FETUIN-A: A NOVEL MARKER FOR OBESITY AND ASSOCIATED COMORBIDITIES

BY

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DISSERTATION

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ABSTRACT

Background: Obesity is a disease characterized by excess adiposity which complicates metabolism, mobility, and multiple systems in the body. Over a third of Americans have a body mass index (BMI) greater than 30 kg/m^2 and therefore, are considered obese. Excess adiposity may be prevented or reduced through behavioral, pharmacological and surgical methods. Bariatric surgery results in significant and sustained loss of body weight and body fat. Many bariatric surgeries have also been termed metabolic surgeries as they result in significant improvements of metabolic abnormalities associated with obesity including dyslipidemia and insulin resistance. It is estimated that 80% of individuals with Type 2 Diabetes Mellitus (T2DM) will experience resolution after bariatric surgeries such as Roux-en-Y gastric bypass and sleeve gastrectomy (SG).

Although individuals with morbid obesity have a greater risk of T2DM, 25% remain insulin-sensitive. To date, it is unclear why some develop insulin-sensitive obesity (Ob_{sen}) and others insulin-resistant obesity (Ob_{res}). A validated biomarker to distinguish these groups may aid in more targeted interventions and monitoring of at-risk populations. Fetuin-A (FetA) has shown promise as a potential marker of insulin resistance yet much about this hepatokine remains unknown.

The overarching goal of this research is to understand whether FetA may be a novel marker of obesity and obesity-associated insulin resistance. Therefore, the objectives of this research were to 1) determine the role genetics play in circulating FetA and metabolic health, 2) investigate how circulating FetA responds to SG, a bariatric procedure known to markedly improve insulin sensitivity, and 3) compare FetA, adipocyte hypertrophy and weight loss trajectories between SG patients with Ob_{sen} and Ob_{res}.

Methods: To determine the genetic influence of FetA, 717 college applicants to the Autonomous University of San Luis Potosi, Mexico (18-25 years old) were genotyped for single nucleotide polymorphisms (rs4917 and rs2518136) in the gene that codes for FetA, alpha2-Heremans-Schmid Glycoprotein (*AHSG*). Circulating lipids, glucose, insulin and FetA were measured in plasma. To investigate the role of FetA in obesity and interventions aiming to improve insulin sensitivity, forty SG patients were recruited and evaluated longitudinally. Participants met with research staff at baseline (T0) (prior to hypocaloric, low-fat preoperative diet), on the morning of surgery (T1) and six weeks following surgery (T2). At each visit, fasting blood and three-day food logs were collected. Body composition was measured via direct segmental multi-frequency bioelectrical impedance analysis. Circulating FetA, insulin, blood lipids and glucose were measured at each visit. Omental adipose tissue was collected at the time of surgery. Formalin-fixed and paraffin-embedded tissues were stained with hematoxylin and eosin, and adipocyte diameter was calculated. To compare individuals with Ob_{res} and Ob_{sen}, the cohort was divided into age- and BMI-matched groups based on homeostatic model assessment for insulin resistance (HOMA-IR) with consideration for diabetes diagnosis and use of anti-diabetic medications.

Results: Individual genetic variation in *AHSG* SNPs rs4917 and rs2518136 explained 8% and 10% of the variability in FetA, respectively. Lower triglyceride values were observed in CC-rs4917 homozygotes than in T-allele carriers ($p<0.05$). In the SG cohort, FetA decreased 12% during the preoperative diet ($p=0.007$) and an additional 26% during the postoperative diet ($p<0.0001$). FetA was negatively associated with age ($r= -0.52$, $p=0.004$). Higher HOMA-IR at all time points was associated with lower FetA at T3 (HOMA-IR T0: $p=0.002$, T1: 0.003 and T2: 0.001). Ob_{sen} and Ob_{res} groups did not differ in baseline BMI or percent body fat. Age-adjusted FetA tended to be higher in Ob_{sen} at T0 and T1 ($p=0.09$ and $p=0.10$, respectively). At T2, age-

adjusted FetA was higher in the Ob_{sen} group than the Ob_{res} group (p=0.004). The Ob_{res} group had significantly more excess body weight loss (adjusted for baseline BMI and time) (p=0.012) and larger adipocytes than the Ob_{sen} group (p=0.0099). Adipocyte size was positively correlated with preoperative change in FetA (r=0.64, p=0.007) but not with reported changes in caloric intake, macronutrient intake, PBF loss or BMI loss.

Conclusions: Variations in the *AHSG* gene influence circulating FetA and are associated with altered triglycerides. The influence of genetic variation was exaggerated in those who were overweight or obese. Individuals with obesity have higher FetA which is significantly reduced by calorie and fat restriction as well as SG. Immediate post-bariatric improvements of FetA have been correlated with rapid improvements in insulin sensitivity. However, decreased FetA may be partially explained by preoperative calorie restriction. Greater reduction in FetA during the preoperative diet was associated with smaller adipocyte size on the day of surgery. Smaller adipocytes have been shown to be more insulin-sensitive. Accordingly, individuals with Ob_{sen} had smaller omental adipocytes than those with Ob_{res}. In contrast to previous reports, those with Ob_{sen} tended to have higher circulating FetA at all time points, lost less weight, and had no appreciable improvements in HOMA-IR. This research demonstrates the potential of FetA as a marker for weight loss and insulin sensitivity improvements following lifestyle and bariatric interventions.

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LIST OF ABBREVIATIONS

AHSG	Alpha2-Heremans-Schmid Glycoprotein
ASMBS	American Society for Bariatric and Metabolic Surgery
BF%	body fat percentile
BMI	body mass index
BW	body weight
CDC	Centers for Disease Control
DNA	deoxyribonucleic acid
EBWL	excess body weight loss
FetA	Fetuin-A
GLC	glucose
GWAS	genome-wide association study
HDL-C	high density lipoprotein
HOMA-IR	homeostatic model assessment for insulin resistance
I-OWLS	Individual Outcomes of Weight Loss Surgery Project
IRB	Institutional Review Board
kcal	kilocalorie
LAGB	laparoscopic adjustable gastric banding
LDL-C	low-density lipoprotein
MetS	Metabolic Syndrome
mRNA	messenger ribonucleic acid
Ob_{res}	insulin-resistant obesity
Ob_{sen}	insulin-sensitive obesity
RYGB	Roux-en-Y gastric bypass
SD	standard deviation
SE	standard error
SG	sleeve gastrectomy
SNP	single nucleotide polymorphism
T2DM	Type 2 Diabetes Mellitus
TC	total cholesterol
TG	triglycerides
TLR4	toll-like receptor 4
UIUC	University of Illinois at Urbana-Champaign
WC	waist circumference

CHAPTER 1. LITERATURE REVIEW[1]

1.1 Obesity

According to the most recent data from the National Health and Nutrition Examination Survey, 37.7% of American adults have obesity (1). Obesity is characterized by excess adiposity or fat accumulation in the body (2). Obesity categories are defined by the body mass index (BMI), which is calculated by dividing an individual's body weight (in kilograms) by height (in meters) squared. Adults with a BMI greater than 30 kg/m^2 are considered obese (3). This disease not only impacts adults but also one-third of children in the U.S. (4). Obesity in children is defined using the child's height and weight compared to a standard growth chart of other age-matched children. These growth charts were created by the Centers for Disease Control and Prevention (CDC) and allow children to be assigned a percentile that reflects how they compare to a reference population that is matched for sex and age. Children who rank above the 85th percentile and below the 95th percentile are considered overweight. Children who are above the 95th percentile are considered obese (5).

Obesity is a complex disease with multiple potential causes. The factors which increase the risk of obesity have been described by Harrison et al. (6). This team of transdisciplinary researchers developed the Six C's model of obesity which suggests that the causes of obesity may be organized into six different spheres including the cell, child (or individual), clan, community, country and

[1] Portions of this literature review were reprinted from Biochimie, 124, Robinson, KN. & Teran-Garcia, M., From Infancy to Aging: Biological and Behavioral Modifiers of Fetuin-A, No. 141-149, Copyright (2016), with permission from Elsevier and from Carle Selected Papers, 59/2, Robinson, KN., Rowitz, B. & Teran-Garcia, M., Weight Loss Surgery: Mechanisms of Action and the Critical Role of Nutrition, No. 15-18., Copyright (2016), with permission from Carle Foundation Hospital.

culture (6). Examples of contributing factors include inadequate physical activity, poor diet quality, insufficient sleep, high parental BMI, aggressive food marketing, and traumatic life events (7–11). Poor diet quality may include inadequate intake of fruits, vegetables and fiber and excessive intake of sugar-sweetened beverages, dietary fat, and alcohol (12). Genetic variations can also predispose some individuals to weight gain (13).

Early twin studies demonstrated a strong correlation between obesity and weight gain in monozygotic twins ($r= -0.7$, $p=0.01$) (14). Genetic factors have been estimated to explain 40-70% of the variation in weight (15,16). Genetic variation in proteins related to feeding regulation, taste preference, adipogenesis, insulin action and metabolism of lipids and energy have been investigated. The effects of these genetic variations depend on the location of the mutation and whether that mutation significantly modifies the protein structure thus leading to altered function. Although rare, it is possible for a single mutation to result in morbid obesity. So called, "monogenic" forms of obesity include mutations in leptin, leptin receptor, melanocortin receptor 4 (MC4R) and Pro-opiomelanocortin (POMC) (17,18). These profoundly impact a pathway essential for weight balance and are characterized by early onset of obesity and severe hyperphagia. In the case of leptin deficiency, treatment with exogenous leptin leads to significant weight loss (17).

While monogenic traits remain rare, polygenic obesity is thought to be more common. It is likely that obesity is promoted by the aggregate effect of multiple genes rather than a single mutation. Genome-wide association studies have identified >150 single nucleotide polymorphisms (SNPs) that are associated with BMI (19). From these findings, multiple genetic risk models have been created to predict obesity (20). Models have been constructed with a wide range of SNPs and have had conflicting results. Beyond obvious differences in the models, it is also possible that genes are

more tightly associated with weight balance over time rather than BMI at a single time point, which is currently the standard in genetic studies of obesity (18,21). Furthermore, GWAS of obesity-associated mutations often assign BMI as the key outcome variable and fail to consider other more sensitive measures of adiposity. Thus, genetics are not currently used to predict obesity, but rather have offered important insight into the mechanisms behind obesity. For example, GWAS of BMI have identified genes with functions related to the brain and hunger cues to be significantly correlated with BMI. Conversely, GWAS of waist circumference have found genes associated with adipocyte biology to be significantly associated. This suggests that total body weight may be more so a result of perturbed satiety/hunger cues and that body composition (as measured by waist circumference) is the result of adipocyte differentiation, metabolism and remodeling.

Obesity is a health concern due to its association with a higher risk for chronic disease, including type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (22). Of individuals less than 55 years old with BMI>30 kg/m^2, men have a 10-fold higher risk of developing T2DM while women had a 2.5 fold higher risk of T2DM than lean counterparts (22). T2DM is a metabolic disorder characterized by high blood glucose (hyperglycemia) and high circulating insulin (hyperinsulinemia). When insulin, a hormone that facilitates glucose uptake, is chronically high in the blood, insulin resistance may result. When cells become resistant to insulin signaling, glucose is not taken up by the cell and therefore, remains in circulation causing a condition known as hyperglycemia and eventually T2DM. Uncontrolled T2DM can result in serious medical complications such as CVD, nephropathy, neuropathy, retinopathy, blindness, amputations, vascular dementia and premature death (23).

Obese adults are more likely to experience risk factors for CVD such as hypercholesterolemia (high cholesterol), hypertriglyceridemia (high triglycerides) and hypertension (high blood

pressure) (24,25). Prevalence of coronary heart disease was two times higher in men with BMI>40 kg/m^2 and nearly three times higher in women with BMI>40 kg/m^2 (22). The association between obesity and chronic diseases was initially discovered in adult cohorts and recently, similar trends have been reported in obese children.

In regards to CVD, a study of obese children found that 70% had at least one risk factor for CVD and 39% had two or more risk factors for CVD (26). The Bogalusa Heart Study found that overweight children were 2.4 times more likely to have high levels of cholesterol, 3 times more likely to have high LDL, 3.4 times more likely to have low HDL, 7.1 times more likely to have high triglycerides and 4.5 times more likely to have high blood pressure than their normal weight peers (27). Additionally, T2DM was initially thought to be an adult onset disease, however, with the rising prevalence of childhood obesity, insulin resistance is being diagnosed earlier in life (28).

Although obesity is associated with a higher risk of comorbidities, it should be noted that not all individuals with morbid obesity develop comorbidities. A quarter of individuals with BMI>35 kg/m^2 remain insulin-sensitive (29). Why this subset remains insulin-sensitive at higher categories of obesity, remains unclear. A review on the topic found several potential differences between individuals with insulin-resistant obesity (Ob_{res}) and insulin-sensitive obesity (Ob_{sen}). In comparison, those with Ob_{sen} consume lower amounts of total fat and saturated fat (30). In addition to these behavioral factors, those with Ob_{sen} also tend to have smaller adipocytes and greater lean body mass (31). However, longitudinal studies have suggested that Ob_{sen} and metabolically healthy obesity (MHO) are transitional periods. With aging, the prevalence of MHO decreases and comorbidities impact this population at a higher rate than their normal weight peers (32). Therefore, treatment options focused on reducing weight and improving body composition, would benefit those with Ob_{res} as well as those with Ob_{sen}.

Obesity Treatment

Behavioral interventions including modifications to diet and physical activity are considered the first-line of obesity treatment. Dietary interventions for weight loss vary greatly and have evolved over the last few decades. This evolution is, in large part, driven by current research. Methods include reducing overall caloric intake, adjusting macronutrient distribution and limiting certain foods (sodas, sweets and dietary fats) (21). Data from the International Food Information Council Foundation suggest that one-quarter of Americans have made an effort to change their diet in the last year and 63% did so to lose weight (33).

Unfortunately, dietary changes result in only modest weight loss. A meta-analysis of 27 self-directed weight loss trials, revealed average weight loss of 2.7 kg at 6 months (34). One year of professional dietary counseling results in only a 1.9 kg/m^2 reduction in BMI (35). Weight loss maintenance following these interventions has also been reviewed. Two to four years after lifestyle and dietary interventions weight loss was <5 kg (36). Likewise, a meta-analysis of 29 structured weight-loss programs found an average weight loss of 3kg five years after intervention (37). Fortunately, metabolic health may be significantly improved with just 5-10% weight loss (38,39).

If behavioral modifications are unsuccessful at producing weight loss and an individual has a BMI>27 kg/m^2, they may be eligible to receive pharmacological interventions. The list of approved weight-loss drugs is ever-changing. Most drugs are aimed at decreasing intake or increasing energy expenditure. When combined with a healthy diet, these medications result in 5-10kg weight loss over a 1-2 year period (36). These medications may also help to control comorbidities. However, side effects may occur including elevated blood pressure, hypoglycemia and gastrointestinal-related adverse events (40,41). If pharmacological interventions continue to

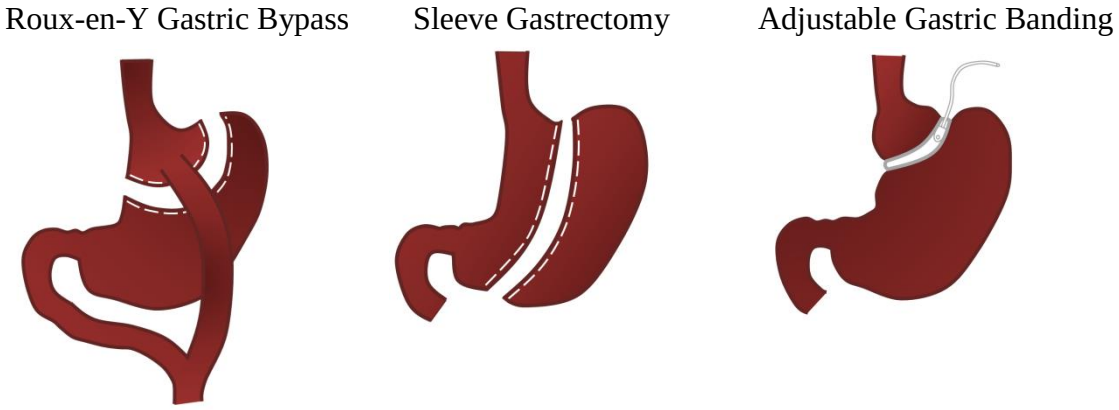
provide only minimal results and an individual has a BMI>40 kg/m^2 or >35 kg/m^2 with comorbidities, they may be eligible for the third level of therapy: weight loss surgery also known as bariatric surgery.

1.2 Weight Loss Surgery

In 2013, 468,609 bariatric surgeries were performed worldwide, which is nearly 38% more than in 2011 (42). The most popular bariatric procedures were Roux-en-Y gastric bypass (RYGB, 45%), sleeve gastrectomy (SG, 37%) and laparoscopic adjustable gastric banding (LAGB, 10%) (42). Of these operations, 154,276 were performed in the US and Canada; 35% RYGB, 43% SG and 10% LAGB. Although dietary and physical activity modifications are still commonly used methods for weight management, bariatric surgery results in greater weight loss and disease resolution especially in individuals with a BMI greater than 40 kg/m^2. Weight loss 2-4 years following surgical intervention range from 25-75 kg (36). Nearly 80% of T2DM cases are resolved following surgery (43–45). Improvements are also seen with blood pressure, sleep apnea and hypercholesterolemia although resolution rates vary based on the type of procedure (43). A recent systematic review highlighted that health-related quality of life is consistently higher in bariatric patients 5-10 years postoperatively (46). Longitudinal data also suggests that 15 years following surgery, patients have a 30% lower mortality risk than individuals with obesity who did not undergo surgery (47).

The surgery which results in the greatest weight loss and comorbidity resolution appears to be RYGB. This procedure involves creating a small gastric pouch which is anastomosed to the jejunum (distal to the ligament of Treitz). Thus following surgery, food bypasses the duodenum, a key site for the absorption of nutrients such as iron, calcium, folate and monosaccharides including

Figure 1.1 Common Bariatric Procedures Worldwide.



glucose (Fig. 1.1). RYGB patients lose an average of 61.6% of excess body weight at two-year follow-up (43). Outcomes are only slightly less effective in SG patients. During SG operations, the stomach is segmented vertically and nearly 80% of the stomach is removed. Therefore, SG patients retain their small intestine but no longer have the majority of the gastric fundus and body. Average excess body weight loss is 55.4% for SG patients (48). Gastric banding procedures are the least effective of these surgeries, however, offer the advantage of reversibility and fewer nutritional deficiencies (49). Gastric bands are FDA-approved, adjustable, tube-shaped devices that are placed around the proximal stomach which restricts the stomach size without altering the anatomy of the gastrointestinal (GI) tract. On average, band patients lose 47.5% of excess body weight (43). It is commonly assumed that weight loss and improvements in comorbidities are the result of dietary restriction; however, longitudinal studies have shown that RYGB and SG patients display unique metabolic changes that significantly differ from matched individuals undergoing a solely dietary intervention. Conversely, LAGB, a restrictive procedure, does not have metabolic effects which consistently differ from traditional weight loss interventions (50).

Mechanisms for Weight Loss and Disease Resolution following Weight Loss Surgery

Traditionally, bariatric surgeries are categorized into three types based on the mechanism of action: 1) malabsorptive; those resulting in alterations of the way the GI tract digests and absorbs food, 2) restrictive; those that reduce the volume of the stomach thus restricting the amount of food that can be consumed, or 3) a combination of both malabsorptive and restrictive. While this categorization may be helpful for teaching purposes, it grossly underappreciates the systemic changes that result following these surgeries. A description of the mechanisms of bariatric surgery (the BRAVE effects) has been published by Ashrafian et al. (51). The BRAVE effects include bile flow manipulation, reduced gastric size, anatomical changes to the GI tract resulting in altered nutrient flow and absorption, vagal signaling modification and enteric hormone alterations (51). While the BRAVE effects refer mainly to RYGB, this model also includes mechanisms of SG.

Both RYGB and SG result in gastric restriction. Reduced gastric size may lead to weight loss by decreasing the size of meals. In response to having a smaller stomach pouch, patients may also alter the macronutrient composition of meals as some nutrients result in a greater perception of fullness. The duodenal bypass in RYGB has been considered a likely cause of diabetes resolution, however, does not explain the resolution rates observed in SG patients. Although SG does not result in anatomical changes to the small intestine, it may alter the speed of transport through the GI tract resulting in altered absorption. An additional consideration following these surgeries is the modification of the vagus nerve. The gastric branches of the vagus nerve, run along the lesser curvature of the stomach and are essential for cross-talk between the GI tract and the brain. It is more likely that the vagus nerve is significantly altered during RYGB rather than SG. This vagal nerve alteration may lead to changes in satiety signaling, GI motility, nutrient sensing and other signals in the gut-brain axis (52,53).

Enteric hormone changes may also explain the metabolic effects of bariatric surgery. One important hormone that is altered in response to bariatric surgery is ghrelin. Ghrelin promotes feelings of hunger and increases during calorie restriction, yet it decreases after bariatric procedures (54–56). Ghrelin appears to decrease more following SG than RYGB (57). One explanation for this phenomenon in SG is that ghrelin-producing cells are found in the portion of the stomach excised during surgery. Partial or complete gastrectomy dramatically reduces the stomach's ability to produce ghrelin and may explain why some patients report no longer feeling hunger following surgery.

Beyond the BRAVE effects, scientists are discovering that changes in gut microbiota may also be an important mechanism in bariatric surgery outcomes. A recent paper suggested that weight loss could be produced simply by transplanting the gut microbiota from rats that received RYGB to obese rats who had not received RYGB (58). Humans with obesity have a higher ratio of firmicutes to bacteroidetes than normal weight peers. Following surgery, the human gut microbiota shifts to reflect a unique profile, unlike individuals in either the obese or healthy BMI range (58,59). The reasons for these shifts and their influence on weight loss rate, weight loss maintenance and comorbidity resolution remain unclear. Because bariatric procedures offer unique benefits beyond weight loss, they may serve as an important model of study for the treatment of long-term obesity and obesity-associated diseases.

Long-Term Efficacy of Bariatric Surgery

According to the Swedish Obesity Study, the largest investigation of long-term bariatric surgery outcomes, maximum weight loss occurs 1-2 years after surgery (60). At this point, RYGB patients lose an average of 32% body weight and variable weight regain follows. On average, weight loss

at ten years post-RYGB decreases to 25%, and at 15 years, patients have lost 20 to 35% (average 27%) of their baseline weight. From these data, it does not appear that weight regain surpasses baseline weight within the 15 years following surgery. Weight loss for LAGB patients reaches a maximum of 20% at 1-2 year follow-up but rises to 14% and 13% weight loss at 10- and 15-year follow-up, respectively (60). Still, at 15 years post-LAGB, weight regain does not exceed baseline. Long-term data for SG procedures is sparse. However, weight loss trends are similar to RYGB. Weight regain of 10 kg above weight loss nadir was seen in 19.2% of SG patients, however, these patients had still lost 20% of their excess body weight at five-year follow-up (61). Two systematic reviews have highlighted that the definition of weight recidivism is not yet standardized and that associated risk factors are still under investigation (62,63).

Maximum weight loss has been positively associated with mandatory preoperative weight loss of 5-10% (64). Preoperative hypocaloric diets are often prescribed in the two weeks before surgery. During the preoperative diet, the liver shrinks 14%, thus optimizing access to the gastroesophageal junction and reducing the risk of anastomotic leakage, abscesses and minor wound complications (65,66). Additionally, postoperative weight loss is negatively associated with preoperative BMI. Weight regain has been linked to preoperative binge eating disorder diagnosis and grazing although replication is needed (67). It remains unclear what factors influence the variability in postoperative weight loss and weight regain. However, a clear understanding is needed to improve patient care and procedure selection. As technology in the field of bariatric and metabolic surgery evolves, it is increasingly important that the nutritional status of these individuals is monitored and maintained. Moreover, early identification of patients at highest risk for postoperative nutritional and medical complications is critical.

Nutrient Deficiencies and Complications of Weight Loss Surgery

Postoperative bariatric patients struggle to achieve adequate micronutrient intake because they consume a decreased amount of food and have GI alterations that modify the digestion and absorption of micronutrients. Extensive reviews have been published; however, common deficiencies include iron, vitamin B_{12}, vitamin D and calcium (68). Following RYGB and SG, iron and B_{12} deficiencies occur in 32% and 11% of patients, respectively (69,70). Iron and B_{12} status is compromised due to inadequate dietary intake, malabsorption, and decreased acidity in the remaining stomach pouch (71). The stomach also contains parietal cells that release intrinsic factor, a necessary cofactor for vitamin B_{12} absorption. When the stomach is partially removed during bariatric procedures, secreted intrinsic factor is either significantly decreased or completely absent 22 months following surgery (72). Thus, post-bariatric patients should aim to consume 150-200 mg of elemental iron each day and B12 should be supplemented either orally (1000 µg per day) or intramuscularly (1000-3000 µg) every 6-12 months (73).

Insufficient circulating vitamin D occurs in 23% of patients before surgery, 32% of patients following SG and 52% of patients following RYGB (74). Postoperative vitamin D deficiency may result if patients have inadequate intake, fat malabsorption or altered bile acid secretion (75). Because vitamin D is necessary to maintain blood calcium concentration, it has been suggested as a potential contributor to postoperative declines in bone mineral density and bone strength. However, rapid weight loss, in general, may also explain decreased bone health (76–78). For these reasons, bariatric specific guidelines suggest vitamin D and calcium citrate supplementation following surgery (73). Long-term monitoring is recommended to optimize patient outcomes and to ensure compliance with nutritional recommendations. However, at one year post-surgery, routine supplement use is only practiced by 26% of RYGB patients (79). Without supplementation

and monitoring, nutrient deficiencies persist and increase the likelihood of long-term complications such as decreased bone health, cognitive deficits and weight regain.

This review of bariatric outcomes and complications highlights the variability inherent to weight loss surgery. Weight loss variability is not unique to bariatric surgery but occurs with all obesity treatments. The causes of variable weight loss are poorly understood making it difficult to predict which patients will experience the best outcomes. Multiple methods have been employed to predict outcomes including the collection of preoperative behavioral data and baseline genotyping. However, metabolomic studies may unveil novel biomarkers and provide a more complete picture of the biological changes underlying bariatric surgery outcomes.

