UNIVERSITY OF CALIFORNIA, SAN DIEGO

Study of Factors Regulating Metabolic Syndrome and Insulin Resistance

A Thesis submitted in partial satisfaction of the requirements
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by

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The thesis of Madlena A. Nalbandian is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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DEDICATION

I dedicate this thesis to Dr. Dorothy Sears for her continuous guidance, support and encouragement.

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ABSTRACT OF THE THESIS

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Madlena Nalbandian

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Metabolic syndrome, also known as syndrome X and insulin resistance syndrome, is a global pandemic that has been exponentially increasing throughout the last two decades. Obesity and insulin resistance are the primary components of metabolic syndrome. Our study aimed to identify factors regulating insulin resistance by further increasing our knowledge of 12/15 lipoxygenase along with a implementing a dietary intervention to reduce insulin resistance. We tested the hypothesis that 12/15 lipoxygenase transgenic mice on

a 45% HFD after 10 weeks would have significantly greater insulin resistance than the wildtype mice. We also hypothesized that a dietary intervention with low glycemic index bread products along with omega-3 and polyphenol supplements for 12 weeks should decrease insulin resistance and lower the risk of developing chronic diseases more effectively than a placebo-controlled diet. The 12/15-lipoxygenase mice study showed that the 12/15 lipoxygenase transgenic mice had significantly higher fasting plasma glucose concentrations, fasting insulin concentrations, plasma triglyceride concentration, and greater gWAT percentage of body weight compared to the wildtype mice. The study also suggested that the 12/15-lipoxygenase mice had greater systemic insulin resistance than the wildtype mice. The dietary intervention study, although incomplete at this stage, showed trends for enhanced insulin sensitivity with the active diet, body weight loss, fat mass loss, waist circumference decrease and improvements in lipid panel were significant in both diet groups. These studies shed new light on mechanisms regulating insulin resistance.

Introduction

Metabolic syndrome is defined as a group of risk factors that can be associated with metabolic diseases and increase the chances of developing diabetes, cardiovascular disease, stroke, and cancer[1]. Metabolic syndrome is also known to cause an increase in chronic inflammation, which can increase the chances of developing diabetes by five times, and the chances of developing heart disease by two times[1]. Although inflammation is necessary to eliminate wastes and destroy pathogens, it can also cause tissue damage during chronic inflammation. To become diagnosed with metabolic syndrome, one must meet specific criteria such as obesity, glucose intolerance, hypertension and dyslipidemia. Obesity and insulin resistance are two of the closest links associated with metabolic syndrome; therefore, the rise of obesity is causing this syndrome to become more prevalent in society. Since 1980, obesity has doubled worldwide; thus, over 200 million men and 300 million women are obese[2]. Obesity is also considered the 5th highest risk for death[2]; hence, preventative measures should be taken to not only improve one's insulin sensitivity but also their Body Mass Index (BMI)[3].

Insulin resistance, a physiological disorder in which the cells are unable to properly respond to insulin, is a major cause of metabolic syndrome. Decrease in the sensitivity of cells can lead to hyperglycemia. Therefore, Beta-cells in the pancreatic islets start producing additional insulin to compensate, which leads to a state of hyperinsulinemia. One of insulin's main tasks is to regulate the delivery of glucose into cells for storage and energy. Insulin resistance can cause the

muscle and fat cells to reduce in their rate of glucose uptake. After a period of time, Beta-cells are unable to secrete sufficient amount of insulin to maintain normal fasting glucose levels, therefore, they might start to fatigue and insulin levels will drop because of Beta-cell death, leading to the progression of diabetes. Insulin resistance may also have detrimental effects on adipocyte's ability to take up lipids in the blood, which will led to an increase in free fatty acids from the hydrolysis of triglycerides. Components of increased insulin resistance, such as hyperinsulinemia may also increase the production of pro-inflammatory signals, shown in **Figure 1.** Overall, insulin resistance can cause hyperglycemia, hyperinsulinemia, dyslipidemia and inflammation.

Obesity promotes insulin resistance, macrophage infiltration, chronic inflammation and ER stress causing dysfunction to systemic tissues, such as the adipose, muscle, pancreatic and liver. High fat diet (HFD) feeding can be performed on mice to induce insulin resistance and inflammation, modeling metabolic syndrome (**Figure 1)**. High fat diets are typically enriched in omega-6 fatty acids, such as linoleic acid (LA) and arachidonic acid (AA), which can be converted into fatty acid metabolites by cyclooxygenase (COX) and lipoxygenase (LOX). However, omega-3 fatty acids such as, α-linoleic acid (LA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) are anti-inflammatory because they can inhibit the action of COX and LOX. In animals and plants, Lipoxygenase enzymes play critical roles in defense responses and inflammation. For example, 12/15-Lipoxygenase (12/15-LO) can help regulate GTPases, MAP kinases along with activation of TLR4 and the production of

MCP-1, IL-6 and IL-8. 12/15-LO is an iron-containing enzyme that catalyzes the deoxygenation of polyunsaturated fatty acids, which cause an increase in pro-inflammatory lipid mediators. 12/15-LO is responsible for metabolizing arachidonic acid to form 12-hydroperoxyeicosatetraenoic acid (12-HPETE), and linoleic acid to 13-hydroperoxyoctadecadienoic acid (13-HPODE). Then they can be further oxidized into 12-hydroxyeicosatetraenoic acid (12-HETE) and 13-hydroxyoctadecadienoic acid (13-HODE), respectively. Although inflammation can be beneficial when fighting infections during necessary times, chronic inflammation, such that in adipose tissue of obese subjects can cause complications such as insulin resistance. These responses can be seen in macrophages, dendritic cells, inflamed muscle cells and endothelial cells, along with tumor cells. As a result, these inflammatory responses induce insulin resistance by the inactivation of insulin receptor substrates (IRS) via cytokine-activated JNK, IKKB and SOCS. Since 12/15 Lipoxygenase (12/15-LO) has a critical role in the development of insulin resistance and diabetes, it may be useful as a therapeutic target for the prevention of metabolic syndrome.

Previous studies from our laboratory and others have shown that 12/15 Lipoxygenase is necessary for the onset of HFD-induced insulin resistance[4]. The requirement for this enzyme was found by knocking out the 12/15-LO gene and placing these mice on a 45% HFD for 2-4 weeks. When comparing these mice with wild type mice, the 12/15 LO/KO were protected from insulin resistance and inflammatory markers. They also found that the 12/15 LO/KO had significantly lower levels of MCP-1 and OPN, which are pro-inflammatory chemokines. By

examining the macrophages in the stromal vascular cells (SVCs) of the adipose tissue, they discovered that the 12/15 LO/KO mice were not significantly different in their value of macrophages from the normal chow diet compared to the HFD chow. They also confirmed that the wildtype had significantly greater adipose tissue inflammation than the 12/15 KO/LO. In conclusion, they had discovered the 12/15 LO/KO mice were protected from insulin resistance. As a result, we wanted to further examine this enzyme by putting the 12/15 LO transgenic and wildtype mice on a 45% HFD for 10 weeks. We hypothesize that they would have developed increased insulin resistance in comparison with wildtype mice. Insulin resistance, inflammation and other metabolic syndrome aspects were assessed, along with the changes in weights and eating patters.

