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| **Study Title:** | Gastrointestinal Hormonal Regulation of Obesity |
| **IRB Protocol Number:** | NCT01146704 |
| **Protocol Date:** | 02/02/2017 |
| **Principal Investigator:** | Joseph Pisegna, MD |

## Project Summary/Abstract

Obesity is a major cause of morbidity and mortality within the VA Healthcare System accounting for the majority of cases of diabetes mellitus, hypertension, coronary artery disease and cerebrovascular accidents. An improved understanding of the mechanisms that regulate body weight in our obese, Veteran patient population will improve their quality of life by avoidance of serious medical complications and by improving novel therapeutic approaches.

The objectives of this study are firstly to establish that a high protein diet is efficacious, safe and beneficial to obese patients by allowing them to curtail food intake resulting in an improvement in body weight in and secondarily to establish the neurohormonal mechanisms of high protein diet-induced early satiety signal in relevant experimental model, focusing on activation of gastric vagal afferents.

We will assess the efficacy of a high protein diet on satiety and pattern of postprandial gut hormone in obese patients. A randomized controlled study lasting 16 weeks will assign volunteer subjects (ages $\leq 30$, BMI 27-40 kg/m2) to: 1) High protein diet (HPD) group, and 2) Standard protein diet (SPD) group as an isocaloric control group. All of the subjects will be followed by a dietitian and determinations made of circulating gut hormone levels and biochemical assays.

Neurohumoral mechanisms through which high protein diet curtailed food intake will be assessed by testing the hypothesis of a potentiating effect of gut peptides released by high protein on vagal afferent satiating signaling to the brain in obese rats using pharmacologic and electrophysiologic approaches. In addition, Fos immunohistochemistry to map brain neuronal activation in response to high protein diet will allow us to establish differential circuitries activated by high vs standard protein diet.

These studies will provide a clinical basis on the weight reducing effect of high protein diet and the associated alterations in the profile of postprandial gut hormones released, and unravel the underlying mechanisms at the neuronal (vagal afferent) level in an experimental model of obesity. The proposed studies will address important pathophysiological questions regarding the mechanisms regulating satiety/body weight as well as provide potentially important clinical treatment strategies.

## NARRATIVE

Obesity is rapidly becoming an epidemic within the VA Healthcare System. It is associated with the development of metabolic syndrome in addition to type 2 diabetes and hypertension. Accordingly, the overarching goals of this proposal are to identify the mechanisms responsible for the development of obesity and to identify potential therapeutic targets that can be used to treat this condition. The utility of a high protein dietary intervention to modulate the vagal nervous system and modulate the gastrointestinal microbiome are being proposed herein. In preliminary studies, we have demonstrated in both rodents and in humans that a high protein diet effectively and safely results in a reduction in body weight and alteration of the gut microbiome to favor an anti-obesity phenotype. Given that the VA Medical Care System is the largest of its type in the USA, and that the 158 medical facilities include over 5 million patients, strategies to reduce the incidence of obesity-related morbidity and mortality are likely to have a beneficial impact not only on patient care through prevention but will result in a significant savings in resources that could be better spent on other aspects of veteran healthcare.

## RESEARCH PLAN
## 1. RATIONALE
### A. Statement of the Problem.

Obesity has become endemic in the United States. It is estimated that about one third of our population is obese.[1,2] The health consequence of obesity involves increased risk of developing adult onset type II diabetes mellitus, dyslipidemia, arteriosclerosis and certain forms of cancer, including colorectal and

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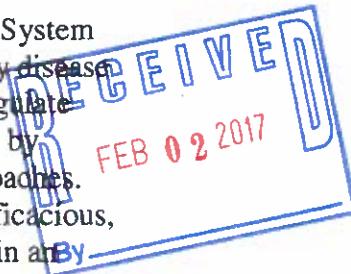
pancreatic cancer.[1] Therefore, obesity represents a major medical problem in the United States and 6% of entire health care costs are spent taking care of obesity-related co-morbidities. A modest 5 to 10% of body weight loss in obese subjects has been shown to result in improvements of these metabolic disturbances. [3] Successful treatment of obesity not only requires an approach that involves a combination of dietary, behavioral modifications and exercise [1] but also requires a greater understanding of the regulation of appetite and satiety. These approaches will lead to innovative therapies to improve treatment. The use of a high protein diet has become a popular method to achieve a reduction in body weight, [4] however the means by which an isocaloric high protein diet influences satiety is not understood.

Evidence indicates that the gastrointestinal (GI) tract acts as nutrient sensor resulting the release of gut hormones or transmitters that play an important role in the control of satiety, feeding behavior and, hence, body weight.[5-9] Neuropeptides and GI hormones regulate the neural pathways interconnecting peripheral stimuli to the brain.[9] Our team, based at the VA Greater Los Angeles Healthcare System, has a long history in the study of GI hormones and their physiological and cellular mechanisms. In addition, we were among the first to provide evidence that GI peptides activate specific brain nuclei involved in the regulation of feeding behavior and the autonomic nervous system [10-12]. Although the focus of our group has been mainly the endocrine, neuronal and chemical regulation of gastric secretion and motility, we are now expanding our focus to obesity, which plays an important role in the health of our veteran patients. Therefore, using our knowledge of GI hormones and gut-brain interactions, the overall objective of this proposal is to further establish that a high protein diet reduces body weight in obese patients and to unravel the physiological basis for the effects of this diet. Specifically, we will test the effect of high protein diet on changes in weight, the microbiome, and neuropeptides released in obese patients and elucidate the underlying physiological mechanisms in animal models.

## B. Hypotheses/Key Questions.

GI signals contributing to energy balance are abnormal in patients with obesity disorders. Levels of hormonal peptides are likely regulated by high protein diets that have been shown to result in weight loss. [5,9] These GI hormones may be regulated by signals from the intestinal microbiome, which we have shown in unpublished data is altered by a high protein diet. We therefore hypothesize that a high protein diet shifts the microbiome to induce a differential pattern of GI hormone release leading to early satiety signaling.

The objectives of this proposal, accordingly, are to corroborate data from our preliminary human studies showing that a high protein diet results in body weight loss. We will correlate the observed clinical findings with an understanding of normal and pathological appetite regulatory mechanisms in experimental animals. In order to accomplish the stated goals of this program, we have brought together interdisciplinary collaborations at our VA Medical Center from diverse backgrounds including: microbiome, cell biology, molecular biology, neurophysiology and human clinical investigations, including health services research to investigate the mechanisms regulating feeding behavior and obesity. Our proposal is focused on addressing three hypotheses linking clinical and translational approaches that are critical to understanding the pathophysiology of satiety/obesity:

1. A higher protein replacement diet will result in early satiety signaling and weight loss in obese patients and a differential pattern of postprandial GI hormone release.
2. Patients on the high protein diet will have shifts in the composition and function of their intestinal microbiome.
3. Abundance of specific fecal microbes can be used to predict response to a high protein diet.

## C. Specific Objectives.

**AIM 1: To Confirm the Hypothesis that a High Protein Diet Reduces Body Weight and Alters the Pattern of Postprandial Gut Hormone Release in Obese Patients.**

**Rationale:** In the first aim, we will evaluate whether an isocaloric high protein diets (1 gram of protein per pound of lean body mass) will improve weight loss in comparison to a standard protein diet based on 0.5 gram of protein per pound of lean body mass. Our preliminary studies support the concept that a higher protein meal replacement will result in significant losses of body mass in obese subjects and that a high endogenous level of ghrelin in patients with neuroendocrine tumors results in increased weight gain. In the study, bioelectrical impedance will be used to determine the protein requirement at the level of 0.5 or 1 gram per pound of lean body mass. Weight loss will be the primary outcome. A secondary variable will include satiation as measured using a standard questionnaire, liver stiffness as determined by transient elastography, and microbiome changes determined by 16S sequencing. In addition, we will establish the changes in the levels of the orexigenic peptide ghrelin including the ratio of ghrelin to des-acyl ghrelin, and anorexigenic peptides PYY, leptin, CCK, GLP-1 and nesfatin-1 following high protein meal ingestion.

## AIM 2. Characterize the changes in the fecal microbiome induced by a high protein diet

**Rationale:** Diet induces rapid changes in microbial composition and is associated with long-term microbial community structure[13,14 30-32]. Recent advances in the microbiome field suggest that intestinal bacteria may be involved in the response to a HPD. Germ-free mice have lower body fat and resistance to diet-induced obesity that are rapidly reversed upon microbial colonization, attributed to increased nutrients from microbial digestion of complex plant polysaccharides and elevated levels of a lipoprotein lipase inhibitor (Fiaf) [15,16]. It was subsequently shown that obese humans and mice have distinct intestinal microbiome composition from controls, characterized at the phylum level by an increased ratio of Firmicutes to Bacteroidetes [17-19]. This ratio decreased in subjects who lost weight on either a carbohydrate-restricted or fat restricted diet [20]. There is now considerable evidence that the composition of the intestinal microbiome influences susceptibility to obesity. Colonization of germ-free mice with the microbiota of obese mice (induced by leptin-deficiency or a Western diet) results in increased body fat accumulation compared to colonization with microbiota from lean controls [18,19]. Similarly, germ-free mice colonized with feces from obese humans had increased adiposity on a high fat diet than germ-free mice colonized with feces from lean humans in weight discordant twin pairs [21]. Moreover, susceptibility of mice deficient in Toll-like receptor 5 and inflammasome components to diet-induced obesity was transmissible to other mice by fecal transplantation [22,23]. Intestinal microbiota that predispose to obesity are believed to increase extraction of calories from ingested food. Supporting this, obese mice have higher levels of bacterial genes involved in complex polysaccharide digestion, decreased residual energy in feces, and increased short chain fatty acids [18]. The obese human microbiome has elevated levels of genes in phosphotransferase pathways involved in bacterial carbohydrate metabolism [24].