1.3 Fetuin-A: Structure and Function

Fetuin-A, also known as alpha2-Heremans Schmid glycoprotein (*AHSG*), was first recognized because of its high concentration in fetal serum (80). Since being discovered, this hepatokine has been assigned multiple functions and circulating levels of Fetuin-A have been associated with numerous chronic diseases; most notably, CVD and T2DM (81–86)

Fetuin-A (FetA) is secreted from the liver as a single chain precursor. The precursor is cleaved during proteolytic processing in the Golgi apparatus to form a heavy and a light chain consisting of 321 and 27 amino acids, respectively. A connecting peptide, which is 40 amino acids in length, is cleaved from the C-terminus of the heavy chain (87,88). The remaining chains are joined by six disulfide bonds. The final (mature) form of FetA has three domains. These domains contain important functional and structural features. The first domain contains a calcium-binding site. Domain 2 has three N-linked glycosylation sites and a phosphorylation site at Ser120. The final domain (Domain 3) contains two O-linked glycosylation sites as well as the second

phosphorylation site at Ser312. It is estimated that 20% of FetA is phosphorylated in at least one site (88). Phosphorylation appears to be essential for activation of FetA (89).

Due to the calcium-binding site in Domain 1, FetA appears to have an important role in mineral transport and utilization. FetA can form complexes with calcium in circulation and, therefore, prevent soft tissue calcification (90). This function of FetA has been widely studied in dialysis patients, where low FetA levels in circulation have been linked to greater risk of cardiovascular events (91,92). At high levels, FetA reduces systemic calcification that contributes to the development of CVD (90,92,93). Alternatively, reduced FetA may also be a sign of inflammation. FetA has been identified as a negative acute phase protein because it is reduced in inflammatory conditions (94). FetA administration following LPS-infection is associated with greater survival risk (95). This is perhaps because FetA competes for binding at toll-like receptor 4 (TLR4) or dose-dependently increases phagocytosis (96). In an environment of chronic inflammation, FetA plays an important role in managing dying cells during apoptosis by enhancing phagocytosis (96). Although these findings support that low FetA is unfavorable, many other studies suggest that high circulating FetA is associated with obesity and T2DM.

In obesity, FetA may also orchestrate macrophage migration and polarization in adipocytes by acting as a chemokine (97). In the pathophysiology of T2DM, FetA alters how muscle and adipose cells respond to insulin by inhibiting autophosphorylation of the insulin receptor (81). Additionally, cell culture and animal models have demonstrated that FetA plays an important role in the development of lipid-induced insulin resistance. Palmitate bound FetA activates TLR4; thus, signaling the release of proinflammatory cytokines, contributing to decreased insulin sensitivity (98). Therefore, it has been suggested that selective inhibition of FetA would lead to increased insulin sensitivity by allowing for autophosphorylation of the insulin receptor and decreased TLR4

activation. For this reason, FetA has been proposed as a target for developing therapies to ameliorate T2DM (83).

Numerous researchers have used cohort studies to demonstrate the relationship between circulating FetA to obesity-associated diseases, mostly T2DM (84,99). Epidemiological evidence suggests that adults with higher FetA are at an increased risk for T2DM. The Nurses' Health Study found that women in the highest quintile of FetA (≥0.57 mg/mL) had an approximately 1.8 fold higher risk of T2DM than women in the lowest quintile of FetA (≤0.40 mg/mL) (99). Similarly, the Health ABC study found that individuals with the highest tertile of FetA (>0.97 mg/mL) had a two-fold higher incident rate of T2DM than individuals with the lowest tertile of FetA (≤0.76 mg/mL) (84).

While FetA has shown association with chronic disease, it has also been associated with other biological and behavioral factors. Biological processes impacting FetA include growth and aging. Current reports suggest that circulating FetA is highest during infancy (near 1mg/mL), declines into childhood (0.5-0.7 mg/mL) and varies in adults (100,101). Furthermore, developmental periods, such as menopause may influence FetA. In a cohort of older adults (median age: 71 yrs.), women taking estrogen had the highest FetA followed by women not taking estrogen. Men had the lowest FetA levels (86,102). Beyond aging, circulating FetA is also affected by genetics and responds to behavioral factors including dietary intake, physical activity and weight maintenance (Fig. 1.2).

Variations in the Fetuin-A Gene

The gene which codes for FetA, *AHSG*, is found on the twenty-seventh locus in the long arm of the third chromosome. Interesting, the 3q27 region has been identified as a T2DM and obesity susceptible region (103,104). Genetic variation may impact circulating FetA levels as well as risk

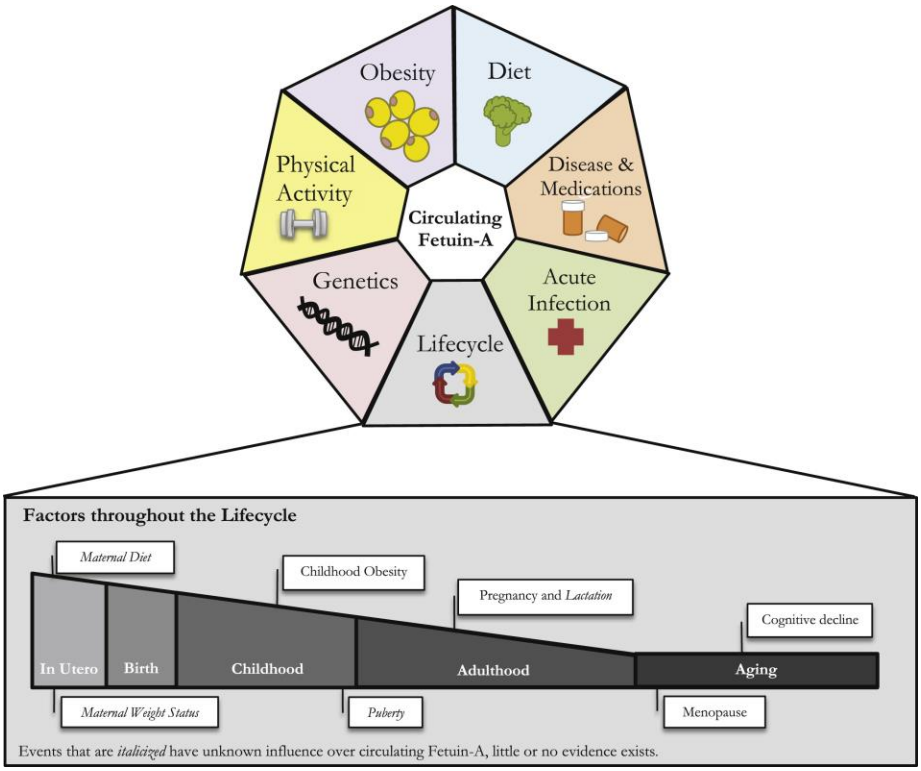
for T2DM and obesity-related phenotypes. Single nucleotide polymorphisms (SNPs) in the *AHSG* gene correlate with plasma FetA (105,106). In a cohort of 2,197 adults, the rs4917 polymorphism explained 21.2% of the variance in FetA with each C-allele increasing FetA by 0.035 mg/mL. To date, four SNPs have been found to be associated with circulating FetA (105).

The correlation between *AHSG* SNPs and body composition is inconsistent (107–110). Multiple *AHSG* SNPs (rs2248690, rs2077119, rs4831, rs4917 and rs11540663) were genotyped in 364 normal weight and obese women and no associations with BMI, percent body fat or waist circumferences were found (108). Another group genotyped rs4917 in 157 patients post-myocardial infarction and a significant correlation with waist circumference and a borderline significant association with BMI was found (p=0.065). When analyzed under a dominant model, excluding individuals with T2DM, carriers of the minor T-allele of rs4917 (T/T or C/T) had significantly lower BMI and waist circumferences than C/C homozygotes (109). In another group of 504 normal weight and obese Swedish men, the G-allele of rs2593813 was associated with leanness. Three *AHSG* SNPs (rs2593813, rs4917 and rs4918) were used to construct haplotypes and variations of these haplotypes were more common in lean versus obese men. Specifically, the combination of a G-allele at rs2593813, a T-allele at rs4917 and a G-allele at rs4918 were 1.9 times more common in men with a BMI less than 25 kg/m^2 (110). Conversely, another research group used magnetic resonance imaging to assess regional body fat and was unable to show associations between body fat and genetic variations in the *AHSG* gene (111). Overall, it appears that SNPs in *AHSG* influence circulating FetA, but these SNPs are inconsistently correlated to BMI. Although some exonic *AHSG* SNPs result in nonsynonymous mutations, it is unclear whether the presence of these mutations alters the ability of FetA to bind to insulin receptor, TLR4, palmitate or other ligands (109).

Behavioral factors impacting circulating Fetuin-A

Behavioral factors that impact FetA levels include physical activity, dietary intake and weight loss. Weight management strategies such as caloric restriction, aerobic exercise and weight loss surgeries have all been shown to reduce circulating FetA while overfeeding and obesity tend to increase FetA levels (112–115). A recent review paper has covered the topic of FetA and adult obesity in great detail and concludes that FetA is positively correlated with obesity and comorbidities and thus, may provide an interesting insight in the study of obesity-associated diseases (107). Circulating FetA is positively associated with obesity and weight gain and negatively associated with weight loss following behavioral intervention and bariatric surgery (112,116,117). In a five-year observational study, baseline FetA was positively associated with a gain of visceral adipose tissue in older adults. More specifically, each 0.42 mg/mL increase in FetA was associated with a 5% gain of visceral adipose tissue (118). In another study, researchers showed that a 34% decrease in BMI caused by Roux-en-Y gastric bypass (RYBP) led to a 19% decrease in FetA levels 16 months after surgery (112). In a different study of RYBP patients, FetA was 27% lower just three days after surgery when compared to FetA levels three days before surgery (116). It is unclear whether other bariatric surgeries cause similar decreases in FetA. Currently, only two studies have evaluated the impact of SG. One study included seven individuals and suggested that FetA decreased following SG although not significantly (116). The other included 22 SG patients and found that FetA was decreased by 13.8% (119). Therefore, the impact of surgery-induced weight loss on circulating FetA merits further investigation. It is also unclear whether the observed changes in FetA are due to bariatric surgery or due to dietary changes resulting from the surgery.

Figure 1.2 Individual Factors that Influence Circulating Fetuin-A.



High-carbohydrate and high-fat diets promote the expression of FetA in hepatocytes and adipocytes and may result in higher circulating FetA levels *in vivo* (97). In one intervention study, plasma FetA was increased after overfeeding 40 healthy adults for 28-days (115). The composition of their diet was increased by 1100 kcals/d from baseline and modified to be composed of 45% energy from fat, 15% energy from protein and 40% energy from carbohydrates. This short report did not provide numerical values for baseline and post-overfeeding circulating FetA, so the magnitude of influence is not known. Furthermore, no descriptive information about the specific fatty acid content, the ratio of saturated fat to monounsaturated and polyunsaturated fatty acids or other nutrition details were reported. As stated previously, FetA preferentially binds the saturated fatty acid, palmitate, and signals the TLR4 inflammatory cascade (97,98). While free fatty acids at high concentrations were positively correlated with circulating FetA in one study, there is no

conclusive evidence on whether the type of dietary fat increased during overfeeding alters the transcription of FetA or the ability of FetA to signal TLR4 (120).

The impact of calorie restriction on FetA levels appears to conflict in two studies of different populations. The first study included overweight women with T2DM. These women were instructed by a dietitian on how to reduce their energy intake by 30% (an average of -586 kcal per day). For 12 weeks, calories were restricted and the result was a 7% decrease in FetA when compared to a control group (0.23 mg/mL and 0.25 mg/mL, respectively, p=0.04) (113). Conversely, the second study included individuals who were considered to have restricted calorie intake due to intentional self-restriction, excessive exercise and low body weight. This type of calorie restriction – short or long-term – had no impact on FetA (121). This report also found no differences in circulating FetA in short-term calorie restriction (72-hour fasting) in men and women with a BMI less than 25 kg/m^2 (121). Therefore, the impact of calorie restriction on circulating FetA may depend on baseline body composition with overweight individuals showing a larger response to calorie restriction than normal or underweight individuals.

Of note, the correlation between FetA and obesity is understudied in children. One existing study found that FetA was not significantly different between normal weight and obese children. However, obese children with non-alcoholic fatty liver disease (NAFLD) had significantly higher circulating FetA (0.35±0.07 mg/mL) than obese children without NAFLD (0.29±0.06 mg/mL) (122). This study may suggest that metabolically unhealthy obesity but not metabolically healthy obesity is positively correlated to FetA.

1.4 Conclusion

In summary, obesity is a complex disease that increases the risk for numerous, life-threatening chronic conditions. Bariatric surgery leads to long-term weight loss as well as improvements in comorbidities. It is not entirely clear why comorbidities such as T2DM resolve following bariatric procedures. As our understanding of the mechanisms behind these diseases evolves, new biomarkers such as FetA may provide novel insight into predicting disease risk, assessing disease progression as well as provide new targets for treatment. FetA is a multifunctional protein that inhibits ectopic calcification and insulin signaling via tyrosine kinase. FetA also promotes inflammation through TLR4. Increased levels of FetA have been linked to greater risk of CVD and incident T2DM. Therefore, it has been proposed as a biomarker for these diseases. To fully understand the clinical utility of FetA as a biomarker for disease, consideration must be given to the biological and behavioral factors influencing FetA. Biological factors include genetics and aging among others.

Behavioral factors that impact FetA levels include physical activity, weight loss and dietary intake. In general, physical activity and weight loss (either by behavioral modification or weight loss surgery) decrease FetA (114,123). Although research concerning the diet's impact on FetA is sparse, it appears that excess calorie intake increases FetA while low-calorie diets reduce FetA. Therefore, if FetA does prove to be an informative clinical marker of CVD or T2DM, it may also be possible to manage FetA through physical activity, weight management and diet in addition to pharmacological means.

CHAPTER 2. OBJECTIVES AND SPECIFIC AIMS

2.1 Rationale and Significance

Obesity is a life-changing disease, which is associated with higher risk of comorbidities including T2DM (25). Uncontrolled T2DM can lead to neuropathy, nephropathy and retinopathy, which cause physical, emotional and financial burden (124). In 2012, the cost attributed to diagnosed cases of T2DM was $245 billion in the U.S. alone (125). Effective treatments for obesity and T2DM would significantly improve the quality of life for millions of Americans.

While weight loss has been shown to improve diabetes-associated markers, behavioral interventions are only marginally effective at producing sustained weight loss. Pharmacological drugs have been modestly successful, yet concerns about side effects and long-term safety exist (126,127). To date, the most successful treatment for obesity is bariatric surgery, which results in significant weight loss as well as resolution of diabetes in 80% of patients (128,129). Due to these dramatic improvements in insulin sensitivity, bariatric surgery has served as an interesting model for the study of diabetes mechanisms. By studying disease resolution, researchers may achieve a different view of disease progression and discover new targets for intervention.

FetA is a novel protein that links obesity and T2DM. By studying FetA, we gain important insights into whether FetA has the potential to serve as a biomarker for these diseases. This research provides is a greater understanding of how FetA relates to obesity while considering multiple biological and behavioral factors such as genetics and dietary intake. By determining how FetA responds to bariatric surgery, we may be able to better understand what role is has in the resolution of T2DM. Circulating FetA has been shown to respond to changes in BMI, however, our study will gather more specific measures of body composition including percent fat mass and lean body

mass. Furthermore, by studying AHSG SNPs in multiple populations, we aim to elucidate whether AHSG polymorphisms relate to differences in T2DM prevalence between ethnic groups.

2.2 Scope of Research

Novel biomarkers have the potential to inform clinical practice and improve patients' lives. Biomarkers may be used as diagnostic tools or therapeutic targets. In this dissertation, we aim to evaluate the potential of FetA as an indicator of insulin resistance in obesity. FetA is just one of many proteins in the body whose function is not fully understood. This cumulative body of work will contribute further clarity on the response of FetA to surgically-induced weight loss and diabetes resolution.

Although FetA has been described in many disease states, there are substantial gaps in the literature. Questions that remain include: how do polymorphisms in *AHSG* influence circulating FetA in ethnic groups with elevated risk for T2DM? If FetA decreases with weight loss, are changes in adiposity or dietary intake more influential? Could changes in FetA partially explain increased insulin sensitivity following bariatric surgery? Overall, this research will add to the understanding of FetA in response to weight loss and describe the influence of AHSG polymorphisms.

2.3 Objectives and Specific Aims

The *overarching goal* of this research proposal is to understand whether FetA may be a novel marker of obesity and obesity-associated insulin resistance and to elucidate the association between obesity, circulating FetA and AHSG polymorphisms. We aimed to understand FetA's role in obesity-associated chronic diseases and to describe the impact of AHSG SNPs on

circulating FetA and blood markers of metabolic health. We explored the impact of surgically-induced weight loss on FetA and determined whether FetA correlates with increasing insulin sensitivity following weight loss surgery. Finally, we sought to determine whether circulating FetA and FetA trajectories differed in bariatric patients with insulin-sensitive and insulin-resistant obesity.

Hypothesis 1: Single nucleotide polymorphisms (SNPs) in *AHSG* will alter circulating FetA and markers of metabolic disease in young Mexican adults.

Chapter 3 highlights metabolic differences in individuals carrying different genetic variants of *AHSG*. Specifically, *AHSG* genotype was associated with circulating FetA and triglycerides but not with BMI or waist circumference in a college-aged Mexican population. Variation in these SNPs explained 8-10% of the variability in FetA.

Hypothesis 2: Significant reductions in FetA following SG begin prior to surgery during the preoperative diet.

Chapter 4 presents evidence of a significant preoperative decline in FetA that compliments the postoperative decline in FetA reported in previous studies. Total change in FetA was associated with percent weight loss. Interestingly, HOMA-IR at all time points was associated with postoperative FetA. Considering the significant change in FetA during this time period and the association with weight change and insulin resistance, we sought to determine whether individuals with insulin-resistant obesity responded differently to bariatric surgery than those with insulin-sensitive obesity.

Hypothesis 3: Individuals with insulin-resistant obesity will have significantly different weight loss and FetA change over the course of SG than individuals with insulin-sensitive obesity.

Chapter 5 summarizes the influence of FetA change over adipocyte hypertrophy and insulin status. Contrary to previous findings, FetA tended to be higher in individuals with Ob_{sen} than those with Ob_{res}. Although baseline BMI and percent body fat did not differ, the Ob_{res} group had significantly more excess body weight loss and larger omental adipocytes. Furthermore, greater preoperative reduction in FetA was associated with smaller adipocytes on the day of surgery.

Chapter 6 reviews the contributions of this research and postulates future areas of interest in the study of FetA, bariatric surgery and obesity. Specifically, a better understanding of how FetA influences adipocyte size and function is needed. Continued pursuits into the mechanisms behind bariatric surgery will shed light on the etiology and resolution of obesity-associated diseases. Also, more research is needed concerning long-term outcomes and the potential use of bariatric procedures in specific populations including adolescents and adults with class I obesity.

CHAPTER 3. A2-HEREMANS-SCHMID GLYCOPROTEIN (*AHSG*) POLYMORPHISMS ARE ASSOCIATED WITH CIRCULATING FETUIN-A AND TRIGLYCERIDES IN YOUNG MEXICAN ADULTS

Abstract

Background: Circulating Fetuin-A (FetA) inhibits insulin receptor signaling and activates the toll-like-4 receptor proinflammatory cascade, thus, may contribute to Metabolic Syndrome (MetS) development. The FetA protein is coded by the Alpha2-Heremans-Schmid Glycoprotein (*AHSG*) gene. Polymorphisms in *AHSG* could influence MetS progression in higher risk ethnic groups. We aim to identify if *AHSG* individual variation relates to biomarkers of metabolic disease and obesity in young Mexican Adults.

Methods: Participants were college applicants to the Autonomous University of San Luis Potosi, Mexico (18-25 years, n=717). Blood was collected for analysis of biomarkers, circulating FetA and genotyping. A sample of this cohort was analyzed five years after baseline visit to assess longitudinal changes (n=74). Two single nucleotide polymorphisms (SNPs) in *AHSG* were genotyped (rs2518136 and rs4917).

Results: Lower triglyceride values were observed in CC-rs4917 homozygotes than T-allele carriers ($p<0.05$). There was no association with *AHSG* SNPs and body mass index or waist circumference. Both SNPs were significantly associated with circulating FetA. Variation in rs4917 and rs2518136 explained 8% and 10% of the variability in FetA, respectively.

Conclusions: Circulating FetA was influenced by *AHSG* polymorphisms. Rs4917 T-allele carriers had higher triglycerides and this relationship was exaggerated in overweight and obese individuals.

3.1 Introduction

Obesity is associated with a higher risk for multiple comorbidities including Type 2 Diabetes Mellitus (T2DM), dyslipidemia and Metabolic Syndrome (MetS) (22). Fetuin-A (FetA) is both a hepatokine and an adipokine which is increased in patients with these conditions. FetA inhibits insulin receptor tyrosine kinase, thus reduces insulin sensitivity via decreased translocation of glucose transporter type 4 (130,131). More recently, FetA has also been shown to bind saturated fatty acids and stimulate inflammatory cytokine release via toll-like receptor 4 (98). Accordingly, circulating FetA has been positively associated with increased risk for T2DM (84). Circulating FetA levels are higher in individuals with T2DM and are positively correlated with fasting blood glucose levels (106). Additional factors associated with elevated FetA such as physical inactivity and dietary intake have been reviewed elsewhere (132).

The gene which codes for FetA, Alpha2-Heremans-Schmid Glycoprotein (*AHSG*), is located in the diabetes-susceptible gene locus 3q27 (103,104). A few SNPs in *AHSG* (i.e. rs2077119 and rs2518136) have been associated with greater risk for T2DM (109,133). Circulating FetA has also been correlated to markers of cardiovascular disease and dyslipidemia (133,134). In the EPIC-Potsdam Study, FetA levels were positively associated with total cholesterol (105). Dahlman et al., also found that three *AHSG* SNPs were correlated to cholesterol (rs2248690, rs2077119 and rs4917) (108). Conversely, other studies have reported no difference in cholesterol concentration between rs4917 genotypes (109,110).

It is unclear at this time what role *AHSG* SNPs have on body composition (108–110). Knock-out mice for *AHSG* are resistant to diet-induced obesity (135). In human studies, *AHSG* SNPs are inconsistently correlated with measures of obesity. The functional *AHSG* SNP, rs4917, is the most

commonly studied. Two studies found evidence to support an association between rs4917 and obesity while two others found no association. In 2005, Lavebratt and collaborators investigated the association between *AHSG* SNPs and obesity. Three SNPs (rs2593813, rs4917 and rs4918) were genotyped in 504 normal weight and obese Swedish men. Lean men were 1.9 times more likely to have a G-allele at rs2593813, a T-allele at rs4917 and a G-allele at rs4918 compared to overweight or obese men (110). In a previous study of 364 Scandinavian women, six *AHSG* SNPs (including rs4917) were genotyped and no association was found with body mass index (BMI), waist circumference or percent body fat calculated from DEXA measurements (108). Mussig et al. measured body fat distribution using magnetic resonance imaging and found that location of body fat was also not correlated with genetic variations in rs4917 (111). Therefore, it appeared that *AHSG* SNP rs4917 was not likely correlated to body composition. Recent evidence from Hungarian researchers, however, demonstrated that the T-allele in rs4917 was in fact, associated with leanness, just as Lavebratt et al. had suggested a decade earlier (109). The reason for the reported inconsistencies remains unknown although population stratification and differences in allele frequencies may play a role.

It is not clear how these polymorphisms are involved in the pathophysiology of obesity-comorbidities. One plausible explanation is the ability of *AHSG* polymorphisms to modify circulating levels of FetA. Three studies have demonstrated that *AHSG* SNPs influence the level of FetA in circulation (105,106,136). In a European population, each C-allele at rs4917 increased FetA by 0.035 mg/mL (105). Polymorphisms at this site explained around 21% of the variance in FetA. The researchers went on to show that three other SNPs, including rs2070633 and rs2070635, were associated with circulating FetA (105). This finding was re-evaluated by Jensen and collaborators. In both Caucasian and African American adults, *AHSG* rs4917 T-allele carriers had

13% and 11% lower circulating FetA, respectively (106). The most recent data to support a connection between *AHSG* polymorphisms and FetA levels comes from the EPIC Study. Among 483 participants, rs4917 C-allele carriers exhibited 16-28% higher plasma FetA (136). Overall, the results of these three studies consistently suggest a role for genetics in the determination of FetA levels. However, the reported difference between ethnicities warrants further investigation.

The associations of FetA with T2DM, dyslipidemia and MetS have been demonstrated in mostly Caucasian cohorts. The SNPs identified in genome-wide association studies (GWAS) of Caucasians are not always consistent with SNPs identified in GWAS of other ethnicities (137). To date, the influence of *AHSG* polymorphisms in other ethnic groups at higher risk for obesity is understudied. Compared to Caucasian groups, Hispanics have higher rates of T2DM and other obesity comorbidities. The Mexican population is one of the groups at highest risk of all Hispanic subgroups. Furthermore, Hispanic and Caucasian populations have different frequencies of minor alleles at *AHSG*, making this a gene of interest in the study of health disparities.

In this report, we aim to identify whether individual genetic variations in *AHSG* relate to biomarkers of metabolic disease as well as circulating FetA in a cohort of young Mexican adults. We will also describe the impact of these markers on longitudinal weight and metabolic changes in a subset of individuals that were followed up to five years after initial recruitment.