According to the National Health Statistics Report, currently 34% of adults and over 47 million Americans meet the criteria to be diagnosed with metabolic syndrome[5]. These numbers are increasing; therefore, A.D.A.M Medical Encyclopedia hypothesized that metabolic syndrome may take the place of smoking and become the leading risk factor for heart disease[1]. Dietary interventions can be used to reduce metabolic syndrome and improve levels of inflammation, if sufficiently effective, diet changes are preferable to pharmaceutical alternatives as they are associated with undesirable side-effects. A few pharmaceutical alternatives include blood pressure medications, such as Lisinopril (an ACE-inhibitor) and Doxazosin (an alpha-blocker), and statins to lower cholesterol levels. Also, since metabolic syndrome involves insulin resistance, Metformin has been used in clinical trials and during the onset of

diabetes, which can have various side effects such as vomiting, stomach pain and diarrhea. Metformin can also have long-term side effect on one's health by decreasing their body's ability to absorb Vitamin B-12. The Diabetes Prevention Program (DPP), a clinical research study, discovered that modest diet and exercise could better protect pre-diabetics from Type-2 Diabetes than metformin[6]. They concluded that participates in the lifestyle intervention group had reduced their risk of developing diabetes by 58%, while individuals taking Metformin had reduced their risk by 31%. Therefore, doctors have begun to advise their patients to take a change of lifestyle approach towards improving their health. Dietary interventions can provide patients with less expensive and more feasible alternatives. Many scientists have begun to do clinical trials with various proposed diets to see which one will provide the most successful approach; however, the exact diet prescription is unclear. The diet intervention described in this thesis is unique compared to others, since it combines three new concepts for metabolic improvements. Therefore, it is believed that with combined efforts a more convincing and stronger thesis can be formed.

The dietary intervention includes low-glycemic index bread products, omega-3 fatty acid fish oil supplements, and polyphenol supplements. It was proposed that this diet should improve metabolic syndrome indicators, such as obesity, insulin sensitivity, and inflammation. The study is innovative, educational and applicable in everyday life. It is educational because it provides individuals with knowledge about improving one's metabolic syndrome with a specific diet intervention. It is innovative because medical professions will be able to utilize

these results in assisting their patients, and if the study proves to be tolerable then it will become more applicable in everyday life. In addition to implementing this strategy into everyday clinical care, it is also beneficial because it will be more cost effective compared to pharmaceutical approaches. Since inflammation is associated with various diseases such as cancer and heart disease, this study will provide various areas of medicine a novel treatment approach.

The components of this diet would work together to not only help individuals lose weight, but also improve their health and well-being. The first component of this diet included low glycemic index bread products. The bread products were provided by Zone labs, and they include items such as bagels, pastas, flat breads, cookies and granola. Glycemic index measures how fast blood glucose levels should rise after eating a specific meal compared to the pure ingestion of glucose or a piece of white bread[7]. Various dietary components found in meals, such as fiber, starch, sugar, protein and fat will affect the level of glycemic index load found in that meal. A meal tolerance test was used to graph glucose concentrations in the blood, where the glycemic index can be determined because it is equal to the percentage value that is equal to the area under a blood glucose curve .The area is determined from the glycemic response to a specific meal. Although two meals can have the same amount of carbohydrates, a high glycemic index meal can cause the glucose and insulin curves to become larger and contain greater spikes, which would cause them to have larger area under the curve values[8]. The curves are referring to the patterns of clearing out the glucose from the circulation into the cells. It has been

hypothesized that after eating a high glycemic index meal, individuals will tend to release a lot of insulin at once to clear out the glucose. However, sometimes this tends to cause hypoglycemia and other times it will cause a strain on one's body, which can lead to great insulin resistance. Hence, it has also been hypothesized that low glycemic index meal products will cause the same amount of glucose to be released, however, through a longer period of time which will leave less of a burden on the body. Therefore, Beta-cells won't get exhausted as quickly from releasing insulin to help compensate for the cells that are becoming more resistant to the insulin that is already available to them. Therefore, to avoid this tread of events, individuals [should be advised] to eat low-glycemic index diet instead of high. A low-glycemic index diet can cause individual to release less insulin and epinephrine, while producing higher amounts of glucagon than a high-glycemic index diet[9]. It has also been shown that in mice, a low-glycemic index diet can cause these rodents to have lower body fat and more lean mass.[10] They can also have an increased glucose absorption rates, lower insulin AUC levels, increased adiponectin levels and lower levels of triglycerides[9]. Lastly, low glycemic index meals can potentially cause individuals to not only improve their insulin resistance, but also increases their level of satiety .[10]

Omega-3 fish oil supplements are also a vital component of the study because they are anti-inflammatory, which can cause a decrease in insulin resistance. High-fat diets, specifically the Western Diet, are filled with a higher ratio of omega-6 to omega-3 fatty acids, which can cause increased inflammation that can be led to increased insulin resistance[11]. High-fat diets, along with

hyperinsulinemia, are known to cause inflammation because of the increase in pro-inflammatory metabolities[11]. Recent studies have shown that fish oil supplements have not been successful in decreasing inflammation and the amount of metabolites alone. These supplements need to be combined with a low-glycemic index diet, which can help decrease insulin values found in the blood. The healthy ratio of omega-6 to omega-3 fatty acids should be from 1:1 to 5:1; however, in reality it is usually about 20 to 50:1[12]. Studies have also shown that increased ratio of omega 6:omega 3 fatty acids can also cause there to be an increase in fat accumulation; therefore, causing individuals to gain more weight.[13,14] Since omega-3 fatty acids are capable of reversing the negative effects of omega-6 fatty acids, the addition of fish oil supplements should help achieve this goal.[15]

Polyphenols are the last nutritional component found in this diet intervention for the improvement of one's metabolic syndrome. Polyphenols are antioxidants found in fruits and vegetables. They are known to reduce hypercholesterolemia, LDL, and pro-inflammatory metabolites[16]. Since high-fat diets are known to increase amounts of omega-6 fatty acids and LDL levels, including polyphenols in one's diet can help control these effects. Also since fatty acid metabolites are known to cause increase inflammation and insulin resistance, polyphenols and fish oil supplements can help decrease them[17]. For example, specific polyphenols from blueberries were found to improve insulin sensitivity and prevent oxidative stress[18]. They inhibited eicosanoids, which is a type of omega-6 fatty acid derivative, by inhibiting the COX/LOX enzymes.

Polyphenols involvement in decreasing metabolic diseases is a new finding that has been shown to be a great asset to the prevention of metabolic syndrome.

The study hypothesis is that a specific dietary intervention should reduce inflammation and increase insulin sensitivity in obese patients with metabolic syndrome. The clinical study will be a single-blinded, randomized controlled 12-week intervention.

The success of this study will depend on the results obtained from the various tests performed during their clinic visits. Some of the specific tests that will be performed will include a meal tolerance test, urine inflammatory marker analysis and calculating HOMA-IR using fasting insulin and glucose levels to determine their level of insulin resistance. Also, specific inflammatory markers such as cytokines, CRP and fatty acid metabolites will be measured. Although clinical trials provide first-hand experiences, they also tend to contain more variability and provide the study with a few difficulties. One of the main challenges with clinical trials are the various behaviors that the subjects are engaging in and after they leave the clinic, such as cheating on their diets and taking medications that have been excluded. The investigators have tried overcome these variables by providing the subjects with added support, such as a weekly call from a dietician and recipes. Compliance was also measured through the weekly questionnaires that they are required to be filled out, along with 3-day food diaries.

Metabolic Syndrome consists of insulin resistance, inflammation and obesity; therefore, it has been a target for improvement in the world of

endocrinology. Both the studies described here aim to modulate and elucidate mechanisms regulating metabolic syndrome. The 12/15 LO transgenic mice study extends previous findings about the involvement of 12/15 LO as a major contributor of metabolic syndrome and a possible target for further studies aiming to alleviate insulin resistance. The human diet study tests the efficacy of a novel diet intervention to improve metabolic syndrome and reduce the risks of Type 2 Diabetes.

