Investigation of the Gut Microbiota in Regulating Nutrient Absorption in Humans

Version 1.5

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About the Protocol:

Uses Ionizing radiation? Yes

Requires IND/IDE? No

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Sponsor:	None
Uses “Durable Power of Attorney”	No
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Multi-Institutional Project?	No
Expedited Review Request?	No

***Denotes those able to obtain informed consent**

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PRECIS

The prevalence of obesity has risen to epidemic proportions in the world, resulting from both excessive energy intake and low levels of energy expenditure. The effect of nutrient absorption on energy balance, that is, the relative amount of nutrients consumed vs. the amount excreted in stool, has been reported only in small studies in which energy waste in feces and urine between lean and obese individuals was not found to be different. New studies have shown that bacteria in the gut may play an important role in calorie absorption. We have recently shown that leaner individuals absorbed more calories when overfed compared to when they were given a diet with just enough calories to maintain their own weight. Our studies have also found that overfeeding also changes the kinds of bacteria found in the gut. In lean individuals, these changes in gut bacterial communities with overfeeding were associated with changes in how many calories were absorbed. Our results are similar to those seen in other studies in animals and humans that suggest a role for gut bacteria in weight gain and obesity. To try to better understand the role of gut bacteria in absorbing food, we propose to investigate 1) whether energy loss (as measured in stool and urine) changes following over- and underfeeding relative to body size and 2) whether changes in the gut bacteria, induced by an antibiotic medication, affect nutrient absorption and glucose tolerance. We plan to study 24 healthy non-smoking volunteers age 18 – 45 years old, not taking any medications (including medications for weight loss, antibiotics or probiotics) for the examination. All participants will be admitted to the Clinical Research Unit for 31 days. During their stay, subjects will be fed a weight maintaining diet for 3 days, followed by two experimental diets (150% and 50% of weight maintaining calories) in a random order. After this, volunteers will be randomly assigned to one of two groups: group 1 will take oral antibiotic medication; group 2 will receive pills that look the same but will not contain any active medication (placebos). Feces (stool) will be collected throughout the study. Additionally, twenty four-hour urine collections will take place each day of

the experimental diet period and when stool is collected on the antibiotics. The energy content of these waste products as well as that of the diet (using duplicate plate analysis) will be measured by bomb calorimetry. Bacterial components in feces will be extracted by repeated fractional centrifugation to obtain bacterial mass and by using 16S rDNA-based oligonucleotide probes to obtain data on gut bacteria. Primary results will examine how many calories remain in stool during relative over- and underfeeding and whether changes in gut bacteria, induced by an antibiotic medication, affect nutrient absorption and glucose tolerance.

INTRODUCTION

Obesity is one of world's greatest health problems. The prevalence of obesity has been steadily increasing in developing as well as industrialized countries (1). In the U.S., more than 60% of the adult population is either overweight or obese (2). Obesity is associated with an increased risk of all-cause mortality with many specific health consequences, including coronary heart disease, hypertension, and diabetes mellitus, among others (1). Excess caloric intake and low levels of energy expenditure are important factors that can cause an energy imbalance, leading to obesity. Energy balance is defined as the equilibrium between energy intake and energy expenditure using the formula below:

$$E_{FD} = E_{RMR}\ E_{TE} + E_{PA} + E_{FE} + E_{UR}$$

where E=energy and the subscripts stand for the quantity of energy in food (FD); the resting metabolic rate (RMR); the thermic effect of food (TE); physical activity (PA); the feces (FE); and, the urine (UR). Many of these components have been widely studied under a variety of conditions (3–10) and in different ethnic groups (11–15).

Energy loss in stool

One frequently overlooked, but potentially important, piece of the energy balance equation is energy loss in stool and urine. In carefully conducted early studies of energy balance, Atwater et al. calculated energy loss from stool and urine as 142 kcal/d and 149 kcal/d, respectively, representing approximately 5% of total daily energy expenditure (16). Stool energy losses, in particular, can be affected by disease states, such as gastrointestinal disorders (17–19), age (20–23), and diet composition.

High fiber diets induce an increase in the quantity and energy content of the stool by causing shortened colonic transit time, thus shortening absorption time, and by increasing stool bulk and water holding capacity, thereby reducing digestive diffusion capacity (24,25). Fecal energy loss is higher in individuals consuming high fiber diets compared to lower fiber diets, even when the caloric content is the same (26–28). Whether the macronutrient content of the diet, apart from fiber, may affect stool energy loss is unclear. A study by Webb found no differences in stool energy loss in individuals consuming diets with different macronutrient composition (29), while other studies indicate that dietary carbohydrate, calcium and saturated fatty acids may affect stool energy loss (21,22,30–34).

Energy content in stool may also be affected by colonic transit time (CTT). Although data from our previous study (35) did not show an association between stool calories and gastrointestinal transit time, previous studies have described differences in CTT by ethnicity (36–38), age (39) and menstrual cycle (39) but not by gender (38,40). The investigations of effect of body size on CTT have produced inconsistent results, however, those studies were limited in the range of the BMI of the study population.

Whether obese individuals may preferentially absorb more nutrients, and hence have lower stool energy loss (which may account for a propensity to gain weight, difficulty losing weight, and/or problems maintaining weight loss) is not clear. To date, only one study has addressed this issue. Webb studied 4 lean and 4 obese individuals under three dietary conditions: a high protein/high fat diet, an 'average' diet, and a high carbohydrate diet. Stool and urine energy loss varied between individuals, but overall, obese individuals had only slightly and not significantly lower stool energy loss compared to lean individuals (178 ± 45 kcal/day vs. 208 ± 100 kcal/day, respectively) (41). Our recently published data in 9 obese and 12 lean individuals also showed no difference in nutrient absorption between lean and obese

individuals. However we did find that lean individuals had lower stool calories, therefore absorbed relatively more calories on a 3400 kcal/day diet compared to a 2400 kcal/day diet (-1.3±1.9%, p=0.04). This was not seen in obese individuals (-0.2±1.2%, p=0.59). However, because of the study design, the degree of overfeeding in obese subjects was much smaller compared to the lean subjects (calculated as a percent of their weight maintaining calories) (35). Therefore, it remains unclear whether only lean individuals have increased nutrient absorption with increased calorie load or whether this was just due to the difference in the degree of overfeeding.

The gut microbiota

Nutrient absorption may also be influenced by enteric bacteria. According to Ley R.E., there is a difference in composition of gut microbial communities between lean and obese mice i.e., ob/ob mice have a 50% reduction in the abundance of Bacteroidetes and a significantly greater proportion of Firmicutes that are the two most abundant bacterial divisions in either mice or human. This was further supported by studies in lean and obese human twins(42). Our data did not show a statistically significant difference in the relative abundance of Bacteroidetes and Firmicutes between lean and obese individuals (22.36±11.58% of 16S rRNA gene sequences (lean) vs. 18.77±15.93% (obese), p=0.56 for Bacteroidetes; 74.85±12.05% vs. 78.69±17.05%, p=0.55 for Firmicutes) (35). However, the observed variation in bacterial abundance on phylum level was consistent with previously published data. Our initial studies did find an association between nutrient load and changes in gut microbial community composition. For instance on the 3400 kcal/day diet we found percent weight maintaining energy needs (%WMEN, [calculated as calories fed/weight maintaining calories]*100) were associated with an increase/decrease in Firmicutes and Bacteroidetes (r=0.47, p=0.04; r=-0.47, p=0.04). As stool samples on each prescribed diet were collected within 2 days of starting the diet, these changes occurred rapidly.