3.2 Methods

The UP-AMIGOS cohort (Universities of San Luis Potosí and Illinois: A Multidisciplinary Investigation on Genetics, Obesity, and Social-environment) included young adult applicants to the Autonomous Universities of San Luis Potosí (USLP) (n=717). The study protocol was approved by the Institutional Review Boards of the University of Illinois at Urbana-Champaign

and USLP. Height to the nearest centimeter, weight to the nearest 0.1 kg and waist circumference (WC) to the nearest 0.1 cm were measured by trained UP-AMIGOS staff. Fasting blood was collected for the analysis of biomarkers and genotyping. Fasting glucose was measured using reagents from BioSystems (Barcelona, Spain) and the Alcyon 300 Auto Analyzer (Abbott Park, IL, US). A colorimetric enzymatic reaction was used to assess triglyceride (TG) levels and high-density lipoprotein (HDL) was measured according to the cholesterol oxidase method. Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated using Matthews' equation (fasting insulin (μU/ml) x fasting blood glucose (mmol/l) divided by 22.5) (138). The UP-AMIGOS cohort also includes a longitudinal arm, which contains 74 individuals from the original cohort that participated in a follow-up appointment five years after admission to college. Plasma samples were collected from these participants and FetA concentrations were determined by enzyme-linked immunosorbent assays provided by BioVendor™ (Asheville, NC). Genomic DNA was extracted from whole blood using a commercially available kit (Gentra Puregene Blood Kit, Qiagen, CA) and stored at -20°C until analysis.

After consideration of minor allele frequency (MAF, minimum of 10%) and location of possible SNPs in the *AHSG* gene, two SNPs were selected (rs2518136 and rs4917). Rs2518136 (IVS6+98C>T) is located in the sixth intron. According to the 1,000 Genomes Project, the C-allele is the minor allele in the majority of populations (Global MAF: 48%). Rs4917 is located in the sixth exon of the *AHSG* gene and the T-allele is the minor allele (Global MAF: 26%). The presence of a C-allele at this site results in a nonsynonymous substitution (Thr230Met). Both of the selected SNPs serve as tag SNPs meaning they are in high linkage disequilibrium with other SNPs of interest and may be used to identify genetic variation at other sites within the *AHSG* locus. Rs2518136 is a tag SNP for rs2070633 and rs2070635. Genetic variation at these locations,

specifically rs2070633-TT and rs2070635-AA have been associated with lower FetA concentrations (105). Rs4917 is a tag SNP for rs2593813 and rs1900618. The rs2593813-GG genotype was two times more common in lean individuals than in overweight or obese counterparts (110). Therefore, our rationale for the selection of these SNPs (rs2518136 and rs4917) is based on previous literature, minor allele frequencies greater than 10%, tag SNP potential and the need to better understand the genetic and metabolic differences between ethnic groups.

Samples of the UP-AMIGOS cohort (n=747) were genotyped at the Functional Genomic Unit of the W.M. Keck Center at the University of Illinois using the Fluidigm® SNP genotyping platform. Genotypes were called using the Fluidigm® Genotyping Analysis Software version 4.1.2 (San Francisco, CA) with a minimum of 95% confidence. Samples that failed genotyping for either marker were excluded (n=30), leaving a final sample size of 717 participants. Comparative MAFs are listed in Table 3.1.

Associations between *AHSG* SNPs, circulating FetA, obesity parameters and comorbidity measures were analyzed using SAS 9.3 (Cary, NC). Generalized linear models and chi-square tests were used to identify associations within genotypic, recessive and dominant models (SAS 9.3). Skewed variables were log-transformed for analysis and back-transformed for interpretation. Models were adjusted for age, sex and BMI, when appropriate.

3.3 Results

A total of 717 participants (44% male) were included in this analysis. Descriptive characteristics are included in Table 3.2. At baseline, the average age of the participants was 18.5±1.3 years and the average BMI was 23.8±4.5 kg/m^2. Of the population, 7.8% were underweight, 59.3% had a BMI within the normal range and 32.9% were categorized as overweight or obese. Both SNPs

were in Hardy-Weinberg Equilibrium according to chi-square analysis ($p>0.05$). Our cross-sectional analysis of the entire cohort showed that neither SNP was associated with BMI or WC (Table 3.3).

The longitudinal cohort included 75 returning participants with an average age of 23.4±1.9 (37% male) at follow-up. BMI was positively correlated with fasting plasma FetA ($r^2=0.12$, $p=0.03$) when adjusted for age. Circulating FetA was not associated with glucose, TG, HDL, LDL or WC. Average weight gain over five years was 8.5±10.9 percent of body weight. The prevalence of overweight and obesity climbed by 9% (from 32% at baseline to 41% at follow-up). Changes in weight and BMI over this time period were not associated with age, sex, baseline BMI or baseline WC. However, changes in WC were associated with sex ($r^2= 0.08$, $p=0.01$) and with baseline WC ($r^2=0.05$, $p=0.05$). At follow-up, WC decreased by 2.9±7.8 cm in females and increased by 2.9±9.4 in males. Neither *AHSG* SNP was associated with changes in weight parameters in the longitudinal cohort (Table 3.4).

Circulating levels of FetA were associated with AHSG rs4917 and rs2518136

The presence of the T-allele at rs4917 was associated with 8% lower circulating FetA (Table 3.5). Each T-allele decreased circulating FetA by 30.0 μg/mL ($p=0.03$). The T-allele at rs2518136 was also associated with lower FetA and each T-allele resulted in an average FetA decrease of 32.5 μg/mL ($p=0.04$).

Circulating triglycerides were associated with AHSG rs4917

Rs4917-CC homozygotes had lower TG values than T-allele carriers (106.5±48.0 vs. 121.1±58.3 mg/dL, $p=0.02$) (Fig. 3.1). When split by BMI category, it appears that obesity amplifies the genotypic influence of this *AHSG* SNP (Fig. 3.2). Homozygotes for the T-allele that were

overweight or obese had higher TG than CC-homozygotes that were overweight or obese (122.5±61.5 vs. 149.8±56.6 mg/dL). Regardless of the *AHSG* genotype, participants whose BMI was within the normal range had lower TG values than participants with a BMI in the overweight or obese category. Interestingly, in the underweight group, the influence of rs4917 on circulating TG was opposite that of the overweight/obese group. Although the sample size is very limited (n=46), it appears that underweight *AHSG* T-carriers had lower TG values than underweight CC-homozygotes. *AHSG* rs4917 was not associated with total cholesterol, HDL or LDL. Rs2518136 genotypes were not associated with TG.

3.4 Discussion

To our knowledge, we are the first to investigate *AHSG* polymorphisms and obesity parameters in a Mexican population. No association was found between *AHSG* SNPs and BMI or WC in either the cross-sectional or in the longitudinal cohort. *AHSG* polymorphisms were, however, correlated with fasting FetA and TG.

Previous findings agree that the *AHSG* rs4917 T-allele promotes lower circulating values of FetA but found that this SNP site explained around 21% of the variance in FetA (105). Jensen and collaborators found that T-allele carriers of rs4917 in Caucasian and African American populations had 13% and 11% lower circulating FetA, respectively (106). In our cohort, we found that rs4917 genotype explained 8% of the variance in circulating FetA. The rs4917 T-allele was associated with an 8% decrease in FetA. In sum, Mexican adults had an average 30.0 μg/mL decrease in FetA per minor allele at rs4917. Each T-allele at rs2518136 was also associated with a 32.5 μg/mL decrease in FetA. Therefore, the decrease in FetA per rs4917 T-allele is lower in our Mexican

cohort as compared to other cohorts suggesting that individual variability may also be influenced by other behavioral and biological factors beyond genetics.

When compared to Caucasian and African American cohorts, there are significant differences in the MAF of these two SNPs in Hispanic cohorts (106). The frequency of the minor T-allele at rs4917 in Mexican adults is 41.4% which makes it more common than in Caucasian and African American cohorts (33% and 30%, respectively). Therefore, if rs4917 genotypes were the main driver of circulating FetA, it would be expected that on average Hispanics have lower circulating FetA than Caucasians. The same inference could be made for rs2518136 because of differences in the minor alleles for populations of Hispanic and Caucasian origin. However, we found that circulating FetA and the variability explained by rs4917 genotypes was lower in Mexican adults than in previous reports of differing ethnicities.

In Caucasians, the T-allele is the minor allele at rs2518136. However, in our cohort of Mexican adults, the minor allele is the C-allele (46%). Thus the risk allele (T) is more common in our sample. Rs2518136, a tag SNP for rs2070633 and rs2070635, was related to circulating FetA. The T-allele at *AHSG* rs2070633 and the A-allele at *AHSG* rs2070635 were linked to lower levels of FetA (105). Therefore it is not surprising that the T-allele at rs2518136, is associated with lower FetA. However, it is of interest that this allele is more common in the Mexican population.

Based on these data, it would be expected that Mexican Adults should have lower circulating FetA in comparison to other ethnicities. However, unpublished data from our lab as well as the results of other research teams have not found this to be the case. Using the same methodology, our lab has found that average FetA was 286.2±61.5 µg/mL in an age-matched cohort of 54 Caucasian students at the University of Illinois (Appendix 1). In the longitudinal arm of the UP-AMIGOS

cohort, we report average FetA values of 368.4±89.8 μg/mL. One study found that FetA tended to be lower in Hispanics than Caucasians, but this did not reach statistical significance (139). Therefore, other biological and behavioral/environmental factors may be more influential in determining circulating FetA in Hispanic populations.

We found no association between *AHSG* rs4917 and HOMA-IR. A similar study by Jensen et al. found no association between *AHSG* rs4917 and incident T2DM or fasting glucose concentration in Caucasian and African American adults (106). Our data revealed that the T-allele at rs4917 was associated with higher fasting TG values in a sample of young Hispanic adults. This association has not been reported in Caucasian cohorts (109,133). Mexican populations have a higher frequency of the T-allele than Caucasian populations. This base mutation at rs4917 results in an amino acid change (Thr230Met) thus, may alter the tertiary structure of FetA and alter the ability of FetA to bind receptors.

As an intronic SNP, rs2518136 is not translated into the final protein. Yet, intronic SNPs may impact mRNA promoter binding, splicing, and transcription (140). Andersen et al. reported that rs2518136 was associated with T2DM. Contrary to our findings, the T-allele was more common in individuals with T2DM in this cohort (133). Jensen and colleagues noted no association of rs2518136 with markers of diabetes (106). We found no association of *AHSG* SNP with fasting glucose or HOMA-IR. These contradictory findings may be explained by the ethnic differences of our cohorts as allele frequencies differ among ethnic groups.

Our cohort included mainly healthy, young adults and therefore, other cohorts with poorer health or older age may yield different conclusions. Attending college represents a unique transition in an adult's life which may include significant dietary and exercise changes. Therefore, follow-up

of this cohort is ongoing with consideration for dietary intake. As weight gain progresses due to age and lifestyle changes, it is possible that genetic associations will shift. Dietary intake, specifically fat consumption, has been shown to significantly influence the association of SNPs with circulating lipid values (141). As mentioned previously, *AHSG* is located in 3q27. This is a diabetes-susceptible locus and has also been shown to influence macronutrient and total calorie intake (104). Thus, it is possible that the association of rs4917 and triglycerides is mediated by dietary intake. A limitation of this study was the relatively small number of samples available for the analysis of plasma FetA. Furthermore, other biological or behavioral differences between the genotype groups in our longitudinal cohort, cannot be ruled out.

3.5 Conclusion

In conclusion, individual genetic variants in the *AHSG* gene impact triglycerides and circulating FetA. Our study is the first to describe these markers in a young Mexican population, which has differing minor allele frequencies than Caucasian populations. Our results suggest that maintenance of a healthy body weight may overcome the deleterious effects of genes related to MetS and T2DM. These findings demonstrate an important public health message, that even in the face of genetic predisposition, health biomarkers may be modified by health behaviors.

Table 3.1. Minor Allele Frequency (MAF) of *AHSG* SNPs reported in the 1000 Genomes Project and in the UP-AMIGOS cohorts

SNPs	1000 Genomes Project		UP-AMIGOS Cohort	UP-AMIGOS Longitudinal
	CEU	MXL	MEX	MEX
N	99	64	714	75
rs4917	0.33 (T)	0.41 (T)	0.425 (T)	0.50 (T)
rs2518136	0.49 (T)	0.46 (C)	0.460 (C)	0.44 (C)

Populations reported include: MXL, Mexican Ancestry from Los Angeles. CEU, Utah residents with North and Western European Ancestry. MEX, Mexican adults living in Mexico from the Universities of San Luis Potosí and Illinois: A Multidisciplinary Investigation on Genetics, Obesity, and Social-environment (UP-AMIGOS) Project.

Table 3.2. Demographic Variables by Sex of the UP-AMIGOS Cohort

Variable	N	Males	N	Females	p-value
Age (years)	312	19.1 ± 2.3	405	19.0 ± 2.1	0.48
BMI (kg/m^2)	312	24.5 ± 4.5	405	23.6 ± 4.7	**0.009**
Waist Circumference (cm)	304	84.2 ± 11.3	396	77.8 ± 11.6	**<0.0001**
GLC (mg/dL)	312	92.0 ± 8.0	405	88.0 ± 8.3	**<0.0001**
TG (mg/dL)	267	118.1 ± 57.8	351	106.4 ± 52.5	**0.002**
TC (mg/dL)	264	170.2 ± 32.4	346	172.5 ± 37.4	0.43
LDL (mg/dL)	247	99.9 ± 27.5	325	99.0 ± 28.9	0.65
HDL (mg/dL)	251	47.2 ± 11.5	328	51.8 ± 11.8	**<0.0001**
HOMA-IR	65	1.6 ± 1.2	64	1.8 ± 1.5	0.10
Fetuin-A (µg/mL)	21	373.0 ± 82.2	34	366.5 ± 96.4	0.74

Values are presented as means ± SD. p-values greater than 0.05 are considered statistically significant. Abbreviations: BMI: Body Mass Index; GLC: Glucose; TG: Triglycerides; TC: Total Cholesterol; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance.

Table 3.3. Association of *AHSG* rs4917 and body mass index in the UP-AMIGOS cohort

Variables	rs4917			p-value for the additive model (CC vs. TC vs. TT)	p-value for the recessive model (CC vs. TC/TT)	p-value for the dominant model (CC/TC vs. TT)
	CC	TC	TT			
Sex (male/female)	93/137	163/202	56/66			
Age (years)	18.8 ± 0.1	19.3 ± 0.1	18.9 ± 0.2	0.017[a]	0.018[a]	0.452[a]
BMI (kg/m^2)	23.7 ± 0.3	23.4 ± 0.2	23.83 ± 0.4	0.821[b]	0.737[b]	0.690[b]
Waist Circumference (cm)	80.6 ± 0.8	81.1 ± 0.6	81.6 ± 1.0	0.735[b]	0.485[b]	0.580[b]
GLC (mg/dL)	90.2 ± 0.5	89.7 ± 0.4	89.4 ± 0.7	0.631[c]	0.381[c]	0.518[c]
TG (mg/dL)	100.2 ± 2.9	100.7 ± 2.4	110.4 ± 4.4	0.058[c]	0.263[c]	0.018[c]
TC (mg/dL)	166.9 ± 2.3	167.6 ± 1.9	172.0 ± 3.2	0.339[c]	0.413[c]	0.155[c]
LDL (mg/dL)	97.5 ± 2.0	96.7 ± 1.6	100.0 ± 2.8	0.602[c]	0.878[c]	0.321[c]
HDL (mg/dL)	48.9 ± 0.8	49.0 ± 0.7	48.0 ± 1.1	0.775[c]	0.743[c]	0.480[c]
HOMA-IR	1.4 ± 0.2	1.3 ± 0.1	1.4 ± 0.2	0.748[c]	0.471[c]	0.993[c]

Values are presented as means ± se. [a]p-values were adjusted by sex. [b]p-values were adjusted by sex and age. [c]p-values were adjusted by sex, age and BMI. Abbreviations: BMI: Body Mass Index; GLC: Glucose; TG: Triglycerides; TC: Total Cholesterol; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance.

Table 3.4. Association of *AHSG* rs2518136 and body mass index in the UP-AMIGOS cohort

Variables	rs2518136			p-value for the additive model (CC vs. TC vs. TT)	p-value for the recessive model (CC vs. TC/TT)	p-value for the dominant model (CC/TC vs. TT)
	CC	TC	TT			
Sex (male/female)	64/83	155/210	93/112			
Age (years)	18.8 ± 0.1	19.2 ± 0.1	19.0 ± 0.2	0.110[a]	0.065[a]	0.721[a]
BMI (kg/m^2)	23.9 ± 0.4	23.6 ± 0.2	23.7 ± 0.3	0.754[b]	0.455[b]	0.869[b]
Waist Circumference (cm)	80.2 ± 1.0	81.3 ± 0.6	81.2 ± 0.8	0.598[b]	0.312[b]	0.811[b]
GLC (mg/dL)	91.5 ± 0.7	89.6 ± 0.4	89.4 ± 0.6	0.521[c]	0.299[c]	0.432[c]
TG (mg/dL)	98.1 ± 3.6	102.8 ± 2.4	104.0 ± 3.2	0.241[c]	0.102[c]	0.355[c]
TC (mg/dL)	165.1 ± 2.9	169.0 ± 1.9	168.8 ± 2.5	0.334[c]	0.138[c]	0.632[c]
LDL (mg/dL)	97.1 ± 2.5	98.7 ± 1.6	96.0 ± 2.1	0.560[c]	0.628[c]	0.453[c]
HDL (mg/dL)	48.2 ± 1.0	48.9 ± 0.7	48.8 ± 0.9	0.935[c]	0.779[c]	0.895[c]
HOMA-IR	1.6 ± 0.2	1.2 ± 0.1	1.5 ± 0.2	0.078[c]	0.047[c]	0.671[c]

Values are presented as means ± se. [a]p-values were adjusted by sex. [b]p-values were adjusted by sex and age. [c]p-values were adjusted by sex, age and BMI. Abbreviations: BMI: Body Mass Index; GLC: Glucose; TG: Triglycerides; TC: Total Cholesterol; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance.

Table 3.5. Body composition changes in the UP-AMIGOS Longitudinal cohort by *AHSG* genotype (n=74)

Variables	rs4917			p-value (CC vs. TC vs. TT)	p-value (CC vs. TC/TT)	p-value (CC/TC vs. TT)	rs2518136			p-value (CC vs. TC vs. TT)	p-value (CC vs. TC/TT)	p-value (CC/TC vs. TT)
	CC	**TC**	**TT**				**CC**	**TC**	**TT**			
N	18	39	17				14	38	22			
Fetuin-A(µg/mL)	393 ± 23	368 ± 16	319 ± 23	0.069[a]	0.145[a]	0.029[a]	396 ± 27	376 ± 16	319 ± 20	0.045[a]	0.142[a]	0.017[a]
Body Weight Δ (%)	7.4 ± 11.4	8.9 ± 10.3	7.8 ± 8.1	0.875[b]	0.373[b]	0.836[b]	6.2 ± 11.0	10.1 ± 10.1	6.3 ± 8.8	0.327[b]	0.412[b]	0.367[b]
BMI Δ(kg/m^2)	1.1 ± 2.7	1.7 ± 2.7	1.5 ± 1.7	0.698[b]	0.439[b]	0.918[b]	0.9 ± 2.7	2.0 ± 2.8	1.2 ± 1.8	0.316[b]	0.272[b]	0.537[b]
WC Δ(cm)	-1.7 ± 9.8	0.8 ± 8.5	-0.3 ± 7.7	0.892[b]	0.750[b]	0.808[b]	-1.3 ± 10.0	1.2 ± 8.5	-1.6 ± 7.8	0.726[b]	0.997[b]	0.448[b]

Values are presented as means ± se. [a]p-values were adjusted by sex, BMI and age. [b]p-values were adjusted by sex and age. Abbreviations: BMI: Body Mass Index; WC: Waist Circumference.

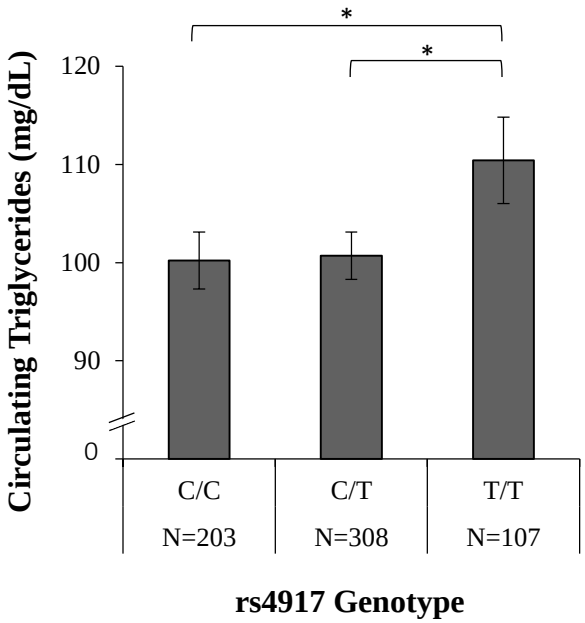


Figure 3.1. Individual variation in *AHSG* SNP (rs4917) was associated with triglycerides in the UP-AMIGOS cohort. C/C homozygotes and heterozygotes had significantly lower triglyceride values than T/T homozygotes (p=0.048 and p=0.045, respectively). Error bars represent the standard error of the mean.

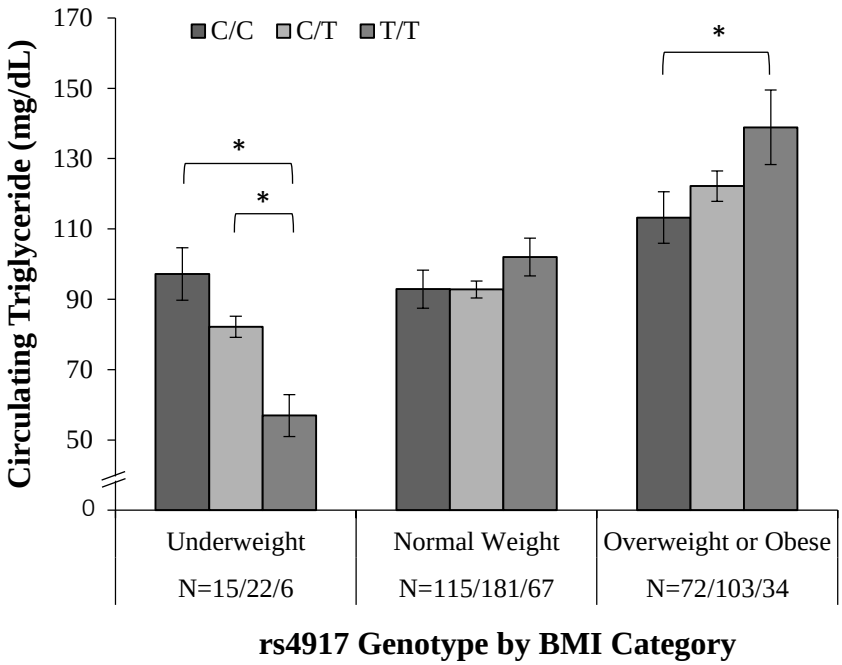


Figure 3.2. The association of *AHSG* SNP (rs4917) with triglycerides was modified by body mass index (BMI) category. Individuals that were T/T homozygotes and were overweight or obese had significantly higher triglycerides than C/C homozygotes who were overweight or obese (p=0.029). Conversely, T/T homozygotes who were underweight had significantly lower triglycerides (56.9 ± 7.3 mg/dL) than carriers of C/T (82.2 ± 5.4 mg/dL) and C/C (97.2 ± 7.5 mg/dL) alleles who were underweight (p=0.018 and p=0.0008, respectively). Individuals with a BMI in the normal range had no significant differences in triglycerides between genotypes. Error bars represent the standard error of the mean.

CHAPTER 4. CIRCULATING FETUIN-A DECREASES DURING PREOPERATIVE CALORIE RESTRICTION AND FOLLOWING SLEEVE GASTRECTOMY

Abstract

Background: Bariatric surgery is an effective treatment for both obesity and type 2 diabetes mellitus (T2DM). Glucose homeostasis is restored within days of surgery independent of weight loss. The hepatokine, Fetuin-A (FetA), has been suggested to play a role in diabetes remission since it dramatically decreases following bariatric surgery. It is unclear why FetA decreases postoperatively and the impact of the preoperative diet has not been previously investigated.

Methods: Plasma was collected from sleeve gastrectomy patients (n=39) before the preoperative diet, on the day of surgery and approximately six weeks postoperatively. At each visit, body composition was collected via bioelectrical impedance analysis.

Results: FetA decreases 12% from baseline to day of surgery (606±170 μg/mL vs. 533±99 μg/mL, $p=0.010$). Compared to baseline, average FetA was 35% lower following the postoperative diet (392±83 μg/mL, $p<0.0001$). Although weight loss was significant in both sexes, the total change in FetA was associated with percent body weight loss in women only ($r=0.39$, $p=0.03$).

Conclusions: The reduction in FetA following bariatric surgery begins during the preoperative diet phase. Therefore, immediate improvements in glucose homeostasis may be partly explained by preoperative caloric restriction. Future studies which seek to evaluate metabolic improvements occurring within days of bariatric surgery should disclose whether participants completed a hypocaloric, preoperative diet.

4.1 Introduction

Bariatric procedures result in significant weight loss and resolution of obesity-associated diseases. In 2013, 468,609 patients with morbid obesity opted for surgical treatment of obesity. The procedure that is growing most rapidly in popularity is the sleeve gastrectomy (SG) which accounted for 37% of all surgeries (42). SG patients lose an average of 62.3% of excess body weight five years after surgery (142). Resolution rates for type 2 diabetes mellitus (T2DM) are near 80% for SG (43–45). Thus, important insights about the pathophysiology of T2DM and novel markers of glucose homeostasis may be gained from understanding the mechanisms behind SG outcomes.

The hepatokine, Fetuin-A (FetA), reduces significantly after bariatric surgery, therefore, it has emerged as a potential novel marker of glucose homeostasis improvements. FetA has been suggested to play a role in glucose metabolism through two mechanisms: 1) FetA inhibits insulin receptor autophosphorylation, therefore, limiting translocation of the glucose transporter (GLUT 4) and 2) when bound to saturated fatty acids, FetA can signal the inflammatory cascade through toll-like receptor 4, thus promoting insulin resistance (81,98). Increased FetA has been linked to adverse health conditions including T2DM and CVD (82,84,120). Thus, FetA reductions following surgery may explain some of the immediate improvements in glucose homeostasis following bariatric procedures.