Recent data suggests that the intestinal microbiome can mediate the response to anti-obesity interventions. Roux-en-Y gastric bypass and vertical banded gastroplasty are associated with long-term alterations of the intestinal microbiome in humans and mice [25-27]. Colonization of germ-free mice with post-bariatric surgery feces resulted in less fat accumulation on standard mouse chow compared to colonization with stool from non-surgical obese patients [26]. Animal models have highlighted the contribution of *Akkermansia*, a genus that is depleted in obese mice and humans but enriched after gastric bypass surgery [25,27,28]. Colonization of germ-free mice with the microbiome of mice following gastric bypass surgery resulted in weight loss and reduced adiposity compared to recipients of sham operated mice, associated with increased *Akkermansia* [27]. Daily gavage of live but not heat-killed *Akkermansia muciniphila* reduced adiposity and hyperglycemia in mice with diet induced obesity [28]. Interestingly, metformin treatment of mice on a high fat diet increases *Akkermansia muciniphila*, which has been proposed as a mechanism of its therapeutic effect [29]. We recently demonstrated in a rodent model of Western diet-induced obesity that a high protein diet induces *Akkermansia muciniphila*, just as had been seen after bariatric surgery. Abundance of *Akkermansia muciniphila* was inversely correlated with body fat mass. Based on our preclinical rodent model data, we hypothesize that a high protein diet will shift the composition of the microbiome of obese subjects, including increased *Akkermansia,* and that this will result in an altered metagenome (abundances of bacterial genes).

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## AIM 3. Identify fecal microbial predictors of response to a high protein diet.

**Rationale**: We hypothesize that subjects will vary in the responsiveness of their microbiota to a HPD based on baseline or post-dietary intervention levels of microbes such as *Akkermansia muciniphila* that respond to the HPD. We will determine the time course of shifts in the microbiome and correlate these changes to weight loss and other metabolic parameters. Further understanding of the link between the microbiome and the beneficial effects of a high protein diet may spur development of therapies for obesity that directly target the microbiome to complement dietary modification.

## 2. BACKGROUND AND SIGNIFICANCE

### A. Background.

### *Diet Influence on Body Weight in Humans*

Obesity and type II diabetes mellitus co-exist in a significant proportion of patients. The long-term health benefits of weight loss in obese patients with type II diabetes have not been clearly defined. Dietary intervention and increased physical activity are the cornerstones of non-surgical management of obesity [30]. A wide range of diets have been studied to treat obesity but a consensus has not been reached on an optimal macronutrient composition. A low-fat, high carbohydrate diet has traditionally been recommended. This was challenged by alternative diets that advocated replacing carbohydrate with protein and fat. An early study from Skov *et al* reported that subjects randomized to *ad libitum* diets with 25% protein intake had greater weight loss (8.9 kg vs. 5.1 kg) and fat loss (7.6 kg vs. 4.3 kg) than subjects on a 12% protein diet over 6 months [31]. Two large-scale dietary studies comparing the performance of diets with varying concentrations of carbohydrate, protein, and fat. Sacks *et al* randomized 811 overweight adults to one of four isocaloric restricted diets with variable protein (15% vs. 25%), carbohydrate (35% vs. 65%), and fat (20% vs. 40%) content. There was a trend towards increased weight loss for those on a high-protein diet who completed the study (0.9 kg, p=0.11) and a greater decrease in insulin in the high vs. average protein diets (10% vs. 4%, p=0.07) [32]. A second study by Larsen *et al* included 773 patients who were randomized after 8 weeks on a calorie-restricted diet to one of five maintenance diets varying in protein intake (13 vs. 25%) and glycemic index [33]. The two high protein diets (HPDs) were associated with 0.9 kg reduced weight regain at 6 months (p=0.003) relative to the normal protein diets. A meta-analysis of smaller randomized studies comparing high protein and normal protein isocaloric restricted diets showed statistically significant decreases in weight, fat mass, and triglycerides in subjects on a HPD compared to control normal protein diets (NPD) [34].

The underlying mechanisms for enhanced weight loss on a HPD are incompletely characterized. HPDs have been shown to promote satiety relative to isocaloric diets with more carbohydrates or fat [35,36]. This effect has been attributed to induction of satiety hormones including glucagon, glucagon-like peptide-1, and peptide $YY_{3-36}$ [37,38]. In clinical studies of *ad libitum* feeding, HPDs are associated with reduced energy intake relative to normal protein diets [31,38]. However, HPDs have also shown efficacy compared to isocaloric diets, indicating that other mechanisms are involved. It has also been proposed that weight loss is secondary to increased thermogenesis and preservation of lean body mass, maintaining resting energy expenditure [34,39,40]. This is supported by a study demonstrating that a high protein diet mitigates the reduction in resting and total energy expenditure that occurs on a calorie-restricted diet [41].

Another important observation has been that increasing body weight is associated with increasing risk for glucose intolerance and type II diabetes [42,43]. Over 70% of patients with type II diabetes are overweight or obese. This is also true within the VA medical care system. The prevalence of type II diabetes in the United States increased, by one-third in the 1990s and in concert with the increasing prevalence of obesity over the past few decades, [44] A modest 5-10% of body weight loss in obese subjects has been shown to result in improvements of these

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metabolic disturbances. [43,45] Weight loss is extremely important to improve glycemic control and to decrease other risks associated with diabetes and obesity. Despite the imperative need for weight management in overweight people with diabetes, weight loss may be more difficult to initiate and maintain than in people without diabetes. [44,41] The long-term effects of diet composition on weight loss and glycemic control in patients with type II diabetes have been controversial beyond decreasing dietary saturated fat and total calories.

## Postprandial Gut Hormone Release and Influence of Appetite Regulation

Several gut hormones and peptides are well established to influence feeding behavior in experimental animals and in humans.[6,7,9]

*i. Ghrelin:* Isolated in 1999, ghrelin is an orexigenic peptide hormone secreted mainly by the X/A-like cells of the gastric fundus and is the endogenous ligand of the growth hormone secretagogue receptor (GHS-R). [46] In humans, plasma ghrelin levels rise prior to meals and fall after eating. [47] Ghrelin has been implicated in both mealtime hunger and the long-term regulation of body weight. Levels are known to negatively correlate with body mass index (BMI) so that levels are increased in both obese patients who have lost weight through dietary restriction and patients with anorexia nervosa [48] Also, patients who have lost weight secondary to gastric bypass surgery have suppressed ghrelin levels. Not only are the levels lower than in those patients who have lost weight by nonsurgical means, but also the meal-related fluctuations and diurnal rhythm are not found after gastric bypass surgery.[49]. There is also a clear negative association between ghrelin and insulin secretion.[47,50] In the rat as in humans, ghrelin is principally synthesized in X/A-like cells in the oxyntic mucosa of the fundus.[46] The major forms of ghrelin include acylated ghrelin, in which serine-3 is octanoylated (a unique biological finding), and non-acylated ghrelin.[3] In blood, non-acylated ghrelin circulates in far greater amounts, accounting for more than 90% of total ghrelin [10]. In rodents, we have shown that the stimulatory action of ghrelin on food intake is linked with the activation of neuropeptide Y (NPY) synthesizing neurons [10] in the arcuate nucleus (Arc) that projects to the paraventricular nucleus of the hypothalamus (PVN) and lateral hypothalamic areas. Both of these hypothalamic nuclei regulate feeding behavior and energy balance. In this way, ghrelin is thought to serve as the missing link between enteric nutrition and central regulation of energy balance.[3] Recently we also established that non-acylated ghrelin can counteract the orexigenic action of ghrelin in rats [51] but showed that obestatin, a newly identified ghrelin associated peptide (GAP) that is putatively derived from post-translational processing of the prepro-ghrelin gene, did not influence food intake contrary to the initial claim.[52]

*ii. Glucagon-like Peptide-1 (GLP-1), Peptide YY (PYY), and Leptin:* are also hormones involved in satiety.[8,12] The effects of a high protein diet on the regulation of these GI hormones has been little investigated.[55] Leptin is secreted directly from adipose cells in proportion to their metabolic activity. Insulin is secreted from pancreatic beta cells in response to increases of circulating glucose. Leptin and insulin are increased in direct proportion to the amount of body fat. Insulin and leptin are both transported through the blood-brain barrier and influence food intake and body weight. When the action of either leptin or insulin is reduced locally within the brain, subjects consume more food, [55, 56] Recently, evidence has emerged that insulin and leptin activate the same intracellular pathways in neurons in the Arc and that they interact to reduce food intake and body weight.[56,67] GLP-1 is secreted in a nutrient-dependent manner in humans *via* either direct interaction of luminal contents with the L cells or indirectly *via* a proximal-distal loop mechanism[58] The processing of proglucagon is different in the pancreas and the intestine; the pancreas contains mainly GLP-1 (1-36) amide or GLP-1 (1-36)-gly, while the intestine contains mainly GLP-1 (7-36) amide and GLP-1 (7-36) gly.[59] The major circulating forms in humans are the intestinal forms, GLP-1 (7-36) amide (henceforth GLP-1) and GLP-1 (7- 36)-gly, and these forms have similar potency for stimulation of insulin and glucagon secretion.[59] The secretion profile for GLP-1 is biphasic with an early peak at 30 min after nutrient ingestion (indirect stimulation of lower bowel by signals from

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upper gut) followed by a more prolonged peak (direct stimulation of lower bowel by luminal contents) for 1-2 h after the meal.[60,61] One recent study indicates that plasma levels of GLP-1 are enhanced by high protein diet in obese humans.[62] In addition, several reports have shown that GLP-1, infused peripherally in normal human subjects, increased satiety and reduced food intake.[63,64] Similar results were observed in type II diabetic patients.[65,66]

### Microbiome Changes Associated with Obesity, Weight Loss and High Protein Diet

Recent advances in the microbiome field suggest that intestinal bacteria may be involved in the response to a HPD. Germ-free mice have lower body fat and resistance to diet-induced obesity that are rapidly reversed upon microbial colonization, attributed to increased nutrients from microbial digestion of complex plant polysaccharides and elevated levels of a lipoprotein lipase inhibitor (Fiaf) [15,16]. It was subsequently shown that obese humans and mice have distinct intestinal microbiome composition from controls, characterized at the phylum level by an increased ratio of Firmicutes to Bacteroidetes [17-19]. This ratio decreased in subjects who lost weight on either a carbohydrate-restricted or fat restricted diet [20]. There is now considerable evidence that the composition of the intestinal microbiome influences susceptibility to obesity. Colonization of germ-free mice with the microbiota of obese mice (induced by leptin-deficiency or a Western diet) results in increased body fat accumulation compared to colonization with microbiota from lean controls [18,19]. Similarly, germ-free mice colonized with feces from obese humans had increased adiposity on a high fat diet than germ-free mice colonized with feces from lean humans in weight discordant twin pairs [21]. Moreover, susceptibility of mice deficient in Toll-like receptor 5 and inflammasome components to diet-induced obesity was transmissible to other mice by fecal transplantation [22,23]. Intestinal microbiota that predispose to obesity are believed to increase extraction of calories from ingested food. Supporting this, obese mice have higher levels of bacterial genes involved in complex polysaccharide digestion, decreased residual energy in feces, and increased short chain fatty acids [18]. The obese human microbiome has elevated levels of genes in phosphotransferase pathways involved in bacterial carbohydrate metabolism [24].