These findings are consistent with several independent studies showing how switching mice (whether with a mouse microbiota or a transplanted human gut microbiota) from a polysaccharide-rich/low-fat to a high-sugar/high-fat diet resulted in a reduced representation of Bacteriodetes and an increased representation of Firmicutes (43–45). Changes in the ratio of Firmicutes to Bacteroidetes in mice and humans may promote adiposity or alternatively represent a host-mediated adaptive response to limit energy uptake/storage (46). In a series of experiments in mice, Turnbaugh et al. demonstrated that the gut microbiota is not only linked to obesity but can also increase the capacity of energy harvest from the gut(43,44,47). In humans, Ley et al noted a correlation between change in % body weight over one year and increase in Bacteroidetes abundance (48) . However, several studies have found no association between specific gut microbiota and adiposity in humans(49). Specifically, Duncan et al using fluorescent in situ hybridization (FISH) did not find any difference in relative abundance of Bacteroidetes between lean and obese individuals, nor did they find any change in relative abundance of Bacteroidetes or Firmicutes with weight loss. In our initial shorter term study, we found that changes in the Firmicutes and Bacteroidetes with altered nutrient load were associated with directly measured stool calories in the lean subgroup ($r=-0.50$, $p=0.02$, $r=0.52$, $p=0.01$, respectively). The magnitude of this effect indicated that this may be a clinically relevant difference in nutrient absorption as a 20% change in the major phylotypes (increase in Firmicutes and decrease in Bacteroidetes) was associated with a 150 kcal/d difference in stool calories.

Effects of antibiotics on gut microbiota

While over (and possibly under) feeding may influence enteric bacteria and nutrient absorption, antibiotic medications will also alter these communities. Recent studies have explored the effect of antibiotics on gut microbial communities using new metagenomic methods

which accurately identify and quantify members of the gut microbiota. Antonopoulos et al. investigated community dynamics of the gut microbiota in mice using 16S rRNA analyses following treatment with metronidazole and amoxicillin and cefoperazone. For the amoxicillin/metronidazole combination, 10 days of treatment, lead to substantial reductions of the representation of Firmicutes, while cefoperazone decreased Bacteroidetes (50). However, these effects were examined only in two and three mice respectively. In humans, ciprofloxacin reduces gut microbiota diversity after 3 days. However the effect on specific taxa appears to be uneven(51). Oral vancomycin has been examined in a model of the distal colon and in mice. Oral vancomycin does cause a decrease in the major phyla with reductions in the relative abundance of both Bacteroidetes and Firmicutes(52–54). In the case of the latter phyla, there is relative preservation of the Lactobacillacea family. Moreover, mice treated with vancomycin were resistant to weight gain despite no changes in calorie intake (53). Mice treated with vancomycin also had lower glucose concentrations although it is not clear if this was due to alteration of gut microbiota or from lack of weight gain. In contrast, intravenous vancomycin has been associated with weight gain in humans treated for endocarditis(55). Oral vancomycin has the advantage of having minimal systemic absorption (in the absence of renal disease and/or clinical colitis) and thereby would be expected to have limited systemic effects.

Several groups have proposed a mechanism by which the microbiota influences glucose metabolism. Cani et al. and others have demonstrated that Lipopolysaccharids(LPS) derived from gram-negative bacteria can pass the mucosal barrier of the gut and induce low-grade inflammation. These processes are mediated by proinflammatory markers such as TNFα and IL-6 which ultimately lead to insulin resistance(56,57). Membrez et al. showed that antibiotic treatment of two weeks lead to reduced circulating LPS and improved glucose tolerance(57).

The mechanism by which gut microbiota influence energy harvest is not clear. In mice, Backhed et al. have shown that gut bacteria affect fat storage not only by increasing delivery of monosaccharides to the liver and increasing transactivation of lipogenic enzymes but also by promoting storage of triglycerides in adipocytes through suppression of intestinal expression of a circulating lipoprotein lipase inhibitor called angiopoietin-like protein 4 (Angptl4)(58). It was shown by Mandard et al. that over-expression of Angptl4 leads to inhibition of fat storage(59).

Even further, Aronsson et al. demonstrated that treatment with certain Lactobacilli was linked to increased expression of Angptl4 and this was associated with decreased LPL activity (60).

The identification of the gut microbiota can be performed using either conventional culturing techniques that are less accurate due to difficulty with culturing anaerobic bacteria or sequence analysis of cloned microbial small-subunit ribosomal RNA genes [16S ribosomal DNA,(rDNA)] which is a more accurate technique (61). There may be some limitations to a single measurement of the gut microbiota. Bacterial gut communities may be affected by diet and health status of their host (62). Measurements of the gut microflora are performed using fecal samples or mucosal tissue achieved from biopsy. Although there are some differences in the representation of gut bacteria measured by fecal sample versus mucosal tissue sample, fecal microbiota is thought to adequately represent the adherent and non-adherent bacterial population(63).

Nutrient absorption may also be regulated by gut and adipocyte hormones. Several investigations in animal models indicate that glucagon-like peptide 1 (GLP-1) reduces lipid absorption (64), whereas GLP-2 increases macronutrient absorption (65). The role of the adipocyte-derived hormone leptin in balancing the intestinal absorption of dietary proteins and fats has also been reported (66). However, information on this aspect in humans is still unclear.

According to Atwater et al(16), energy loss in urine and stool respectively is approximately 5% of total energy expenditure. Our preliminary data demonstrated that mean stool energy loss in lean and obese individuals is close to this (4.9±1.8%, 4.8±1.4%). Energy content in urine may also be affected by dietary calorie content (21,22,29,41) and age (21–23). However based on our preliminary data, urine calories were not different between lean and obese individuals, but further information about energy loss in urine in relation to obesity is needed (35).

We propose to study both stool and urine energy loss in 24 individuals on two experimental diets (50% increased and 50% reduced nutrient load relative to body size) in a random cross-over design. Following this over/underfeeding, volunteers will also be randomly assigned to a placebo versus oral antibiotic medication arm. This study will extend our previous findings by investigating whether 1) nutrient absorption changes upon similar increases/decreases in relative nutrient load and 2) whether manipulation of gut microbial communities with antibiotics alters nutrient absorption and 3) how these changes may affect glucose tolerance and fat storage.

AIMS OF THE STUDY:

A. Primary objectives

1. To investigate energy loss in stool and urine when volunteers are fed diets in which the calories are 50% and 150% of their weight maintaining calorie needs.

2. To investigate the effects of oral antibiotic vancomycin on nutrient absorption.

B. Secondary objectives

1. To investigate the effect of over and underfeeding on the gut microbial community structure

2. To investigate the effect of the oral antibiotic vancomycin on the gut microbial community structure

3. To investigate whether antibiotic manipulations of gut microbiota affect fat storage by measuring lipoprotein lipase activity and Angptl4

4. To investigate whether manipulation of the microbiota by over and underfeeding affects Angptl4 secretion.

5. To investigate whether antibiotic manipulations of the gut microbial community structure are associated with changes in 24 hour energy expenditure.

6. To examine whether gastrointestinal transit time is altered when volunteers are fed 50% and 150% of their weight maintaining calories and whether it is altered by antibiotic treatment with vancomycin

7. To examine plasma concentrations of glucagon-like peptides 1 and 2 (GLP-1, GLP-2) and leptin in relationship to stool energy loss.

HYPOTHESES

In the course of this study we will test the following hypotheses:

Primary Hypotheses:

1. Percent stool calories will increase and decrease respectively when individuals are fed diets that are 50% and 150% of their weight maintaining energy requirements.

2. Oral vancomycin treatment will be associated with decreased nutrient absorption as measured by an increase in stool calories.

Secondary Hypotheses:

1. Antibiotic induced changes in gut microbiota will decrease lipoprotein lipase activity

2. Higher relative nutrient load decreases Bacteroidetes/Firmicutes ratio and lower nutrient load increases Bacteroidetes/Firmicutes ratio.