To date, three studies have evaluated changes in FetA following weight loss surgery. Although study lengths and protocols varied, all found that when compared to preoperative FetA, postoperative FetA was significantly lower. Sixteen months after Roux-en-Y Gastric Bypass (RYGB), average BMI was decreased by 34% and FetA decreased by 19% (143). FetA was also

significantly reduced one year after RYGB, mini-gastric bypass, and SG. Overall, the average change was 10% but the largest change (near 14%) was seen in the group receiving SG (119). In a metabolomic analysis, FetA was one of only two proteins to significantly decrease three days after RYGB when compared to three days before surgery. In this short time period, plasma FetA decreased by 27% in the absence of significant weight loss (116). These studies suggest that bariatric procedures result in the rapid and sustained reduction of FetA. However, it is unclear whether this reduction in FetA is unique to surgery or can be partially explained by dietary restriction.

Circulating FetA responds to dietary changes. Exposure to high-fat diets increased FetA *in vitro* and in animal models (144). Hepatocytes exposed to saturated fatty acids; specifically palmitate, secreted FetA in a dose-dependent manner and had higher expression of FetA mRNA (145). FetA is also reduced by caloric restriction (113). Thus, when attempting to study the response of FetA to surgery, it is important to consider the role of the diet.

In the weeks preceding bariatric surgery, patients are often prescribed a hypocaloric, low-fat diet. The preoperative diet is recommended to decrease liver size and thus, improve access to the stomach and gastro-esophageal junction (146). In the three studies mentioned previously (112,116,119), the timing of the initial FetA measurement was often unreported except one, which measured baseline FetA three days before surgery (116). Details about the preoperative diet protocol were also not consistently described. Thus, the patient's baseline FetA level may have already been influenced by preoperative diet regimen. Therefore, our objective is to describe changes in FetA during the preoperative diet as well as the weeks immediately following SG.

4.2 Methods

Data Collection

Forty-five patients receiving SG at Carle Foundation Hospital (Urbana, IL) were recruited for the longitudinal cohort. Patients were excluded if they were current smokers, below the age of 18 or had a pacemaker or implanted defibrillator. Participants attended three visits; baseline (T0) took place approximately two weeks (median: 18 days, mode: 15 days) before surgery prior to the preoperative diet. The second appointment (T1) occurred following the two-week preoperative diet on the morning of surgery. The final appointment (T2) occurred approximately six-weeks (median: 41 days, mode: 41 days) following surgery. The Individual Outcomes of Weight Loss Surgery (I-OWLS) Study protocol received approval from the Institutional Review Boards at both Carle Foundation Hospital and the University of Illinois at Urbana-Champaign.

A Registered Dietitian Nutritionist advised each patient on the pre- and postoperative dietary protocol. During the two weeks before surgery, men were advised to consume 1000 calories and 70-90 grams of protein per day. Women were advised to consume 800 calories and 50-60 grams of protein per day. All patients were encouraged to drink 64 ounces of water each day and to split daily calories into six small meals. The postoperative diet was a standard transitional diet: full liquid for postoperative weeks 1-2, pureed diet for postoperative weeks 3-4 and regular foods during postoperative week 5. Three-day food logs (including two weekdays and one weekend day) were collected at each appointment to assess dietary adherence. Food logs were analyzed using the Nutrition Data System for Research software developed at the University of Minnesota (147).

Participants were asked to arrive at each appointment fasted, to avoid alcohol for 24 hours prior to the visit and to avoid exercise for 12 hours prior to the visit. All completed a survey which inquired

about demographic variables and medical history. Height, weight and body composition (InBody230, Biospace, Cerritos, CA) were measured by trained research staff (K.R.). Laparoscopic SG was performed by one of two surgeons (B.R. and U.O.) at Carle Hospital following protocols previously established at the Carle Bariatric Surgical Unit.

Blood analysis

Fasting blood was collected by trained phlebotomists at Carle Hospital. Blood was centrifuged within 30 min at 10,000g for 10min, separated into aliquots and stored at -80°C until further analysis. Plasma FetA and insulin were measured by enzyme-linked immunosorbent assays (ELISA) (BioVendor™, Asheville, NC and EMD Millipore, Billerica, MA). Blood lipids were measured by LabCorps (Dublin, OH) and glucose was measured in duplicate by glucometer (Trividia Health, Fort Lauderdale, FL). Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated using Matthews' equation (138).

Statistics

Variables were evaluated for errors and normality. Non-normal variables were transformed for analysis and back-transformed for interpretation. The effect of preoperative diet and SG on FetA was assessed by calculating the change (Δ) between T0 and T1 and between T1 and T2 appointments, respectively. Total weight loss was calculated during the preoperative diet, during the postoperative diet and across the entire study length using BMI. Ideal body weight was estimated based on a reference BMI of 25 kg/m^2 and used to calculate excess weight (EBW) at baseline and excess weight loss percentage at T1 and T2. To assess rate, FetA change was averaged over the days between appointments. Time was treated as a within-subject factor. Thus, repeated measures of time were considered. Differences between time points were compared using mixed

models and contrast statements as appropriate. All statistical analysis was performed in SAS 9.4 (Cary, NC).

4.3 Results

Demographics

The study protocol was completed by 39 participants (80% female) (Table 4.1, Fig. 4.1), of which, 86.8% were non-Hispanic white and 13.2% were non-Hispanic black. Average baseline BMI was 47 kg/m^2 and baseline percent body fat (PBF) was 52% for females and 47% for males. Prediabetes or T2DM diagnosis was reported by 30% of participants. Baseline average calorie intake was 1757±121 kcal per day with a macronutrient distribution of 43% carbohydrate, 19% protein and 38% fat. When stratified by sex, the only significant difference was greater ($p=0.04$) fat consumption in men (42±7.3%) when compared to women (37±6.3%).

Caloric intake, excess weight and HOMA-IR were significantly reduced during the pre- and postoperative period.

Dietary intake differed ($p<0.0001$) across time points. During the preoperative and postoperative diet, intake decreased to 960±42 and 751±54 calories per day, respectively. No sex differences were found in reported dietary intake at either T1 or T2. Macronutrient distribution of the preoperative diet was 61% carbohydrate, 24% protein and 15% fat whereas distribution at six-week follow-up was 34% carbohydrate, 31% protein and 35% fat (Fig. 4.2).

Significant weight change was observed during the study (Table 4.2). Following the preoperative diet, men lost 10.8±3.3% of EBW while women lost 9.9±4.6% of EBW. On average, postoperative EBW loss was 19.5±8.5% in men and 18.2±6.9% in women. During the preoperative diet,

significant improvements in fasting HOMA-IR were noted ($p<0.0001$). At baseline, average HOMA-IR was 2.75±0.26. On the morning of surgery, HOMA-IR was 1.15±0.11. At six-week follow-up, average HOMA-IR was not significantly lower than on the morning of surgery (1.12±0.10, $p=0.797$).

Response of circulating FetA to the preoperative diet and sleeve gastrectomy

FetA was significantly reduced during both the preoperative diet and during the six-weeks following surgery (Fig. 4.3). At baseline, average FetA was 589.7±113.5 μg/mL in males and 610±185 μg/mL in females ($p=0.620$ for sex). Older age was associated with lower baseline FetA in women only ($r= -0.52$, $p=0.004$). Baseline FetA change was not significantly correlated with sex, HOMA-IR, BMI or PBF. Following the hypocaloric, preoperative diet, FetA declined from baseline in both men (491.9±79.1 μg/mL) and women (545.8±101.83 μg/mL). At T1, FetA tended ($p=0.052$) to be lower in men. In both sexes, the change in FetA was associated with baseline FetA, in that; those with the highest baseline FetA had the greatest reductions in FetA during the preoperative diet ($r=0.69$, $p<0.0001$). In women, preoperative FetA change was significantly correlated with percent weight change ($r=0.39$, $p=0.03$), BMI change ($r=0.38$, $p=0.04$) and EBWL ($r=0.37$, $p=0.04$), but not with PBF change or time between T0 and T1. In men, preoperative FetA change was not significantly associated with any of the aforementioned weight variables (Fig. 4.4, A-B) (Appendix 3).

Following surgery, FetA further decreased to 367.7±85.7 in males and 399.9±85.7 in females ($p=0.433$). In women, this change was related to BMI change ($r=0.48$, $p=0.008$) and body weight percentage ($r=0.43$, $p=0.018$) change. Again, change in FetA in males was not related to change in body composition (Fig. 4.4, C-D). FetA changes during the postoperative period were correlated

with changes during the preoperative diet (r= -0.53, p=0.0005). Additionally, the rate of FetA reduction (total FetA change divided by the time interval in days) was not significantly different during the pre- and postoperative periods. FetA and HOMA-IR were not associated at baseline or on the day of surgery. However, HOMA-IR at T0, T1 and T2 were significantly associated with FetA at follow-up (p=0.002, 0.003 and 0.001, respectively) (Table 4.3, Fig. 4.5).

Circulating FetA was not associated with reported caloric intake or macronutrient distribution at baseline or during the preoperative diet. During the postoperative diet, individuals who had greater reductions in dietary fat had greater reductions in FetA (r=0.35, p=0.039). This association was strongest in women (r=0.43, p=0.027). No further correlations with caloric intake or macronutrient distribution were observed during the postoperative diet.

4.4 Discussion

The preoperative diet regimen offers an important period for studying how individuals with morbid obesity respond to acute dietary changes prior to receiving weight loss surgery. However, the literature often fails to account for this time period. In the case of FetA, we found that FetA was significantly reduced following the preoperative diet and during the weeks immediately following SG. Although the time period during the preoperative diet and the postoperative period differed, the rate of FetA change was not significantly different. In females only, FetA change was associated with change in BMI, but not with change in body composition as measured by bioelectrical impedance. Caloric intake was significantly reduced over the course of this study. However, calories alone did not explain changes in circulating FetA. Some evidence emerged to suggest that dietary fat intake is correlated with postoperative FetA in females, but replication is needed. Although the pre- and postoperative diets both allow additional calories for men, there

was no reported difference in daily caloric intake between the sexes. Food logs may be limited due to reporting errors or bias.

It remains unclear why FetA reduces following bariatric surgery. In women, change in BMI, age and baseline FetA were associated with greater reductions in FetA. Although pre- and postoperative FetA changes were not associated with changes in dietary intake, we have demonstrated the importance of monitoring changes during the preoperative period. A consistent association between FetA and HOMA-IR was not seen at each time point, though we did observe that HOMA-IR at baseline, on the morning of surgery and at follow-up was significantly associated with postoperative FetA. Specifically, higher HOMA-IR at these time points predicted lower FetA at six-week follow-up. Other potential mechanisms for the reduction in FetA include changes in liver composition and hepatic functioning that were not measured in this cohort.

Like other studies of bariatric patients, our sample was primarily female. Further studies in male populations are warranted. Phosphorylated forms of FetA were not accessed, but may yield a different response to surgery and may be a more sensitive indicator of FetA function. Nevertheless, our results suggest that the preoperative diet should be considered during the study of bariatric outcomes. We found that FetA, a marker of relevance to T2DM which quickly improves following surgery, actually begins to improve preoperatively. Thus, it should be standard procedure for all studies in this area to report whether preoperative diets were completed, as well as the duration and composition of the preoperative diet.

4.5 Conclusion

Metabolic and longitudinal cohort reports have found that FetA decreases after bariatric surgery and therefore may account for immediate improvements in insulin sensitivity (112,116,119).

However, preoperative diet protocol is not considered or not reported in these studies. We found that the reduction in FetA following bariatric surgery is already present following the preoperative diet. FetA reduction is most strongly correlated with baseline FetA as well as HOMA-IR at all previous time points. Therefore, improvements in circulating FetA may be partly explained by behavioral modifications that occur prior to SG. The preoperative diet offers an opportunity to evaluate the impact of a behavioral intervention on outcomes of interest in post-bariatric glucose improvements. Studies reporting short-term improvements in glucose homeostasis should disclose whether patients were or were not advised to complete a preoperative dietary restriction.

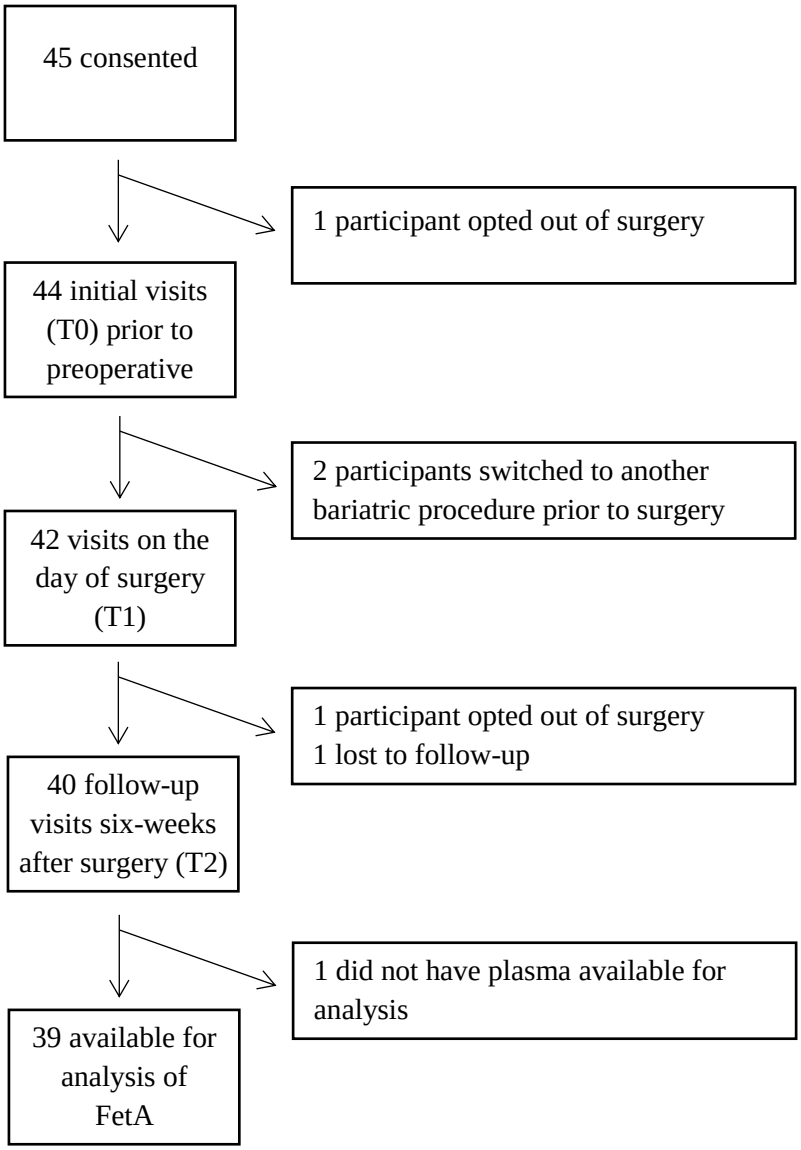


Figure 4.1. Participant flow diagram for the I-OWLS cohort. Participants were recruited June 2015 through March 2016. Of the 45 individuals who were consented for the study, 89% completed all three scheduled appointments.

Table 4.1. Baseline characteristics of the I-OWLS cohort

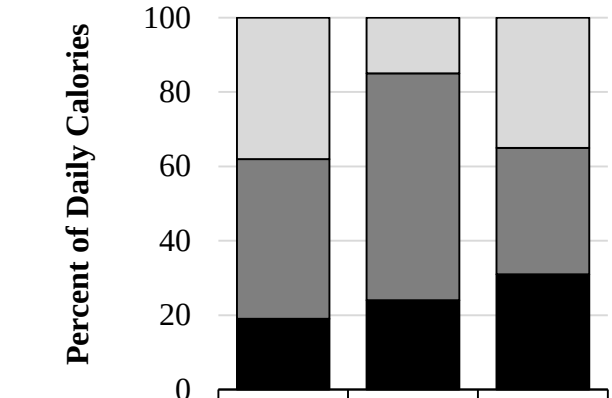
Variable	Females	Males	p-value
N	**31**	**9**	
Age (years)	43.7 ± 1.7	45.8 ± 3.3	0.58
BMI (kg/m^2)	47.4 ± 1.2	46.9 ± 2.7	0.85
Percent Body Fat	52.1 ± 0.5	46.9 ± 2.2	**0.0009**
TG (mg/dL)	149.3 ± 14.3	137.9 ± 17.7	0.69
TC (mg/dL)	180.3 ± 6.9	160.7 ± 10.5	0.18
HDL (mg/dL)	43.5 ± 2.5	35.0 ± 2.7	0.11
LDL (mg/dL)	108.5 ± 6.1	98.2 ± 10.5	0.42
Glucose (mg/dL)	103.4 ± 2.4	107.8 ± 1.78.1	0.56
HOMA-IR	2.8 ± 0.6	2.8 ± 0.3	0.92
Fetuin-A (µg/mL)	611.0 ± 33.8	589.7 ± 37.8	0.75

Values are presented as means ± SE. p-values greater than 0.05 are considered statistically significant. Abbreviations: BMI: Body Mass Index; TG: Triglycerides; TC: Total Cholesterol; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance.

Table 4.2. Weight loss trajectory of the I-OWLS cohort

Variable	Females (n=31)			Males (n=9)			p-value for change between time points adjusted by sex
	T0	T1	T2	T0	T1	T2	
Age (years)	43.2 ± 1.7	43.3 ± 1.8	43.3 ± 1.8	45.8 ± 3.3	45.6 ± 3.3	45.9 ± 3.2	NS
Weight (kg)	131.9 ± 3.9	125.9 ± 3.8	115.1 ± 3.5	148.2 ± 8.4	141.3 ± 7.9	127.6 ± 7.7	<0.0001
BMI (kg/m^2)	47.3 ± 1.2	45.2 ± 1.2	41.3 ± 1.1	46.9 ± 2.7	44.7 ± 2.6	40.5 ± 2.5	0.0007
Body Fat (%)	52.1 ± 0.5	52.3 ± 0.6	50.2 ± 0.7	46.9 ± 2.2	46.0 ± 2.7	43.5 ± 2.7	<0.0001

Values are presented as means ± SE. Change across time was analyzed using generalized linear model adjusted for sex. p-value less than 0.05 are considered statistically significant. Abbreviations: BMI: Body Mass Index.



	T0	T1	T2
Fat	38	15	35
Carbohydrate	43	61	34
Protein	19	24	31

Figure 4.2. Reported macronutrient distribution range of sleeve gastrectomy patient by visit. Three-day dietary records were collected from participants at each visit. Logs were analyzed in Nutrition Data System for Research software. Macronutrient distribution was calculated by dividing average total calories of fat, protein and carbohydrate by average daily caloric intake.

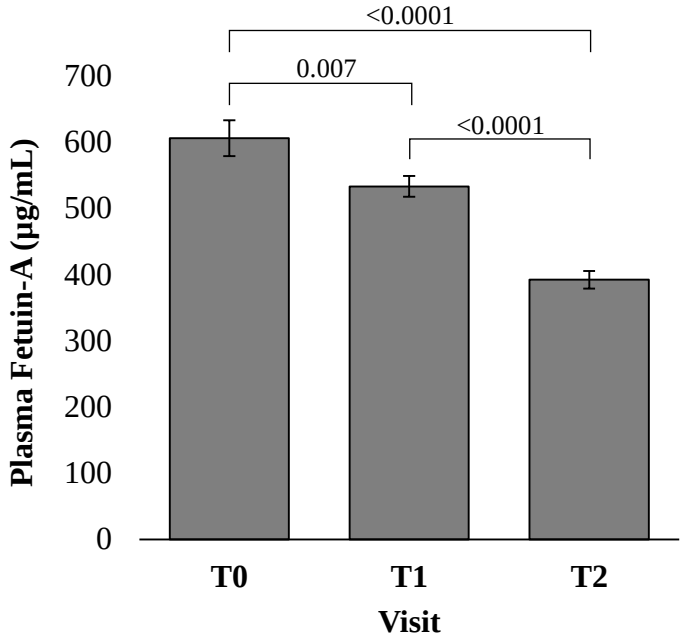


Figure 4.3. Fasting plasma Fetuin-A concentrations in sleeve gastrectomy patients at baseline (T0), on the morning of surgery (T1) and at six-week follow-up. Fasting plasma Fetuin-A was significantly reduced on the morning of surgery and at six-week follow-up compared to baseline. Data are adjusted for age and presented as mean ± SE.

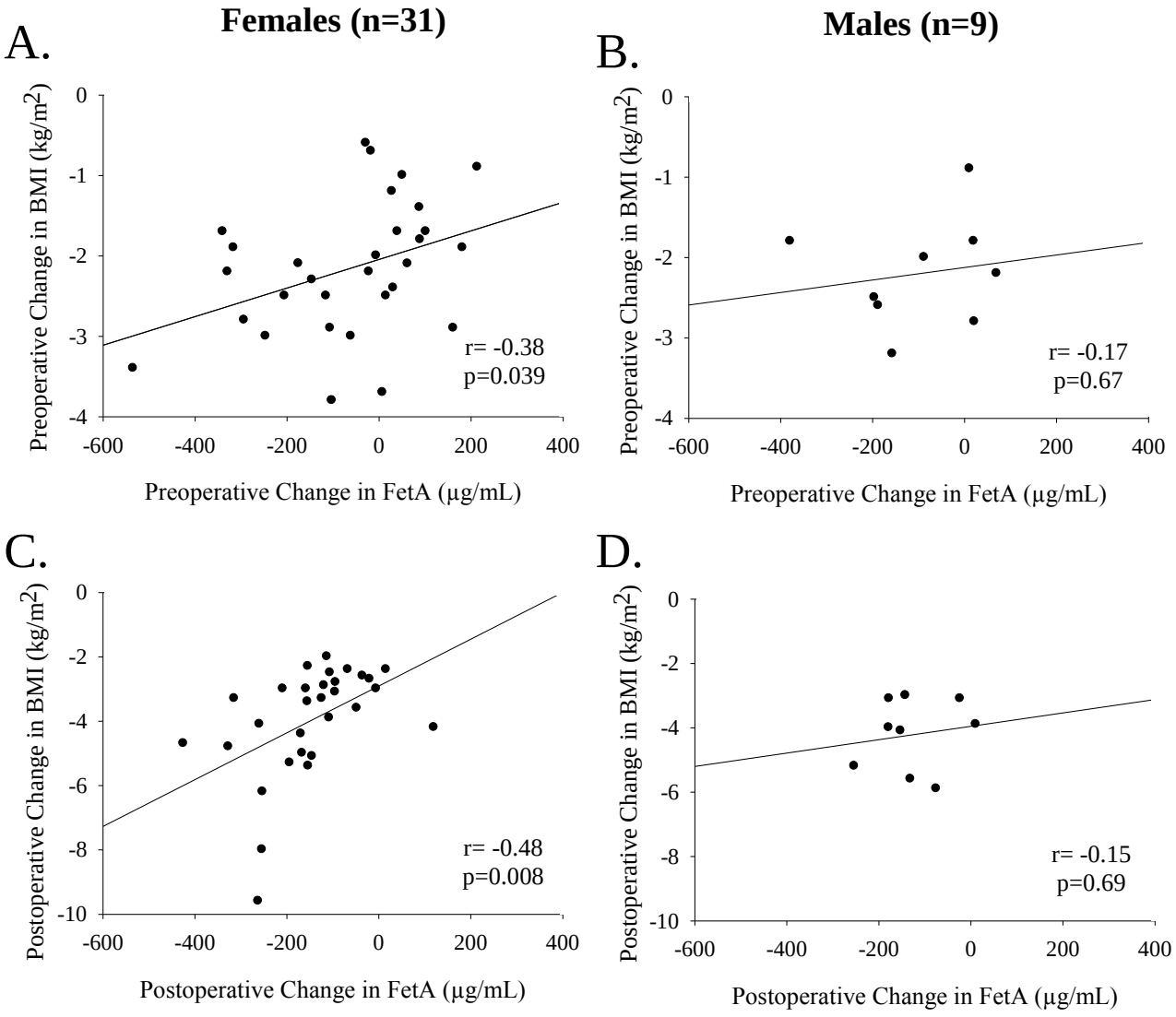


Figure 4.4. Correlation of fetuin-A change with body mass index (BMI) change during the pre- and postoperative period by sex; A. preoperative correlation in females; **B.** preoperative correlation in males; **C.** postoperative correlation in females; **D.** postoperative correlation in males. p-value less than 0.05 are considered statistically significant.

Table 4.3. Correlation of HOMA-IR and Fetuin-A by visit

Variable		Fetuin-A		
		T0	**T1**	**T2**
HOMA-IR	**T0**	r= -0.27 p=0.111	r= -0.13 p=0.427	r= -0.48 **p=0.003**
	T1	r= -0.33 **p=0.044**	r= -0.23 p=0.168	r= -0.47 **p=0.003**
	T2	r= -0.30 p=0.068	r= -0.14 p=0.420	r= -0.50 **p=0.001**

Associations were evaluated using Pearson Correlation Coefficient. p-value less than 0.05 are considered statistically significant.