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Diet induces rapid changes in microbial composition and is associated with long-term microbial community structure[28]. In particular, animal protein intake greatly impacts the microbiome after as little as one day of consumption[31].

## B.    Significance.

Obesity is associated with early mortality in the United States. It has been estimated to result in about 280,000 deaths per year in U.S. adults and the expenses related to obesity are in excess of $80 billion.[2] Obesity is a major cause of morbidity and mortality within our VA medical system

accounting for the majority of cases of diabetes mellitus, hypertension, coronary artery disease and cerebrovascular accidents.[1,2,44] The proposed studies will address important physiological questions regarding the mechanisms of gut peptides regulating satiety and food intake, as well as provide potentially important clinical treatment strategies. The release of GI hormones in response to meal stimuli plays an important role in the regulation of body weight homeostasis. [9,55]. The neural pathways interconnecting gut sinaling of satiety to the brain in response to nutrient intake are regulated by neuropeptides and GI hormones. [9]

Our investigators have a long history in the study of GI hormones. In the current application, we plan to elucidate the impact of a high protein diet on the profile of gut hormones released postprandially in obese subjects and the underlying changes at the neuronal (vagal afferent) level that take place in response to a high protein diet in a relevant experimental model. Understanding the regulatory mechanisms involved in satiety will provide clues for existing and novel forms of therapies. We also propose a randomized clinical study to characterize the effects of a high protein diet on the intestinal microbiome of obese subjects. In this proposal, we will determine the time course of shifts in the microbiome and recapitulate these changes in humanized gnotobiotic mice. Further understanding of the link between the microbiome and the beneficial effects of a high protein diet may spur development of therapies for obesity that directly target the microbiome to complement dietary modification. Studies may also provide insight into underlying mechanisms responsible for weight loss induced by gastroplasty and bariatric procedure used for the treatment of obesity.

## C.    Relevance to the VA Patient Care Mission.

Obesity is an escalating medical problem in the VA Healthcare System. [67] It is a major risk factor for the development of chronic diseases seen in our patient population.[1] These illnesses include arteriosclerosis, diabetes mellitus, and certain forms of cancer, therefore, accounting for significant morbidity and mortality.[1] A study, reported in 2000, [67] established that among 93,290 female American veterans, 68.4% were at least overweight with a BMI >25 kg/m$^2$ and 37.4% were classified as obese with a BMI over 30 kg/m$^2$. Of 1,710,032 men 73% were defined as overweight and nearly 33% were classified as obese. Since the prevalence is increasing in the VA Healthcare System, interventions to reduce obesity are likely to result in positive outcomes for our patient population. Given that the VA Medical Care System is the largest of its type in the USA, and that the 158 medical facilities include over 5 million patients, strategies to reduce the incidence of obesity-related morbidity and mortality are likely to have a beneficial impact not only on patient care through prevention but will result in a significant savings in resources that could be better spent on other aspects of veteran healthcare.

## 3. WORK ACCOMPLISHED
### 3.1. Toward Aim 1
**A. A High Protein Meal Reduces Body Weight in Human Subjects with Adult Type II Diabetes:** We have performed a preliminary study comparing the effects of high protein meal replacement (MR) *vs.* individualized diet plans (IDP) as recommended by the American Diabetes Association on weight loss and metabolic profile. A total of 104 obese patients with type II diabetes on oral hypoglycemic agents (51 male and 53 female), with obesity as defined as BMI >25 kg/m$^2$, were randomized and 77 patients completed the 12-month study. The percentage of weight loss in the MR group ($4.57 \pm 0.81\%$) was significantly greater ($p<0.05$) than in the IDP group ($2.25 \pm 0.72\%$), Fasting glucose was significantly reduced in the MR group ($126.4 \pm 4.9$ mg/dL) compared with the IDP group ($152.5 \pm 6.6$ mg/dL, $p<0.0001$) at 6 months but not at 12 months.

Controlling for baseline levels, HgbA1c level, a long term marker for efficiency of glucose control, was improved by $0.49 \pm 0.22\%$ for those receiving MR relative to the IDP group ($p<0.05$). Considerably more patients in the MR group reduced their use of sulfonylureas

(p<0.0001) and metformin (p<0.05) as compared to the IDP group. In the 77 patients who completed the 12-month study, highly sensitive C-reactive protein (hs-CRP) was measured. While the two groups had the same baseline hs-CRP level (MR: $3.70\pm0.75$ mg/L vs. IDP: $3.66\pm0.69$ mg/L), significant decreases in hs-CRP were observed in the MR group (-26.3%, p=0.019) but not in the IDP group (-7.06%, p=0.338) at 6 months as well as 12 months (MR: -25.0%, p=0.019; IDP: -18.7%, p=0.179).

This preliminary study suggests that high protein meal replacement is a safe and viable strategy for weight reduction in obese type II diabetes mellitus patients resulting in beneficial changes in measures of glycemic control and reduction of medications. The marked reduction in inflammatory marker hs-CRP following weight loss may confer additional cardiovascular benefit. These studies are relevant to the current proposal in that they clearly demonstrate that in patients with type II diabetes there is a significant reduction in weight in subjects ingesting a high protein diet compared to a normal protein diet.

## B. High Protein Meal Induced Weight Loss and Satiety in Obese

Subjects: In a second study, we investigated the effects of an isocaloric high protein diet in non-diabetic obese subjects. In order to determine the effects of isocaloric increased protein meal replacements, 100 obese volunteers were recruited to participate in a 3-month study of meal replacements with either 0.5 g/lb lean body mass/day or 1.0 g/lb lean body mass/day. There were a total of 75 females (41 in group A and 34 in group B) and 25 males (9 in group A and 16 in group B) in the preliminary study. The meal replacements were isocaloric with carbohydrate substituted for protein and dietary fat held constant at 20%. Sixty percent of the subjects have completed the study. The 86 subjects who finished the study are included in this analysis. Mean baseline BMI was 33.63 kg/m$^2$ (range 27 to 43); mean baseline age was 49.7 years (range 24 to 71). Participants were randomized to either the HP MR treatment group (n=42) or standard protein (SP) MR group (n=46). Both groups received dietary counseling at baseline, as well as at 2, 4, 8, and 12 weeks. 12-week follow-up data were available in 86 subjects (42 HP, 44 SP). At 12 weeks, the HP and SP groups both lost significant amounts of weight (males: $235.96\pm7.28$ to $221.73\pm9.34$ lbs in HP, $231.5\pm8.09$ to $217.69\pm7.73$ lbs in SP; females: $198.66\pm4.68$ to $188.98\pm4.53$ lbs in HP, $194.19\pm4.58$ to $182.34\pm 5.33$ lbs in SP). In the HP versus the SP group, there were no statistically significant differences in changes in weight (-11.1 vs. -11.9 lbs; p=0.80), waist circumference (-3.9 vs. -3.0 cm; p=0.34), hip circumference (-2.0 vs. -1.08 cm; p=0.94), bioelectrical impedance analysis (BIA) fat mass (-7.4 vs. -4.3 lbs; p=0.17), total cholesterol (-17.5 vs. -6.3 mg/dL), LDL cholesterol (-11.3 vs. -8.3; p=0.77), and HDL cholesterol (+1.3 vs. +0.5 mg/dL; p=0.62). A statistically significant reduction in the HP group was observed with regard to fat mass (-1.82±0.66 lbs for HP and 0.24±0.68 for SP, p=0.0395) as shown in Fig. 2 and triglyceride concentration (-42.5 vs. -0.3 mg/clL). This analysis demonstrates the potential of increased protein supplementation to result in retention of lean body mass at equivalent weight losses, but longer-term studies are needed to expand on these findings.

## C. Alteration of Ghrelin Plasma Levels in Patients' with Neuroendocrine Tumors:

In patients with cancer cachexia, it has been postulated that lack of appetite and weight loss despite elevated ghrelin levels results from partial ghrelin resistance, not unlike that seen in diabetes. However, exogenous ghrelin given to these patients has been shown to increase appetite and caloric intake by about 30% in both healthy controls and in those with cancer cachexia.[48] In this study, we hypothesized that elevated endogenous ghrelin



Plasma Ghrelin Values in Patients with and without Hepatic Metastases
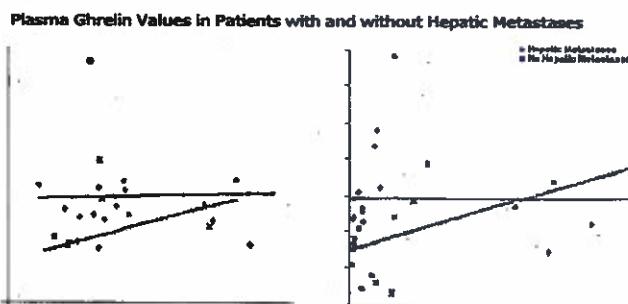
Figure 1. Plasma Ghrelin Values in Patients with Neuroendocrine tumors with or without Hepatic Metastases. These results indicate that in patients with hepatic metastases there is an increase in the amount of circulating ghrelin and consequent maintenance of body weight.