3. Antibiotic-induced changes in the gut microbiota will improve glucose tolerance (lower fasting glucose and decrease glucose area under the curve).

4. Changes in the microbial community structure induced by antibiotics will be associated with changes in 24 hour energy expenditure

5. Circulating GLP-1 and GLP-2 will be associated with nutrient absorption

METHODOLOGY

Study design

This is a controlled diet intervention study involving random ordered over and underfeeding followed by double blind randomized placebo controlled assignment to administration of oral vancomycin or placebo.

Subjects

Seventy, non-diabetic (67) individuals aged 18-45 y (in order to minimize the effect of aging on nutrient absorption) and on no medications (see details below) will be admitted for this study. All subjects will be recruited from the Phoenix greater metropolitan area by newspaper/internet advertisement. The study will end once 24 subjects have completed the entire in-patient part of the study.

Eligibility criteria

Inclusion Criteria

- Free of acute and chronic diseases (especially GI disorders) as determined by medical history, physical examination and laboratory tests.
- Individuals may be taking laxative drugs but they must be discontinued 3 or more weeks before admission.
- Age 18-45 y (in order to minimize the affect of aging on nutrient absorption)

Exclusion Criteria

Because it is unclear how chronic illnesses or substance abuse could affect nutrient absorption we will exclude volunteers with chronic diseases or current substance abuse. This is especially important because the limited number of study subjects in this study will make it hard to control for these confounders. We will therefore exclude subjects with a history or clinical manifestation of:

- Current smoking
- Type 2 diabetes (according to the World Health Organization diagnostic criteria) (67)
- Endocrine disorders, such as Cushing's disease, pituitary disorders, and hypo- and hyperthyroidism
- HIV infection (self-report), due to effects on weight and body composition of HIV and medications used to treat HIV
- Active tuberculosis (self-report)
- Asthma on active daily treatment with medications
- Pulmonary disorders including physician diagnosed chronic obstructive pulmonary diseases and obstructive sleep apnea syndrome
- Cardiovascular diseases, including coronary heart disease, heart failure, arrhythmias, and peripheral artery disease

- Hypertension (according to the World Health Organization diagnostic criteria) (68), treated or uncontrolled

- Gastrointestinal disease, including inflammatory bowel diseases (e.g. Crohn's disease and ulcerative colitis), malabsorption syndromes (e.g. celiac disease), gastric ulcer (active) and irritable bowel syndrome.

- Lactose intolerance

- Anemia (defined as hemoglobin < 11 mg/dl), leucopenia (defined as white blood cell count < 4,000/μL) or thrombocytopenia (defined as platelet count < 150,000/μL)

- Liver disease, including non-alcoholic fatty liver disease or current elevated liver enzymes over 1.5 times the normal range for AST, ALT or GGT or a history and physical exam that indicates a potential liver disease as describe by Giannini et al(69)

- Evidence of chronic renal disease as defined by estimated glomerular filtration rate of < 60 ml/min or evidence of overt proteinuria on urine dipstick. (70)

- Central nervous system disease, including previous history of cerebrovascular accidents, dementia, and neurodegenerative disorders

- Cancer requiring treatment in the past five years, except for non-melanoma skin cancers or cancers that have clearly been cured or in the opinion of the investigator carry an excellent prognosis

- Behavioral or psychiatric conditions that would be incompatible with a safe and successful participation in the study (such as major depression, schizophrenia and presence of psychotic symptoms)

- Eating disorders such as anorexia nervosa, bulimia or binge eating syndrome
- Taking weight loss drugs
- Weight change of more than 5% of total body weight in the 3 months before admission
- Use of any antibiotic or probiotic agents within 6 months prior to minimize the potential effects of these substances on the gut microbiota.
- Use of antacids (Proton pump inhibitors, H2 antagonists or aluminum/magnesium hydroxide) 3 months prior to the study assessed by self-report because a modified gastric pH might affect the gut microbiota as well
- Evidence of alcohol and/or drug abuse (more than 3 drinks per day and use of drugs, such as amphetamines, cocaine, heroin, or marijuana)
- Pregnant (due to use of research related radiation) and breastfeeding women (because of the prolonged inpatient stay and use of medication) are also excluded.

The following exclusion criteria are necessary because of the substances given or tests performed during the study

- Known allergies to vancomycin
- Known allergies to heparin or a history of heparin-induced thrombocytopenia
- Personal history or evidence of a bleeding disorder

All individuals will be fully informed of the aim, nature, and risks of the study prior to giving written informed consent. The study's informed consent will be obtained by a principal or

associate investigator, research physician or physician assistant working in the clinical research unit. Should we enroll a non-English speaking subject, we will obtain IRB approval to use the short form consent process as outline in SOP 12.

Experimental design:

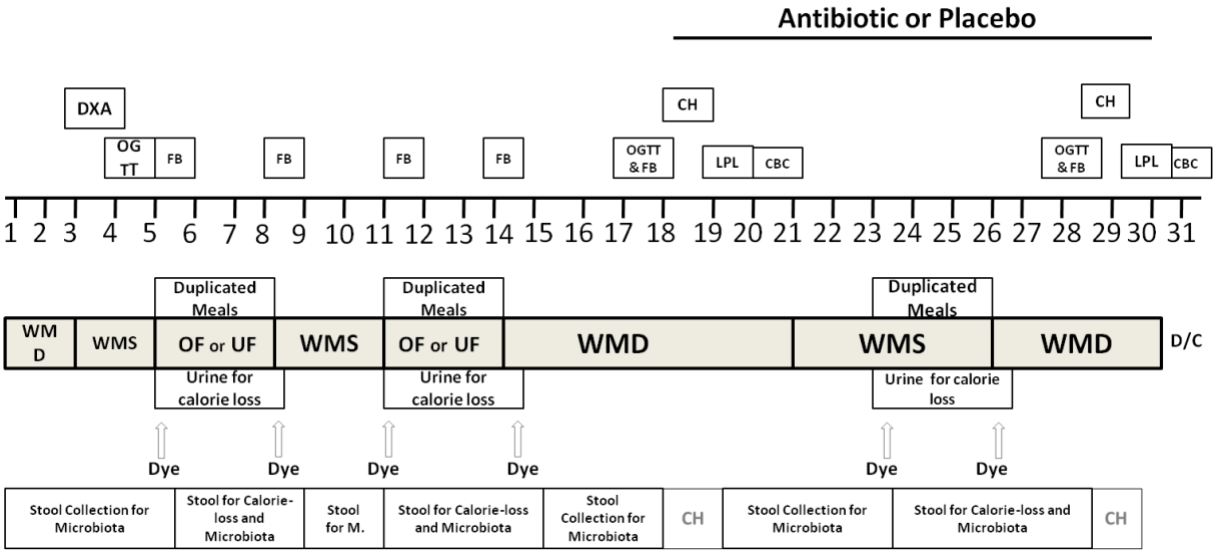
Individuals will be admitted to the Clinical Research Unit for approximately 31 days. On the day of admission, volunteers will be instructed to limit their physical activity during the study. Specifically, volunteers will be asked not to exercise and activities will include only those permitted on the research unit such as playing pool, board games, and occasional supervised outings from the unit which involve a short walk. From admission, all stool samples will be collected both for 16S rRNA sequencing of the gut microbiota and during the period of diet manipulation and antibiotic for direct measurement of stool calories (stool will not be collected when volunteers are in the metabolic chamber). Initially, volunteers will be placed on a weight maintaining diet for three days. During this time body composition will be measured using dual-energy x-ray absorptiometry and on day 3 volunteers will undergo a 75 g oral glucose tolerance test. On day 4, volunteers will begin consuming diets consisting of 50% and 150% of their weight maintaining calories in random order. Each intervention diet will last 3 days interrupted by a 3 day weight maintaining phase to eliminate potential carry-over effects. Volunteers will consume blue dye markers at the beginning and end of each experimental diet. Precisely the dye capsules will be taken before breakfast on day 1 of each experimental diet, and before breakfast on the day following each intervention diet. Stool for caloric content measurements will be collected from appearance of the first dye marker in stool (dyed stool included) until appearance of the second dye marker (dyed stool excluded). Because the dye marker does not