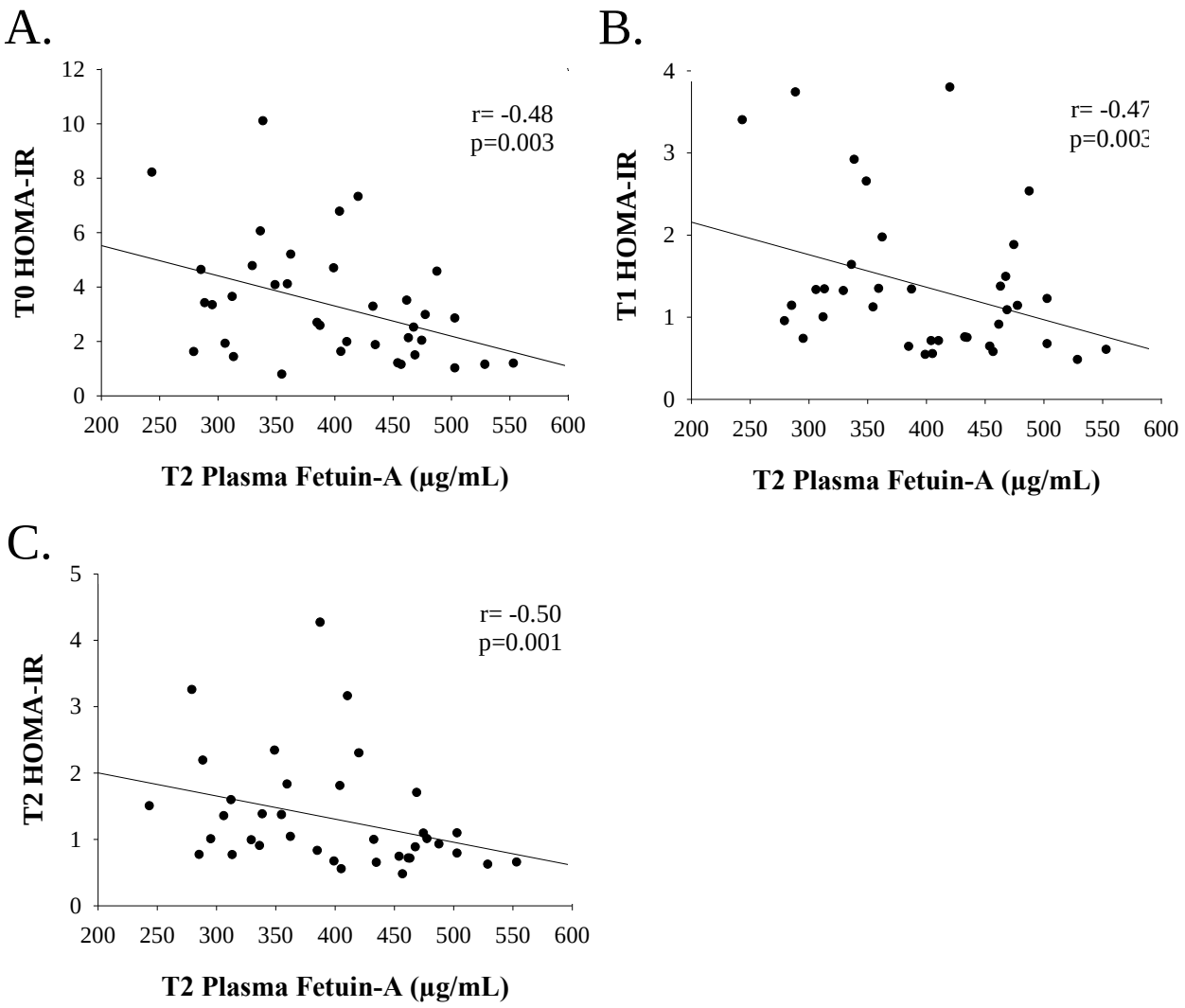


Figure 4.5. Correlation of postoperative Fetuin-A with HOMA-IR; A. at baseline; **B.** on the morning of surgery; **C.** at six-week follow-up.

CHAPTER 5. ADIPOCYTE SIZE, CIRCULATING FETUIN-A AND SURGERY-INDUCED WEIGHT LOSS IN INDIVIDUALS WITH INSULIN-RESISTANT OBESITY AND INSULIN-SENSITIVE OBESITY

Abstract

Background: Although excess adiposity is associated with increased risk for diabetes, there is a subset of adults who remain insulin-sensitive in the face of morbid obesity. Although consensus is limited, cross-sectional studies have elucidated several behavioral and biological factors including the hepatokine Fetuin-A (FetA), which may differentiate individuals with insulin-resistant obesity (Ob_{res}) from their insulin-sensitive (Ob_{sen}) counterparts. It is unclear whether these groups respond differently to sleeve gastrectomy (SG), a weight loss surgery which leads to improved insulin sensitivity and sustained weight loss.

Methods: Candidates for SG (n=40) were evaluated at baseline (T0), on the morning of surgery (T1) and six weeks following SG (T2). Three-day food logs, body composition data and fasting blood for the measurement of FetA, insulin, lipids and glucose were collected at each visit. Adipocyte diameter was calculated from omental adipose tissue collected during surgery. To compare Ob_{res} and Ob_{sen}, the cohort was divided into age- and sex-matched groups (n=10 each) based on homeostatic model assessment for insulin resistance values with consideration for diabetes diagnosis and use of anti-diabetic medications.

Results: The Ob_{sen} group tended to have higher FetA than Ob_{res} at all time points (T0: p=0.09; T1: p=0.10 and T2: p=0.004). Baseline BMI did not differ between groups yet, the Ob_{res} group had greater total excess body weight loss (-29% vs. -26%, p= 0.01). Those with Ob_{res} had larger adipocytes than Ob_{sen} (71±2 vs. 61±3 μm, p=0.01). This relationship was exaggerated when

adjusting for genetic differences in toll-like receptor 4 (rs4986790). Adipocyte size was positively correlated with preoperative change in FetA (r=0.64, p=0.007) but not with changes in dietary intake, body fat loss or BMI loss.

Conclusions: Participants with Ob_{sen} tended to have higher FetA at all time points. Greater reduction in FetA during the preoperative diet was associated with smaller adipocyte size on the day of surgery. Declining FetA may suggest reduction in adipocyte size which correlates with improved insulin sensitivity, however, longitudinal studies of histology changes are needed.

5.1 Introduction

Excess adiposity increases the risk of developing insulin resistance and T2DM. Four percent of individuals with a normal BMI have T2DM while 15% and 26% of individuals categorized as obese class II and class III have T2DM, respectively (148). As morbid obesity rates climb, parallel increases in T2DM incidence are also reported. Long-term consequences of T2DM include reduced quality of life, high financial burden and devastating health consequences such as renal failure, cardiovascular disease, and peripheral neuropathy (149–151). Fortunately, not all individuals with obesity demonstrate impaired glucose tolerance. To date, it is unclear why some with higher BMI experience delayed onset of T2DM or do not develop T2DM at all. Therefore, a greater understanding of those susceptible to T2DM may help facilitate more targeted healthcare interventions in at-risk individuals.

Recently, the term "metabolically healthy obesity (MHO)" has been suggested to describe an individual who has a BMI greater than 30 kg/m^2 with no additional metabolic abnormalities associated with MetS. Specific criteria for MetS have been defined by the Adult Treatment Panel III (ATP III) and include the presence of three or more of the following: elevated triglycerides,

high blood pressure, hyperglycemia, elevated waist circumference and reduced HDL cholesterol (152). Although this definition exists for MetS, the definition for categorizing MHO and metabolically unhealthy obesity (MUHO) is heterogeneous in the literature (153,154). For example, O'Connell et al. used presence of any MetS criteria to identify MUHO subjects while Meigs et al. used presence of three or more MetS criteria (ATP III) to identify MUHO subjects (155,156). The use of MetS criteria to define MHO/MUHO has also been challenged. Some research suggests that MHO is not a stable period and that MHO individuals tend to be younger (≤40 years old) and eventually transition into MUHO (32). Furthermore, because MetS includes a spectrum of diseases, it is difficult to compare phenotypes. Therefore, it has been proposed that individual diseases, such as insulin resistance, be considered rather than a cluster of diseases such as MetS.

Epidemiological data suggests that nearly 25% of individuals with a BMI >35 kg/m^2 retain normal glucose tolerance (insulin-sensitive obesity (Ob_{sen})) (157). The definition of normal glucose tolerance in this study was based on a HOMA-IR values <75th percentile of participants without diabetes however, diagnostic criteria from the American Diabetes Association and glucose infusion rate >70 μmol/kg^{-1}/min^{-1} have also been used (31,154). Individuals with obesity are considered insulin-resistant (Ob_{res}) if they meet diagnostic criteria from the American Diabetes Association, HOMA-IR is >75th percentile of participants without diabetes or glucose infusion rate <60 μmol/kg^{-1}/min^{-1}.

Numerous factors have been investigated in an attempt to understand why some develop Ob_{sen} and others develop Ob_{res}. Thus far, there is evidence to suggest that Ob_{res} individuals have higher dietary fat intake than Ob_{sen} individuals (30). At a biological level, circulating adipokines, specifically FetA, and inflammatory cytokines tend to be higher in individuals with Ob_{res} while

circulating ghrelin is reduced in individuals with Ob_{res} (31,158). Characteristics of adipose tissue may also differ significantly. For example, individuals with Ob_{res} tend to have higher fat content in the liver, more visceral adipose, greater macrophage infiltration of visceral adipose tissue and larger adipocyte size (31,159,160).

Adipocyte size is influenced by obesity status, metabolic health and genetic factors. Adipocyte size has been positively correlated with body fat percentage and insulin resistance (161,162). Change in adipocyte size may lead to altered expression of surface level receptors. For example, large adipocytes had three times more glucose transporter 4 (GLUT4) protein than small adipocytes (163). Large adipocytes also expressed 40 fold more P85 regulatory subunit of phosphatidylinositol 3-kinase (P85-PI3K) mRNA than small adipocytes (163). Expression of GLUT4 is upregulated by insulin receptor activation and p85 (when not bound to p110) inhibits PI3K, a downstream target of insulin receptor activation (164). Knockout of P85 in mice led to increased insulin sensitivity, thus elevated P85 in large adipocytes support the findings that larger cells are insulin-resistant (165,166). Although not yet evaluated, FetA may be associated with adipocyte size.

FetA is a ligand for both insulin receptor and toll-like receptor 4 (TLR4), which are receptors of importance for determining adipocyte size. FetA alters the ability of p85-PI3K to bind IRS-1, thus, inhibiting insulin receptor action and preventing GLUT4 translocation to the cell wall (81). FetA may also influence adipocyte size via inhibition of peroxisome proliferator-activated receptor-γ (PPAR-γ) via Wnt3a signaling (167). Finally, FetA has been suggested to be elevated in individuals with obesity and T2DM (31). Thus, we sought to understand the relationship between circulating FetA and adipocyte size. Because larger adipocytes are associated with insulin resistance, a description of the association between circulating FetA and adipose hypertrophy may

clarify whether FetA has potential as a biomarker for insulin sensitivity (162). We hypothesize that circulating FetA and adipocyte size will differ based on presence of comorbidities. Specifically, we expect that individuals with Ob_{res} will show greater adipocyte size than Ob_{sen}. We also sought to gather preliminary data concerning the influence of individual gene variants on adipocyte size. We hypothesized that single nucleotide polymorphisms (SNPs) in key genes such as *PPAR-γ*, *TLR4*, Fat Mass and Obesity (*FTO*) associated-gene and FetA (*AHSG*) would alter adipocyte size.

To date, most studies comparing Ob_{sen} and Ob_{res} groups have been cross-sectional in nature. It is unclear whether these groups respond differently to interventions that improve insulin sensitivity. Diabetes control can be improved with just 5-10% (168). A recent meta-analysis found that rate of weight loss on very-low-calorie and low-energy liquid formulas was similar in adults with Ob_{sen} and Ob_{res} (169). Bariatric surgery is a highly effective intervention for diabetes treatment that results in glucose improvements just days after surgery (170). It is unclear whether individuals with Ob_{res} respond differently to bariatric surgery than individuals with Ob_{sen}. We seek to understand whether the presence of Ob_{sen} or Ob_{res} influences longitudinal outcomes of SG including changes in BMI, PBF, FetA and HOMA-IR. We also aim to understand how preoperative changes in weight and diet associate with adipocyte size on the day of surgery.

5.2 Methods

Data Collection

The Individual Outcomes of Weight Loss Surgery (I-OWLS) study consisted of SG candidates (n=40) which were recruited from the Carle Foundation Hospital according to protocol approved by the Carle Hospital Institutional Review Board. Participants were excluded if they were a current

smoker, pregnant or attempting to become pregnant, under the age of 18 or if they had a pacemaker or implanted defibrillator. All participants met with research staff before beginning a required hypocaloric, preoperative diet intended to reduce the liver size. Visits took place at baseline (T0), on the morning of surgery (T1) and approximately six weeks following surgery (T2). At each of the three time points, fasting blood, three-day dietary recall and anthropometric measures (InBody230, Biospace, Cerritos, CA) were collected.

Laparoscopic SG was performed by one of two surgeons at Carle Hospital following protocols previously established at the Carle Bariatric Surgical Unit. During surgery, approximately 1-2 grams of visceral adipose tissue was collected from the omentum along the greater curvature of the resected stomach. This adipose tissue was immediately fixed with formalin and paraffin-embedded for histological analysis.

Blood analysis

Plasma FetA and insulin concentrations were detected by Enzyme-linked immunosorbent assay (ELISA) method (BioVendor, Asheville, NC and EMD Millipore, Bellerica, MA). Blood lipids were measured by LabCorps (Dublin, OH) and glucose was measured in duplicate by glucometer (Trividia Health, Fort Lauderdale, FL). Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated using Matthews' equation (138). Genomic DNA was extracted from whole blood using a commercially available kit (Gentra Puregene Blood Kit, Qiagen, CA) and genotyped using the Fluidigm® SNP genotyping platform at the Functional Genomic Unit of the W.M. Keck Center at the University of Illinois. Genotypes were assigned using the Fluidigm® Genotyping Analysis Software version 4.1.2 (San Francisco, CA) with a minimum of 95% confidence.

Tissue histology

Omental adipose tissue was available from 30 participants. Formalin-fixed and paraffin-embedded tissues were cut into 5 micron slices and mounted on slides by the Comparative Biosciences Histology Laboratory at the College of Veterinary Medicine at UIUC. Hematoxylin and eosin (H&E) stained adipocytes were scanned at 20x by Nanozoomer slide scanning system (Hamatsu, Hamamatsu City, Japan). The area and diameter of adipocytes was calculated using Adiposoft software plugin for Image J (NIH, Bethesda, MD). Following analysis in automated mode, each individual image was evaluated for improper gating. Adipocytes that touched the field border were removed. Average adipocyte diameter and area were calculated from 100 randomly selected adipocytes from three fields within each individual tissue sample.

Statistics

Data was evaluated for errors and normality and transformed when appropriate. Food logs were analyzed using Nutrition Data System for Research software (99). Participants were stratified into two groups based on whether they had Ob_{res} or Ob_{sen}. Participants were categorized as Ob_{res} if they had a baseline HOMA-IR>2.5, a documented diagnosis of prediabetes or diabetes or a current prescription of anti-diabetic medications per electronic medical chart review. If these characteristics were not present, participants were categorized as Ob_{sen}. The majority of the cohort met criteria for Ob_{res} (66%) while 34% of the cohort met criteria for Ob_{sen}. To minimize potential confounding factors, patients with Ob_{res} were matched by age, sex, and BMI to Ob_{sen} patients. The distribution of males to females was not significantly different between Ob_{sen} and Ob_{res} groups ($p= 0.67$). However, since our cohort was 78% female, only women were included in this analysis. Participants taking Thiazolidinediones (TZDs) or other antidiabetics which alter PPAR-γ and thus,

may alter adipocyte size were excluded. After matching and exclusions, there were ten participants available for comparison in each group (Appendix 5). Ideal body weight was estimated based on a reference BMI of 25 kg/m^2 and used to calculate excess body weight loss (EBWL) during the pre- and postoperative period. General linear models and t-tests were used to test the difference between Ob_{sen} and Ob_{res} groups. Correlations between other variables such as anthropometric data, FetA and HOMA-IR were evaluated using Spearman Correlation Coefficients. Fisher's exact test was used for categorical analysis.

5.3 Results

Insulin-sensitive and insulin-resistant obesity

At baseline, the Ob_{sen} and Ob_{res} groups (n=10 women each) were not significantly different in age, BMI, percent body fat, triglycerides or LDL-cholesterol (Table 5.1). Individuals with Ob_{sen} had greater (p=0.005) baseline HDL than Ob_{res} (51.3mg/dl vs. 35.5mg/dl). As expected, those with Ob_{res} had elevated HOMA-IR at baseline. Although HOMA-IR declined during the preoperative diet, the Ob_{res} group still had higher (p=0.008) average HOMA-IR on the day of surgery than the Ob_{sen} group. Following SG, there was no longer a significant difference in HOMA-IR between groups (Fig. 5.1). The time lapse between appointments was not significantly different between the groups (22±12 days between T0 and T1 and 44±11 days between T1 and T2). Reported intake at baseline was not different between groups except for intake of polyunsaturated fatty acids which was higher (p=0.03) in individuals with Ob_{sen} than their counterparts (9.1±0.9 vs. 6.8±0.6% of total calorie intake) (Table 5.2). Dietary intake during the preoperative diet and postoperative period did not differ between groups except protein intake during the preoperative diet (Appendix

6). Individuals with Ob_{res} reported consuming greater (p=0.03) percent protein on T1 (24.9±1.1 vs. 21.3±1.1%).

The most significant predictor of excess body weight loss and BMI change was baseline BMI and time. Unadjusted preoperative, postoperative and total weight loss over the course of this study was not significantly different between Ob_{sen} and Ob_{res} groups. However, when adjusted for baseline BMI and time, the Ob_{res} group had more (p= 0.012) EBWL than the Ob_{sen} group (-29% vs. -26%) (Table 5.3). The Ob_{res} group also had greater (p=0.006) decline in BMI (-6.0 vs. -5.2 kg/m^2).

Adipocyte size

Adipocyte size was not significantly altered by SNPs in *PPAR-γ* (rs1801282 and rs2279525) *FTO* (rs8057044) or *AHSG* (rs4917 and rs2518136). Carriers of the G-allele at rs4986790, a SNP in *TLR4*, had larger (p=0.025) adipocyte diameter than non-carriers regardless of baseline BMI (62.8±2.4 vs. 73.7±3.6 µm). This finding was replicated in the entire cohort (Appendix 7). Adjusting for this association, we found that the Ob_{res} group had larger (p=0.0099) adipocytes than the Ob_{sen} group (70.6±2.2 vs. 60.6±2.5 µm) (Fig. 5.2). The genotype distribution of rs4986790 was not significantly different between Ob_{sen} and Ob_{res} groups (Fisher's p=0.37). Adipocyte diameter was positively associated with baseline HOMA-IR (r=0.50, p=0.05) (Fig. 5.3).

Circulating fetuin-A in insulin-sensitive and insulin-resistant obesity

FetA was strongly correlated with age (r= -0.59, p=0.006). Age-adjusted FetA tended (p=0.09) to be higher in Ob_{sen} than Ob_{res} at T0 (667±42 vs. 562±42 µg/mL). On the morning of surgery, FetA did not differ (p=0.10) between groups (Ob_{sen}: 577±24 vs. Ob_{res}: 515±25 µg/mL). However, the Ob_{sen} had a greater (p=0.01) reduction in age-adjusted FetA when compared to Ob_{res} (-89±40vs. -

47±40 µg/mL). Change in FetA following SG, was not significantly different between groups (Ob_{sen}: -117±32 vs. Ob_{res}: -151±32 µg/mL p=0.19). FetA was higher (p=0.004) in the Ob_{sen} group than the Ob_{res} group at T2 (461±20 vs. 364±20 µg/mL) (Fig. 5.4).

Fetuin-A and adipocyte size

Adipocyte size was strongly correlated (p=0.007) with preoperative change in FetA (r=0.64). Achievement of greater FetA reduction during the preoperative diet was associated with smaller adipocytes on the day of surgery (Fig. 5.5, A). Postoperatively, there was an opposite trend (p=0.07) between FetA and adipocyte size (r= -0.47). Individuals with larger adipocytes on the day of surgery had greater postoperative change in FetA (Fig. 5.5, B). Change in preoperative FetA was not associated with reported changes in caloric or macronutrient intake or preoperative changes in PBF or BMI. Postoperative change in FetA was not associated with reported postoperative dietary changes or changes in PBF. However, postoperative FetA change was positively associated (p=0.012) with change in BMI (r=0.55).

5.4 Discussion

Insulin-sensitive and insulin-resistant obesity

In agreement with Leslie et al., we found that diet-induced preoperative weight loss did not differ based on insulin sensitivity status (169). However, postoperative weight loss was significantly greater in those with Ob_{res}. Changes in HOMA-IR were not consistent between the groups. Individuals with Ob_{res} had greater decreases in HOMA-IR during the preoperative diet. This reduction was maintained during the postoperative period. Interestingly, HOMA-IR in the Ob_{sen} group remained stable over the course of the study, showing little improvement over time. Karelis et al. demonstrated a similar phenomenon when they found greater improvements in insulin

sensitivity in Ob_{res} following diet-induced weight loss (171). These findings suggest that individuals with Ob_{res} experience superior short-term outcomes in terms of weight loss and improvements in insulin sensitivity.

A limitation to this finding is that hyperinsulinemic-euglycemic clamp data was not available for this cohort. Although HOMA-IR is commonly used in the study of insulin resistance, there are no established cut-points for insulin resistance. Also, HOMA-IR correlates more so to hepatic glucose production rather than glucose utilization and insulin sensitivity as measured by a hyperinsulinemic-euglycemic clamp (154). Thus, future studies should consider additional markers for insulin sensitivity beyond HOMA-IR.

In comparing the dietary intake of adults with Ob_{res} and Ob_{sen}, we replicated previous reports of altered fat consumption (30). Specifically, we found that those with Ob_{sen} consumed less saturated fat ($p=0.106$) and more ($p=0.031$) percent of calories from polyunsaturated fat at baseline. Previous studies of calorie restriction and overfeeding have demonstrated simultaneous reductions and elevations of FetA, respectively (113,115). However, in the I-OWLS cohort, change in FetA was not correlated with change in reported calorie or macronutrient intake. The lack of correlation between dietary change and change in FetA in our cohort was unexpected. Although a portion estimation tool and detailed instructions were provided to I-OWLS participants before collection of three-day food logs, it is possible that dietary reports may not have been accurate.

Adipocyte Size

Adipose tissue expands to accommodate excess fatty acids by either 1) hyperplasia, the increase in adipocyte number or 2) hypertrophy, the increase in adipocyte size. Hypertrophied adipose cells have been associated with insulin resistance and greater cytokine and lipid release (162,172). Like

previous reports, we found that HOMA-IR was positively associated with omental adipocyte size and that individuals with Ob_{res} had larger omental adipocytes (31,155). In contrast to previous studies, we were able to evaluate the influence of SNPs in genes such as *PPAR-γ*, *TLR4*, and *FTO*, which may predispose an individual to have larger or smaller adipocytes. We are the first to show a correlation between adipocyte size and the *TLR4* polymorphism, rs4986790. However, carriers of the G-allele at rs4986790 have been shown to have greater liver, visceral and total body fat (173). By adjusting for rs4986790, a larger difference of average adipose diameter between the Ob_{res} and Ob_{sen} groups was reported. Although adipose tissue is difficult to obtain and adipocyte measurement is labor-intensive, this finding warrants replication in larger cohorts. Furthermore, the present study evaluated adipocyte size using H&E staining. Other technology, such as particle size analyzers, would allow for the measurement of 3D cell size rather than a singular plane.

Circulating Fetuin-A in Insulin-Sensitive and Insulin-Resistant Obesity

Individuals with Ob_{sen} had higher circulating FetA than those with Ob_{res} which conflicts with results from previous reports (31,119,174). One exception found that FetA was not different between groups (175). These reports measured circulating FetA in serum, whereas plasma was used in the current study. The same commercial kit was used for all but one (119) of the studies (BioVendor™). Importantly, the studies above evaluated FetA in different populations. Reinehr et al. evaluated adolescents (174), Yang et al. evaluated Asian bariatric patients (119) and Kloting et al. evaluated a convenience sample of adults with similar age and BMI to our cohort, however, the participants were undergoing elective surgeries, including cholecystectomy, exploratory laparotomy and SG (31). Our population strictly included SG participants.

FetA is recognized as a negative acute phase protein, however, the response of FetA to chronic inflammation is less clear. In dialysis patients, greater circulating C-reactive protein (CRP) is associated with lower FetA (92,176). Thus, our finding that Ob_{res} had lower FetA than Ob_{sen}, may be due to the chronic inflammation associated with insulin-resistance. Additionally, omega-3 supplementation has been shown to increase FetA in dialysis patients and decrease FetA in patients with T2DM (177,178). The Ob_{sen} group consumed greater PUFAs than the Ob_{res} group which may suggest improvements in inflammatory status leading to higher circulating FetA. Although non-alcoholic liver disease increases circulating FetA, information concerning liver status was not available in this cohort (83, 122).

Fetuin-A and Adipocyte Size

Greater preoperative reduction in FetA was associated with smaller adipocytes on the day of surgery while reported changes in dietary intake, body fat loss or BMI loss were not associated with adipocyte size. The mechanistic relationship between FetA and adipocyte size is not entirely clear. Two possible explanations include: 1) the relationship of FetA with PPAR-γ and 2) the complex interaction of FetA with inflammatory markers (Fig. 5.6-5.7).

PPAR-γ is a nuclear receptor that plays an important role in adipocyte differentiation and lipid metabolism (179). PPAR-γ is primarily found in the adipose tissue. Upon forming a heterodimer with retinoid X receptor, PPAR-γ may be stimulated by ligands including but not limited to TZDs and fatty acids (180). Contrary to FetA which preferentially binds saturated fatty acids, PPAR-γ preferentially binds polyunsaturated fatty acids (180,181). Activated PPAR-γ increases transcription of proteins such as adiponectin, lipoprotein lipase and adipocyte binding protein and decreases cytokines such as TNFα and IL-6 (182–185). Thus, PPAR-γ activation is associated with

greater lipid uptake and adipogenesis (167,186). Accordingly, PPAR-γ knockout mice have smaller adipocytes and are more insulin-resistant following a high-fat diet (187). PPAR-γ activity is inhibited via Wnt signaling. For adipogenesis to occur, suppression of Wnt signaling is essential (188). Agarwal et al. recently demonstrated that FetA upregulates Wnt3A thus decreasing PPAR-γ (167). Therefore, greater reductions in FetA may relieve suppression of PPAR-γ and allow for hyperplasia. However, if FetA remains elevated, Wnt-suppression of PPAR-γ may lead to adipocyte hypertrophy and unopposed proinflammatory cytokine release (185,189)

Because omental adipocyte diameter was only measured at one time point during this study, it is also possible that change in adipocyte size influences circulating FetA. In fact, activation of PPAR-γ via TZDs increases insulin sensitivity and reduces FetA expression in liver (190). Chronic use of TZDs leads to a greater risk for osteoporosis, a condition also associated with reduced FetA (191,192). It is possible that activation of PPAR-γ leads to a reduction of hepatic FetA output. Thus, the directionality of this relationship remains unclear and warrants further investigation.