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levels helped to maintain body weight in patients with. hepatic metastases. We have recently demonstrated that in patients with metastatic neuroendocrine tumors, there is maintenance of body weight despite widespread disease.

As demonstrated in Fig. 1, ghrelin levels are greater in patients with hepatic metastases than in those without metastases at every BMI observed. We also see that ghrelin levels increase with BMI in both groups revealing a positive correlation that is observed in all the subgroups. These data indicate that the release of ghrelin, in this example from tumors derived from GI endocrine tumors, results in an increase in body mass index and provides support for our ability to correlate changes in gastric hormones with alterations of body weight in the proposed studies.

### 3.3. Toward Aims 2 and 3:
### A. A High Protein Diet Induces Fat Loss and Changes in Microbial Composition in Rats with Western Diet-Induced Obesity

We have investigated the mechanisms of weight loss on a HPD using a rat model of obesity induced by a Western diet (WD, 45% fat, 20% protein, 35% carbohydrate including 17% sucrose). After 12 weeks, half were switched to an isocaloric HPD (9% fat, 52% protein, 39% carbohydrate; n=11) for 6 weeks and the other half remained on the WD (n=10). Age-matched control rats (n=8) were fed a normal diet (ND, 9% fat, 29% protein, 62% carbohydrate) for the same period (18 weeks). The body fat mass of rats that remained on a WD was 77.7% higher than that of rats on a ND (Fig. 1A). Rats switched to a HPD for 6 weeks had a 29% reduction in body fat (p<0.01) compared to rats remaining on a WD. Rats switched from WD to HPD had equivalent caloric intake to rats maintained on a WD, indicating that reduced body fat mass is not attributable simply to reduced energy intake.

We then compared the microbiome of the WD and HPD groups. There was a highly significant difference in composition by weighted UniFrac analysis ($p<10^{-5}$) (Fig. 1B). At the genus level, the HPD microbiome was characterized by expansion of *Akkermansia, Ruminococcus, and Bacteroides* and depletion of *Lactobacillus* and *Turicibacter* (Fig. 1C). The HPD group had statistically significantly increased abundance of 114 operational taxonomic units (OTUs) selected at 97% similarity (corresponding approximately to species) after adjustment for multiple hypothesis testing (q-values). Of these, *Akkermansia muciniphila* (q=0.008) and an unclassified Clostridiales (q=0.04) had a statistically significant inverse correlation with fat mass after adjustment for diet. The correlation of *Akkermansia muciniphila* with reduced fat is consistent with findings in gastric bypass patients and studies indicating that *Akkermansia muciniphila* reduces adiposity and hyperglycemia (25, 27-29). There was decreased abundance of 188 OTUs, of which an unclassified RF39 (q=0.0001) and a *Phascolarctobacterium* (q=0.02) were correlated with fat mass. Interestingly, RF 39 is a member of the Mollicutes class, which is dramatically increased in mice on a Western diet [31].
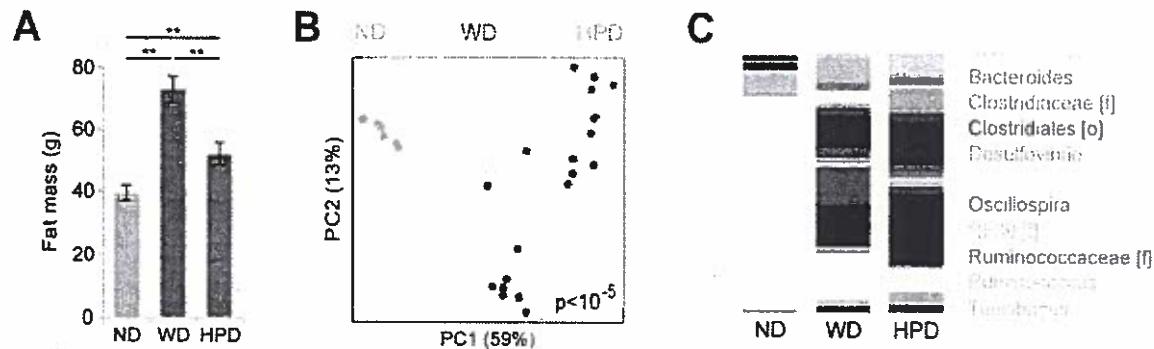
**Figure 2. A high protein diet induces fat loss and changes in microbial composition in rats with Western diet-induced obesity.** A) Body fat mass of rats kept on a WD or switched to a HPD. Age-matched control rats on a ND are shown for comparison. ** $p<0.01$ B) Principal coordinates analysis of the cecal luminal microbiome colored by diet. P-value across groups was calculated using Adonis. C) Mean abundance of common genera. Some reads were identified only at the family (f) or order (o) level.

## 4. WORK PROPOSED
## A. AIM 1: To Test the Hypothesis that a High Protein Diet Reduces Body Weight and Alters the Pattern of Postprandial Gut Hormone Release in Obese Patients.

*Rationale:* High protein intake has been associated with reduced caloric intake, and meal replacements are an effective strategy for weight loss and weight maintenance in obese subjects. [4] The primary objective of this study is to evaluate whether the high protein diets based on 1 gram of protein per pound of lean body mass will improve weight loss in comparison to a standard protein diet based on 0.5 gram of protein per pound of lean body mass. In all arms of the study, bioelectrical impedance will be used to determine the protein requirement at the level of 0.5 or 1 gram per pound of lean body mass. The prescribed calories will be the same on all meal replacement weight loss plans. Weight loss will be the primary outcome. Secondary variables will be satiation as measured using a standard questionnaire, bioelectrical impedance analysis, transient elastography, 16S rRNA sequencing and shotgun metagenomics. A third variable will be multi satiety hormone responses as measured before and after a meal.

The study described below is also unique in its design in that in the same subjects we will assess the changes in gut hormone release and the microbiome (Aims 2 and 3). This is the first study to investigate the effects of meal composition on satiety in connection with the microbiome. The result of this study will guide future clinical trials on weight loss with high protein diet in subjects.

*Recruitment:* Subjects will be recruited through the use of IRB-approved flyers which will be distributed in the primary care and subspecialty clinics. Online advertisement on ClinicalTrials.gov will also be used to disseminate information about this study to make an outreach effort that spans the entire community. In addition, those patients that are being evaluated as part of our MOVE program, a national weight management program designed by the VA NCP, would be provided with information regarding the study.

Approximately 200 healthy volunteers age 30 years and older between BMI of 27 to 40 kg/m² will be able to take part in this study. Patients must fulfill all of the following conditions or characteristics in order to be considered for study enrollment:

Inclusion criteria:
a. Age 30 years and older at screening.
b. BMI of 27 to 40 kg/m² inclusive.
c. Subjects must be in good health as determined by medical history, physical examination, and screening clinical laboratory including chemistry panel and CBC.
d. Must have stable smoking habits (or be non-smokers) for at least 6 months prior to screening and agree not to intend to change such habits during the course of the study.

e. Subjects requiring the regular use of any prescription medication may be admitted to the study providing the dose is stable.

f. Subjects must sign. the VA Greater Los Angeles Healthcare System Institutional Review Board-approved written informed consent prior to the initiation of any study specific procedures or randomization.

Exclusion Criteria:

g. Weight stability: Subjects reporting weight change of >3.0 kg in the month prior to screening.

h. Any subject who has been on a very low calorie diet (<800 kcal/day) for a period of 4 months or more in the 12 months prior to screening, or who has lost >10 kg in the 6 months prior to screening.

i. Any subject who has a history of diabetic gastroparesis or gastric emptying disorder.

j. Use of any other investigational drug(s) within 8 weeks prior to screening.

k. Abnormal laboratory parameters: Serum creatinine >1.6 mg/c1; Liver function tests, ALT, AST, Bilirubin results >2.0 times the upper limit of normal; Triglycerides >500 mg/dL; total cholesterol >350 mg/dL; TSH outside of normal range.

l. Subjects who drink >1 alcoholic beverage per day.

m. Has an implanted cardiac defibrillator.

Pregnant women or women likely to become pregnant during the course of the study may not participate in this study. Female subjects must not be able to conceive by reason of surgery, radiation, 1 year past the onset of menopause, or an approved method of contraception. No vulnerable subjects will be included in the study.

## Procedures:

*i. Study Design:* This randomized controlled study will assign approximately 200 subjects (100 each) to the following arms (Appendix 1): 1) High protein diet group based on 1 gram of protein per pound of lean body mass, and 2) Standard protein diet group as control based on 0.5 gram protein per pound of lean body mass with same calories. All participants will meet with a registered dietitian, physician or nurse practitioner to assist them with their diet efforts in all the arms. In the study, the percent energy from fat will be held constant at 30% and the differences in the diets relate only to the protein and carbohydrate contents (30% protein and 40% carbohydrate, and 15% protein and 55% carbohydrate respectively). At the initial visit patient will not be calorie restricted but will have standardization of their nutritional macronutrient intake based on their group randomization. At the 2 week visit patient will then begin a 1500 calorie restricted diet with the same macronutrient composition (ie. 30% protein and 40% carbohydrate, and 15% protein and 55% carbohydrate respectively for the high and standard protein groups).

*ii. Number of Subjects:* Approximately 200 healthy volunteers age 30 years and older with a BMI between 27 to 40 kg/m$^2$ will be able to take part in this study.