appear in urine, urine collections for caloric content measurements will begin right after breakfast on day 1 of the first experimental diet and stop before breakfast of the day after the 3 day intervention diet. The gastrointestinal transit time for the two intervention period will also be recorded as time between ingestion of the dye marker and appearance in stool. Weight maintaining and experimental diets will be similar in their relative macronutrient content for each volunteer to allow for comparison of stool energy content between diets and intervention groups. During the experimental diets and during 3 days under antibiotic/placebo, meals will be prepared in duplicates with one meal being chosen at random for consumption by the volunteer and the other meal for exact caloric content measurement via bomb calorimetry. The gastrointestinal transit time for this period will also be recorded. In case the gastrointestinal transit time of the dye marker takes longer than expected, we will extend stool collection time as well as study period to make sure volunteers complete the study. Following the over/underfeeding diets, volunteers will again be placed on a weight maintaining diet for 3 days. Following this (on study day 16), they will undergo a second OGTT. The next day they will enter the metabolic chamber at 0800 for approximately 23.5 hours for measurements of energy expenditure and substrate utilization. In the morning of the next day after exiting the metabolic chamber, volunteers receive a heparin bolus in the fasting state with subsequent blood sampling 15 minute later for measurement of lipoprotein lipase activity. Then while remaining on the weight maintaining diet, volunteers will be randomly assigned to either receive vancomycin 125mg qid or identical placebo pills for 12 days. Volunteers and investigators including nursing staff will be blinded to the randomization. The randomization and release of the medication will be managed by the pharmacy of the Phoenix Indian Medical Center. After 5 days on the vancomycin or placebo volunteers , while continuing the weight maintaining diet, will undergo measurement of stool and urine calories as described above over the next 3 days. Following

this, (and while still on vancomycin or placebo), volunteers will undergo a third OGTT. One the day after the third OGTT, they will have a second 23.5 hour stay in the metabolic chamber. On exiting the metabolic chamber, volunteers will undergo a repeat measurement of lipoprotein lipase activity post heparin bolus as described above. After this 12 day period vancomycin/placebo will be discontinued. The following morning, after checking their platelet count, volunteers will be discharged. Volunteers will be asked to return with stool samples, one week, one month and 6 months after discharge. Weight and height will be recorded at the 6 month visit.

During the inpatient stay, fluid consumption may affect the outcome of the study by changes in gastrointestinal transit time. Fluid intake, therefore, will also be controlled during the study. Each volunteer will be allowed to drink up to 2000 ml of non-carbonated or carbonated, non-caffeinated, non-caloric beverages or water plus fluids brought with each meal. The liquid drunk for the oral glucose tolerance test will not be counted toward the 2000 ml daily fluid. We will also record total fluid intake and urine output. The experimental design is shown in Figure 1.

Figure 1: Overview of the Study



WMD: weight maintaining diet, OF/UF : (overfeeding/underfeeding) experimental diet with either 150% or 50% of weight maintaining calories, CH: 24 hour measurements of energy expenditure in metabolic chamber, DXA: dual-energy x-ray absorptiometry; OGTT: oral glucose tolerance test; FB: Fasting blood sample for measurements of GLP1&2 and Leptin; LPL: Measurement of Lipoproteinlipase activity. CBC; complete blood count for assessment of platelet count after Heparin injection. Duplicated Meals: experimental meals are provided in duplicates with one meal for consumption by the volunteer and the other chosen at random for measurement of caloric content,

Detailed experimental design

DAY 1:

- Admission, medical history, physical examination, nutritional history (Block questionnaire(71))
- Resting 12-lead electrocardiogram
- Blood tests: complete blood cell count, platelet count, prothrombin time, partial thromboplastin time, glucose, electrolytes, plasma urea nitrogen, plasma osmolarity, creatinine, SGOT, SGPT, gamma-GT, bilirubin, calcium, phosphorus, total protein, albumin, alkaline phosphatase, TSH
- IgA anti tissue transglutaminase for celiac disease
- Lactose intolerance screening (using volunteer's self- report)
- Urine drug screening for Cocaine, Opiates, Meth-Amphetamine, THC and Nicotine (if the results of the urine drug screen are positive, the volunteer will be ineligible for the study. The volunteer will be informed confidentially of their results and referred to their primary care provider to get counseling or treatment. The results of the positive urine drug screen will be

part of the volunteer's medical records. This information will be treated with the same protection and confidentiality as all other medical records)

- Begin 24-hour stool collection
- Fecal tests for parasites and occult blood
- Begin regular weight maintenance diet (50% carbohydrate, 30% fat and 20% protein) for weight stabilization

DAY 2 - 3

- Continue weight maintaining diet
- Measurement of body composition (DEXA)
- Fecal tests for parasites and occult blood
- Explanation of the changes in stool color when the marker is used
- Continue 24 hour stool collection
- Start special weight maintaining diet on Day 3

DAY 4

- Oral glucose tolerance test (OGTT) for diabetes status determination
- Continue special weight maintaining diet

DAY 5-7

- Fasting blood collection
- Begin first intervention diet (under/overfeeding diet, random order) with dye capsule administered just prior to breakfast
- Continue 24-hour stool collection; before having breakfast, volunteers will be asked to urinate, thereafter begin 24-hour urine collection for caloric measurement during first experimental diet
- Prepare duplicate meals

DAY 8-10

- Fasting blood collection on day 8
- Before having breakfast, volunteers will be asked to urinate and this urine sample will complete the urine collection for the first diet period
- Administer dye capsule administered just prior to breakfast of day 8
- Begin 3 day weight maintaining diet.
- Continue 24-hour stool collection; finish first stool collection for caloric measurement with appearance of the second marker in stool
- No duplicate meals

DAY 11-13

- Fasting blood collection on day 11

- Begin second intervention diet (under/overfeeding, random order) with third dye capsule administered just prior to breakfast

- Continue 24-hour stool collection; before having breakfast, volunteers will be asked to urinate, thereafter begin 24-hour urine collection for caloric measurement during second intervention diet

- Prepare duplicate meals

DAY 14-15

- Fasting blood collection on day 14

- Before having breakfast, volunteers will be asked to urinate and this urine sample will complete the urine collection for the second diet period
- Begin weight maintaining diet with fourth marker prior to breakfast meal of day 14
- No duplicate meals

- Finish stool collection for caloric measurement for second experimental diet period with appearance of the fourth marker in stool

- Continue 24-hour stool collection for microbiota

DAY 16

- Oral glucose tolerance test

- Assessment of blood electrolytes and creatinine
- Continue weight maintaining diet
- Continue 24-hour stool collection for microbiota

DAY 17

- Measure 24h energy expenditure in the metabolic chamber
- Discontinue stool collection during 24h energy expenditure measurements in metabolic chamber
- Continue weight maintaining diet

DAY 18

- Fasting blood collection
- Administration of first heparin bolus with subsequent blood collection after 15 min.
- Start oral vancomycin or placebo in the morning after LPL measurement
- Continue weight maintaining diet

DAY 19-22

- Blood collection to assess platelet count on day 19
- Continue weight maintaining diet
- Continue oral vancomycin or placebo
- Continue to collect stool samples for microbiota

- Special weight maintaining diet on day 20 and 21

DAY 23-25

- Administer dye capsule prior to breakfast
- Start 24 hour urine collections for measurement of urine calories; collect stool samples
- Duplicate meals
- Continue weight maintaining diet
- Continue oral vancomycin or placebo
- Continue to collect stool/urine samples.

