At 2 weeks after discharge, subjects will arrive to the MCRU at 0700 h after an overnight fast. At that time body composition will be measured by DEXA and air-displacement plethysmography, and total body water content will be measured by bioelectrical impedance. At 0800 h, subjects will enter the metabolic chamber for measurement of 24h energy expenditure. The content and calories of food fed in the chamber will be the same as that fed to the subjects while in the chamber during the baseline period and physical activity will also be identical to that in the chamber during the baseline period. Subjects will continue to wear the physical activity monitors, the bite counter and the automatic ingestion monitor while in the chamber. At 6 weeks after discharge subjects will again arrive to the MCRU at 0700 h after an overnight fast and body composition will be measured by DEXA and air-displacement plethysmography, and total body water content will be measured by bioelectrical impedance. At 0800 h, resting energy expenditure and fat oxidation rate will be determined by indirect calorimetry. At 10 weeks after discharge, subjects will again arrive to the MCRU at 0700 h after an overnight fast and, after collection of a baseline urine sample, subjects will be given a dose of DLW (urine samples will again be collected twice more during the week and three times during the following week for measurement of tracer decay). Following the consumption of the DLW dose, body composition will be measured by DEXA and air-displacement plethysmography, and total body water content will be measured by bioelectrical impedance. At 0800 h, resting energy expenditure and fat oxidation rate will be determined by indirect calorimetry. Before leaving the MCRU, subjects will again be given a handheld indirect calorimeter to measure and record resting metabolic rate at home each morning for the next two weeks and will also be given physical activity monitors, a bite counter and an automatic ingestion monitor to wear continuously during these two weeks. Before each visit at week 2, 6, and 10, subjects will be required to consume a standard meal (i.e. 50% carbohydrate, 35% fat, 15% protein) the night before their arrival that will be provided by the clinical center metabolic kitchen. Two weeks after their visit during week 10, subjects will arrive at the MCRU at 1700 h and be admitted to the hospital and given a standard meal for the evening. At 2200 h, subjects will be administered a dose of a sodium bromide solution for measurement of extracellular water content. Subjects will be weighed, and a blood sample will be collected before the sodium bromide dose is given. At 0700 h the following morning, DEXA and air-displacement plethysmography measurement of body composition will be taken and total body water content will be measured by bioelectrical impedance. Following measurement of body composition, a blood sample will be taken for measurement of sodium bromide dilution. At 0800 h, subjects will enter the metabolic chamber for measurement of 24h energy expenditure, and hunger and satiety will be assessed by visual analog scale and questionnaire. Conditions within the chamber (i.e., food fed and physical activity) will again be identical to the chamber conditions during the baseline period. At 1100, the POMS questionnaire will be administered,

and at 1800 h, subjects will again undergo assessment of liking and wanting of food by computer presentation of four separate food categories. The next morning, after leaving the metabolic chamber, subjects will have a blood sample taken, undergo PET imaging, followed by the delay discounting computer procedure, a blood sample at 1730 h, and at 1800 h subjects will undergo an fMRI scanning comprised of the required clinical scan, a resting-state scan, food rating scans, and an anatomical scan. At 2200 h on, an intravenous catheters will be inserted for infusions during the "metabolic study" (i.e. tracer infusion, indirect calorimetry, muscle and adipose tissue biopsy) that will occur the following morning. The "metabolic study" procedures will again be optional and if a subject chooses not to participate, they will be discharged following the fMRI session the night before. The "metabolic study" procedures and timeline will be identical to those outlined in Figure A2, minus the IVGTT. Following the conclusion of these procedures, subjects will be discharged.

Longitudinal follow-up of obese subjects that completed the outpatient weight loss phase