*iii. Estimated Study Duration:* Accrual period is 3-4 years; anticipated dropout rate is 20%; and total study duration is approximately 5 years (taking into account data analysis).

*iv. Subject Eligibility:* Subjects will be assigned an enrollment number after signing the informed consent form. The subjects will complete all the screening tests and procedures as listed. When a subject completes the screening tests and procedures, the subjects will be assigned a randomization number. Subjects will be randomized into one of the two diet groups as described in Appendix 1.

The practitioner at screening will obtain each subject's date of birth, sex, race, medical history, current medications, and smoking and alcohol history. The physician will perform complete physical examinations. Height will be measured using a standardized stadiometer, while the subject stands

erect with feet together and head level; height will be measured to the nearest 0.5 cm. Weight will be measured to the nearest 0.25 lbs using a balanced scale. BM will be calculated as weight in kilograms divided by the square of the height in meter. Waist and hip circumferences will be measured in the standing position. The subject should be wearing unrestricting undergarments with a hospital gown being optional. Waist circumference will be measured at the level midway between the lateral lower rib margin and the iliac crest. Hip circumference will be measured at the level of the trochanters through the pubic symphysis.

*v. Study Visits:* Participants in both groups will meet with registered dietitians every 2 to 4 week throughout the Intervention Phase and the dietary intake patterns will be reviewed. A variety of visual tools including food models and common household measuring utensils will be used during the interview to help participants gauge amounts accurately.

a. Assessment will be performed at weeks 0, 2, 4, 6, 8, 12, 16.
1. Vital signs
2. Height
3. Weight
4. Vital Signs
5. Fasting Labs: CBC/Metabolic Panel, Hemoglobin a1c, TSH, Cholesterol, TG, HDL, blood sugar, hormone panel (leptin, CCK, GLP-1, PYY, ghrelin, insulin, nesfatin-1)
6. Body Composition and estimated resting metabolic rate
7. Hepatic transient elastography
8. Stool collection for fecal short chain fatty acids and microbiome analysis
9. Satiety Scale
10. 3-Day food record
11. Food frequency questionnaire
12. Adverse Event Tracking

Compliance with consumption will be assessed using check lists that will be turned in during clinical visits. Minimal accepted compliance will be set at 80% of the assigned diet. A participant will be withdrawn once cumulative consumption levels fall to the point that 100% compliance cannot get them over the 80% threshold by the final day of the intervention.

**Study Schedule**

| Procedures | Screen Week -1 | Baseline Week 0 | Week 2 | Week 4 | Week 6 | Week 8 | Week 12 | Week 16 |
|---|---|---|---|---|---|---|---|---|
| Informed Consent | X | | | | | | | |
| Medical History | X | | | | | | | |
| Physical Examination | X | | | | | | | |
| Labs: Serum Pregnancy Test (if needed) | X | | | | | | | |
| Urine Pregnancy Test (if needed) | X | | | | | | | |
| Height | | X | X | X | X | X | X | X |
| Weight | | X | X | X | X | X | X | X |
| Vital Signs | | X | X | X | X | X | X | X |
| Labs: CBC/Metabolic Panel, Hormones, | | X | X | X | X | X | X | X |

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| Hemoglobin a1c, TSH, lipids | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Body Composition BIA | | X | X | X | X | X | X | X |
| Liver Stiffness Fibroscan | | X | X | X | X | X | X | X |
| Product Dispensing | | X | X | X | X | X | X | X |
| Stool collection | | X | X | X | X | X | X | X |
| Satiety Scale | | X | X | X | X | X | X | X |
| 3-Day food record | | X | X | X | X | X | X | X |
| Food frequency questionnaire | | X | X | X | X | X | X | X |
| Satiety Scale | | X | X | X | X | X | X | X |
| Adverse Event Tracking | | X | X | X | X | X | X | X |

### vi. *Nutritional assessments:*

• <u>Food Frequency Questionnaire (FFQ):</u> The FFQ is an assessment tool useful in monitoring trends in dietary patterns over time, with adequate test and retest reliability. We will be utilizing the Diet History Questionnaire (DHQ) FFQ tool developed by the National Cancer Institute and National Institutes of Health (NIH) (https://epi.grants.cancer.gov/dhq2/about/). It is an online semi-quantitative food frequency questionnaire, which is cost-effective and rapid, with minimal participant burden requiring approximately 45 minutes to 1 hour to complete. The FFQ is self-administered and does not require specialized training for the administrator. The extensive nutrient composition database that supports the questionnaire allows estimations of the intake of a wide range of nutrients, including macronutrients (e.g., fats), micronutrients (e.g., caroteneids), as well as intake of specific food groups such as fruits, vegetables and grains. The FFQ will be administered to participants in all groups at baseline, and weeks 2, 4, 6, 8, 12 and 16. The FFQ will query the previous two-week recall for the average frequency of consumption of the list of food items.

<u>Three-Day Food Record (3DFR):</u> The three-day food record consists of detailed documentation of food eaten on three consecutive days including three weekdays and one weekend day (Saturday or Sunday). We will be utilizing the Automated Self-Administered 24-hour recall system 3DFR developed by the National Cancer Institute and National Institutes of Health (NIH) (https://epi.grants.cancer.gov/asa24/). The main objective of the 3DFR is to obtain a complete record of a participant's dietary intake during the given time period. This method provides an assessment of individual nutrient intake that is less subject to underreporting than other methods. Food records will also provide information regarding the use of meal replacements, which other assessment tools, such as food frequency questionnaires, would not include. Participants will keep 3DFR's at baseline, and weeks 2, 4, 6, 8, 12 and 16.

<u>Satiety Questionnaire:</u> Satiety will be measured by using a visual analog scale. Participants will be asked to rate the degree to which they felt hungry by placing a mark on a visual analog scale numbered 0 to 10 (0 indicating "not hungry at all" and 10 indicating "as hungry as I have ever felt"). Participants will complete the scales at baseline and weeks 2, 4, 6, 8, 12 and 16.

***Dietary Interventions:*** All participants will receive individual consultation on their respective diet plans, lifestyle modification, and physical activity with a registered dietitian or healthcare provider (i.e. nurse practitioner or physician) throughout the study. At the initial visit patient will not be calorie restricted but will have standardization of their nutritional macronutrient intake based on their group randomization. At the 2 week visit patient will then begin a 1500

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calorie restricted diet with the same macronutrient composition (i.e. 30% protein and 40% carbohydrate, and 15% protein and 55% carbohydrate respectively for the high and standard protein groups). Participants in the high protein groups will receive a diet plan based on 1 gram of protein per pound of lean body mass, while the standard protein group will receive 0.5 gram of protein per pound of lean body mass. All subjects will be given guidelines to balance the rest of their caloric and nutritional needs with fruits, vegetables, and whole grains. All participants will meet with a dietitian or healthcare provider at baseline and weeks 2, 4, 6, 8, 12 and 16. Participants will also be weighed at each of these visits and will pick up meal replacement products at that time. All participants will be advised to keep self-monitoring daily food diaries to help with diet compliance and behavior change. Subjects will also be asked to complete the visual analog scale to assess fullness with the overall diet at the same time the 3DFR is being kept. Adherence with strict dietary controls will be performed by reviewing daily patient logs and telephone calls.

*vii.* **Laboratory Studies:** Blood samples will be obtained by venipuncture. Subjects will be required to fast at least 8 h and not more than 14 h prior to venipuncture. The laboratory tests will consist of the following: Chemistry Panel, Complete Blood Count (CBC), HbAl c, Lipid Panel, Liver Function Panel, Thyroid Stimulating Hormone (TSH) and C-Reactive Protein (CRP) performed by the laboratory.

Insulin/Glucose: Insulin is measured using a commercial double antibody $1^{125}$ method. Glucose is measured using spectrophotometry at 505 nm absorbance.

Lipids — Cholesterol, High Density Lipoprotein (HDL) Cholesterol, Triglycerides: Plasma cholesterol and triacyiglycerol are determined using standard enzymatic methods. The interassay coefficients of variation are less than 4% and interassay variation is usually less than 2%. These levels of precision are required for the types of nutritional studies carried out by the core in recent years demonstrating statistically significant changes in total cholesterol with adherence to a low fat diet and modest weight loss. Concentrations of alpha cholesterol are determined enzymatically. The alpha cholesterol is derived from the measurement on the supernatant following the precipitation of apo B containing lipoproteins with Heparin and $MnCl_2$. The so-called low density lipoprotein (LDL) or beta lipoprotein cholesterol is estimated from these data using the Friedenwald equation. For seven years, the laboratory has participated with the Centers for Disease Control in the standardization program for measuring cholesterol, HDL cholesterol and triacyiglycerol and it continues to meet the criteria required by the center for disease control in the analyses of these plasma lipids. The laboratory has been assigned a Laboratory I.D. No. LSP 266 for the ODC-NHLB1 Lipid Standardization Program.

*viii.* **Hormonal and Biochemical Assays:** The ability to conveniently and rapidly profile a diverse set of peptides has valuable applications. The comparison of a biomarker profile is important both for the better understanding of pathophysiology and for the development of improved clinical diagnostics. In a step toward further enabling such a capability, we propose to utilize a biomarker kit based on Phoenix Pharmaceuticals. This kit is able to measure levels of GLP-1, $PYY_{3-36}$, adiponectin, IGF-1, interleukin (IL)-6, leptin, acyl ghrelin, CCK, gastrin and tumor necrosis factor (TNF)-alpha. We intend to measure leptin, IGF-1, TNF-alpha, and IL-6 concentrations because recent studies have demonstrated a strong positive correlation of serum levels with percentage of body fat.