Materials/Methods: Part A

Animals

10 Male C57Bl/6J wild type mice were purchased from Jackson Laboratories. Male 12/15LO transgenic mice, backcrossed with C57Bl/6J mice were gifted to Dr. Yury Miller from Dr. Catherine C. Hedrick (University of Virginia). The wildtype mice (1-10) were 17 weeks of age, 12/15LO TG (58-62) were 28 weeks of age and 12/15LO TG (70,72,74,76) were 23 weeks of age when they started on their High Fat Diet (45%kcal from fat; D12451, Open Source Diets) for 10 weeks. The HFD contained 45% kcal of fat, 20% kcal of protein and 35% kcal of carbohydrates. The fat composition of the HFD was 18.5% polyunsaturated fatty acids, which is mainly composed of linoleic and arachidonic acids. Specifically, the C18:2 linoleic fatty acid was 14.9% of the polyunsaturated fatty acids, however, C18:3 linoleic acid and C20:4 arachidonic acid made up 1.93% and 1.57% of the polyunsaturated fatty acid composition, respectively. Compared to the normal chow mice, the 45% HFD provided the mice with 10X more arachidonic acid, while providing 1.8X more linoleic acids. Mice were fed and weighed twice a week with 4-5g of available food per mouse per day. Mouse food was also weighed twice a week to calculate food consumption per cage. The cages were changed twice a week, and mice with individual cages were supplied with nestlets. WT Mice (1-5) were caged together in a single house for 10 weeks. WT mice (6-10) were caged together in a single house for 3 weeks, after which, they were all separated into separate cages until week 10 because of aggressive activities. TG Mice (58-62) were housed together in a single cage

for 10 weeks. TG Mice (70,72) were housed together in a single cage for 10 weeks. TG Mice (74,76) were housed together in a single cage for 10 weeks. Mice were housed under controlled light (12:12 light:dark) and climate conditions with unlimited access to food and water. All procedures were performed in accordance with the *Guide for Care and Use of Laboratory Animals* of the National Institutes of Health and were approved by the University of California, San Diego, Animals Subjects Committee

Plasma and Tissue Analyses

After a 12-hour fast, mouse were anesthetized with carbon dioxide and sacrificed after 10 weeks on the HFD. Mice were perfused with PBS and tissues were harvested and distributed for a variety of experiments, most of which are not presented in this report. However, the gonadal white adipose tissues (gWAT) and peri-renal white adipose tissues (rWAT) were weighed, rinsed in PBS and minced in DPBS+5% BSA to be analyzed by FACS. Blood was collected from the vena cava, plasma could be extracted to determine levels of insulin, triglycerides (TG) and free fatty acids (FFAs).

Fluorescence-activated cell sorting (FACS) of adipose tissue SVCs

Freshly harvested fat pads, specifically gWAT and rWAT, were rinsed/minced and transferred to be centrifuged at 500xg to remove erythrocytes and free leukocytes. They were treated with 2mg/mL type II collagenase (Sigma, St.Louis, MO) and transferred to a 37° shaking water bath. Adipose tissue cell

suspensions were filtered through a 70um mesh filter using 2X rinses of DPBS+5%BSA, and centrifuged to separate floating adipocytes from the SVC pellet. The SVC pellets were resuspended in RBC lysis buffer (eBioscience, San Diego, CA) for 5 minutes, and resuspended in DPBS+2%BSA. The adipocytes, and part of the SVC samples were repacked with centrifugation and lysed with Trizol for RNA preparation. Cell concentrations were determined for the SVC samples, and controls were created with equal volumes of each sample. Cocktails of anti-mouse monoclonal antibodies with fluorescent tags such as, FITC, PE, PI and APC were prepared for sample staining. Samples were transferred to DPBS with Live/Dead Stain, washed two times, and fixed in 3.7% formaldehyde in PBS. The presence of fluorescent stains in the SVCs were analyzed using a FACS Calibur flow cytometer (BD Biosciences). Controls for the SVCs preparation included unstained cells, PI-only stained cells, and fluorescence-minus-one (FMO) stained cells. The percentage of M1-type cells out of total macrophages found in the SVC can be calculated by dividing the number of live cells that have F4/80+,CD11c+ and CD11b+ markers on them and dividing it by the number of F4/80+,CD11b+ cells.

Glucose Tolerance Test, Insulin ELISA Assay

GTT was performed after 8 weeks on a 45% HFD chow, involving 10 Wildtype mice and 9 12/15-Lipoxygenase transgenic mice. The mice were fasted for 6 hours prior to the start of the Glucose Tolerance Test (GTT). After the 4.5 hrs., the mice were weighed, and the insulin doses were calculated for 1g/kg of body

weight. Fasting blood glucose concentrations were measured, along with the collection of blood for an insulin ELISA assay. The mice were left to fast for an additional 1.5hours to ensure that the GTT values weren't elevated due to the blood collection. Before the IV injection of glucose into the peritoneal region of the mouse, blood glucose was measured at time 0 and continued to be measured at time intervals of 30,60, and 90 minutes with a glucometer. Blood was collected by tail snipping. Blood for an additional insulin ELISA assay at 15 minutes was collected using EDTA coated capillary tubes, however, this included 6 individuals from each strain of mice. After which, the blood was spun down at 5,000xg for 15 minutes and the plasma was collected for the ELISA assay.

Insulin Tolerance Test

ITT was performed after 9 weeks on a 45% HFD chow, involving 9 wildtype mice and 9 12/15-Lipoxygenase transgenic mice. One of the wildtype mice was excluded from the study after insulin-induced hypoglycemia of 25 mg/dL. The mice were fasted for 6 hours prior to the start of the Insulin Tolerance Test (ITT), since it was reported that it is the optimal fasting period to ensure adequate gastric emptying without inducing starvation in the mice[19]. After the 6 hrs, the mice were weighed, and the insulin doses were calculated for 0.4U/kg of body weight. Blood glucose concentrations were measured at time 0, before the IV injection of insulin into the peritoneal region of the mouse. Blood glucose concentrations were measured at time intervals of 15, 30, 60, 90, and 120 minutes with a glucometer. Blood was collected by tail snipping.

Statistical Analyses

Unpaired t-tests on Prism and Excel were performed for statistical analyses. A p-value cutoff of 0.05 was used to determine statistical significance.

Materials/Methods: Part B

Intervention Guidelines

Subjects were randomly assigned to the control diet, also known as the placebo diet, or the experimental diet, also known as the active diet. The study was single-blinded, and weekly disbursements of food, shakes, recipes and capsules were mailed to the subjects. The control group was given placebo supplements of corn oil and starch with higher glycemic index foods. The experimental group was given low-glycemic index foods with fish oil and polyphenol supplements. Both groups were limited to 1500 calories/day, and the subjects were required to provide their own side dishes of mainly vegetables. Recipes were provided to help them during the process. They also received guidance on the phone from a dietician every week, and completed food diaries and weekly questionnaires to assess for compliance.

Recruitment

Subjects were recruited by advertisements displayed at various locations, such as the internet, specifically Craigslist, libraries, coffee shops, hospitals, and various campuses in the San Diego area.

Pre-Screening/Screening Visits

Subjects were pre-screened on the phone for eligibility into the study according to specific exclusion and inclusion criteria. If they passed the pre-screening, they came for a screening visit at the UCSD Clinical and Translational Research Institute (CTRI), where they had to give informed consent, medical history, height, weight, waist circumference data, along with a blood lipid panel. If they passed the screening visit, they were asked to enroll in the study.

Inclusion Criteria

1. BMI of 30.0-40.9
2. Age 21-55
3. Metabolic Syndrome
    a. Waist circumference of greater than or equal to 102 cm if they were male and greater than or equal to 88cm if they were female
    b. Two of these criteria: triglycerides 150mg/mL or greater; HDL less than or equal to 40mg/dL for males and HDL less than or equal to 50 mg/dL for women; fasting blood glucose between 100 and 125 mg/dL or acanthosis nigricans; blood pressure greater than or equal to 130/85 but less than or equal to 180/100.

Exclusion Criteria

1. Various diseases such as diabetes, inflammatory diseases, eating disorders, kidney disease, coronary disease, gastrointestinal or peripheral artery disease.
2. Currently pregnant, smoke or engage in regular physical activities
3. Taking fish oils (greater than 500mg of EPA/DHA per day) or polyphenols regularly
4. Exhibited excess weight changes within the last three months
5. Allergic to fish or unable to swallow large capsules
6. Currently taking medications for lipid lowering, such as fibrates and statins. Also, if they are taking any anti-inflammatory or diabetic medications.