The relationship between FetA and adipocyte size may also be related to inflammatory status. When bound to saturated fat, FetA may stimulate the inflammatory cascade via TLR4 (98). FetA has been shown to promote macrophage migration into the adipose tissue *in vitro* (34). Adipose tissue macrophages contribute additional inflammatory cytokines to the environment (193). Researchers have shown that macrophage infiltration and inflammation decreases after bariatric surgery in mice (194,195). It is possible that these results are related to the postoperative reduction in circulating FetA.

The present study is unable to determine whether the reduction in circulating FetA after bariatric procedures is due to a reduction in adipose- or liver-derived FetA. However, adipose expression

of FetA does appear to be related to adiposity status. Adipose from obese adults with T2DM had three times higher FetA expression than adipose tissue from normal weight humans without T2DM (97). Obese rats had increased FetA expression in visceral AT compared to lean rats (196). Thus, it is possible that weight loss led to reduced expression of FetA from the adipocyte. Another limitation was that the present study collected only omental fat at the time of surgery. Perez-Sotelo et al. demonstrated that visceral and subcutaneous FetA production responded differently to fasting. (196). Specifically, omental fat expression of FetA was reduced by fasting while subcutaneous fat expression of FetA was not. It is possible that subcutaneous tissue responds differently to obesity, insulin resistance and changes in FetA.

5.5 Conclusion

Previous studies found that FetA was elevated in adults with Ob_{res} supporting the potential role of FetA as a biomarker for distinguishing Ob_{res} from Ob_{sen}. Contrary to these studies, we found that participants with Ob_{sen} had higher FetA which remained higher than their Ob_{res} peers following preoperative diet and SG. The rationale for this conflicting finding is not clear. It is possible that chronically elevated inflammatory status common in Ob_{res} may play a role in reducing FetA which is also a well-known negative acute phase protein. Regardless of insulin-sensitivity group, greater reduction in FetA during the preoperative diet was associated with smaller adipocyte size on the day of surgery. Therefore, decreasing circulating FetA may suggest a reduction in adipocyte size which correlates with improved insulin sensitivity. Because this study only measured adipose tissue at one time point, longitudinal studies of histology changes are needed to support our finding further.

Table 5.1. Baseline characteristics of individuals with insulin-sensitive and insulin-resistant obesity

Variable	Insulin-Resistant Obesity (n=10)	Insulin-Sensitive Obesity (n=10)	p-value
Age, years (range)	44.2 ± 2.1 (36-57)	44.5 ± 2.0 (37-55)	0.920
BMI (kg/m^2)	46.1 ± 1.7 (39-55)	45.9 ± 1.7 (40-54)	0.940
Body Fat (%)	51.9 ± 0.6 (48-54)	52.0 ± 0.6 (48-55)	0.869
TG (mg/dL)	151.4 ± 17.6	134.0 ± 19.4	0.637
TC (mg/dL)	172.3 ± 11.2	207.6 ± 13.4	0.058
HDL (mg/dL)	35.5 ± 3.3	51.3 ± 4.8	**0.005**
LDL (mg/dL)	103.2 ± 9.5	124.9 ± 12.3	0.179
Glucose (mg/dL)	112.2 ± 5.18	95.5 ± 2.2	**0.003**
HOMA-IR	4.0 ± 0.5	1.4 ± 0.1	**<0.0001**

Values are presented as means ± SE. p-values greater than 0.05 are considered statistically significant. Abbreviations: BMI: Body Mass Index; TG: Triglycerides; TC: Total Cholesterol; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance.

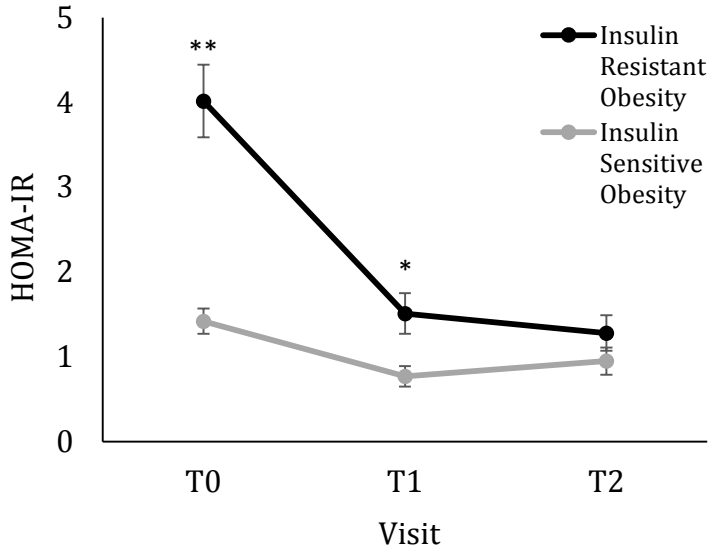


Figure 5.1. HOMA-IR trajectory by insulin resistance status. HOMA-IR was transformed for analysis and back-transformed for graphical representation. Values are adjusted for time and presented as LSmeans ± SE. **p=<0.0001, *p=0.008, p-value for T2:0.22

Table 5.2. Reported baseline dietary intake of individuals with insulin-sensitive and insulin-resistant obesity

Variable	Insulin-Resistant Obesity (n=8)	Insulin-Sensitive Obesity (n=9)	p-value
Total Kcal	1512 ± 215	1908 ± 246	0.302
Kcal/kg	12 ± 1.9	15 ± 2.1	0.244
Carbohydrate (% of total kcal)	41.7 ± 2.7	42.6 ± 2.0	0.777
Protein (% of total kcal)	19.9 ± 0.9	19.3 ± 2.2	0.798
Fat (% of total kcal)	37.6 ± 2.2	37.0 ± 2.3	0.844
Saturated Fat (% of total kcal)	14.0 ± 1.3	10.8 ± 1.2	0.106
PUFA (% of total kcal)	6.8 ± 0.6	9.1 ± 0.9	**0.031**
MUFA (% of total kcal)	13.0 ± 1.1	13.3 ± 0.8	0.820

Values are presented as means ± SE. p-values greater than 0.05 are considered statistically significant. Abbreviations: Kcal: kilocalorie; Kg: kilogram; PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids.

Table 5.3. Weight loss in insulin-sensitive and insulin-resistant obesity

Variable	Insulin-Resistant Obesity (n=10)	Insulin-Sensitive Obesity (n=10)	p-value
Preoperative EBWL	-10.3 ± 1.3	-9.5 ± 1.3	0.675
Postoperative EBWL	-18.4 ± 1.3	-17.1 ± 1.3	**0.0006**
Total EBWL	-29.0 ± 1.5	-26.4 ± 1.5	**0.012**
Preoperative PBF Δ	0.4 ± 0.4	0.4 ± 0.4	0.114
Postoperative PBF Δ	-1.9 ± 0.4	-1.8 ± 0.4	0.904
Total PBF Δ	-1.6 ± 0.4	-1.4 ± 0.4	0.768
Preoperative BMI Δ	-2.3 ± 0.2	-2.0 ± 0.2	**0.045**
Postoperative BMI Δ	-3.7 ± 0.3	-3.3 ± 0.3	**0.025**
Total BMI Δ	-6.0 ± 0.3	-5.2 ± 0.3	**0.006**

Data presented as mean ± SE. Values are adjusted by time and baseline BMI. Abbreviations: EBWL: excess body weight loss based on an ideal BMI of 25 kg/m^2; PBF: percent body fat calculated by direct segmental multi-frequency bioelectrical impedance; BMI: body mass index.

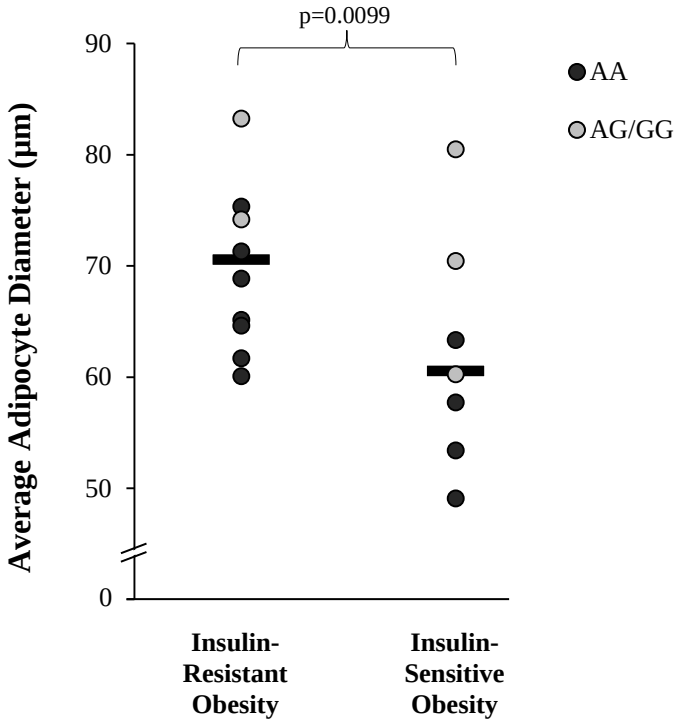


Figure 5.2. Adipocyte diameter by insulin resistance status and toll-like receptor 4 (*TLR4*) (rs4986790) genotype. Group average is represented by black bar. When adjusted for *TLR4* rs4986790 genotype, those with insulin-resistant obesity had larger average adipocyte diameter (70.6 ± 2.2 µm) than those with insulin-sensitive obesity (60.6 ± 2.5 µm).

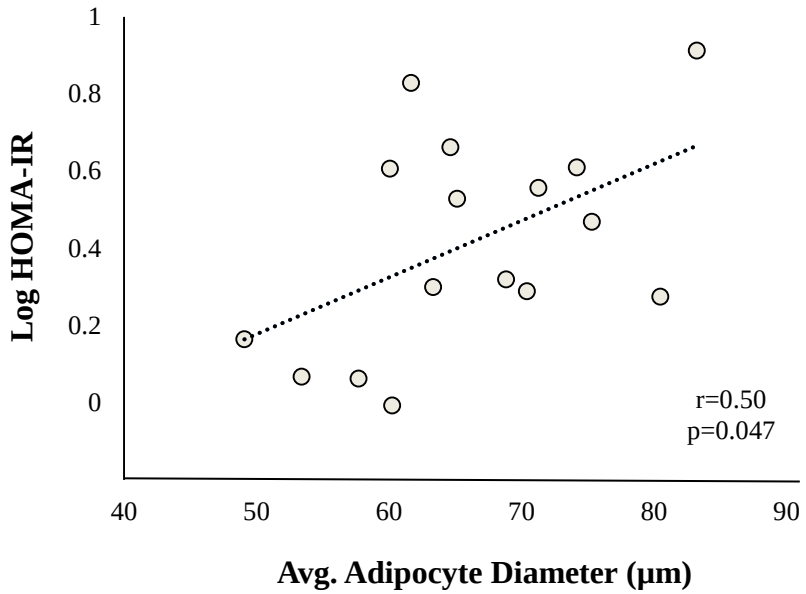


Figure 5.3. Association of adipocyte diameter and baseline HOMA-IR.

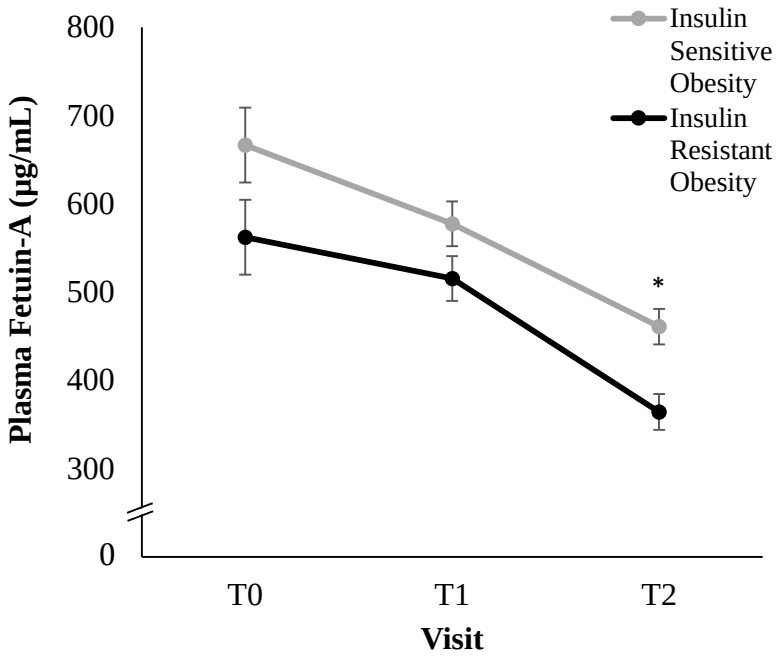
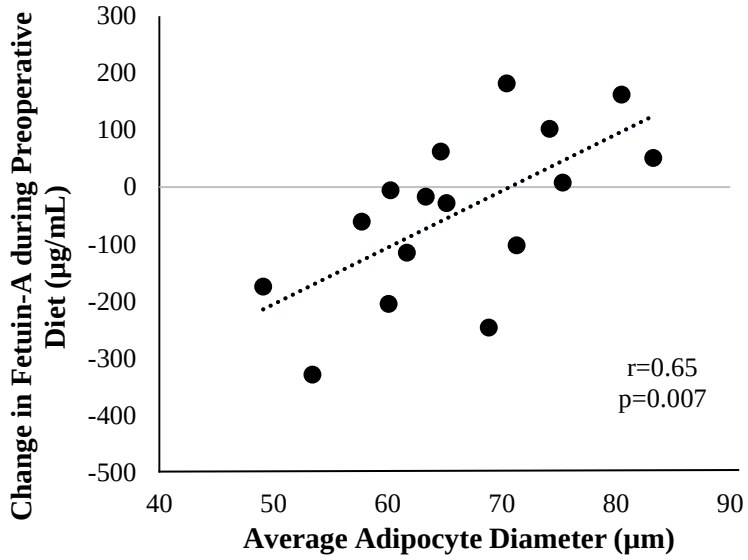


Figure 5.4. Fetuin-A trajectory by insulin resistance status. Data are adjusted for age and presented as LSmeans ± SE. p-value for T0: 0.09, p-value for T1: 0.10, p-value for T2: 0.004.

A.



B.

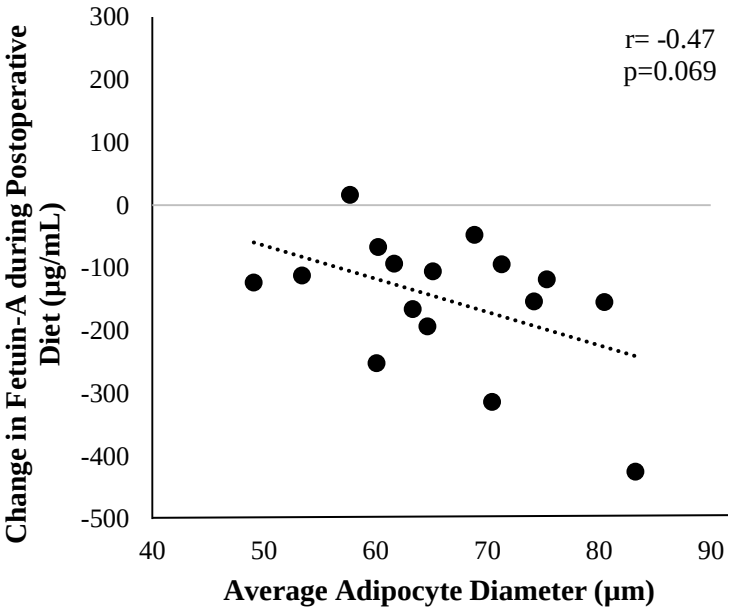


Figure 5.5. Association of adipocyte size on the day of surgery with preoperative and postoperative change in Fetuin-A. Data includes a subset of the I-OWLS cohort which were age- and sex-matched for insulin sensitivity status. For complete cohort data see Appendix 8.

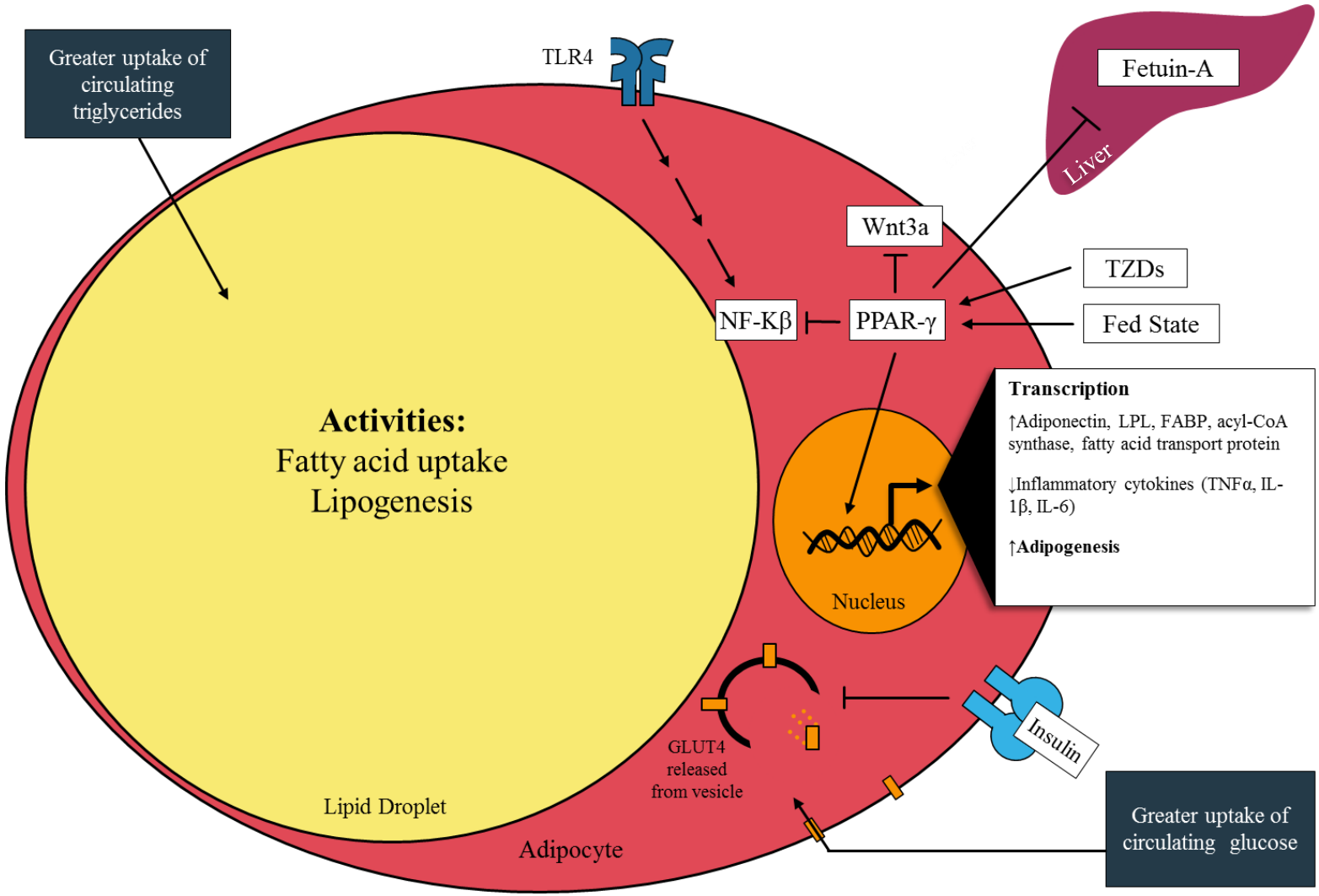


Figure 5.6. Activation of PPAR-γ within the adipocyte. PPAR-γ is activated during the fed state and by Thiazolidinediones (TZD). Activated PPAR-γ inhibits NF-Kβ and WNT3a activity and also stimulates transcription of genes related to lipid transport and metabolism. Thus, fatty acid uptake and lipogenesis in the cell are increased. Transcription of inflammatory cytokines is suppressed. Activation of PPAR-γ increases adipogenesis and reduces hepatic transcription of Fetuin-A. In the fed state, insulin signals glucose transport 4 (GLUT4) to translocate to the cell wall allowing glucose to enter the adipocyte. Figure not drawn to scale. Abbreviations: LPL: lipoprotein lipase; FABP: fatty acid binding protein, TNFα: tumor necrosis factor-alpha; IL: interleukin.

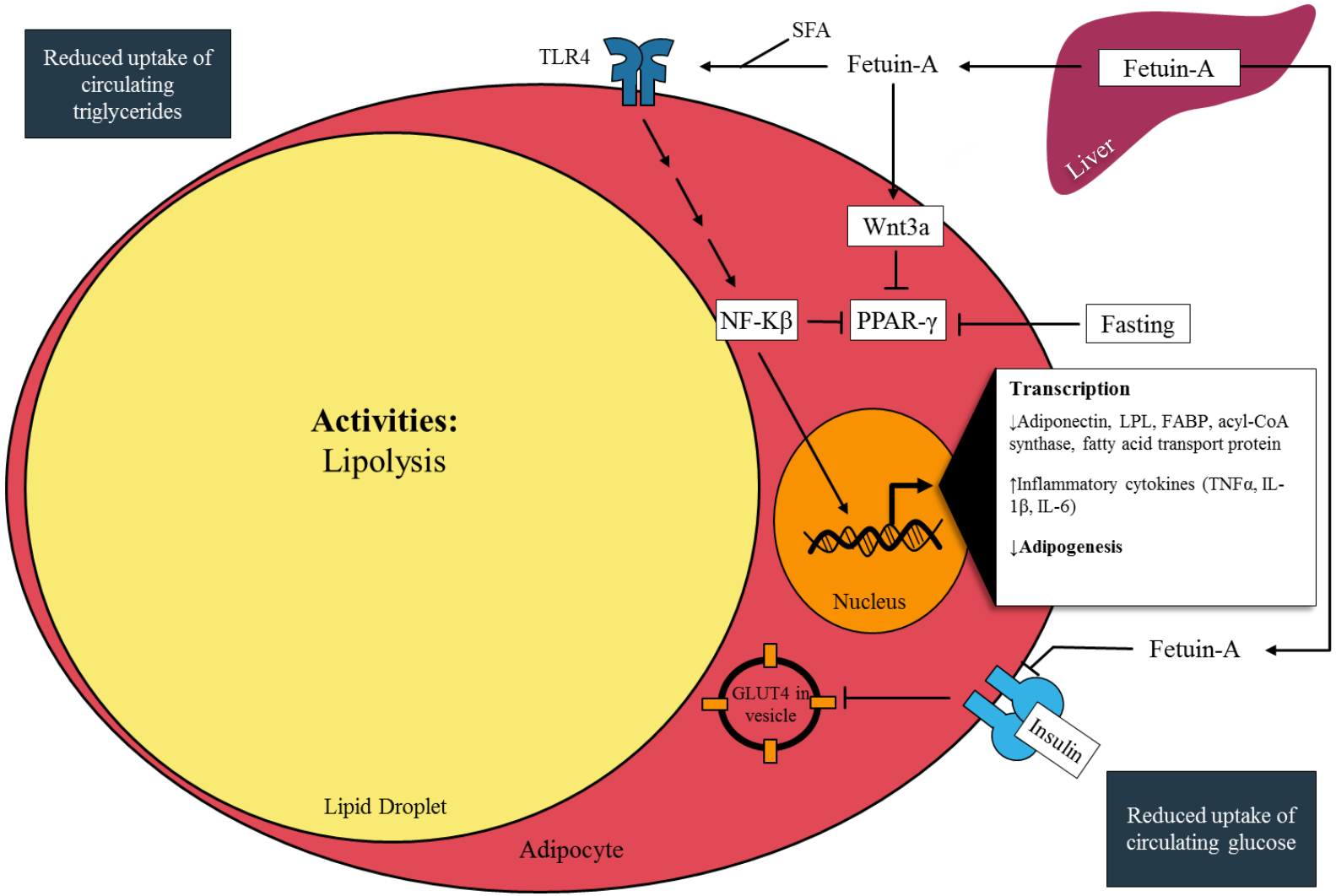


Figure 5.7. Inhibition of PPAR-γ within the adipocyte. PPAR-γ is inhibited during fasting and in response to elevated NF-Kβ and WNT3a signaling. Thus, transcription of genes related to lipid transport and metabolism are reduced. Inhibition of PPAR-γ reduced adipogenesis and allows for greater hepatic release of Fetuin-A (FetA). FetA stimulates Wnt3a which further inhibits PPAR-γ. FetA inhibits insulin receptor tyrosine kinase which prevents glucose transporter (GLUT4) translocation and limits glucose uptake. FetA binds saturated fatty acids (SFA) and stimulates a proinflammatory cascade via toll-like receptor 4. Cytokine release and elevated FetA attract additional monocytes and macrophages into the cellular environment. Overall, this leads to reduced adipogenesis and greater lipolysis, gluconeogenesis and inflammation in adipose tissue. Figure not drawn to scale.

CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

Summary

The *overarching goal* of this research was to understand whether FetA may be a novel marker of obesity and comorbidities. Specifically, we investigated the association between obesity, circulating FetA and *AHSG* polymorphisms and evaluated change in FetA following bariatric surgery. We found that *AHSG* genotype was associated with circulating FetA and triglycerides but not with BMI or waist circumference in a college-aged Mexican population. Genetic variation in *AHSG* (rs4917 and rs2518236) explained 8-10% of the variability in FetA. Next, we investigated the response of FetA to SG, an effective treatment for T2DM. Circulating FetA was significantly reduced following both the preoperative and postoperative period. Total change in FetA was associated with percent weight loss. Unexpectedly, we found that HOMA-IR at all time points was strongly associated with postoperative FetA rather than FetA at the corresponding time point. Contrary to previous studies which suggest a positive association, we found that higher HOMA-IR was associated with lower postoperative FetA. One possible explanation is that individuals with the greatest HOMA-IR are more responsive to changes in FetA whereas those with lower HOMA-IR do not have the same capacity for change.

Considering the significant change in FetA during this period and the association with weight change and insulin resistance, we sought to determine whether individuals with Ob_{res} responded differently to bariatric surgery than those Ob_{sen}. We found that baseline BMI and PBF did not differ between groups, yet, the Ob_{res} group had significantly more excess body weight loss, lower

FetA and larger omental adipocytes. Preoperative reduction in FetA was associated with smaller adipocytes on the day of surgery.