To assay the levels in hormones that are released in the high protein *versus* standard protein meal, we intend to use the "Obesity Peptide Biomarker Chip" from Phoenix Pharmaceuticals (Appendix 2). From the designed antibodies immobilized on the nitrocellulose coated slide, the results from this assay can qualitatively indicate the concentration of these obesity-related hormones from serum/plasma, adipocytes, or conditioned medium. This kit contains a two-pad glass slide. Each pad has been spotted with 36 to 40 kinds of capture antibodies to detect the corresponding sample antigens. First, the test serum is biotinylated and purified. Following purification, the nonspecific binding sites of the capture antibodies are blocked with blocking

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solution. Biotinylated antigens in the sample or in the standard solution can then bind to the capture antibodies immobilized on the nitrocellulose pads. Streptavidin-horseradish peroxidase is added and will subsequently catalyze the chemiluminescence substrate solution. The enzyme-substrate reaction is imaged by CCD camera, chemiluminescence imager, or X-ray film. Spot intensity will be counted and is directly proportional to the amount of antigen in the test sample. A direct comparison between sample and standard will give the biomarker profile in question. After knowing which marker is up or down regulated, the conventional ELISA will be used for accurate quantitation of those biomarkers. We first obtain the baseline of hormone release before starting the meal replacement, at fasting, 1 hour, 2 hour, and 4 hour postprandial time-points. At the first week after initiation of the meal replacement, the subjects repeat the above tests. The test interval is then extended to one month to two months.

### ix. Bioelectrical Impedance Analysis (BIA), Hepatic Transient Elastography (Fibroscan) and Anthropomorphic Measurements:

Bioelectrical Impedance Analysis will be obtained at baseline and at weeks 2, 4, 6, 8, 12 and 16 by a physician, nurse practitioner or trained research dietician in our department of gastroenterology at VAGLAHS. This noninvasive tool permits the assessment of both visceral and total fat composition. It is anticipated that this assessment will take approximately 5-10 minutes to complete. The data will be saved using VA equipment to ensure that patient privacy. A visceral/extremity fat content, body fat percentage and basal metabolic rate will be measured. Hepatic Transient Elastography will be obtained at baseline and at weeks 2, 4, 6, 8, 12 and 16 by a certified technician in our department of gastroenterology at VAGLAHS. This noninvasive tool uses ultrasound to characterize the liver stiffness and fat composition of the liver. It is anticipated that this assessment will take approximately 5-10 minutes to complete. The data will be saved using VA equipment to ensure that patient privacy. A liver stiffness score and fat composition will be measured.

Body anthropometry will be performed using the Gulick ll 180 centimeter measuring tape with tensiometer (Country Technology, Wisconsin) that will be performed by the nutrition service. The landmark for the' waist circumference is approximately below the lowest rib. All measurements will be taken at the end of expiration. Hip circumference will be measured at the maximum extension point around the buttocks. Height will be assessed using a stadiometer and performed in bare feet. Weight will be measured in all subjects using the same scale to ensure standardization.

### viii. Fecal Sampling

Stool samples will be collected on day 3, 7, 14, 17, 21, 28, 42, 56, 84 and 112. Subjects will be provided with kits for home sampling. The subjects will urinate first then defecate into the Fisherbrand Commode Specimen Collection System. Subjects will use a spoon to transfer freshly defecated feces to a Para-Pak stool collection cup prefilled with 95% ethanol to fix the samples, allowing storage at room temperature for up to 2 weeks. The remaining feces will be frozen. This involves removing the cup from the toilet hat, tightly closing the lid, and transferring the cup to a Ziploc bag for storage in the subject's freezer. Frozen samples will delivered to the VA clinic during scheduled study visits using a Styrofoam box and frozen packs. Subjects who forget to bring samples will be provided with prepaid envelopes to mail their samples to the coordinator. Samples will be stored at -80°C. Ethanol-fixed and fresh frozen samples give comparable metagenomics results to samples stored in preservatives such as RNALater while minimizing subjects' risk of exposure to toxic chemicals [33].

### Statistical Analysis:

Statistical Design: This is a 2-group randomized nutritional study to evaluate whether the high-protein diet will improve weight loss in comparison to a standard (control). diet. Eligible subjects will be randomly assigned into one of the two groups in a ratio of 1:1.

Sample Size Determination: The sample size is determined based on the Specific Aim 1 to significantly improve weight loss, as observed by comparing the high-protein diet to the standard diet. The primary outcome of interest is weight loss at 16 weeks (the end of the study). An evaluable sample size of at least 165 (i.e., 55 per arm) will achieve 80% power to detect an effect size of 0.245 using an F test with a two-sided 0.05 significance level when comparing each of the two high-protein diets to the standard diet, assuming that the mean weight loss for the standard diet is 14 lbs with the common standard deviation within group of 10 (from preliminary data), and the size of the variation in the means is 2.45. To account for an attrition rate of up to 20%, we will enroll a total of 200 subjects (66 per arm). Preliminary data suggest that the estimated effect size ranges from 0.23-0.34 when comparing the high-protein diet to the standard diet.

Data Analysis: Descriptive statistics (mean, standard deviation, median, inter-quartile range, and frequency distribution) of demographic information and baseline assessments will be generated to characterize the study participants. Patient characteristics and baseline measurements of the three diet groups will be compared using analysis of variance (ANOVA) for continuous variables, and Chi-square test for categorical variables to evaluate the appropriation of the randomization. If the distribution of the variable is skewed, an appropriate variable transformation will be made before the comparison. Nonparametric methods, such as Kruskal-Wallis one-way analysis of variance, will also be used for comparison.

Primary Endpoint: the primary endpoint is change in body weight, which is calculated as the difference between Day 1 (baseline) and 16 weeks. The primary objective is to compare the weight loss between the high-protein diet and the standard diet. We will use ANOVA to compare the change in weight among the three groups (through contrast), and also use ANCOVA to compare the weight loss among these groups, adjusting for the baseline values and gender. if the primary endpoint is non-normally distributed, the Kruskal-Wallis one-way analysis of variance will be used.

Secondary Endpoints: The secondary endpoints are (1) efficacy variables: nutrition assessments (FFQ, 3DFR, and satiation questionnaire), anthropometric profiles (e.g., waist and hip circumferences), laboratory and biochemical variables (e.g., insulin, lipid levels, HbAlc), body composition (e.g., body fat), liver stiffness, microbiome changes, and level of hormones; (2) safety variables: incidence, severity and duration of adverse events, vital signs, concomitant medications and physical examination results.

Secondary Statistical. Analyses: All analyses will be performed using SAS software (SAS Institute Inc., Cary, NC). First, linear mixed-effects regression model (or GEE model) will be constructed to examine the study effect on the weight loss over time among the two diet plans, referred to as Model 1. Covariates include gender, diet group (high-protein and standard), time that the measurement is taken, and an interaction between diet plan and time. Models also include the subject-level random effects to account for the correlation between repeated measures at baseline and subsequent follow-up visits occurring at 2 week to 1 month intervals. Next, mixed-effects regression models will be used to evaluate the differences in hormonal patterns among the three diet plans for various hormones of interest (e.g., CCK, GLP-1, etc). Since the hormonal level over time is expected to be nonlinear, the covariates in the model include diet group (normal vs. high-protein), time (in days), and smooth-spline function of time by group interaction. Next, we will also construct linear mixed-effects regression models to examine the study effect on the continuous efficacy variables and safety variables (e.g., vital signs) over time among the three diet plans using PROC MIXED. The continuous efficacy variables include nutritional assessments (FFQ, 3DFR, and Satiety), selected laboratory and biochemical variables (e.g., HbAlc, insulin), body fat, etc. Models include the same fixed effects in Model 1, and the subject-level random effects, which account for the correlation between repeated measures at baseline and pre-defined time points for different variables (e.g.,

baseline and at weeks 2, 4, 6, 8, 12 and 16 for the nutritional assessments). Lastly, generalized linear mixed-effect regressions will be used to model *any* dichotomous and categorical (e.g., severity of adverse events) secondary variables, respectively, in the PROC GLIMMIX or GENMOD. All tests will be two-sided, and significance level is $p < 0.05$.

***Expected Results/Pitfalls/Significance:*** We do not anticipate problems in conducting the proposed human experimental studies. Our preliminary data indicate that an isocaloric high protein diet is effective at achieving a reduction in BMI in obese subjects. It is therefore likely that the results will parallel those of the preliminary studies due to the long-term study design and larger study population. It is expected that there is inhibition of the release of ghrelin, which will also be reflected through satiety questionnaires which will be performed during the study.

## B. Aim 2. Characterize the changes in the fecal microbiome induced by a high protein diet.

*Rationale and hypothesis:* Based on our preclinical rodent model data, we hypothesize that a high protein diet will shift the composition of the microbiome of obese subjects, including increased *Akkermansia*, and that this will result in an altered metagenome (abundances of bacterial genes).

*Study design:* This is a randomized study using the same subjects described in Aim 1. Nutritional assessment, physical examination, bioelectrical impedance, laboratories will be performed at baseline, and weeks 2, 4, 6, 8, 12 and 16. Stool samples will be collected on day 3, 7, 14, 17, 21, 28, 42, 56, 84 and 112.

*Study timeline:* Data collection will be completed at week 16. The stool samples collected during the study period will be used for 16S rRNA sequencing, shotgun metagenomics, and data analysis.

*Fecal sampling:* Subjects will be provided with kits for home sampling. The subjects will urinate first then defecate into the Fisherbrand Commode Specimen Collection System. Subjects will use a spoon to transfer freshly defecated feces to a Para-Pak stool collection cup prefilled with 95% ethanol to fix the samples, allowing storage at room temperature for up to 2 weeks. The remaining feces will be frozen. This involves removing the cup from the toilet hat, tightly closing the lid, and transferring the cup to a Ziploc bag for storage in the subject's freezer. Frozen samples will delivered to the VA clinic during scheduled study visits using a Styrofoam box and frozen packs. Subjects who forget to bring samples will be provided with prepaid envelopes to mail their samples to the coordinator. Samples will be stored at -80°C. Ethanol-fixed and fresh frozen samples give comparable metagenomics results to samples stored in preservatives such as RNALater while minimizing subjects' risk of exposure to toxic chemicals [70 33].