Specific procedures during long-term follow-up

On an approximately yearly basis for 5 years, subjects will be given the option to return to the MCRU and complete many of the procedures that were performed over the last 2 weeks of the outpatient weight loss phase. Specifically, subjects will arrive at the MCRU at 0700 h after an overnight fast and, after collection of a baseline urine sample, subjects will be given a dose of DLW (urine samples will again be collected twice more during the week and three times during the following week for measurement of tracer decay). Following the consumption of the DLW dose, body composition will be measured by DEXA and air-displacement plethysmography, and total body water content will be measured by bioelectrical impedance. At 0800 h, resting energy expenditure and fat oxidation rate will be determined by indirect calorimetry. Before leaving the MCRU, subjects will again be given physical activity monitors, a bite counter and an automatic ingestion monitor to wear continuously during the next 2 weeks. Subjects will be required to consume a standard meal (i.e. 50% carbohydrate, 35% fat, 15% protein) the night before visiting the MCRU and will be provided by the clinical center metabolic kitchen. Subjects will return to the MCRU 2 weeks later at 1700 h and be admitted to the hospital and given a standard meal for the evening. At 2200 h, subjects will be administered a dose of a sodium bromide solution for measurement of extracellular water content. Subjects will be weighed, and a blood sample will be collected before the sodium bromide dose is given. At 0700 h the following morning, DEXA and air-displacement plethysmography measurement of body composition will be taken and total body water content will be measured by bioelectrical impedance. Following measurement of body composition, a blood sample will be taken for measurement of sodium bromide dilution. At 0800 h, subjects will enter the metabolic chamber for measurement of 24h energy expenditure, and hunger and satiety will be assessed by visual analog scale and questionnaire. Conditions within the chamber (i.e., food fed and physical activity) will again be identical to the chamber conditions during the baseline period. At 1100, the POMS questionnaire will be administered, and at 1800 h, subjects will again undergo assessment of liking and wanting of food by computer presentation of four separate food categories. The next morning, after leaving the metabolic chamber, subjects will have a blood sample taken and undergo PET imaging beginning at 0800 h, followed by the delay discounting computer procedure at 1400 h, a blood sample at 1730 h, and at 1800 h subjects will undergo an fMRI scanning comprised of the required clinical scan, a resting-state scan, food rating scans, and an anatomical scan. At 2200 h on, an intravenous catheters will be inserted for infusions during the "metabolic study" (i.e.

tracer infusion, indirect calorimetry, muscle and adipose tissue biopsy) that will occur the following morning. The "metabolic study" procedures will again be optional and if a subject chooses not to participate, they will be discharged following the fMRI session the night before. The "metabolic study" procedures and timeline will be identical to those outlined in Figure A2. Following the conclusion of these procedures, subjects will be discharged. These procedures will be repeated on a yearly basis for 5 years following the weight loss phase of the study.

Table A1. Example of standard and restricted diets for hypothetical subject with estimated total daily energy intake of 2000 kcal/day.

	Standard Diet	**Carbohydrate Restricted Diet**	**Fat Restricted Diet**
Breakfast	- 6 oz fat-free blueberry yogurt - Bagel w/ 21 g cream cheese - 210 g (~ ¾ cup) orange juice - 120 g (~ ½ cup) fruit salad	- Scrambled eggs: · 70 g egg white (~ 3 egg) · 35 g egg yolk (~ 2 ½ egg) · cooked in 4 g butter - 3 slices bacon - 1 slice wheat toast w/ 4 g butter - Water	- 35 g Corn Pops (~ ¾ oz) - 200 g skim milk (~ ¾ cup) - 1 medium banana - ¼ cup scrambled egg substitute
Lunch	- Turkey sandwich: · 2 slices multigrain bread · 45 g turkey breast (1½ oz) · 30 g provolone cheese (1 slice) · 1 leaf lettuce · 2 slices raw tomato · 10 g Dijon mustard - Salad: · 40 g lettuce · 40 g tomato · 30 g cucumber · 43 g Italian salad dressing - 1 oz potato chips - 120 g red grapes (~ ½ cup) - Water or diet beverage	- Turkey wrap: · 1 small tortilla · 80 g turkey breast (~ 3 oz) · 30 g American cheese (1 slice) · 1 lettuce leaf - Salad: · 40 g lettuce · 40 g tomato · 40 g cucumber · 45 g Italian salad dressing - 100 g red grapes (~ ½ cups) - Water or diet beverage	- Turkey sandwich: · 2 slices multigrain bread · 60 g turkey breast (2 oz) · 1 leaf lettuce · 1 slice raw tomato · 10 g Dijon mustard - Salad: · 40 g lettuce · 30 g tomato · 40 g cucumber · 50 g fat-free Ranch salad dressing - 100 g red grapes (~ ½ cup) - 130 g lemonade (~ ½ cup)
Dinner	- 100 g salmon (~ 3½ oz) cooked w/ 5 g (1 tsp) olive oil - 150 g brown rice (~ ¾ cup) - 100 g broccoli (~ ¾ cup) - Chocolate chip cookie - Water or diet beverage	- Chicken stir fry: · 40 g (~ 1 oz) stir fry sauce · 130 g stir fry vegetables · 75 g chicken breast (~ 2½ oz) · cooked in 13 g olive oil - 120 g brown rice (~ 5/8 cup) - 100 g peaches (~ ½ cup) - Water or diet beverage	- 100 g chicken breast (~ 3 oz) - ½ cup cooked carrots - ¾ cup brown rice - Salad: · 50 g lettuce · 40 g tomato · 40 g cucumber · 50 g fat-free French salad dressing - 1 slice angel food cake - 100 g tropical fruit salad - Water or diet beverage
Total	**2000 calories** **75 g protein (15%)** **78 g fat (35%)** **250 g carbohydrate (50%)**	**1405 calories** **75 g protein (21%)** **78 g fat (50%)** **101 g carbohydrate (29%)**	**1405 calories** **75 g protein (21%)** **12 g fat (8%)** **250 g carbohydrate (71%)**