Baseline/Midpoint/Endpoint Visits

The subjects had three additional visits to the CTRI every 6 weeks from their baseline visit. The subjects had to fast for 12hrs prior to each visit. At each visit specific pieces of data was collected from them. At the baseline visit; vitals, waist circumference, meal tolerance test, DEXA body composition, blood glucose, insulin, c-peptide, CRP, cytokines, monocyte gene expression, lipid panel, hemoglobin A1C, urine inflammatory markers and specific information for genotyping were collected. At the midpoint visit; vitals, blood glucose, insulin, c-peptide, CRP, cytokines, monocyte gene expression, lipid panel, hemoglobin A1C and urine inflammatory markers were collected. At the endpoint visit; vitals, waist circumference, meal tolerance test, DEXA body composition, blood

glucose, insulin, c-peptide, CRP, cytokines, monocyte gene expression, lipid panel, hemoglobin A1C, and urine inflammatory markers were collected.

Inflammatory Markers and Fasting Glucose/Insulin

Lipid panel, fatty acids, TXB2 and 11-dehydrothromboxane B2 were measured with HPLC and gas chromatography-mass spectrometry (GC-MS). Fasting insulin and glucose concentrations were measured with an ELISA.

Meal Tolerance Test

The subjects were asked to fast for 12 hours, and arrived at the CTRI for the exam. The subjects were given an Ensure Plus Shake (11g of fat, 13 g of protein, 50 g of carbohydrates), and IV was used to collect samples of blood for glucose and insulin level analysis. Blood was collected at baseline (0 minutes), 30 minutes, 60 minutes, 90 minutes, and 120 minutes.

Statistical Analyses

Paired and Unpaired t-tests were performed on Prism and Excel for statistical analyses. A p-value cutoff of 0.05 was used to determine statistical significance.

Results: Part A

Chapter 1: Mouse Strain Characteristics

In **Chart 1,** the wildtype and 12/15 LO TG mice characteristics such as, body fat percentage, gWAT fat pad weights, fasting glucose concentrations, fasting insulin concentrations, free fatty acids, triglycerides, and M1 cell concentrations are presented at their average endpoint values with the standard deviation. The chart also compares their average weights from baseline to endpoint.

Chapter 1A: Mice Weight Changes

Since obesity is a major contributor to insulin resistance and inflammation, it is imperative to monitor the average weights of the mouse strains and assess whether they are significantly different. The mouse weights were calculated, compared twice a week for 10 weeks, and graphed with respect to average weights and individual weights, shown in **Figure 2 (A-D).** In **Figure 2C,** the total average weights of WT and TG mice are represented on line graph. At 8,9 and 10 weeks, the WT and TG mice were not significantly different in their total weights, indicated by an arrow. During these 3 weeks, the GTT, ITT and harvesting of the tissues were performed. After 3 weeks of being housed together, mice in WT Cage #2 (WT#6-10) were separated into individual cages for the remainder of the study because of aggressive fighting. In **Figure 2A,** the mice in Cage #2 are presented with dashed lines, while the rest of the mice are represented with a solid line. In the **Figure 2A**, the mice in cage #2 on average

weighed less than the mice in cage #1, however, the average weights at the end of the study were not significantly different between the two cages. In **Figure 2B,** we illustrated the average weights of the transgenic mice. In **Figure 2D,** an overview of the average total weights of both groups are compared together in a scatter plot, and no significant differences are found between them. In **Figure 2C,** it clearly depicts that there are no significant weight differences between the two groups. As a result, these conclusions suggest that weight should not alter the results obtained in individual experiments comparing the mouse strains.

Chapter 1B: Eating Patterns/Food Intake

Both the TG and WT mice were fed 45% HFD food for 10 weeks, approximately 4-5grams of food per mouse per day was provided. Individual food intake was calculated twice a week, shown in **Figures 3**. At every single time point, the WT mice ate more than the TG mice. These values suggest that although the WT mice were separated for aggressive behavior, they still ate significantly more than the TG mice on 12/20 of the time points. In **Figure 3,** a line graph depicts the average food intake per mouse per day for both strains of mice. According to **Figures 2C,** the TG mice gained more weight than the WT mice at every time point, however, the opposite was true about their eating patterns. These patterns would suggest that the TG mice experienced reduced energy expenditure the entire time that they were placed on the diet. According to previous studies, reduced energy expenditure along with a HFD will cause obesity and insulin resistance in the muscle, liver and adipose tissue[20]. To further

confirm our predictions, future studies should place these mice in metabolic cages to measure energy expenditure, physical activity, calorimetry, and individual food/water intake.

Chapter 1C: Images of WT and TG mice/ Body Fat Compositions

In **Figure 4A,** the TG and WT mice on Week 10 of their diet are shown in two different views. The first view represents the mouse before harvesting the tissue, and second view is after the mouse has been perfused with PBS. Even though both mice look the same size, it is clearly indicated in the picture that the transgenic mouse, on the right, has a larger gWAT fat pad than the wildtype mouse, on the left. In **Figure 4B,** the gWAT body fat percentages of both mice groups are compared to each other. The transgenic mice had a significantly higher average body fat percentage than the wildtype mice, specifically 5.5% for the transgenic mice and 3.5% for the wildtype mice. Interestingly, their average body weights were not significantly different from each other. In **Figure 4C,** the gWAT fat pad sizes are compared to each other, and the transgenic mice have a significantly larger gWAT average fat pad size than the wildtype. Since we suggested that the transgenic mice were experiencing lower energy expenditure than the wildtype mice, it would also suggest that they would have larger fat pads than the wildtype mice. Also, since we postulate that there might be increased inflammation in the transgenic mice compared to the wildtype mice, this might also have an effect on the size of the fat pads because they might be more inflamed.

Chapter 2: Glucose Tolerance Test, Insulin ELISA assay

Glucose Tolerance Tests (GTTs) are performed to classify a subject's level of glucose tolerance, specifically if they have normal glucose tolerance, impaired glucose tolerance or diabetes. It can also provide information about a subject's level of insulin sensitivity and Beta-cell function by analyzing how efficiently post-loaded glucose can be cleared out from their blood.

The data revealed that the both strains of the HFD-fed mice had developed impaired glucose tolerance since they had impaired fasting glucose levels. Specifically, mice that have fasting blood glucose concentrations of more than 150mg/dL are considered hyperglycemic. In addition, there was also a significant difference between the average fasting blood glucose levels of the WT mice compared to the 12/15 TG mice, specifically 202.3mg/dL for WT compared to 238.7mg/dL for TG, indicating that the TG mice have greater impaired fasting glucose than the WT mice.

At the start of the GTT, the basal glucose concentrations were taken again with a 90-minute interval from the first blood collection to ensure that stress from the blood collection hadn't affected their glucose concentrations. There were significant differences between the two basal blood glucose collections. For example, the 0 minute time point shown in **Figure 5A** has an average basal glucose concentration of 187.8mg/dL for WT and 247.2mg/dL for TG mice. Since TG mice have significantly higher basal glucose concentrations than the WT, two hypothesises can be made about these observations. The first hypothesis is that

they have reduced insulin production, therefore, they are in a state of hyperglycemia. The second hypothesis is that the TG mice are in a greater state of increased gluconeogenesis because they have hepatic insulin resistance. Normally insulin acts to inhibit gluconeogenesis, however, if the liver has become resistant to insulin, the liver over produces glucose. Observing the insulin concentrations at baseline has shown us that both mice are in a state of hyperinsulinemia, which is a state of increased insulin secretion to maintain glucose concentrations. However on **Figure 5B,** the insulin concentration of the TG mice at baseline are significantly higher than the WT, specifically 2.67 ng/mL for WT and 3.83 ng/mL for TG. Therefore, it possibly suggests that their state of hyperglycemia at baseline is not due to decreased insulin production, but instead to increased hepatic insulin resistance. Technically, hyperinsulinemia should have caused the basal glucose values to be maintained at normal blood glucose levels in insulin resistant mice because of compensation, however, this was not the case since the TG mice were unable to lower their glucose levels with the additional insulin that their Beta-cells were supplying. This event would indicate that the increased compensation was insufficient enough, which can cause Beta-cell failure down the line.