### 2.1 Kinetic analysis of shifts in microbial composition in obese subjects (BMI 27-40) on a high protein diet compared to an isocaloric normal protein diet.

*Experimental protocol:* DNA will be extracted from fecal samples collected in ethanol using the MoBio Powersoil kit. Sequencing of the 254 base pair V4 region of 16S ribosomal DNA will be performed as we previously described using the HiSeq 2500 to a depth of >200,000 reads per sample [71 34]. Microbial diversity will be compared between the two dietary groups at each time point using the Chao1 index, Shannon index, and phylogenetic diversity. Significance will be determined using a two-sided t-test. Microbial composition will be compared across all samples using weighted UniFrac (a phylogenetic distance metric) and principal coordinates analysis. Significance of differences in composition will be determined using Adonis.

Microbial abundance will be compared between baseline data for the two groups to confirm that randomization was effective in abolishing a pre-existing microbial difference between the

two groups. The data for all time points will then be fitted to a multivariate model under a negative binomial distribution using DESeq2 with diet group, time point, interaction between diet and time point, baseline BMI, age, sex, and smoking status as covariates. This model will be compared to a reduced model that does not include the diet:time point interaction [70]. A likelihood ratio test will be used to identify microbes with differential abundance by diet at time points after baseline. Estimates of significance will be adjusted for multiple hypothesis testing [71]. OTUs that are differentially abundant on a HPD will be grouped by hierarchical clustering, which we anticipate will highlight distinct kinetic patterns of microbial change.

*Expected results/potential pitfalls:* We do not anticipate problems with the collection or transportation of fecal samples to our clinical research unit. We predict that *Akkermansia* will significantly increase on a HPD as was seen in our rodent studies. We further predict that most HPD-responsive microbes will have altered abundance within the first 3 to 7 days as has been observed in other dietary studies [72,73]. We estimate a 20% dropout rate based on our high protein meal replacement study, leaving approximately 80 subjects per group. This compares favorably to published studies reporting dietary effects on the microbiome using sample sizes of 10-15 [72,73,74]. We anticipate that a HPD will have a similar magnitude of effect as these previously studied diets and hence we expect to have sufficient numbers to detect compositional differences.

### 2.2 Identification of metagenes affected by a high protein diet.

*Experimental protocol:* We will assess the functional consequences of altered microbial composition by sequencing the genes carried by intestinal microbes. Shotgun sequencing of DNA extracted from fecal samples will be performed using the HiSeq 3000 with a target depth of 10 GB data per sample, comparable to the 11.7 GB depth used by the Human Microbiome Project for stool samples [75]. Due to the costs of sequencing at this depth, we will only sequence baseline samples and samples from day 112.

Shotgun reads will be inputted into HUMAnN2 for annotation of gene families and identification of the microbial source of these genes [76]. Individual genes will be aggregated into functional pathways using the MetaCyc database [77]. Gene and pathway abundance for the HPD and NPD groups at baseline will be compared to confirm that randomization prevented skewing of the functional profiles of the two groups. Pathway abundance will be fitted to a multivariate model using DESeq2 with diet group/time point, baseline BMI, age, sex, and smoking status as covariates to identify functional pathways impacted by a high protein diet after adjusting for covariates. Genes belonging to HPD-associated pathways, stratified by the genus of their microbial source, will then be individually evaluated using DESeq2. This approach will reduce the number of statistical tests which might be overwhelming given the greater than 5 million genes within the intestinal metagenome [75]. HPD-associated genes will be visualized in pathway maps to highlight the specific metabolic shifts in the microbiome at the DNA level that occur on a HPD.

*Expected results/potential pitfalls:* We predict that a HPD will reduce the abundance of genes in phosphotransferase pathways involved in bacterial carbohydrate metabolism, which are elevated in obese humans [24]. Our estimated final sample size of 80 subjects per group would be much larger than existing metagenomics studies of obese patients which have used 6-7 subjects per group at lower sequence depths (3 GB per sample in the most recent study) [24,26]. We anticipate that the magnitude of effect of a high protein diet will be less than that in those comparisons (obese vs. lean, gastric surgery vs. non-surgically managed) but that the 10 fold larger sample size will provide sufficient power to detect metagenomic shifts.

### C. Aim 3. Identify fecal microbial predictors of response to a high protein diet.

*Rationale and hypothesis:* The second aim of this proposal focuses on the relationship of the microbiome to clinical endpoints including weight loss, body fat loss, lipids, and

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hyperglycemia. We hypothesize that subjects will vary in the responsiveness of their microbiota to a HPD based on baseline or post-dietary intervention levels of microbes such as *Akkermansia muciniphila* that respond to the HPD. The impact of these microbial changes on diet-induced weight gain will be assessed in humanized gnotobiotic mice.

*Study design:* This is a randomized study using the same subjects described in Aim 1. Nutritional assessment, physical examination, bioelectrical impedance, laboratories will be performed at baseline, and weeks 2, 4, 6, 8, 12 and 16. Stool samples will be collected on day 3, 7, 14, 17, 21, 28, 42, 56, 84 and 112.

*Study timeline:* Data collection will be completed at week 16. The stool samples collected during the study period will be used for 16S rRNA sequencing, shotgun metagenomics, and data analysis.

*Bioinformatics protocol:* Patient characteristics (physical measurements, lean body mass, laboratories, demographic traits) at baseline will be compared using a 2-sided t-test (for numerical variables) or chi-square test (for categorical variables) to evaluate the randomization. 3DFR and FFQ performed at each timepoint will be used to assess the predicted protein content of the patient's diet compared to baseline to evaluate the success of the dietary intervention. Clinical response endpoints significantly associated with HPD in Aim 1 will then be correlated with the abundance of microbes found to be enriched or depleted on a HPD in Aim 2.1. These endpoints will be fitted to multivariate models to adjust for baseline clinical covariates including BMI, age, sex, and smoking status. Spearman correlations will then be calculated for all combinations of residuals for the clinical endpoints and for microbial abundance (using DESeq2 fitted multivariate models from Aim 2.1 to adjust for covariates). Separate correlation analyses will be performed at baseline and each of the time points during treatment.

Microbes that are independently associated with clinical response to a HPD will then be used to create random forests classifiers to predict response. Subjects in the HPD group will be identified as responders if they had a decrease in any of the clinical endpoints that exceeded the predictions of the multivariate model for the NPD group. The sensitivity and specificity of the classifiers will be estimated using 10-fold cross-validation. Classifiers constructed from baseline microbial abundance and microbial data at each time point will be compared to determine the earliest time point when accurate predictions of response are possible.

The above analytic pipeline will then be applied to the shotgun metagenomics data obtained at baseline and day 112. Gene families and/or metabolic pathways that shift in response to a HPD may be more consistent across diverse populations than abundance of specific microbes such as *Akkermansia* which could vary greatly from person to person [78]. The performance of classifiers constructed from metagenes will be compared to those built from microbial abundance to identify the approach that is most predictive for response.

*Expected results/potential pitfalls:* We predict that a subset of the HPD-associated microbes and metagenes will be associated with response in the HPD. We also expect that baseline and/or post-dietary intervention abundances of microbial features can be used to generate accurate random forests predictors for clinical response. We used similar bioinformatics approaches to identify the inverse correlation of *Akkermansia muciniphila* with body fat in our rodent model and to predict IBD status in families with pediatric inflammatory bowel disease. Accordingly, we do not anticipate problems performing these analyses.


## Human Subjects
### 1. Risk to Subjects
### (a)        *Human Subjects Involvement and Characteristics:*
Approximately 198 healthy volunteers age 30 years and older between BMI of 27 to 40 kg/m$^2$ will be able to take part in this study. Pregnant women or women likely to become pregnant during the course of the study may not participate in this study. Female subjects must not be

able to conceive by reason of surgery, radiation, 1 year past the onset of menopause, or an approved method of contraception. No vulnerable subjects will be included in the study. (See the detail it the Research Plan, Proposed Work, Aim 1, Sections of Recruitment, Procedures, and Statistical Analysis)

*(b)* ***Sources of Materials:*** See the detail in the Research Plan, Proposed Work, Aim 1, Section of Procedures.

*(c)* ***Potential Risks:***

Blood Draw: There are other possible risks or discomforts that may be experienced during this study. Subjects may experience pain, bruising, and/or burning at the site where blood is taken. Blood clot formation, infections, or bleeding may also occur, but these are rare. Subjects may also experience fainting during or shortly after having blood drawn. If subject experiences fainting symptoms, such as dizziness or light-headedness, he/she should lie down immediately to avoid possible injury caused by falling and should notify study personnel immediately.

Women of Child-Bearing Potential: There might be unknown risks to the unborn child if the subject is or if becomes pregnant during the study. Due to these risks, subjects must not participate in this study if they are pregnant, or plan to become pregnant during the research study period, or are breast-feeding a child. If the subject is a woman of child-bearing potential: by signing the consent form, they confirm to the best of their knowledge that she is not pregnant now and does not intend to become pregnant during this study. A pregnancy test will be done to confirm that she is not pregnant before she takes part in this study and she must avoid becoming pregnant during the study by using an acceptable form of birth control as described below. If at any time during this study the subject thinks she might be pregnant, or later learn that she was pregnant during the study, she must contact the study doctor immediately for further instructions about her participation in this study and fallow-up. The following forms of birth control are considered acceptable: intrauterine device, implantable progesterone device, progesterone intramuscular injection, oral, transdermal, or vaginal contraceptive (started at least 1 month before Visit 1 and continuing for entire study) condoms with spermicide, Single-barrier methods alone (such as condoms without the use of spermicide) and rhythm methods are not acceptable methods of birth control. Since subjects will not be receiving the study drug for the treatment of a health problem, alternative treatment does not take part in this research study.