DAY 26 and 27

- Before having breakfast, volunteers will be asked to urinate and this urine sample will complete the urine collection for the antibiotic/placebo period
- Administer blue dye capsule prior to breakfast
- Continue weight maintaining diet
- Continue stool collection for microbiota

DAY 28

- Oral glucose tolerance test
- Collecting blood for complete blood count

- Control of blood electrolytes and creatinine
- Continue weight maintaining diet
- Continue oral vancomycin or placebo
- Continue to collect stool samples until dye marker is present.
- End stool collection for caloric measurments study after dye marker appears
- End stool collection for microbiota on evening of day 28

DAY 29

- Measure 24h energy expenditure in the metabolic chamber
- Continue weight maintaining diet

DAY 30

- Volunteer exits metabolic chamber
- End oral vancomycin or placebo after morning dose was taken
- Collect fasting blood sample

- While fasting receives heparin bolus and then 15 minutes later has blood drawn.

DAY 31

- Blood collection for complete blood count, if normal, volunteer can be discharged home.

DAY 6-8 after discharge

- Blood collection for complete blood count
- Volunteer is asked to return with most recent stool sample (dated and timed)

DAY 30 after discharge

- Volunteers will be asked to return with most recent stool samples (dated and timed)
- All volunteers will asked about possible side effects and general state of health

Six Months after discharge:

- Volunteer will be asked to return with most recent stool sample
- Volunteer will have weight and height recorded.

TESTS AND ANALYSES

Study diets:

Weight maintaining and experimental diets have similar macronutrient profiles (Protein 20%, Fat 30%, Carbohydrates 50%). Daily calorie needs for the weight maintaining diets are calculated as: body weight (in kg) x 9.5 + 1973(72). In order to diminish the effect of consumed food on the gut microbiota, the menu and order of the meals will be kept exactly the same over the course of the study; only the quantity of food will differ. This will ensure that volunteers eat proportionally the same amount of macronutrients and fiber during the study. In addition, in

order to minimize the effect of lactose intolerance during the study which will only be screened by patient self report, the meal will be made to minimize the quantity of dairy products. Experimental diets are characterized by either 150% or 50% of weight maintaining calories and will be offered in random order. During the experimental diet period and during 3 days under antibiotic/placebo, meals will be prepared in duplicates and chosen at random to either be offered for consumption to the volunteer or used for caloric measurement by bomb calorimetry. The same diet will be fed to volunteers (but at 100% of weight maintaining calories) during the 3 day stool collection period while on vancomycin or placebo.

Dye marker:

An inert colored dye, brilliant blue (FD&C blue) will be used to define the dietary periods. This colored dye is not absorbed in transit, does not undergo secondary metabolism by gastrointestinal bacteria, and thus is recovered fully in the stool. In addition, the average time between ingestion and the first appearance in stool of brilliant blue is 34 hours and complete passage of this dye takes 85 hours (73). However, this dye does not appear in urine. The dye marker can be purchased commercially.

Body composition by DEXA:

DEXA will be used to accurately measure body composition. DEXA is an X-ray device which non-invasively assesses both skeletal density and soft tissue composition by region with a precision of <1% for bone and 4-5% for soft tissue densities. Total body scan requires 10-50 minutes depending on the stature and thickness of the subject. The major limitation of the apparatus is the width of the scanning table. We validated the use of a half-body scan as an indicator of whole body composition in obese subjects who do not fit entirely on the scanning

area. This half body method is valid for individuals weighing as much as 350 pounds (74). During the procedure, the subject is asked to lie flat on the table and to remain motionless, since the precision of the measurement can be compromised by subject's movement during the scan. Proper subject positioning is also important. Once the proper alignment is determined, the subject's ankles and knees are loosely strapped to maintain the position. When the machine is turned on, a 150 mA current flows through the X-ray tube to produce X-rays. As the scan table arms move from the top of the subject's head downward towards the subject's feet, the shutter opens and a narrow beam of radiation projects upward through the table top and subject. After the scan, the arm clears the subject's feet, the scanner stops, and the source shutter closes. The software then calculates body composition in grams of fat tissue and lean tissue and percentage of body fat. The operator remains in the room with the subject during the scan.

Oral glucose tolerance test:

Oral glucose tolerance tests will be used to assess glucose metabolism. After an overnight fast, an intravenous catheter will be placed in the forearm vein for blood withdrawal. Each subject will ingest 75 g of glucose over 2 minutes. Blood will be drawn at -15, 0, 30, 60, 90, and 120 minutes for assessment of plasma glucose and insulin.

Twenty four hour energy expenditure and basal metabolic rate:

The respiratory chamber is a comfortable, air-tight room (approximately 10 feet x 8 feet x 7 feet) constructed as a large open-circuit indirect calorimeter. It is furnished with a toilet, sink, bed, desk, chair, color television and radio. The air temperature is maintained constant at 75 ^{o}F

by an air conditioning system. Visual contact with the subject is possible through one of the windows. Food is provided into the chamber through an airtight interlock. Spontaneous physical activity is continuously monitored by a radar system based on the Doppler Effect. The flow rate through the chamber and the CO_2 and O_2 concentrations of the out-flowing air are continuously computed and calculations of oxygen consumption, carbon dioxide production, respiratory quotient and spontaneous physical activity are made every minute. The subject will enter the chamber at 7:45 am for 23.5 hours.

Antibiotic treatment and placebo:

Volunteers will be randomly assigned to either oral antibiotic or placebo medication for 12 days. The antibiotic treatment group will receive vancomycin 125mg orally four times per day starting in the morning of day 18. The last dose will be given in the morning of day 30. The duration of the vancomycin or placebo phase is a combination of a loading phase of five days (to allow for changes in gut microbiota to change and stabilize) followed by a series of tests that will be done over the next 7 days.(24 hour energy expenditure measurement, collection of stools for caloric measurements over 6 days, OGTT and LPL measurements). Placebo medication will be given in the exact same manner. All investigators including nursing personnel as well as the volunteer him-/herself will be blinded to the randomization code. The randomization and release of the medication/placebo compounds will be managed by the pharmacy of the Phoenix Indian Medical Center.

Heparin boli and post-heparin blood sampling: Lipoprotein lipase(LPL) is anchored to endothelial cells and is needed to remove triglycerides (TG) from chylomicrons and very low density lipoproteins and then transfer these TG into parenchymal cells. A bolus of heparin

releases LPL from the endothelium and allows the measurement of LPL activity in a blood sample(75). On day 18 before starting the antibiotic/placebo medication, volunteers will be given an intravenous bolus of 75 IU/kg heparin after a 12-hour overnight fast. This method has been applied previously by our collaborators (76–78). Before the injection of the heparin bolus, 10ml venous blood from the cubital vein will be drawn into a Monoject© sodium heparin collection tube and 10ml will be drawn into a Monoject© 15% EDTA collection tube. 15 min after the heparin bolus another 10ml will be drawn into a Monoject© sodium heparin collection tube. This procedure will be repeated on day 26. Directly after the blood sampling, collection tubes will be kept cold on ice until centrifugation and subsequently stored at -70ºC until measurement of the lipoprotein lipase (LPL) activity in Dr. Gloria Vega's lab. Briefly, total lipase activity will be measured in the presence of apoC-II at physiological NaCl concentration. LPL activity will be calculated as total LPL activity subtracted by salt-resistant LPL activity (hepatic lipase). Details to this method are described elsewhere (79).