The 15-minute time point is not only critical for the assessment of glucose tolerance, but also insulin secretion levels. Both the WT and TG mice peaked at 15 minutes, however, TG peaks at a significantly higher concentration than the WT, with a plasma glucose concentration of 531.4 mg/dL compared to 338.5 mg/dL, respectively. According to the insulin concentrations, there is no

significant difference between the two groups at the 15-minute time point. However, there is a significant difference between the baseline and 15-minute insulin concentrations for the WT mice, indicating that the WT mice are producing additional insulin to control their glucose levels. Unfortunately, the TG mice don't increase in insulin production between the two time points. The insulin concentrations in conjunction with the GTT at the 15-minute time point suggest that the TG mice have great insulin resistance than the WT, which is causing them to have increased hyperinsulinemia continuously for the first two time points because they are not able to clear out the glucose effectively. Although the data strongly suggests that the state of hyperglycemia is due to the increased insulin resistance, it can also suggest that they have a greater degree of Beta-cell defects. Since, increased basal hyperinsulinemia and reduced cell sensitivity to insulin can cause disturbances in the release of insulin from the Beta-cells. As a result, periods of prolonged hyperinsulinemia can led to Beta-cell defect.

The trend continued throughout the GTT as the TG continue to have significantly higher glucose concentrations than the WT at the 30, 60 and 90-minute time point, These points would reconfirm our hypothesis that the TG mice have greater glucose intolerance than the WT. At the 90-minute time point, the WT mice returned to their basal glucose concentrations, however, the TG mice were still significantly higher than their basal concentrations and needed additional time to return to baseline. This could further suggest that the TG mice were in a state of greater gluconeogenesis, which caused them to not be able to suppress their glucose levels and they were still greatly insulin resistant. Not only

does the GTT provide evidence to suggest abnormalities in the response to glucose and the production of insulin, but it also can help identify specific tissues that are insulin resistant by observing the various time points. Previous studies have identified that the 90 to 120-minute time point is a result of insulin resistance in the liver. [21] As a result, this provides us with additional confidence of what tissues are insulin resistance.. Since there is a significant difference between both groups at all the time points on the GTT, it suggests that there is insulin resistance throughout the whole body. Additional tests need to be conducted to confirm these predictions.

In **Figure 5C,** the AUC of plasma glucose concentrations were calculated after the glucose tolerance test was performed. The TG mice had a significantly higher AUC than the WT mice, specifically 1840for TG and 1028 mg/dL(min) for WT. During the GTT, the AUC would correlate with the ability to dispose of glucose administered to the mice by insulin action. Since the AUC for the TG is greater than the WT, it would indicate that the TG have greater insulin resistance because they are unable to efficiently utilize the insulin provided by their bodies for disposal purposes. Overall, the AUC-glucose provides a way to view and assess the GTT curves as a whole.

Chapter 3: Insulin Tolerance Test

Insulin tolerance test (ITT) is another test for the determination of insulin sensitivity in mice, since the fall of glucose is representative of the insulin action of the mice. The ITT is also a representative of total body insulin sensitivity,

which includes tissues such as the muscle, liver and fat. The insulin tolerance test is independent of B-cell phenotype as insulin is exogenously supplied.

During the ITT, insulin was injected into the mice to measure insulin-induced hypoglycemia, while blood glucose concentrations in the plasma were measured at specific time intervals of 15, 30, 60, 90 and 120 minutes. Since there were significantly different baseline glucose levels between the TG and WT mice, a graph of the ITT with percent baseline of the blood glucose concentrations is shown in **Figure 6B** alongside the ITT of the absolute glucose concentrations in **Figure 6A.** The 15-minute time point was the most conclusive towards the determination that the TG mice were less insulin sensitive than the WT mice. At this time point, the TG mice had significantly higher blood glucose concentrations than the WT with an average blood glucose concentration of 213.5mmol/L compared to 111.2mmol/L. TG mice's percentage change from basal blood glucose concentrations also differed greatly compared to the WT mice with a -7.6%% change compared to a -34.7%% change, respectively. These results indicated that the TG mice were less insulin sensitive than the WT, since the TG mice required a longer period of time before their bodies were able to respond to the additional insulin that was injected. However, there are no other significant differences between the groups after the 15-minute time point.

Overall, the ITT turned out to be inconclusive, since we believe that the amount injected into the mouse was too high. Insulin acts to suppress glucagon secretion, however, when there is an overload of insulin injected into a mouse, the insulin receptors become saturated, and the alpha-cells secrete glucagon to

maintain glucose concentrations and prevent hypoglycemia. Previous studies identified that counter regulatory hormone responses such as epinephrine, corticosterone and glucagon increased in mice at glucose levels concentrations near 80mg/dl[22]. This response was seen at the 15 and 30 minute time intervals for both groups as their average glucose levels dropped significantly, seen in **Figure 6A.** As a result, during this regulatory period, no significant differences were seen between the groups. When performing an ITT, the amount of insulin injected is due to various factors including body weight, composition and diet. Usually, 0.3u/kg of insulin is usually injected into a normal chow mouse and 0.5u/kg of insulin into 60% HFD mice, therefore, we decided to use 0.4u/kg of insulin for our 45% HFD mice. Other studies have shown that occasionally different brands of insulin are more active than others, so we hypothesis that this might have also contributed to the overdose of insulin injected.

Chapter 4: Dyslipidemia Characteristics

Chapter 4A: Free Fatty Acid Concentrations

The metabolism of fats and carbohydrates are intertwined together because of insulin's role in the body. Increased insulin affects the liver by causing the conversion of glucose into glycogen. During hyperglycemia, the liver becomes saturated with glycogen and excess glucose becomes converted into free fatty acids (FFAs), which are esterified into triglycerides. FFAs are a link between obesity and insulin resistance because obesity can increase fasting plasma FFAs, which can increase insulin resistance. It has been found that

increased FFAs can cause specific organs to not respond to the insulin as well. Insulin also plays a role in lipolysis by inhibiting its action, while glucagon promotes it. However, in insulin resistance adipocytes, insulin isn't able to inhibit lipolysis, therefore, triglycerides broken down and cells over-release FFAs. Hyperinsulinemia attempts to maintain the level of glucose at normal fasting concentrations, however, the TG mice have significantly higher fasting glucose concentrations then the WT, suggesting impaired glucose tolerance. Since we predict that we have insulin resistance cells, we can also predict that we would have a higher concentration of free fatty acids (FFAs) in the blood. In **Figure 7A,** FFAs are not significantly different between the two groups, suggesting that hyperinsulinemia is able to suppress fasting concentrations of FFAs effectively. FFAs are good indicators of adipocyte insulin sensitivity. To estimate the level of adipocyte insulin sensitivity, the mice can be given a glucose challenge and the FFA concentrations determined throughout the normal time point of the GTT.