Bioelectrical Impedance Analysis (BIA): This noninvasive tool is a commonly used to measure body fat. The impedance measurement is considered safe because of several factors. One factor is that currents at a low frequency are reported to be unlikely to stimulate nerves or cardiac muscle. There have been no reports of adverse events caused by BIA, even in the course of thousands of individuals undergoing measurement. Relatively small current magnitudes are involved, which are less than the threshold of perception. Furthermore, the use of batteries or low-voltage power sources greatly diminishes risks from a shock. Patients with implanted cardiac defibrillator will be excluded from the study to prevent potentially incorrect defibrillator responses.

Hepatic Transient Elastography: Sound waves are used to determine the density of tissues based on the amount of water content in the body. Transient Elastography is thus noninvasive and does not use any radiation. Patients may experience slight discomfort related to positioning the ultrasound probe along the abdomen. They will be advised to minimize discomfort through positioning themselves and visiting the exam room in advance or using relaxation techniques.

Fecal collection: Collection involves passing a bowel movement into the provided toilet hat and using a plastic spoon to store a sample in an insulated container. Other than discomfort in collecting stool samples, there is minimal risk to potential subjects.

## 2. Adequacy of Protection from Risks

*(a) Recruitment and Informed Consent:* All subjects who are considered for this protocol will be evaluated by the PI or co-PI. Following the explanation of the protocol and the consent form, the subjects will be asked about their level of comprehension of the study. Subjects may be asked to restate study purpose and their role in participation to verify understanding. Only when the study doctor is assured that the prospective subject understands all aspects of the study and is consenting to participate freely and without coercion, will the study doctor have the prospective subject sign the informed consent form. Potential subjects that are not able to understand the protocol will not be considered to be eligible for entry into the clinical study.

*(b) Protection against Risk:* All procedures will be fully explained to the subjects prior to the start of the screening. All questions will be answered as needed for the subject reassurance. All procedures will be conducted by trained and qualified medical personnel. Subjects will be asked to report all side effects and adverse experiences. Blood drawing supplies are sterile and single use only. The staff is trained and certified in Phlebotomy (blood withdrawal). If there is any evidence of an allergic reaction or adverse effect from the protein meal, the subject will be monitored, and all reactions will be followed up with the PI until resolution. The subject may be discontinued from the study if deemed necessary by the study doctor. Subjects will be provided with a telephone number to contact the study staff in the event of emergency or adverse events.

## 3. Potential Benefit of the Proposed Research to the Subject and Others.

Subjects will not experience any direct health benefits during or after completing this study. A potential medical benefit subjects may derive from participating in this study is the detection of an unsuspected medical condition from tests performed (for example, physical exam and blood tests). In addition, the information about the study may benefit others in the future. The information learned from this study will enable doctors and scientists to understand the regulation of GI hormones in obesity.

## 4. Importance of the Knowledge to be Gained.

Metabolic syndrome is a common problem in the VA and, thus, the overall direction of this project is well suited for the VA. Strategies to help veteran patients lose weight would definitely decrease morbidity and mortality and would be cost saving for chronic care of complications from obesity.


## ADVERSE EXPERIENCES

Patients will be monitored and questioned regarding the occurrence and nature of any adverse experiences. An adverse event is any change in the physiological or psychological state, other than the primary condition, that qualifies the patient for this study. Adverse events are first graded according to seriousness and then severity. Additionally, the Investigator must decide if the occurrence of the adverse event is related to the study product. A description of the event and its relationship to study product must be reported on the adverse events case report form for each adverse event recorded in the patient's chart.


## Definition of serious adverse event

A serious adverse event, as defined in Title 21 of the code of federal regulations, Part 312, subpart ID, Section 312.32, means any adverse experience occurring at any dose that results in any of the following outcomes: death, a life-threatening adverse drug experience, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require in-patient hospitalization may be considered a serious adverse experience when, based on appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. An adverse event is usually considered by the Sponsor to be

serious if severity for the event is graded as 3 (severe) or 4 (immediately life-threatening or fatal).

### Data Safety Monitoring Procedure

Any adverse event (AE) will be recorded as described below. All AEs will be reviewed by the investigator and co-PI for the proposal. if it is deemed to be a serious AE these will be recorded and submitted to the IRB for review.

#### Severity

For evaluation and reporting purposes, the clinical severity of the event is stratified into four classes. The four classes of Severity are 1-Mild, 2-Moderate, 3-Severe, or 4-Immediately Life-Threatening/Fatal.

#### Life-Threatening Adverse Experience

A life-threatening adverse experience is defined as any adverse experience that places the subject, in the view of the investigator, at immediate risk of death from, the reaction as it occurred, i.e., it does not include a reaction that, had it occurred in a more severe form, might have caused death.

#### Association with the use of Study Product

Association with the use of the study product means that there is a reasonable possibility that the adverse event may have been caused by the product under investigation. All adverse events are graded with regard to their association with the use of the study product. The classifications used include Not Related, Unlikely Related, Probably Related and Definitely Related.

#### Unexpected Adverse Event

An unexpected adverse event is any adverse product experience that is not consistent with the current nutritional product insert in specificity or severity.

#### Adverse Event Reporting Procedure

The Investigator must report to the occurrence of any *serious*. adverse events, regardless of relationship to study product, or the first occurrence of any unexpected or previously unknown clinical event (regardless of Grade). A written report (Report for Serious/Life Threatening Events) of the event must be faxed within 5 working clays to The VA GLAHS Institutional Review Board (IRB).

## PREMATURE WITHDRAWAL OF PATIENTS

Whenever possible, patients will be studied for a minimum of 2 weeks. The patient's participation in the protocol will terminate under any of the following circumstances:
- At any time for any reason.
- Physician determination that patient's further participation in the protocol is not in the patient's best interest.
- At the determination of the sponsor.
- For any discontinuation, the investigator will obtain all the required details and document the date of and the reason for the discontinuation. If the reason for removing a patient is an AE, the specific event or the main laboratory abnormality will be recorded in the source.

*THE INVESTIGATOR WILL MAKE THOROUGH EFFORTS TO DOCUMENT THE OUTCOME*
**GOOD CLINICAL PRACTICES/ETHICAL CONSIDERATIONS**

This study will be conducted in accordance with Good Clinical Practices, Title 21 of the Code of Federal Regulations, Part 50, Subparts A and B; Part 56; and Part 312, Subpart D, and the 1996 International Conference of Harmonization (ICH) *Guideline on Good Clinical Practices.*

Data Collection
Source documents will be utilized for each patient entered into the study. Study participants will NOT be identified by name on any study documents. Patients will be identified by a patient identification number (PIN). investigators will keep a patient code list accessible. All data collected will be stored in a secure locked file cabinet in accordance with the VA's Data Safety policies.

## *ETHICAL CONSIDERATIONS*
*The investigator will ensure that the study is conducted in full conformance with the FDA standards for human research as specified in 21 CFR 312.*
Institutional Review Board (IRB1
The VA GLANS operates a Department of Health and Human Services-approved Institutional Review Board with a Federal Wide Assurance (FWA) agreement in place since the 2003 (FWAA00004642).

### *Institutional Review Board Approval*
An Institutional Review Board (IRB), which is duly registered with the FDA and operating in accordance with Title 21 of the Code of Federal Regulations Part 56, must approve the Clinical Protocol and corresponding Informed Consent Document and agree to monitor the conduct of the study and periodically review its progress at regular intervals.

### *Informed Consent*
All study participants must sign an informed consent. The investigators will inform all subjects as to the nature, aims, duration, potential hazards, and procedures to be performed during the study and that his or her medical records, may be reviewed by the FDA. This protocol must receive approval by the Institutional Review Board prior to implementation. The investigator must also explain that the patients are completely free to refuse to enter the study or to withdraw from it at any time. The protocol will be discussed in detail with all potentially eligible patients. All revisions of the protocol must be reflected in the consent form and reviewed by the IRB.

### *Patient Confidentiality*
All reports and patient samples will be identified only by a coded number to maintain patient confidentiality. All records will be kept confidential to the extent permitted by law. The investigator should keep a separate log of patients, codes, names, and addresses. Documents which identify the patient by name (informed consent) should be kept in strict confidence.

### *Patient's Financial Responsibilities during the Study*
Patients will not be financially responsible for any procedures or tests of any investigational nature.

## GENDER AND MINORITY INCLUSION
In accordance with Part VIII of the Federal Register, NIH Guidelines on the Inclusion of Women and Minorities as Subjects in Clinical Research (NIH, March 1994), women and members of minority groups and their subpopulations will be included in this research.

## Multiple PD/PI Leadership Plan

Joseph R. Pisegna, MD, and Jonathan Jacobs, MD, PhD, are co-PIs for the proposed project. Dr. Pisegna has expertise in molecular/cellular biology studies and human clinical studies and thus will have the principal responsibility for directing the human clinical studies and molecular biological studies. He will oversee the experimental design assessment of methodological approaches, review the data, plan new projects, follow the advancement of the field in the areas of the project and be responsible for publications of the results derived from the work. Dr. Jacobs has had extensive experience planning and implementing microbiome studies in patient cohorts and in animal models, including a rat model of Western diet-induced obesity that responds to a high protein diet. He will oversee the processing and sequencing of stool samples and the bioinformatics analysis of the microbiome sequence data. The co-PIs will have monthly laboratory meetings to review and interpret data from the proposed studies with a focus on translating the results obtained from basic science projects to the design of human trials as well as to translate data obtained from the proposed human clinical data to confirmatory testing in the animal models core. The co-PIs have worked together on projects for several years and thus have developed collaborative projects previously. Any budgetary issues will be resolved by the ACOS Research and Development, Dr. Dean Yamaguchi. It is unlikely that there will be differences in how the budget will be executed since the co-PIs have had a close working relationship and given that the purpose of this proposal is to develop new and innovative research directions focused ultimately on improving the healthcare of veterans with obesity disorders.