When our results are considered along with the current body of evidence, it appears that FetA is a marker at the interface of insulin resistance, obesity and inflammation. Biomarkers can be used clinically to 1) screen and identify at-risk patients, 2) diagnose patients or 3) indicate disease progression or influence treatment intervention (197). At this time, FetA does not appear to fit strictly in any one of these categories. However, FetA decreases in response to weight loss and insulin sensitivity improvements, and greater FetA change is correlated with smaller adipocyte size, a known contributor to adipose insulin sensitivity (162). These data support the potential role of FetA as a biomarker of insulin sensitivity improvement following lifestyle and bariatric intervention. FetA may also be a promising addition to cumulative risk scores including other known biomarkers of obesity and insulin resistance. As with all research endeavors, the present research has revealed gaps in our understanding of not only FetA but also obesity and bariatric surgery.

Future Directions

Future directions for FetA research

Future objectives in FetA should include establishing reference ranges for FetA and improving our understanding of FetA and inflammation. Because FetA is influenced by many disease states such as T2DM, non-alcoholic fatty liver disease and end-stage renal disease, it is challenging to set standard values and identify disease-specific cut points. An additional complication to our understanding of "normal" FetA is the complex interactions of FetA and inflammation. FetA is a negative acute phase protein that also promotes proinflammatory cytokine release from adipocytes,

acts as a chemokine and reduces expression of adiponectin, a known anti-inflammatory agent (94,97,98,167,198). Furthermore, the active form of FetA, phosphofetuin, has seldom been measured. One study demonstrated that phosphofetuin, but not FetA was significantly reduced by four days of exercise. Thus, it is possible that our results may have differed if we were able to measure phosphofetuin (199).

While the results presented in Chapter 5 concerning the association of FetA change and adipocyte size are thought-provoking, the longitudinal correlation of FetA with adipose size has not been established. Although adipose is difficult to obtain, especially omental adipose, trials of at least two time points would better clarify whether adipocyte hypertrophy is the cause or consequence of FetA change. Chapter 4 concluded that FetA was significantly reduced following preoperative diet. This decline was not attributed to any specific macro- or micronutrient. Due to the possibility of dietary reporting errors, the use of controlled feeding trials and metabolomics may elucidate this mechanism and dietary correlates. We presented evidence that FetA is reduced six weeks following SG. Although FetA has been shown to remain lower than baseline for up to a year postoperatively, the trajectory of FetA after this point remains unknown (119).

Future directions for bariatric surgery

Bariatric surgery is a rapidly evolving practice (42). Further clinical studies are needed to evaluate long-term outcomes, efficacy in specific populations and novel procedures and devices. Recently approved weight loss devices such as the ReShape Dual Balloon System and the AspireAssist obesity device, offer additional, reversible options to patients with morbid obesity. However, studies of long-term safety and efficacy are needed. In general, the study of bariatric outcomes would benefit from studies which seek to evaluate health benefits beyond weight loss.

Research to date has not uncovered preoperative factors which predict successful weight loss. One topic of interest to all weight loss interventions is how chronic weight cycling influences an individual's ability to lose and keep off excess weight. A recent animal study demonstrated that weight cycling may influence fat distribution (200). Thus, making this an important variable to study in the understanding of adipocyte function.

To understand weight cycling history in the I-OWLS cohort, we collected follow-up surveys from a subset of participants one year postoperatively (n=22). On average, these patients reported trying 12.4±5.0 different weight-loss strategies before surgery. The most common weight loss strategies attempted were counting calories, reducing intake of sweets and limiting portions (Appendix 10). Risky behaviors such as taking diet pills (59%), completing cleanses (27%), taking laxatives (23%), or smoking more cigarettes (9%) were also reported. I-OWLS participants reported purposely losing 10 pounds or more 9.8±8.9 times in their life. They also reported regaining as much as 10 pounds they had previously lost 10.3±8.6 times in their life. These data suggests that weight cycling, also known as yo-yo dieting, is common practice in bariatric populations. To date, it is not clear whether previous dieting attempts influence ability to lose weight following bariatric surgery.

An additional area of uncertainty is the influence of genetics on post-bariatric outcomes. Genetics may influence outcomes by altering key proteins involved in the mechanisms of action for bariatric surgery. As suggested in the literature review, over 150 SNPs have been associated with human obesity (19). Many of these genetic variants were identified in cross-sectional analyses of large cohorts. Evidence for the influence of genes on weight loss trajectories remains sparse. Available studies focus primarily on change in BMI and fail to consider change in body composition, insulin sensitivity and other markers of metabolic health. If genetic variants are found to be useful

predictive variables, genetic analysis could be incorporated into preoperative care to improve procedure selection, maximize patient outcomes and avoid unnecessary risk in those who may have less success. Genetic markers that are found to be associated with bariatric outcomes may also reveal important insights into the mechanisms of action behind bariatric procedures. One example is the post-surgical reduction in the hunger hormone, ghrelin.

Ghrelin is secreted by the stomach and thus, is decreased following SG (57). This may contribute to the reported reduction in hunger postoperatively (55). To determine the role of ghrelin polymorphisms in surgery-induced weight loss, we conducted a preliminary, exploratory analysis in the I-OWLS cohort described previously. Genomic DNA was extracted from white blood cells and total plasma ghrelin was measured using a commercially available kit (EMD Millipore, Billerica, MA). We found that T/T-homozygotes at the ghrelin polymorphism rs27647 lost 1.3±0.6 BMI units more than C/T heterozygotes and 1.4±0.7 BMI units more than C/C-homozygotes postoperatively (p=0.04) (Appendix 11). Postoperative body fat loss was significantly higher in carriers of the rare rs696217 G/T genotype (p=0.05) (Unpublished data presented by Robinson et. al, ObesityWeek 2016). Although the sample size of the I-OWLS cohort was not adequately powered to detect genetic associations, the association of rs696217 with greater weight loss was recently replicated in a larger cohort of RYGB patients (201).

Bariatric research is also moving toward a better understanding of efficacy in specific, unique populations. As more children are developing morbid obesity, studies concerning the safety and efficacy of bariatric procedures in children and adolescents are warranted (1). It is estimated that the number of bariatric procedure performed on adolescents tripled between 2000 and 2003 and has remained steady since this time (202). Specifically, 2.4 per 100,000 adolescents received bariatric surgery in 2009. Of which, 68% selected RYGB. Although large observational studies

such as Teen-Longitudinal Assessment of Bariatric Surgery (Teen-LABS) are in progress, long-term follow-up remains a challenge especially in this transitional population (203).

The present study found that six weeks following SG, individuals with ob_{res} had significant improvements in HOMA-IR. In fact, individuals with ob_{res} had similar HOMA-IR values as those with ob_{sen} at this time point. Large trials have concluded that 80% of patients who receive SG experience diabetes resolution (44,45). Bariatric procedures have even been suggested to be the "cure" for diabetes (204). Thus, the use of bariatric surgery in individuals with T2DM and a lower BMI (30-35 kg/m^2) has been proposed. One study found that the use of RYGB in patients with class I obesity led to an 88% resolution of diabetes six years postoperatively (205). This demonstrates that bariatric surgery is an effective treatment for T2DM in adults with class I obesity.

Current eligibility criteria (>35 kg/m^2 with comorbidities or >40 kg/m^2) may not be appropriate for all ethnic groups. At a BMI of 30 kg/m^2, diabetes incidence is 11% of Caucasians, 25% of African Americans, 30% of Asians and 40% of South Asians (206). If BMI category cut points are adjusted for ethnic groups to match the diabetes incidence risk of Caucasians at a BMI of 30 kg/m^2 (11%), BMI cut points would be 26 kg/m^2 for African Americans, 25 kg/m^2 for Asians and 24 kg/m^2 for South Asians (206). Therefore, although these individuals are at a greater risk for diabetes, they may not have the option of bariatric surgery based on current eligibility criteria from the American Society for Metabolic and Bariatric Surgery (207,208).

Criticism of the BMI is common in obesity research. Many question whether diagnostic criteria for obesity should rest solely on BMI (209). As we and others have demonstrated, metabolic health can vary even at a similar BMI values (31,154,159). BMI fails to account for body composition

(lean body mass to fat ratio), fat distribution and ethnic-specific differences (209,210). Additionally, it is now widely recognized that adipose tissue possesses the ability to influence other organs in the body through the release of cytokines and adipokines and through mechanical stress (211). It is not entirely clear why some individuals can evade these processes while other experience their deleterious effects. However, strong evidence connects adipocyte hypertrophy with suboptimal adipocyte function (162,163). Future research should seek to uncover non-invasive biomarkers which predict omental adipocyte size in an attempt to better understand adipocyte health. Although much remains to be discovered, the present research contributes meaningful data towards a better understanding of FetA in insulin sensitivity, response to bariatric surgery and adipocyte health.

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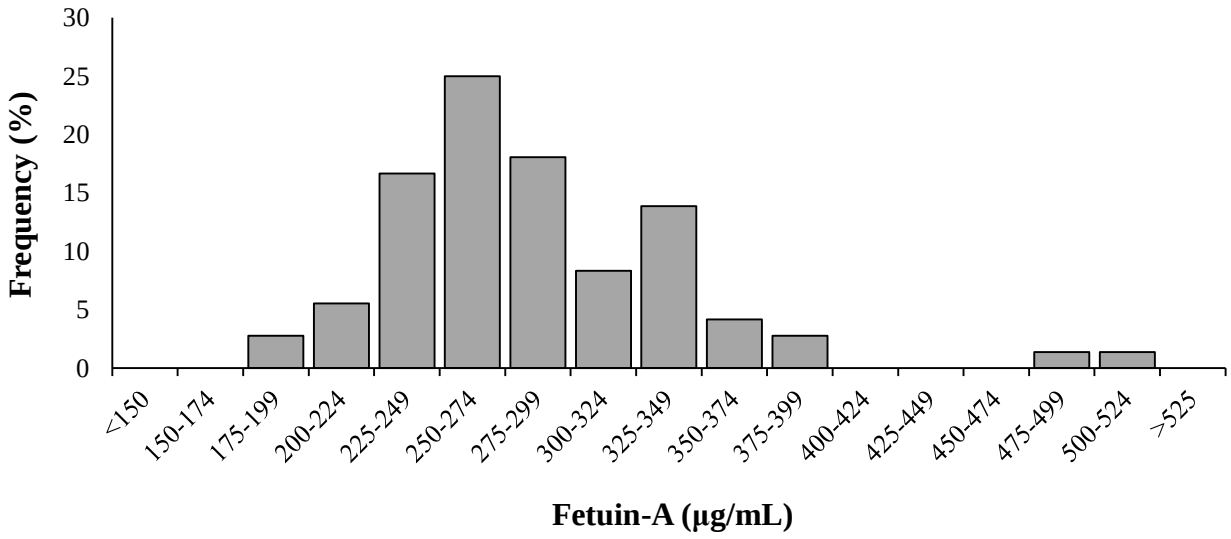
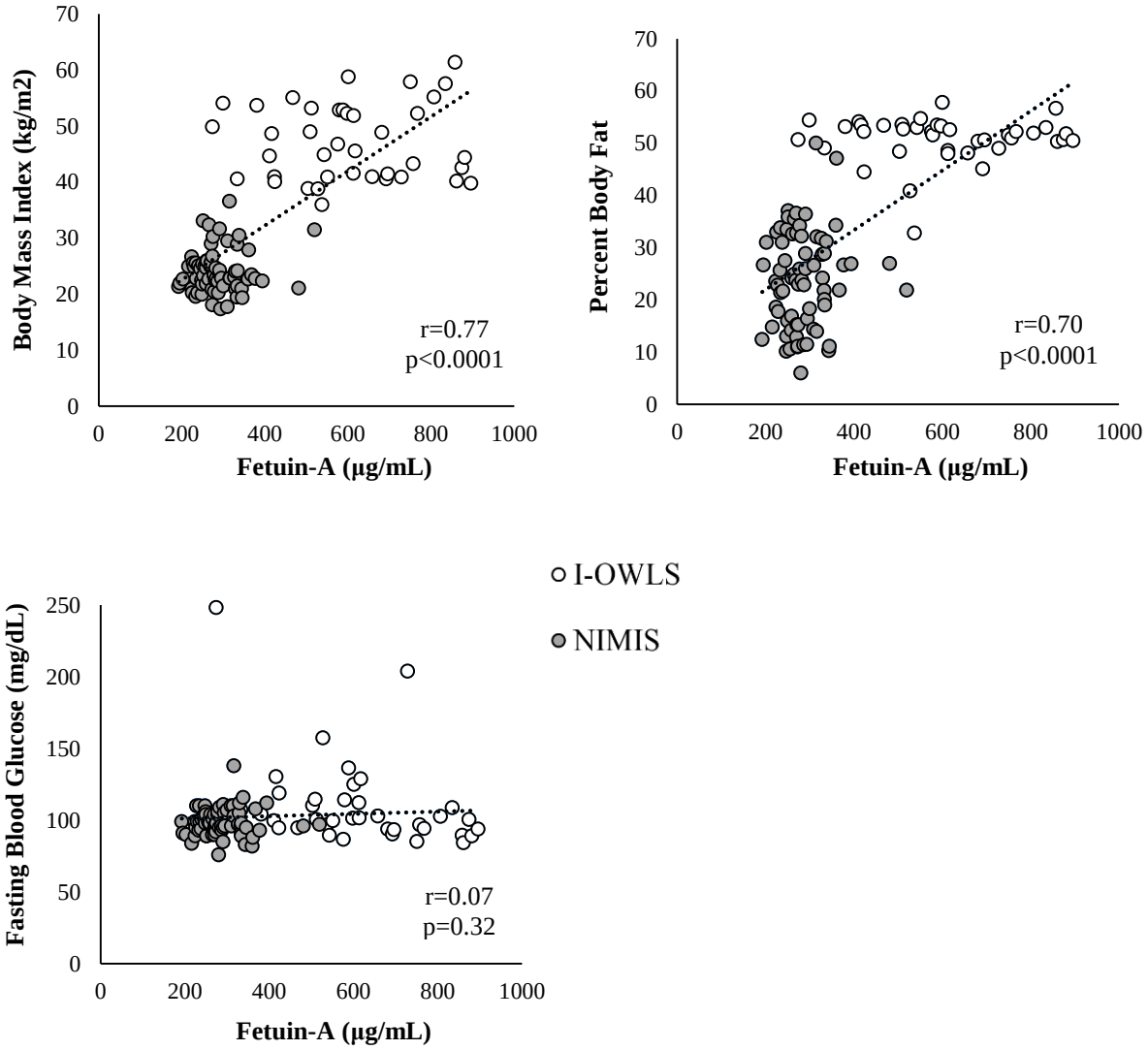
APPENDIX 1. DISTRIBUTION OF FETUIN-A IN NORMAL WEIGHT POPULATION



Fetuin-A distribution in normal weight population. The Non-Invasive Measures of Insulin Sensitivity (NIMIS) cohort included 72 non-diabetic adults (37 males) recruited from the University of Illinois at Urbana-Champaign. Age ranged from 18-35 years old. Fetuin-A was measured using a commercially-available enzyme-linked immunosorbent assays from BioVendor™ (Asheville, NC). Average (± SD) Fetuin-A was 287 ± 57 µg/mL.

APPENDIX 2. BODY MASS INDEX, PERCENT BODY FAT, FASTING BLOOD GLUCOSE AND FETUIN-A IN NORMAL WEIGHT AND OBESE POPULATIONS



Association of body mass index, percent body fat and fasting blood glucose with Fetuin-A in normal weight and obese population. The Non-Invasive Measures of Insulin Sensitivity (NIMIS) cohort included 72 adults (37 males) recruited from the University of Illinois at Urbana-Champaign. Age ranged from 18-35 years old. The Inflammatory Outcomes of Weight Loss Surgery (I-OWLS) study included 39 sleeve gastrectomy (SG) candidates prior to preoperative diet and prior to SG. In both studies, Fetuin-A was measured using a commercially-available enzyme-linked immunosorbent assay from BioVendor™ (Asheville, NC). Body fat percentage was measured using bioelectrical impedance analysis (InBody230, Biospace, Cerritos, CA).

APPENDIX 3. CORRELATION TABLE OF PREOPERATIVE AND POSTOPERATIVE CHANGES IN FETUIN-A AND BODY COMPOSITION

Women (n=31)

Preoperative Change

Preoperative Change	BMI	Percent BW	Percent EBWL	PBF	FetA	Time
HOMA-IR	0.11 0.576	0.17 0.378	0.19 0.308	0.17 0.381	-0.08 0.661	0.19 0.316
BMI		0.92 **<.0001**	0.73 **<.0001**	0.00 0.996	0.38 **0.039**	0.28 0.128
BW			0.94 **<.0001**	0.15 0.429	0.39 **0.032**	0.26 0.161
EBW				0.30 0.097	0.37 **0.045**	0.21 0.263
PBF					0.04 0.813	0.27 0.141
FetA						-0.03 0.867

Postoperative Change

Preoperative Change	BMI	Percent BW	Percent EBWL	PBF	FetA	Time
HOMA-IR	0.02 0.897	0.01 0.944	0.10 0.608	-0.10 0.599	-0.18 0.343	-0.20 0.273
BMI		0.84 **<.0001**	0.58 **0.001**	0.21 0.257	0.48 **0.008**	-0.70 **<.0001**
BW			0.91 **<.0001**	0.15 0.422	0.43 **0.018**	-0.54 **0.002**
EBW				0.18 0.321	0.27 0.141	-0.43 **0.016**
PBF					0.05 0.804	-0.21 0.269
FetA						-0.15 0.418

Men (n=9)

Preoperative Change

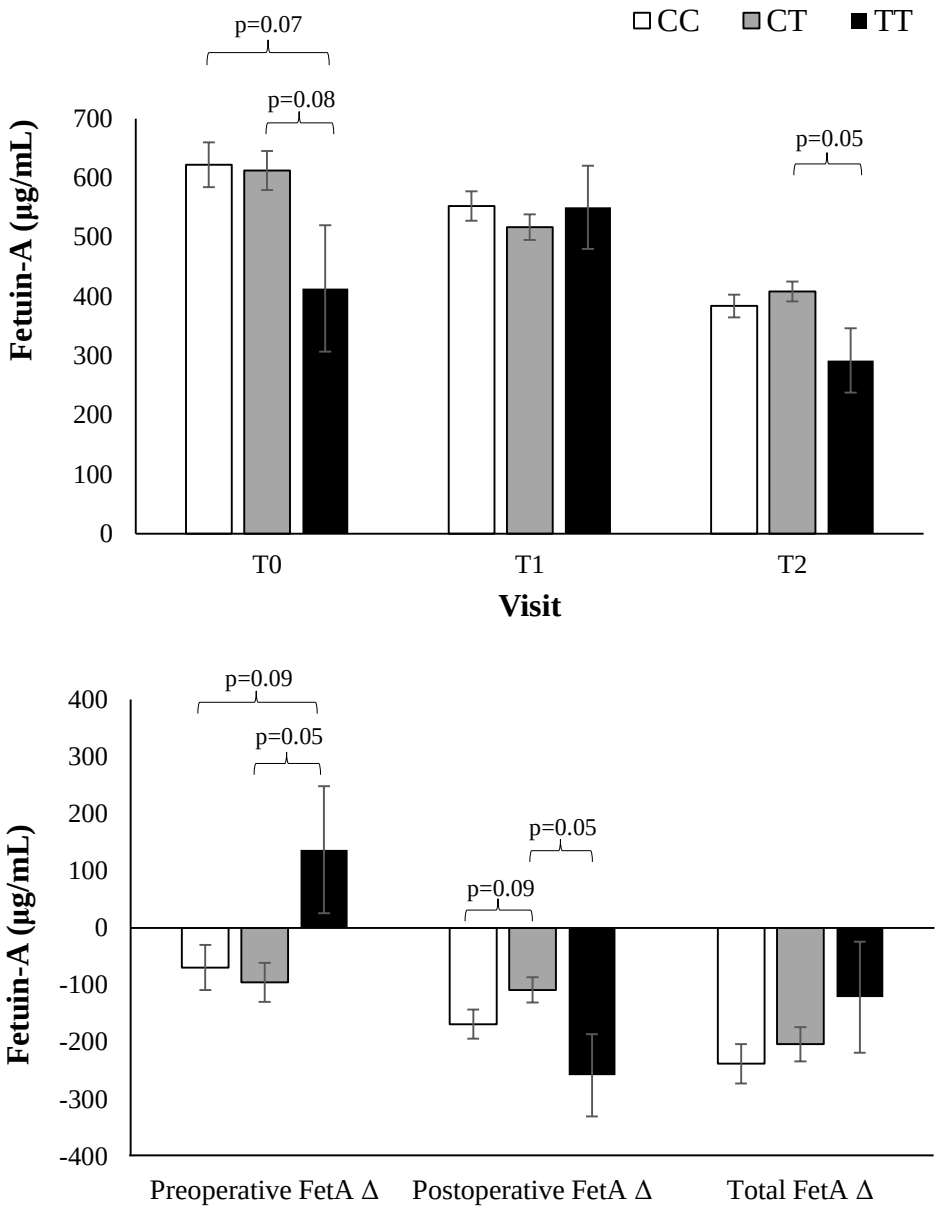
Preoperative Change	BMI	Percent BW	Percent EBWL	PBF	FetA	Time
HOMA-IR	0.13 0.756	0.18 0.668	0.32 0.491	0.23 0.586	-0.57 0.141	-0.40 0.326
BMI		0.86 **0.003**	0.16 0.713	-0.86 **0.003**	0.17 0.668	-0.02 0.969
BW			0.78 **0.022**	-0.70 **0.035**	0.02 0.969	-0.36 0.345
EBW				0.06 0.882	-0.37 0.361	-0.66 0.074
PBF					-0.42 0.259	-0.30 0.431
FetA						0.16 0.682

Postoperative Change

Postoperative Change	BMI	Percent BW	Percent EBWL	PBF	FetA	Time
HOMA-IR	0.63 0.097	0.23 0.576	0.23 0.618	0.30 0.465	0.15 0.719	-0.50 0.204
BMI		0.78 **0.013**	0.46 0.257	0.21 0.583	0.15 0.691	-0.68 **0.046**
BW			0.91 **0.002**	0.36 0.338	0.39 0.295	-0.80 **0.010**
EBW				0.95 **0.0004**	0.23 0.588	-0.83 **0.010**
PBF					-0.25 0.515	-0.70 **0.035**
FetA						-0.27 0.485

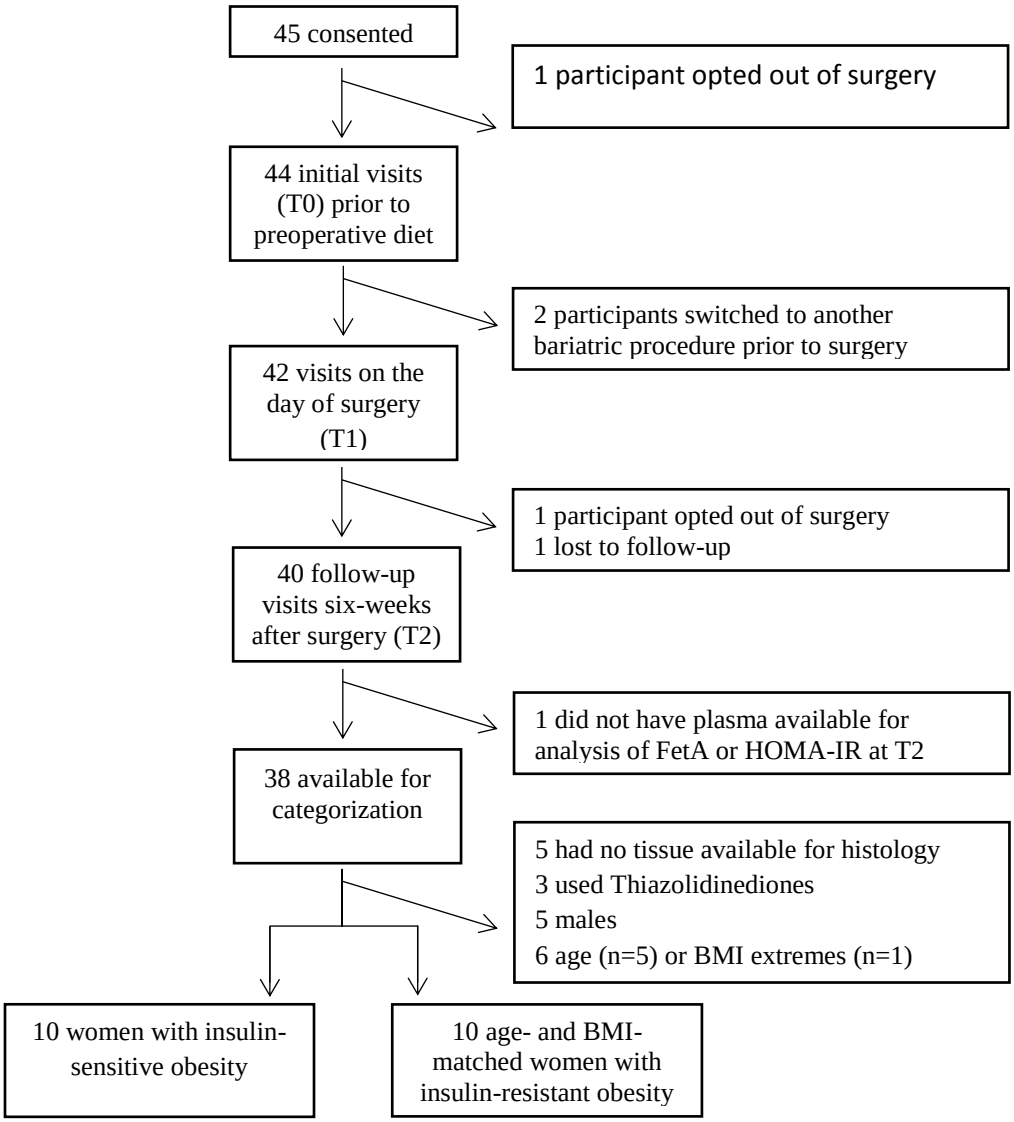
Each cell contains the Pearson correlation coefficient (r) followed by the p-value for the correlation. P<0.05 were considered statistically significant. Abbreviations: HOMA-IR: homeostatic model assessment for insulin resistance; BMI: body mass index; BW: body weight; EBWL: excess body weight loss; PBF: percent body fat; FetA: Fetuin-A. Time is a measure of days between visits.