Chapter 4B: Triglycerides

Triglycerides are esters made up of a glycerol bounded to fatty acids, which get broken down by lipolysis to release FFAs. Although triglycerides play vital roles in metabolism, increased levels are associated with metabolic syndrome. During a state of hyperglycemia, the insulin is unable to clear out the glucose from the blood, therefore, excess amounts of glucose become converted into triglycerides. Also, the liver is consistently taking up triglycerides for the body to function properly, however, it can develop fatty liver where there is an

abnormal amount of triglycerides forming fat droplets. During insulin resistance, the body uses fatty acids for energy because it can't use the glucose properly. Therefore, the fatty acids become released from the adipose tissue, and become transported to the liver. Increased levels of triglycerides in the liver can cause inflammation, hence, increased levels of triglycerides in the liver can be related to insulin resistance. Since both strains of mice were on a HFD, they should both have elevated triglycerides, since wildtype normal chow mice have triglyceride concentrations around 31.0 mg/dL [6.] However, the TG and WT mice are in the range of 70-90mg/dL, which is expected for HFD-induced mice. Since we suggest that the 12/15 LO TG mice are more insulin resistant than the WT, we predicted that they would have increased levels of fasting plasma triglycerides. Triglycerides were measured at week 10, and the TG have significantly higher levels than the WT, shown in **Figure 7B.** Therefore, these results suggest that transgenic mice have greater hepatic insulin resistance than the WT

Chapter 5: Fluorescence-activated cell sorting (FACS) of adipose tissue SVCs (M1 cell concentrations)

Flow cytometry separates a population of cells according to specific fluorescent labels, therefore, it is able to count cells, sort cells and detect specific biomarkers. During HFD, the adipose tissue becomes concentrated with increased amount of macrophages and inflammation, therefore, we examined the stromal vascular cells (SVCs) from the gWAT for macrophage content. Since the SVC cells are composed of a variety of different cell types, such as endothelial

cells, pre-adipocytes and immune cells, FACS is a great method because it is sensitive enough to differentiate between different types of specific cells, and the entire adipose tissue fat pad can be used for analysis. When analyzing the cells, we looked for the percentage of live cells to make sure that our methods for preparing and fixing the cells had not destroyed our SVCs. Also, we had a set of controls staining for specific antibodies, and they helped us identify non-specific binding of antibodies. Then we looked for specific markers found on the macrophages to identify, which macrophages were M1 cells (CD11c+). M1 cells are pro-inflammatory because they produce a high amount of cytokines, and metabolites such as nitric oxide. Hence, we measured the amount of live adipose tissue SVCs that expressed F4/80, CD11b+ and CD11c+, since macrophages that contain all three of these markers together are considered pro-inflammatory. Then we found the percentage of those cells over the total number of macrophages, which are cells that contained CD11b+ and F4/80+. This percentage gives us an accurate representation of how many of the macrophages were M1 cells. In **Figure 9,** the amount of M1 cells in TG and WT SVCs is represented. Since these mice were both on a HFD, there should be a significant amount of M1 cells in both groups, however, since the TG mice have an up-regulated LOX enzyme they should have an increased amount of eicosanoids, suggesting that they would have a higher concentration of pro-inflammatory macrophages. The TG have more M1 cells, as predicted, than WT mice, however, it is not significantly higher unless an outlier is removed from the calculation. If an outlier is removed, the p-value will change from 0.06 to 0.04.

Results: Part B

Chapter 6: Subject Profile Characteristics

The diet intervention study includes 41 subjects, and 27 of these subjects are represented in my data. Out of the 27 subjects, 10 of them are male and 17 are female. There were 12 subjects on the placebo diet, and 15 subjects on the active diet. Specific criteria for metabolic syndrome such as age, weight, body mass index, waist circumference, fasting glucose concentration, hemoglobin A1C, and lipid panel are compared in **Table 2.** There is a great deal of variety between the subjects, since the subjects met the criteria for metabolic syndrome according to various categories. The subjects were randomly assigned, and the subjects from control group compared to the experimental group were not significantly different in any of the categories, except for their levels of triglycerides. Therefore, percentage change from baseline analyzes were used when comparing the progress of the two groups.

Chapter 7: Changes in Adiposity, Weight and Glucose Parameters

Metabolic syndrome is characterized as a group of risk factors that pre-disposes individuals to various diseases. These risk factors make up a list of criteria that subjects must have met to have been enrolled in this dietary intervention study. After 12 weeks on the diet, the percent change for these characteristics was measured to evaluate the specific effects of the diet. The percent change represents the change seen at the endpoint visit to baseline visit,

divided by the baseline visit. The percent change was calculated for changes in body mass index (BMI), waist circumference, hemoglobin A1c (HbA1c), fasting glucose concentrations, fat mass, and lean mass. These characteristics are illustrated on scatterplots in **Figure 9(A-D).** According to **Figure 9A**, the active and placebo groups are not significantly different in their percent changes for BMI and waist circumference. This data suggests that weight won't play a significant role when comparing the effects the diet had on the groups. Since both groups lost about the same amount of weight they should see similar results in many of the categories tested. The diet invention is specifically targeted to insulin resistance and all of the risk factors of metabolic syndrome. In fact, there is a significant difference between baseline and endpoint for BMI and waist circumference in both groups. The diets had significant effects on decreasing the subjects' BMI and waist circumferences, hence, it suggests that each works well as a method for losing weight.

In **Figure 9C**, percent changes for HbA1c are represented with a scatterplot to compare average changes in the groups along with individual changes. The active diet tends to lower HbA1c to a greater extent than the placebo. Since HbA1c reflects the average glucose concentration in the blood over a period of three months, 12 weeks may be a too short of a time to see significant changes induced by the diet intervention. If the subjects had stayed on the diet for a longer period of time, we might see more significant changes since there are trends forming amongst the groups. In **Figure 9D**, no significant differences in fasting plasma glucose are shown between the active and placebo

groups, although the active group's fasting glucose levels tended to decrease more than the placebo group's levels. However, the scatterplot reveals an outlier that is present in the active group. If the outlier were removed, there would be a significant difference between the active and placebo groups, specifically an average of -3.65% change in the active group and a 4.4% change in the placebo. Decreasing fasting glucose concentrations would suggest that the subjects were becoming more insulin sensitive, since the insulin is able to be clear out the glucose from the blood more efficiently. However, since the data for the insulin concentrations is not currently available, results can't be confirmed. In **Figure 9B**, the results from the DEXA (Dual X-ray absorptiometry) scan for the percent change of fat and lean mass are shown. No significant differences were observed between the two groups for changes in fat mass, specifically a -4.6% change for the active group and a -4.1% for the placebo group. Also, no significant differences were observed between the two groups for changes in lean mass, specifically a -2.8% change in the active group and a -2.3% change in the placebo group. However, there was a significant difference in both groups for changes of fat mass from baseline to endpoint, indicating that the diets had helped both groups lose a significant amount of fat mass. Since both groups lost a significant amount of weight and fat mass, there was no significant differences between the groups. Overall, these results suggest that both diets had similar effects on certain profile characteristics, such as BMI, waist circumference and fat loss.

Chapter 8: Lipid Panel Assessment/ Atherogenic Dyslipidemia Index

Dyslipidemia occurs when there is an abnormal amount of lipids found in the blood, which can be due to a poor diet and hyperinsulinemia. A lipid panel is performed to assess if the diet intervention is examining changes seen in Low-density Lipoprotein (LDL), High-density lipoprotein (HDL,) Triglycerides (TG), TG/HDL, non-HDL, and Total Cholesterol (TC). In **Figure 10(A-C)**, scatterplots illustrate an overview of the lipid panel data. No significant changes were found between the active and placebo group in any of the lipid panel assessments. In **Figure 10A,** a scatterplot is utilized to depict percent changes seen in LDL and TG/HDL. High levels of LDL can cause damage to the body by blocking blood vessels, which can lead to atherosclerosis and heart attacks. TG/HDL, termed “atherogenic index”, is closely related to a body’s level of insulin resistance. The active group tends to exhibit greater decreases in LDL and TG/HDL that are greater than the placebo group, however, the differences are not significant. Both groups have a significant decrease in TG/HDL from the baseline to endpoint visit. However, only the active group decreases significantly in LDL from the baseline to endpoint visit. In **Figure 10B,** total cholesterol and HDL are depicted on the scatterplot bar graph with no significant differences found between the active and placebo groups. Although, both groups decrease in their levels of total cholesterol significantly, however, neither group experience significant changes in HDL. In **Figure 10C,** TG and non-HDL levels are depicted, although, no significant differences are found between the two groups, both groups decrease significantly in their levels of triglycerides and non-HDL. Triglycerides are often

highly elevated in insulin resistance patients and consuming omega-3 fish oil capsules has been proven to decrease their concentration of TG in the blood, however, no differences between the groups are depicted in these results. Weight loss alone can play a significant role on the lipid panel, such as decreasing triglycerides in the blood. We suggest that since both groups lost a significant amount of weight, weight loss has primarily attributed to the improvement of lipid panel characteristics such as, triglycerides, total cholesterol, non-HDL, TG/HDL and LDL, in the study subjects.