Stool and urine collection and storage:

The first stool sample obtained after admission will be used as baseline measure of the microbiota. During the inpatient stay all stool will be collected for determination of the microbiota. Additionally to that we will assess caloric content of the stool during both under and overfeeding as well as for 3 days during the antibiotic/placebo part of the study. These periods will be marked using a dye marker. Right after receiving the first experimental meal with the dye marker, stool and urine collections for caloric content measurements will begin. The urine collection will be stopped before the volunteer has breakfast on the first day after each 3 day intervention diet. Before this meal the volunteer will swallow the second dye marker The two intervention diets will be interrupted by a 3 day weight-maintaining diet to wash out the effects of the prior diet. The stool collection of the first experimental diet period will be stopped as the

second dye marker appears. These processes for stool and urine collection will be repeated for the second experimental diet period and during three days of stool collection for caloric measurements while the volunteer is on vancomycin/placebo. Only the stool samples collected between the appearance of the first marker and before the appearance of the second marker of each diet period and the urine samples collected during the these 3-day periods will be used for analysis of energy content. A small aliquot to determine the microbiota will be obtained from every stool sample from day one till day 31. This will not be more than several grams of stool. The aliquot will be frozen at -70° directly after collection of the stool. Volunteers will defecate into plastic bag that is held by a special container. The bag can easily be detached from the container and will be sealed and labeled by the volunteer. Then the plastic bag is put into a second container and will be given to our nursing stuff immediately who will then obtain the small aliquot for determination of the microbiome. Sample for bomb-calorimetry will be frozen at -20°C. The procedure of high throughput sequencing does not require anaerobic conditions. Bacterial overgrowth of aerobic bacterial species will be prevented by freezing samples immediately after collection.

Calorie content of food during experimental diet periods (gross energy intake):

During the marked diet period, volunteers will eat under supervision and they will be encouraged to eat all food provided in each experimental meal. Moreover, to determine the energy content of the consumed food, each meal consumed during the over-/underfeeding part of the study and during three days of the weight maintaining diet will be duplicated. One meal will be given to the volunteer. The other meal will be homogenized then burned to determine the heat released after complete combustion using bomb calorimetry methodology. Volunteers who

are unable to consume 90% of provided calories will not continue in the study. In addition, any unconsumed calories will then be subtracted from the total calories to determine the overall intake.

Calorie content in stool and urine:

Three days of stool collection on each experimental diet will be pooled. The samples will be homogenized and freeze-dried. Three days of urine collection on each experiment diet will also be pooled and freeze-dried prior to analysis. Urine and fecal energy content of these pooled samples will be obtained using bomb calorimetry methodology.

Preparation and measurement of calorie content of stool:

Prior to analysis, distilled water, equal to the weight of the feces, will be added. The feces-water mixture will be homogenized then. Because some of the energy containing fecal compounds are volatile in the pH ranges of normal stool and hence may be lost during the sample preparation, fecal alkalization will be employed before lyophylization(80). The preparation process will be done using up-to-date methods (80,81). After preparation, the energy content in stool will be determined by measuring the quantity of heat that is produced upon complete combustion of the sample, using bomb calorimetry methodology.

The microbial flora in stool will be enumerated using 16S rRNA probes that has been described previously (46). In brief, 16 S rRNA amplicons will be generated using universal bacteria-specific primers that target most enteric bacteria, allowing us to detect a broad, unbiased survey of the enteric bacterial population in the study cohort. However, the majority of gut microbes are expected to belong to either the Bacteroidetes or the Firmicutes phyla (46). The primers that will be used are 8F and 338F. Samples selected for microbiota analyses will be

sent to and performed inDr. Peter Turnbaugh's laboratory at the G.W. Hooper Research Institute at University of San Francisco, CA. . Stool for metagenomic analyses will be collected throughout the study period with exception of two 24-hour periods in the metabolic chamber and the discharge day. The total number of stool samples collected will vary with the defecation frequency of the volunteer during the study period.

Block Questionnaire:

Will be administered on admission. The Block Food questionnaire will assess usual nutrient intake over the preceding year.

Blood analyses

Glucagon-like peptide (GLP-1,2) and leptin measurements

GLP-1&2 and leptin will be measured using commercial radioimmunoassay kit and will be drawn at 6 time points during the study:

1. Day 5 before start of the first intervention diet (GLP1&2, leptin)
2. Day 8 after the first 3 day intervention diet (GLP-1&2, leptin)
3. Day 11 before the start of the second intervention diet (GLP-1&2, leptin)
4. Day 14 after the second 3 day intervention diet (GLP-1&2, leptin)
5. Day 18 before LPL measurment (GLP 1&2, leptin)

6. Day 30, before LPL measurment (GLP 1&2, leptin)

Blood samples for GLP1&2 will be collected in ice-cooled tubes and will be collected using heparin as an anticoagulant (10 IU heparin/ml blood).The appropriate amount of DPP-IV inhibitors will be intermediately added for each collection. Tubes will be mixed and stored in an ice bath, then centrifuged at 1000 x g for 10 minutes in a refrigerated centrifuge. The specimens will be stored at -70°C until analyzed. All blood samples will be taken in the fasting condition.

DATA ANALYSIS

This is a, cross-over intervention study in volunteers consuming two different caloric diets followed by a double blind randomized placebo controlled phase investigating the effect of antibiotics . The main outcomes of the study are stool energy content (nutrient absorption) and genetic identification of gut microbial communities during over/underfeeding and during antibiotic or placebo intervention. Results of the study will be expressed as kcal/day for energy content in stool, % of calories in stool relative to ingested calories and relative abundance of gut bacteria. A paired t-test will be used to compare the changes in percent stool calories and relative abundance of gut bacteria during the over and underfeeding diets. Pearson or Spearman correlation will be used to investigate the association of change in the relative abundance of major gut microbiota phyla with stool calorie loss, the association of stool calorie loss with gut transit time, and the association of change in relative abundance of the major phyla. Student's t-Test will be applied to determine the difference in the outcomes of stool and urine calories between the antibiotic versus placebo groups. Changes in fasting plasma glucose, area under the curve for glucose and insulin during the OGTT and lipoprotein lipase activity will be compared using paired t-test. In addition, mixed models will be applied to

investigate changes in the gut microbiota over time. The defined age range will minimize possible differences in ages between the groups. However, if the mean age is significantly different between the two groups, general linear regression models accounting for age will be used to compare energy loss and relative abundance of gut bacteria in stool between the intervention groups

Sample size

There will be two primary outcomes for this study. The first will be the difference in nutrient absorption between the experimental diets (50% and 150% of WMEN) within the groups. The second will be to examine the difference in nutrient absorption in those receiving antibiotic versus placebo. In our initial study, we found a significant difference in nutrient absorption in lean individuals fed a 2400 kcal/day versus 3400 kcal/day diets of 1.3 ± 1.9%. Assuming a similar difference during under versus overfeeding, using a correlation between stool calories for each diet as determined in the initial study of r=0.45, with 24 individuals we will have a power of 0.86, at an alpha of 0.05 to detect a difference in stool calories. This should be considered a conservative estimate as the power may be higher as the difference in calories fed during the over/underfeeding will be larger (nearly double) than in the initial study.

For the comparison between placebo versus antibiotic groups: Murphy et al demonstrated that treatment with oral vancomycin resulted in a 16% decrease in Firmicutes. Our previous results in humans showed that a 20% decrease in the abundance of Firmicutes was associated with a decrease in nutrient absorption of ~150 kcal/d. Based on this, a 16% decrease in Firmicutes would be expected to decrease nutrient absorption by 120 kcal/day. Even assuming a more modest antibiotic-induced change in the relative abundance of Firmicutes by only 10% resulting a 75 kcal per day difference with a standard deviation of 48.9

kcal/d, 12 subjects per intervention group (n=24 total) would be sufficient to achieve 95% power to detect a difference between intervention groups at a p-value less than 0.05. To account for participants who are unable to complete the study protocol, we plan to recruit 40 volunteers.