APPENDIX 4. FETUIN-A CHANGE BY ALPHA2-HEREMANS-SCHMID GLYCOPROTEIN (*AHSG*) POLYMORPHISM



Change in Fetuin-A (FetA) by *AHSG* (rs4917) genotype. In chapter three, *AHSG* genotype was shown to be associated with circulating FetA. Thus, I-OWLS participants were stratified by rs4917 genotype for this exploratory analysis of the association between *AHSG* genotype and age-adjusted FetA change (Δ). P-values<0.1 are included in the figure although only p-values<0.05 are considered statistically significant. Genotype distribution was: CC (n=16), CT (n=22) and TT (n=2) thus, replication in larger cohorts is needed.

APPENDIX 5. PARTICIPANT CATEGORIZATION AS INSULIN-SENSITIVE OR INSULIN-RESISTANT



Categorization of I-OWLS participants as insulin-sensitive or insulin-resistant. Participants were considered to have Ob_{res} if they had a baseline HOMA-IR>2.5, a documented diagnosis of prediabetes or diabetes or current prescription of anti-diabetic medications. If these characteristics were not present, participants were considered to have Ob_{sen}. Patients with Ob_{res} were matched by age, sex, and BMI to Ob_{sen} patients. Participants taking Thiazolidinediones (TZDs) which alter PPAR-γ and thus, may alter adipocyte size were excluded.

APPENDIX 6. COMPARISON OF PRE- AND POSTOPERATIVE DIETARY INTAKE BETWEEN INSULIN-SENSITIVE AND INSULIN-RESISTANT OBESITY

Preoperative diet comparison by insulin-sensitive and insulin-resistant obesity status

Variable	Insulin-Resistant Obesity (n=10)	Insulin-Sensitive Obesity (n=10)	p-value
Total Kcal	914 ± 43	1059 ± 93	0.166
Kcal/kg	7.6 ± 0.4	8.7 ± 0.8	0.241
Carbohydrate (% of total kcal)	60.0 ± 1.7	63.3 ± 2.1	0.238
Protein (% of total kcal)	24.9 ± 1.1	21.3 ± 1.1	**0.033**
Fat (% of total kcal)	15.1 ± 2.1	15.4 ± 1.7	0.907
Saturated Fat (% of total kcal)	3.3 ± 0.4	4.0 ± 0.4	0.178
PUFA (% of total kcal)	3.6 ± 0.9	3.3 ± 1.0	0.810
MUFA (% of total kcal)	6.0 ± 1.2	5.6 ± 0.8	0.772

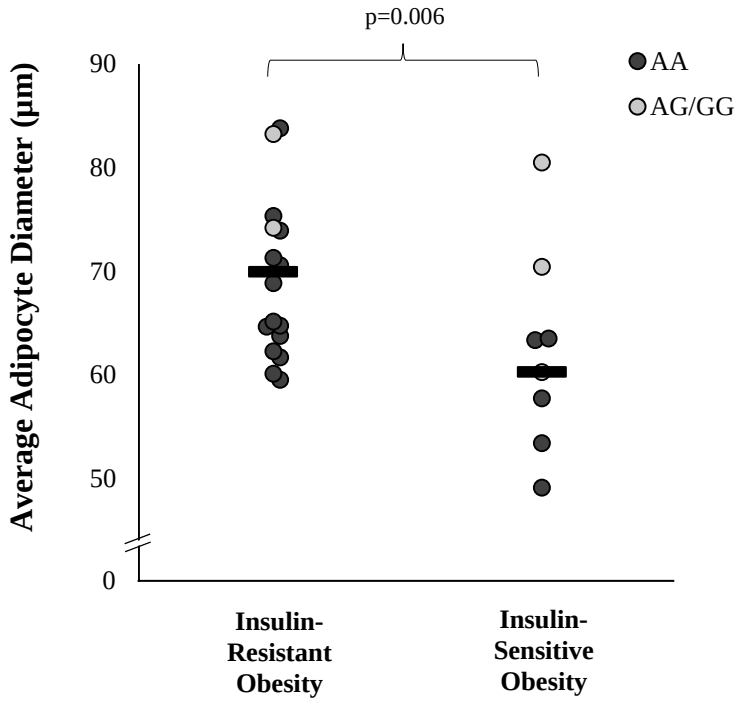
Three-day food logs were collected on the day of surgery. Values are presented as means ± SE. p-values greater than 0.05 are considered statistically significant. Abbreviations: Kcal: kilocalorie; Kg: kilogram; PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids.

Postoperative diet comparison by insulin-sensitive and insulin-resistant obesity status

Variable	Insulin-Resistant Obesity (n=10)	Insulin-Sensitive Obesity (n=9)	p-value
Total Kcal	640.6 ± 89.7	867.1 ± 134.6	0.166
Kcal/kg	5.9 ± 1.0	7.7 ± 1.2	0.281
Carbohydrate (% of total kcal)	34.5 ± 5.1	32.2 ± 3.8	0.726
Protein (% of total kcal)	32.5 ± 2.9	30.6 ± 2.4	0.623
Fat (% of total kcal)	32.4 ± 3.3	37.1 ± 2.8	0.292
Saturated Fat (% of total kcal)	10.3 ± 1.9	11.1 ± 1.9	0.779
PUFA (% of total kcal)	4.9 ± 0.9	7.6 ± 1.2	0.076
MUFA (% of total kcal)	12.2 ± 1.1	13.7 ± 1.4	0.373

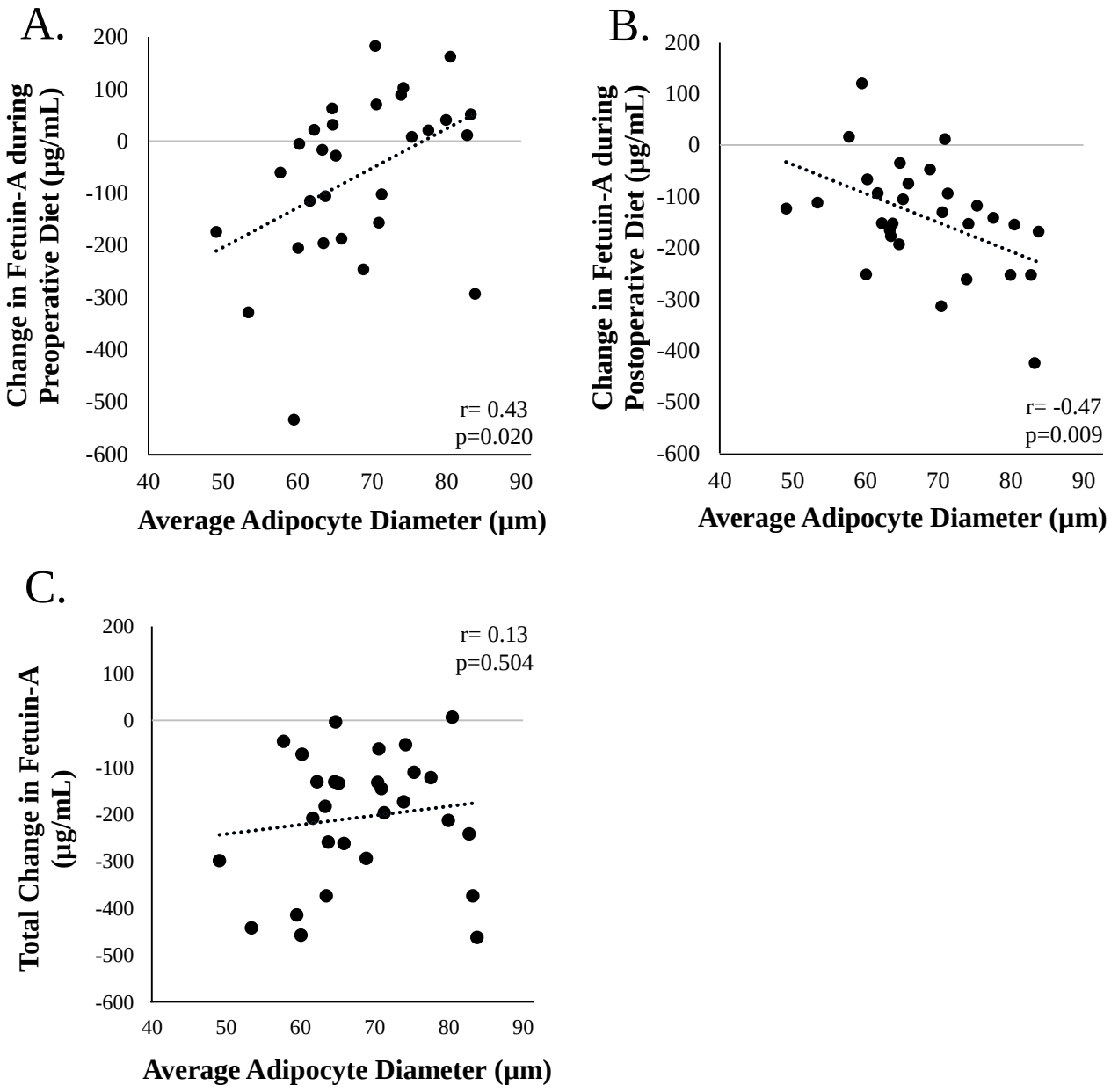
Three-day food logs were collected six weeks following surgery. Values are presented as means ± SE. p-values greater than 0.05 are considered statistically significant. Abbreviations: Kcal: kilocalorie; Kg: kilogram; PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids.

APPENDIX 7. ADIPOCYTE DIAMETER BY INSULIN RESISTANCE STATUS AND TOLL-LIKE RECEPTOR 4 GENOTYPE



Adipocyte diameter by insulin resistance status and toll-like receptor 4 (rs4986790) genotype in the I-OWLS cohort. Participants with incomplete data or current prescription for Thiazolidinediones were excluded from this analysis. Thus, hematoxylin and eosin stained tissue samples were available from 24 participants in the I-OWLS cohort. Data represents all available samples stratified by insulin status but unmatched for other demographic variables such as sex, age or BMI. Group average is represented by black bar.

APPENDIX 8. ASSOCIATION OF OMENTAL ADIPOCYTE DIAMETER AND CHANGE IN FETUIN-A.



Association of adipocyte size on the day of surgery with preoperative, postoperative and total change in Fetuin-A. Data includes all participants of the I-OWLS cohort which had omental adipose tissue available for H&E staining (n=29).

APPENDIX 9. SURVEY MEASURES COLLECTED FROM THE I-OWLS LONGITUDINAL COHORT

Measure	T0	T1	T2	T3
Demographics Ethnicity, sex, age	X			
Self-reported weight	X	X	X	X
Current diagnosis of common obesity-associated diseases	X	X	X	X
Family history of common obesity-associated diseases	X			
Perceived age and cause of excess weight gain	X			
Children's Body Image Scale and Adult Body Size Guide [212,213]	X			
Health beliefs and perceived barriers [Modified from (214)]	X			X
International Physical Activity Questionnaire [215]	X	X	X	X
Patient Health Questionnaire (PHQ-9) [216]	X	X	X	X
Three-day food log and nutritional supplement use	X	X	X	X
Additional demographics Marital status, education level				X
Dieting strategies [Modified from (6)]				X
Weight cycling patterns [217]				X
Bariatric support group attendance				X
Outcome satisfaction survey				X
Sleep Quality Assessment [218]				X
Taste and Smell Questionnaire [Modified from (219,220)]				X
Three-Factor Eating Questionnaire [221]				X

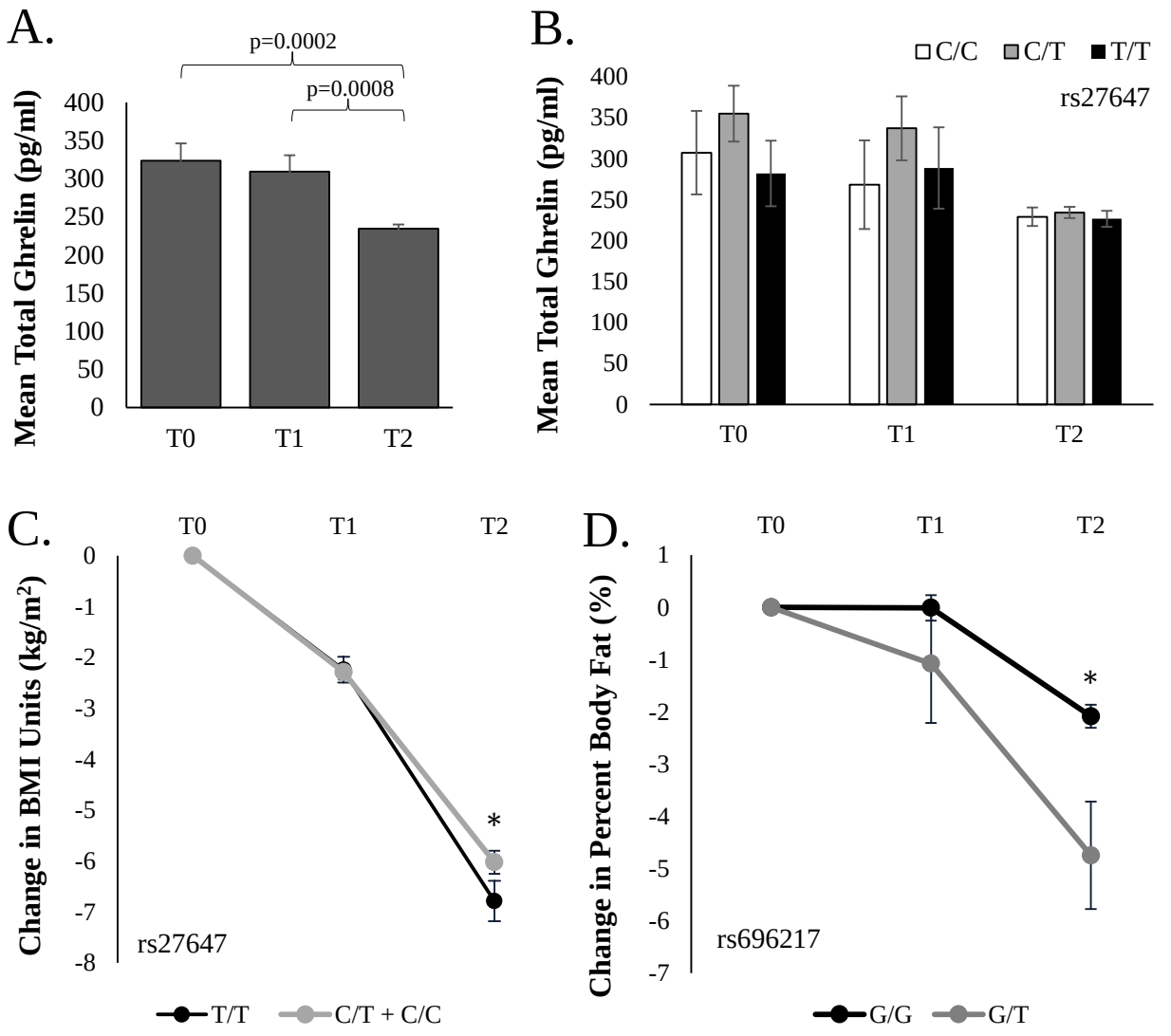
An "X" indicates that this survey or measure was collected from participants at the given time point: T0: baseline, T1: the morning of surgery, T2: six-weeks postoperatively and T3: one year postoperatively.

APPENDIX 10. WEIGHT-LOSS STRATEGIES USED BY I-OWLS COHORT PRIOR TO BARIATRIC SURGERY

Weight-Loss Strategy	N	Percentage
Counting calories	19	86%
Reducing intake of sweets	19	86%
Limiting portions	18	82%
Consuming low-calorie foods	15	68%
Reducing intake of carbohydrates	15	68%
Avoiding eating after a certain time of night	15	68%
Eating packaged diet meals	15	68%
Keeping a food log	14	64%
Decreasing fat intake	14	64%
Reducing snacking	14	64%
Skipping meals	14	64%
Taking diet pills	13	59%
Increasing physical activity or exercise	12	55%
Increasing fruit and vegetable consumption	11	50%
Using an appetite suppressant	11	50%
Consuming a liquid diet	7	32%
Eating slower	6	27%
Fasting for a day	6	27%
Completing a "cleanse" or detox	6	27%
Taking caffeine pills	6	27%
Taking laxatives	5	23%
Reducing intake of meat	4	18%
Reducing alcohol intake	4	18%
Fasting for more than a day	3	14%
Using diuretics	3	14%
Increasing cigarette smoking	2	9%
Purging	1	5%

Retrospective report of weight-loss strategies used by I-OWLS cohort prior to sleeve gastrectomy. One year following sleeve gastrectomy, participants of the I-OWLS cohort were asked to complete an online survey concerning preoperative dieting strategies and weight cycling. On average, survey respondents (n=22), attempted 12.4(±5.0) different weight-loss strategies prior to surgery (range: 4-21 strategies). Respondents reported purposefully losing 10 lbs or more 9.8(±8.9) times in their life and regaining as much as 10 lbs they had previously lost 10.3(±8.6) times in their life.

APPENDIX 11. WEIGHT LOSS FOLLOWING SLEEVE GASTRECTOMY IS INFLUENCED BY GHRELIN GENE POLYMORPHISMS



Exploratory analysis of circulating ghrelin, weight loss and ghrelin gene polymorphisms in the I-OWLS cohort (n=39). Fasting total ghrelin was measured in plasma using enzyme-linked immunosorbent assays. Participants were genotyped for two ghrelin gene polymorphisms (rs27647 and rs696217). Values are presented as means ± SE. **A.** Ghrelin was significantly reduced following sleeve gastrectomy but not preoperative diet. **B.** When stratified by ghrelin genotype (rs27647), circulating ghrelin was not significantly different at each time point although variability was reduced at T2. **C.** T/T-homozygotes at rs27647 lost 1.3 ± 0.6 BMI units more than C/T heterozygotes and 1.4 ± 0.7 BMI units more than C/C-homozygotes postoperatively (p=0.04). N=31. Values were adjusted by sex, age, time interval and baseline BMI. **D.** Postoperative body fat loss was significantly higher in carriers of the rare rs696217 G/T genotype (p=0.05). Values were adjusted by sex, age, time interval and baseline percent body fat. This data was presented at the ObesityWeek conference in 2016 (New Orleans, LA) and received first place in the Early Career Education Theater and was the rated top abstract in the Clinical Management of Obesity Section.

APPENDIX 12. PROTOCOL FOR THE MEASUREMENT OF PLASMA FETUIN-A USING COMMERCIALLY-AVAILABLE ENZYME-LINKED IMMUNOSORBENT ASSAYS

Department of Food Science and Human Nutrition	Protocol No.	SOP-MT-102
Standard Operating Procedure Title: **Measuring Plasma Fetuin-A using ELISA**	Version No. Issue Date	002 4/6/2015
Written by: Katie N. Robinson		

1. **OBJECTIVE**

1.1. This procedure outlines the measurement of circulating Fetuin-A using the BioVendor ELISA kit. The kit measures Fetuin-A in serum and plasma (collected in EDTA, citrate or heparin tubes). Samples should be assayed immediately after collection or should be stored at -80°C.

NOTE: Blood is considered a potentially infectious material therefore caution shall be excised when handling samples

2. **SCOPE**

2.1. This SOP is applicable to all research collaborators.

3. **RESPONSIBILITY**

3.1. It is the responsibility of the principal investigator (PI) in conjunction with the research collaborators to ensure that all blood samples are handled in a proper manner.

3.2. It is the responsibility of the PI in conjunction with research collaborators to ensure that all research participants are adequately instructed on the procedures to be followed for analyzing blood samples.

3.3. All reagents should be stored at 2-8°C prior to use. Opened reagents are stable for 3 months. Check expiration date prior to use.

4. **PREPARATION of MATERIALS**

4.1. Label 1.5mL tubes for the dilutions (two tubes for each sample, labeled A and B) and for the standard curve (one tube for each: 40 ng/ml, 20 ng/ml, 10 ng/ml, 5 ng/ml and 2 ng/ml).

4.2. Thaw reagents for one hour at room temp. Thaw samples on ice.

4.3. **Dilute Dilution Buffer Concentrate (10x)** ten-fold in distilled water to prepare a 1x working solution. Check the refrigerator for previously-made buffer.

4.3.1. In a 250ml glass container, with a lid, you will add 20 ml of Dilution Buffer Concentrate (10x) + 180 ml of distilled water for use in all 96-wells.

4.3.2. Add half of the distilled water, then the dilution buffer. Rinse the dilution buffer container with remaining distilled water. Invert to mix. Avoid making bubbles.

4.3.3. Stability and storage: The diluted Dilution Buffer is stable 1 month when stored at 2-8°C.

4.4. **Prepare samples.** Mix thoroughly thawed samples just prior to the assay and avoid repeated freeze/thaw cycles, which may cause erroneous results. Make note of samples with hemolysis or lipemia.

4.4.1. First, add 990 µl of Dilution Buffer into each 1.5mL tube.

4.4.2. Dilute samples 10 000x with Dilution Buffer just prior to the assay in two steps as follows:

4.4.3. Dilution A (100x): Add 10 µl of sample into the "A" set of tubes (pre-filled with 990 µl of Dilution Buffer) and mix well (not to foam). Vortex is recommended.

4.4.4. Dilution B (100x): Add 10 µl of Dilution A into the "B" set of tubes (pre-filled with 990 µl of Dilution Buffer) to prepare final dilution (10 000x) and mix well (not to foam). Vortex is recommended.

4.4.5. Stability and storage: Do not store the diluted samples.

4.5. Refer to the Certificate of Analysis for current volume of Dilution Buffer needed for reconstitution of standard.

4.6. **Reconstitute the lyophilized Master Standard** with Dilution Buffer just prior to the assay with 1.3ml of dilution buffer to obtain a concentration of 100ng/ml. Invert to mix. Let it dissolve at least **15 minutes** with occasional gentle shaking (not to foam).

4.7. **Quality Controls HIGH and LOW.** Refer to the Certificate of Analysis for current volume of Dilution Buffer needed for reconstitution and for current Quality Control concentration.

4.7.1. Reconstitute each Quality Control (HIGH and LOW) with Dilution Buffer just prior to the assay.

4.7.2. QC HIGH - Add 1.6ml of dilution buffer to the vial.

4.7.3. QC LOW – Add 1.6ml of dilution buffer to the vial.

4.7.4. Let dissolve at least 15 minutes with occasional gentle shaking (not to foam). Do not store the reconstituted Quality Controls.

4.8. **Wash Solution Conc. (10x)** Dilute Wash Solution Concentrate (10x) 10-fold in distilled water to prepare a 1x working solution.

4.8.1. Add 100 ml of Wash Solution Concentrate (10x) + 900 ml of distilled water for use in all 96-wells. Add half of the water to a 1000mL glass container. Then add the wash solution. Rinse wash solution container with remaining water.

4.8.2. Stability and storage: The diluted Wash Solution is stable 1 month when stored at 2-8°C.

4.9. Prepare the set of standards using the **Master Standard** (which should now be at 100ng/ml). Follow the table below:

Volume of Standard	***Dilution Buffer***	***Concentration***
Stock	-	100 ng/ml
200 µl of stock	300 µl	40 ng/ml
250 µl of 40 ng/ml	250 µl	20 ng/ml
250 µl of 20 ng/ml	250 µl	10 ng/ml
250 µl of 10 ng/ml	250 µl	5 ng/ml
200 µl of 5 ng/ml	300 µl	2 ng/ml

5. PROCEDURE

5.1. Pipet 100 µl of diluted Standards, Quality Controls, Dilution Buffer (=Blank) and samples, preferably in duplicates, into the appropriate wells. See example from BioVendor.

	strip 1+2	strip 3+4	strip 5+6	strip 7+8	strip 9+10	strip 11+12
A	Standard 100	QC LOW	Sample 8	Sample 16	Sample 24	Sample 32
B	Standard 40	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	Standard 20	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	Standard 10	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	Standard 5	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	Standard 2	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	Blank	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
H	QC HIGH	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39

Figure 1: Example of a work sheet.

5.2. Incubate the plate at room temperature (25°C) for 1 hour, shaking at 300 rpm on an orbital microplate shaker.

5.3. Wash the wells 3-times with Wash Solution (300 µl per well).

5.3.1. Aspirate wells by flicking the contents into the sink.

5.3.2. Pipet 300 µl Wash Solution into each well (use multichannel or electric pipette). Aspirate wells. Repeat this process twice.

5.3.3. After final wash, invert and tap the plate strongly against paper towel. Make certain that Wash Solution has been removed entirely.

5.4. Add 100 µl of Conjugate Solution into each well.

5.5. Incubate the plate at room temperature (25°C) for 1 hour, shaking at 300 rpm on an orbital microplate shaker.

5.6. Wash the wells 3-times with Wash Solution (300 µl per well). After final wash, invert and tap the plate strongly against paper towel.

5.7. Add 100 µl of Substrate Solution into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with the silver container that the plate came in or aluminum foil is recommended. Label with the current time and the stop time.

5.8. Incubate the plate for 10 minutes at room temperature. The incubation time may be extended [up to 20 minutes] if the reaction temperature is below than 20°C. Do not shake the plate during the incubation.

5.9. In the meantime, turn on the laptop and VICTORx5 plate reader.

5.10. Stop the color development by adding 100 µl of Stop Solution.

5.11. Determine the absorbance of each well using a microplate reader set to 450 nm, preferably with the reference wavelength set to 630 nm (acceptable range: 550 - 650 nm). Subtract readings at 630 nm (550 - 650 nm) from the readings at 450 nm. The absorbance should be read within 5 minutes following step 5.10.

Additional laboratory standard operating procedures which were developed or amended by Katie Robinson include:

1. General Laboratory Safety
2. Laboratory Waste Management and Autoclave
3. Proper Use of the Biological Safety Cabinet
4. Saliva Collection from Adults
5. Breath Collection from Children and Proper Storage
6. Bioelectrical Impedance Analysis using InBody 230
7. Agarose Gel Preparation
8. RNA Extraction from Adipose Tissue and rtPCR
9. Genotype Analysis using Fluidigm Software
10. Calculating Adipocyte Diameter Using Adiposoft