Chapter 9: Meal Tolerance Test (MTT), Glucose Excursions

Meal Tolerance Test (MTT) was performed at the baseline and endpoint visits to determine if the subjects had improved their insulin resistance through the dietary intervention. MTT is useful for assessing glucose tolerance in the context of a mixed meal and determining if the provided diet is a good therapeutic method for improving one's level of insulin sensitivity. In previous studies, the Meal Tolerance Test (MTT) has be a preferred method over the Oral Glucose Tolerance Test (OGTT) for the induction of glucose and insulin responsiveness because it provides a carbohydrate, lipid and protein challenge to the subjects[23]**.** After a 12hr fast, an Ensure Plus shake was provided to the subjects, and their blood was collected every 30 minutes for 2 hours. Then, the blood is assessed for glucose and insulin concentrations. The insulin concentrations are currently unavailable because there are two more subjects that have not completed the diet study and measures of insulin will be collected

all at one time. Therefore, I will present data on the plasma glucose concentrations. The MTT AUC for glucose and insulin, along with HOMA-IR values are good indicators of insulin resistance. An increase in AUC of glucose can be correlated with an increase in insulin resistance because the subjects are in a state of hyperglycemia for a longer period of time since they are unable to respond to the insulin secreted by their Beta-cells.

With the glucose concentrations provided, a curve was illustrated for each subject, and the area under the curve (AUC) was calculated using a trapezoidal method on PRISM with a baseline of 50mg/dL. In **Figure 11 (A-B),** a curve is illustrated with the average at each time point of the subjects. In **Figure 11A,** the average glucose excursion curve for the active group is shown. There are no significant differences within any of the points, however, it is possible to see that the area under the curve has decreased with the diet intervention. In **Figure 11B,** the average glucose excursion curve is shown for the placebo group. At 60 minutes, there is a significant difference between the baseline and endpoint curves. The graph clearly shows that there was an area under the curve increase from the baseline to the endpoint visit. Also, the average endpoint visit curve has a higher glucose concentration at all time. In **Figure 11C,** the glucose AUC of the placebo and active groups are compared to determine if the diet has positively impacted their level of glucose tolerance and insulin resistance. In **Figure 11C,** a bar graph is utilized for the comparison of the average AUCs for the placebo and active groups during their baseline and endpoint visits. The average baseline AUC for the active group is not significantly different from the average baseline

AUC of the placebo group, specifically a p-value of 0.31. To be able to compare their changes in AUC, it is important to show that their baseline values are not significantly different. The active group tended to decrease in AUC from baseline to endpoint visit, however, it is not a significant change. An outlier is present in the active group that has a dramatic increase in glucose AUC, shown in both **Figure 11D** and **Figure 11E.** If the outlier was removed, there would be a greater difference between the baseline and endpoint visits since the p-value would drop to 0.09. The placebo group increased in AUC significantly from baseline to endpoint visit, specifically from 5815 to 7233.6 mg/dL(min). These scatterplot graphs allow for the presentation of individual change from baseline to endpoint visit. In **Figure 11 (E-F),** a scatter plot is shown to demonstrate changes in AUC in the placebo and active groups. The change in AUC for the placebo group is significantly higher than the change in AUC of the active group, specifically a p-value of 0.01. In **Figure 11D**, a Before and After graph, is presented to link the subject's baseline to endpoint visit. In the active group, 10/15 subjects decrease in their glucose AUC. However, in the placebo group, only 2/12 subjects decrease in AUC, while 10/12 increase in their glucose AUC. Overall, these figures suggest that the placebo and active groups are changing in their glucose AUC differently in response to the specific diets that they were given. It also suggests that it might be helping the active group become less insulin resistant and more glucose tolerant than the placebo group, however, predictions can't be confirmed without comparing these MTT glucose curves with their respective insulin curves.

Since conclusions cannot be made about the changes seen in the overall groups without the insulin concentrations, a few individual subject graphs have been constructed to discuss trends that have occurred after the intervention. Two subjects from each group have been selected and depicted in **Figure 12 (A-B)** and **Figure 13(A-B)**. According to the American Diabetes Association (ADA), fasting glucose concentrations between 100-125mg/dL indicate pre-diabetes[4]. Also according to the National Institutes of Health (NIH), it has been suggested that during an OGTT, normal subjects should peak at 30-60 minutes, and after 2 hours they should return to their fasting glucose concentrations[5]. However, during a MTT, individuals tend to peak and respond to the increase in glucose concentration a little later since they are also supplied with fats and proteins in their meal. The diagnosis for metabolic syndrome, pre-diabetes, includes a list of risk factors that subjects are not required to pass every point to become diagnosed. As a result, not all of our subjects have meet all of these requirements set by the NIH and ADA for diagnosis of metabolic syndrome, such as fasting glucose concentrations over 100mg/dL. However, we presumably assume that the subjects have same impairment of glucose tolerance that could potentially be further improved with a diet intervention.

Compared to the GTTs presented for the 12/15 LO TG study, the human curves have a greater degree of variability because of human diversity. Therefore, they need to be individually assessed with their respected insulin concentrations to form a confident conclusion about their levels of insulin resistance. However, speculations can be made by examining the glucose

excursion test curves. For example in **Figure 12A**, the glucose tolerance curve is shown for MS34, an active subject. MS34's fasting glucose concentration from the baseline visit is 106mg/dL, which is at a state of impaired glucose tolerance and pre-diabetes. However, after the diet intervention it has decreased to 94.8mg/dL, which is under the pre-diabetic fasting glucose concentration set by the ADA. Also, the subject peaks earlier and at a lower glucose concentration at the endpoint visit, which suggests that her insulin sensitivity has increased and she is better able to clear out the excess glucose provided to her. Also, after the peak, the subject decreases in glucose levels quickly suggesting that he/she is better able to respond to the insulin provided to her. Again, these predictions can't be confirmed without comparing them to the insulin concentrations. Since at the baseline visit the subject doesn't return to fasting glucose concentrations, she is considered to have impaired glucose tolerance. However, at the endpoint visit, the subject returns to fasting glucose at 120 minutes. Overall, the glucose AUC of MS34 decreased from 8175 to 5720mg/dL(min), presented in **Figure 12B,** which also presumably suggests that the diet helped MS 34 decrease in her insulin resistance.

In **Figure 12C**, the glucose tolerance curve is depicted for another active subject, MS 25. The subject did not have high fasting glucose concentration, and there was a decrease from 90.4 to 84.7mg/dL from the baseline to endpoint visit. Although, MS 25 had normal fasting glucose levels, he peaked at 90 minutes, which is considered abnormally late for a normal individual since they usually are unable to return to fasting glucose levels. However, at the endpoint visit, he/she

peaked at 60 minutes and at a lower glucose concentration. Also, since MS 25 peaked later at baseline, he was unable to return to fasting glucose levels after 2 hours, which suggests that he also had impaired glucose tolerance. At the endpoint, MS 25 was able to return to normal fasting glucose levels after 2 hours, which suggests that the diet has improved his level of insulin resistance and glucose tolerance Interestingly, his AUC decreased slightly from 7536 to 6759 indicated in **Figure 12D,** which suggests that even a slight decrease in AUC can cause a subject to see improvements in his health. MS 25 did not lose any weight or fat mass during the study, which possibly suggests that improvements were seen because of the types of food he was consuming and not on weight loss.