BENEFITS

The expected benefits of this study include obtaining valuable knowledge to whether gut bacteria are causally related to the development of obesity. Individuals may not directly benefit from this study, although each individual will receive a physical examination and laboratory testing that may be relevant to their health.

PROTECTION OF HUMAN SUBJECTS

Written informed consent

All individuals will be fully informed of the aim, nature, and risks of the study prior to giving written informed consent. The study's informed consent will be obtained by staff of the clinical research unit who have reviewed and are familiar with the protocol and compliant with human subjects training; these include the principal investigator or other designated qualified protocol investigators (listed on the protocol's face page). Should we enroll a non-English speaking subject, we will obtain IRB approval to use the short form consent process as outline in SOP 12.

Rationale of human selection

As mentioned previously, there is limited information about energy loss in stool and urine in humans. Factors which influence energy loss (such as nutrition, absorption rate, gastrointestinal transit time and the gut microbiota) are also affected by several factors i.e., aging (21,23,82) ethnicity (36–38), menstrual cycle (39), fluid consumption and food patterns. We will include both male and female 18-45 years of age. However we will assess the phase of the menstrual cycle of our female volunteers both through medical history and hormone measurements on the day of admission.

Possible risk and hazards

Radiation exposure due to DXA:The DXA scans may take place on one of two comparable machines: the GE Lunar Prodigy or the GE Lunar iDXA. The radiation exposure for a 150 mA scan in the slow mode using the GE Lunar Prodigy may be as much as 2 mrem, as indicated by a

January 1997 internal radiation exposure review. The radiation exposure for a 188 mA scan using the GE Lunar iDXA may be as much as 1 mrem as indicated by a January 2014 internal radiation exposure review. According to the manufacturer, the exposure at the fast or medium scan speeds is likely to result in one-third (fast) to one-half (medium) the radiation exposure of the slow scan speed. Typical exposure from this procedure is equivalent to 1.22 (iDXA) to 2.5(Prodigy) days of exposure to natural background sources, such as the sun or radioactive materials found naturally in the earth's air and soil. For subjects who move excessively during the scan, a complete or partial repeat of the scan procedure may be necessary. Thus, subjects could receive a maximum of 2 mrem per procedure for the iDXA (equivalent of 2.5 days of exposure) or 4 mrem per procedure for the Prodigy DXA (equivalent to 5 days of exposure from natural sources). In our study, only one baseline DXA scan will be performed resulting in a possible maximum exposure of 4 mrem if the Prodigy DXA is used for the scan and a repeat is required(equivalent to 5 days of exposure from natural sources). This is well within the NIH-Phoenix Radiation Safety Committee's Guidelines for research subjects of 3,000 mrem to any organ or tissue in a 13-week period and 5,000 mrem per year. The total radiation dose due to the DXA scan in this study, if the iDXA is used and a repeat is necessary, will be 2 mrem. The radiation exposure from a DXA scan increases the lifetime percentage cancer risk from 25% to 25.00016% for the Prodigy DXA or 25.00008% for the iDXA. Pregnancy will be excluded using measurement of hCG in urine prior to the DXA scan. Oral glucose tolerance test: This test carries the risk of having an indwelling catheter in place for three hours. This risk includes hematoma, ecchymoses, and infection.

Twenty-four hour energy expenditure measurement: There is a minimal risk associated with this measurement. At anytime, subjects can be in contact visually or by phone with the nurses. The nurses also check the temperature in the room as well as the different parameters listed on our computer screen every hour. In the case of any discomfort, the volunteer can ask to be taken out of the chamber.

Oral antibiotic treatment: Oral Vancomycin is an approved antibiotic which is used for Clostridia Difficile diarrhea and Staphylococcal enterocolitis. Vancomycin inhibits bacterial cell wall synthesis and is bactericidal against a number of aerobic and anaerobic bacteria. Potential adverse events, which are predominantly associated with the intravenous form of vancomycin include hypokalemia (13%) and abdominal pain (15%), diarrhea (9%), nausea (17%) and vomiting (9%). There is also risk of nephrotoxicity (5%) (67). Intravenous vancomycin is also associated with "red man syndrome". Oral vancomycin, in the absence of ongoing enterocolitis, is very poorly absorbed with only 0.76% appearing in the urine (67). Contraindications to oral vancomycin are hypersensitivity to previous vancomycin use. Given the poor oral absorption potential side effects are expected to be minimized in these otherwise healthy volunteers. Volunteers will all be on the inpatient unit during vancomycin administration and will be closely observed. In addition, a chemistry panel including measurement of serum potassium and serum creatinine will be performed on admission, and on day #13 (prior to starting vancomycin) and day # 24. The long-term effect of Vancomycin on the gut microbiota is unclear. There are several studies showing that oral antibiotic treatment can lead to changes in the gut microbiota that can persist for months(83). One study showed an increased risk of developing obesity after treatment with intravenous Vancomycin compared to other antibiotic treatments(55) however oral and not intravenous Vancomycin will be used in this study. However, specifically whether oral vancomycin has a persistent effect on gut microbiota is not clear.

Intravenous heparin boli: Contraindication to intravenous heparin injections are active or uncontrollable bleeding, severe thrombocytopenia, known allergies to heparin or a history of heparin-induced thrombocytopenia. Common adverse events of heparin injections are local skin irritation, erythema, hematoma, increased liver aminotransferase levels, transient thrombocytopenia defined as a platelet count < 150,000/ml which occurs in up to 30% of patients that received heparin, and heparin induced thrombocytopenia requiring treatment with an

alternative anticoagulant (1-10%). Severe adverse events are hemorrhage, heparin-induced thrombocytopenia with thrombosis (less than 1%), and anaphylaxis or increased immune hypersensitivity reaction (84). The anti-coagulation effect half-life is 1.5 hours and plasma half-life 0.687 to 2.478 hours dependent on heparin dose.1 Day and 7 days after the heparin injection we will check platelet count to look for signs of heparin induced thrombocytopenia as described by Warkentin et al(85). Throughout the treatment period tests for occult blood in stool will additionally be performed to periodically check for occult GI bleedings. Of note the dose we are using (75 IU/kg) is within the recommendations for heparin bolus for clinical use which is 80 IU/kg(86).

Twenty-four hour stool and urinary collection for energy and bacterial content: There are minimal risks related to this procedure but it is inconvenient. The volunteer will be trained all the necessary skills and equipped with collection devices, storage bags and gloves.

Digestion of dye marker*:* There are minimal risks associated with this procedure. Individuals may feel uncomfortable by the changes in color of their feces. These dyes have proven to be excellent indicators, creating sharp color demarcations (73).

Disclosure of medical conditions

The participants will be informed of any medical conditions uncovered by the screening blood work performed and referred to their primary care provider. The information will also be entered into their medical records and at the patient's request, sent to their physicians. All information from the study will be made available to the patients and their physicians at the patient's request. Information will be treated with the same protection and confidentiality as all other medical records. All information from the study may be made available to insurance companies if the information is requested by the insurance company and the subject signs a release of information. This could affect future insurability and employment opportunities.

Data and safety monitoring

Except for abdominal discomfort, nausea and headache, potential adverse effects from this study are expected to be infrequent. This is, in part, a randomized double blind placebo controlled study. Oral vancomycin is an approved FDA medication but its' use in this study (to promote changes in the gut microbiota) is off-label. Participants will be on oral vancomycin for only 12 days and during this time, they will be observed on the inpatient clinical research unit. Therefore the principal investigator will act as the data and safety monitor for this study. Should an serious unanticipated problem occur (defined below), the principal investigator (PI) will have access to the randomization code. The PI will report all serious unanticipated problems in writing as soon as possible, but within seven calendar days of learning of the event. Not serious unanticipated problems will be reported within 14 days after the PI learns of the event to the NIDDK IRB.

All AEs reported spontaneously by the subject, as well as those noted by the investigator or study site staff, will be recorded. In order to avoid vague, ambiguous, or colloquial expressions, the AE term should be recorded using standard medical terminology rather than the subject's own words. Every attempt will be made to describe the AE in terms of a diagnosis. Whenever the investigators are confident in making a unifying diagnosis, all related signs, symptoms and abnormal test results will be grouped together and recorded as a single AE.

All subjects who have AEs, whether or not the AEs are considered associated with the use of the study medication, will be monitored until the AE resolves, stabilizes or becomes chronic. The clinical course of the AE will be followed according to accepted standards of medical practice, even after the end of the observation period, until a satisfactory explanation for the AE is found or the investigator considers it medically justifiable to terminate follow-up.

All AEs will be evaluated for intensity and causal relationship with use of the study medication or study procedures by the investigator.

Study procedures will be subject to audits and/or monitoring visits to ensure compliance with the protocol and applicable regulatory requirements consistent with the NIDDK quality assurance program plan. Audit and/or monitoring visit results will be reported to the Principal Investigator for further reporting as appropriate. Study documents and pertinent hospital or clinical records will be reviewed to verify that the conduct of the study is consistent with the protocol plan.

Event Characterization and Reporting to the IRB and Clinical Director (CD)

Adverse events, protocol deviations, unanticipated problems (UP), Unanticipated Adverse Events (UADEs), serious adverse events, and serious are defined as described in NIH HRPP SOP 16 ("Reporting Requirements for Unanticipated Problems, Adverse Events and Protocol Deviations"). All adverse events occurring during the study, including those observed by or reported to the research team, will be recorded. Serious unanticipated problems, Unanticipated Adverse Device Effects and serious protocol deviations, will be reported to the IRB and CD as soon as possible but not more than 7 days after the PI first learns of the event. Not serious unanticipated problems 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. Not serious protocol deviations will only be reported to the IRB (within 14 days after the PI first learns of the event) if they represent a departure from NIH policies for the conduct of human subjects research, adversely affect the health care of the

subject(s) or compromise the interpretation or integrity of the research. Non-serious protocol deviations that result from normal subject scheduling variations or technical issues associated with sampling that does not impact the health of the subject or the interpretation of the study data will not be reported.

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

Research Use of Stored Human Samples, Specimens or Data

Blood (stored as plasma or serum), urine and stool samples not immediately used for study purposes will be stored for future measurements. Some of these samples will be sent to a commercial laboratory to measure GLP-1, GLP-2, leptin (plasma samples). Stool samples selected for analyses of the gut microbiota will be sent to Dr.Peter Turnbaugh at G.W. Hooper Research Foundation at University of San Francisco, CA. , as specified in the study. Blood received before and after the heparin boli will be sent to Dr. Gloria Vega at the UT Southwestern Medical Center in Dallas for measurement of lipoprotein lipase activity. Blood (stored as plasma or serum), urine, and stool samples not immediately used for study purposes will be stored for future measurements. All samples will be stored in freezers located on the premises at the NIH in Phoenix.

Stored samples and specimens will be used only to measure factors that relate to diabetes, obesity and their complications. Stored samples, specimens or data may be sent to collaborators for specific measurements or analyses. All stored samples, specimens, and data will be coded so that when sent for measurements the identity of the volunteer remains confidential. Identification of coded samples will be kept in a secure password protected database accessible only to investigators, but will be identifiable in case specific tests yield clinical information of importance to a particular volunteer or samples can be destroyed per volunteer request (see below). Samples will be used only for research and not for commercial purposes. Research volunteers will not be informed of individual results from analyses performed specifically for research purposes, unless there is clear evidence accepted by the medical community that these results will impact the volunteer's individual medical care or future health. Samples will be stored until used unless the volunteer requests in writing that the

samples be destroyed. All samples lost or destroyed secondary to volunteer request will be included in the annual renewal report.

COMPENSATION

Subjects will be receiving a total amount of $2635 if they complete the clinical part of the study. The amount is calculated as stated in the table below. For each follow-up visit subjects will receive another $25 per visit for a total of 75 dollars. If subjects are discharged prior to completion of the inpatient portion of the study for any reason including ineligibility based on initial testing, side effects of the used treatments, inability to perform testing on the unit (for instance due to non-operational equipment) or personal reasons they will receive a rate of $20 per day completed. These are the standard compensation rules for all studies on our unit.

Daily payment @ $35.00/d x 31 days	$1085.00
One inconvenience unit for the OGTT x 3	$75.00
Three inconvenience units for the respiratory chamber for 23.5 hours x 2	$150.00
One inconvenience unit for fasting blood collection/CBC x 6	$150.00
One inconvenience unit for intake of study medication daily x 12	$300.00
One inconvenience unit per day of 24h-urine and/or stool collection x 31	$775.00

Two inconvenience units for intravenous heparin injection x 2	$100.00
Total	**$2635.00**

Follow up:

One inconvenience unit will be paid for each stool sample for follow up x 3	$ 75.00

Appendix A:

Participants enrolled in a related study of nutrient absorption at the Children's Hospital of Philadelphia (CHOP). This study entitled "Diagnosing Pancreatic-Based Malabsorption in Patients with Chronic Pancreatitis CHOP IRB# 16-013001" has been reviewed and approved by the CHOP Institutional Review Board. Dr. Virginia Stallings is the principal investigator. In this study, participants with chronic pancreatitis and controls collect stool over 72 hours as part of a study to investigate the alterations in nutrient absorption in participants with chronic pancreatitis. These stool samples will be assessed for both amount of fecal fat and calories/gram of stool. To measure stool calories, approximately 50 grams of stool will be sent to our clinical research unit. We expect to receive approximately 72 samples from 48 participants (24 with chronic pancreatitis and 24 controls). We will then freeze dry and make one gram pellets and perform bomb calorimetry on these samples for measurement of calories/gram.

Samples will be coded and without any personal identifiers. Participants are aware that coded samples will be sent to our facility for these measurements. Once the measurements are made, we will send the researchers at CHOP the results. Remaining sample will not be returned, and will be destroyed once the measurements are made and reviewed. Researchers at CHOP will use this data for research analysis only, and results will not be communicated to the participants.

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SCHEDULE OF PROCEDURES

PROCEDURES	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY	DAY
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Lab test	X																														
CBC	X																		X									X			X
Fasting Blood					X			X			X			X				X												X	
Fecal test	X	X																					X								
Weight maintenance diet	X	X	X(s)	X(s)				X(s)	X(s)	X(s)				X	X	X	X	X	X	X	X(s)	X(s)	X(s)	X(s)	X(s)	X	X	X	X	X	X
DXA		X														X															
OGTT				X																								X			
Vanco/Placebo																		X	X	X	X	X	X	X	X	X	X	X	X	X	
Experimental Diets					X	X	X				X	X	X																		
Stool collection for Microbiota	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X			
Urine collection					X	X	X				X	X	X										X	X	X						
Duplicate Meals					X	X	X				X	X	X										X	X	X						
Stool collection for caloric loss					X	X	X	X	X		X	X	X	X	X								X	X	X	X					
Dye marker					X			X			X			X									X			X					
24-h EE																	X												X		
Heparin Bolus																		X												X	
Discharge																															X

Blood Drawing

1)	Screening lab work	32 ml
2)	CBC x 2	10 ml
2)	OGTT (fasting, 2h)	3 ml
3)	OGTT (3-hr) x 2	90 ml
4)	Fasting blood samples x 6	180 ml
5)	LPL measurements	82 ml
TOTAL for study		**397 ml**