In **Figure 13C,** the glucose tolerance curve is illustrated for a placebo subject, MS 5. The fasting glucose concentration for MS 5 remains about the same from baseline to endpoint visit, and it was at a normal level at the baseline visit to begin with. At the baseline visit, MS 5 peaks at 30 minutes, however, it seems as though there was a large amount of insulin secreted, since her glucose levels dropped close to a state of hypoglycemia at 71.75mg/dL at 90 minutes. A state of clinical hypoglycemia is at a threshold of 70mg/dL for humans, and counter regulatory compensation threshold is at 60-70mg/mL[26]. Since the subject drops quickly to near hypoglycemia levels, it can be inferred that hormones such as glucagon and norepinephrine are acting to raise the subject's glucose levels. These hormonal actions are due to counter regulatory responses set up by the body to secrete various hormones to subsequently raise the subject's glucose

levels to their fasting glucose concentrations. The baseline curve suggests that MS 5 is insulin resistant, therefore, the body continuously responds to an increase of glucose with a great deal of insulin, which can put her at a state of hypoglycemia. During the endpoint visit, the glucose concentrations peak much later, specifically at 90 minutes with a higher glucose concentration of 135 mg/dL. However, he/she returns to fasting glucose levels after 2 hours without hypoglycemia. Also, MS 5's glucose AUC increased from baseline to endpoint visit, specifically from 5067 to 7830, shown in **Figure 13D**. Since we don't have the insulin concentrations, we can't conclude if the placebo diet had a positive or a negative outcome on MS 5. MS 5 doesn't go into a state of hypoglycemia during the endpoint visit, which could potentially be a positive outcome of the diet, however, he/she is at a state of hyperglycemia for a longer period of time. Increased exposure to glucose, like the ones seen in large glucose curves can have potentially negative outcomes. MS 5 lost 8% of her baseline body weight, which suggests that losing a large amount of weight on the placebo diet isn't necessarily associated with improved glucose tolerance.

In **Figure 13A**, the glucose tolerance curve is illustrated for another placebo subject, MS 20. The subject started and ended the study with the same fasting glucose concentration of about 99mg/dL, which is a borderline concentration to be considered pre-diabetic. However, the glucose curves after differ significantly. At the baseline visit, MS 20 peaked at 30minutes and it is assumed that she was at a state of hyperinsulinemia, which caused the subject to become hypoglycemic for about 30-60 minutes. MS 20's body was unable to

compensate as well MS 5's counter regulatory hormones did, therefore, she didn't return to fasting glucose levels at the end of 2 hours. At the endpoint visit, MS 20 peaked at 60 minutes, but at a higher glucose concentration. The subject's glucose levels slowly declined, but did not reach fasting glucose levels at 2 hours. The glucose AUC also increased from 4286 to 8850, shown in **Figure 13B.** Since the AUC increased for the subject, she never returned to fasting glucose concentrations, and her glucose exposure was greater at the endpoint than the baseline. Without the insulin levels, it can't be determined if the individual's state of hyperglycemia is better or worse than her state of hypoglycemia, however, this could possibly suggest that the individual was still at a state of impaired glucose tolerance and highly insulin resistant at the end of the study.

Overall, the evaluation of the active group's individual glucose curves allow us to suggest that the diet intervention improved their levels of insulin resistance, however, the same can't be said about the placebo group. More information is necessary to infer if the placebo group was negatively affected by the diet.

Discussion

Metabolic Syndrome has become increasing prevalent throughout the years; however, an appropriate solution has not been discovered yet. Since metabolic syndrome encompasses a vast array of risk factors, such as insulin resistance, obesity and inflammation, it has become increasing difficult to target all of its elements. The human diet intervention study and the 12/15-Lipoxygenase transgenic mouse study aimed to reduce insulin resistance for the alleviation of metabolic syndrome.

The 12/15 LO TG mouse study aimed to expand on the current knowledge of 12/15 lipoxygenase enzyme because it has been hypothesized as being a therapeutic target for the treatment of HFD-induced insulin resistance. Since recent studies have proven that 12/15LO KO can protect against insulin resistance, we took on a group of transgenic mice to see if they would have increased levels of insulin resistance after being put on a HFD for 10 weeks. We first began to examine weight changes and eating patterns, since constant weight gain between the experimental and the control group is a vital component to our results. WT mice ate more grams of food than the TG mice at every time point during the 10-week diet. However the TG mice tended to weigh more than the WT mice, but the weight gain was not significantly different at the end of the study. Therefore, we speculate that the TG mice experienced lower energy expenditure than the WT, which pre-disposed them to obesity and insulin resistance. In future studies, metabolic cages can be used to assess these mice for low energy expenditure. At the end of the study, we also found that the TG

mice had larger gWAT fat pads than the WT mice. Since their body weights weren't significantly different, the TG mice developed a significantly higher body fat percentage than the WT mice. We also looked at their levels of free fatty acids, since insulin resistant adipocytes tend to have an increased FFA secretion into the plasma because the insulin is unable to inhibit lipolysis. However, we didn't see a significant difference between the two groups. Although the FFAs were not significantly different, we assessed for triglycerides, which provided us with evidence that there is an increased amount of hepatic insulin resistance in the TG mice compared to the WT. Liver TG content is currently being assessed in these mice by our collaborators. Lastly, FACS was used to measure levels of M1 cells in the adipose tissue for the assessment of inflammation. The TG mice had higher levels of M1 cells in the stromal vascular cells of the gWAT, however, their levels weren't significantly higher.

The Glucose Tolerance Test (GTT) and the Insulin Tolerance Test (ITT) provided us with evidence that the 12/15 TG mice were significantly more insulin resistant than the WT mice. At every single time point on the GTT, the TG mice expressed great hyperglycemia than the WT mice along with increased hyperinsulinemia. The glucose AUC for the TG mice was significantly larger than the WT mice, which meant that they were unable to utilize their insulin efficiently to clear out the excess glucose. The ITT might have been overall inconclusive, however, the 15-minute time point provided us with greater evidence that the transgenic mice were having difficulties responding to the insulin provided to them. The percent baseline of the blood glucose concentrations and absolute

glucose concentrations were significantly greater in the TG mice than the WT, suggesting that the TG mice had greater insulin resistance. Since the TG mice were significantly greater at every time point on the GTT, we suggested that it was due to systemic insulin resistance. Although we suggested that the insulin resistance was on the whole body, additional experiments can be performed to further confirm this hypothesis. For example, performing a hyperinsulinemic-euglycemic clamp to assess for insulin sensitivity in individual organs of the mice. The clamp can be conducted by infusing the mice with radiolabeled-[^{3}H]-glucose and insulin to see which specific tissues are producing or taking up the glucose. Lastly, another experiment that could further our knowledge about the specific tissues that are more insulin resistant than others would be an Acute Insulin Response Test, where glucose would be injected into the mouse's vena cava and tissues would be quickly taken and assessed at specific time points for activation of insulin signaling. Insulin signaling would be assessed by probing for phosphorylated proteins, such as Akt, since it is a rate-limiting step in the insulin signaling pathway. This experiment would provide data identifying which tissues were the most insulin resistant. These experiments would hopefully further confirm our hypothesis that TG mice have greater whole body insulin resistance than the WT mice.

However, since the TG mice experienced higher fasting glucose concentrations than the WT, it could also be suggested that it was also due to Beta-cell defects, which led to reduced insulin production. Examining the insulin concentrations at the baseline vs. 15-minute time point of the GTT, the TG didn't

increase insulin significantly, which can also be a result of Beta-cell defects. Therefore, to confirm if our results were due to primarily insulin resistance or to Beta-cell death, we could repeat the GTT and collect blood additional time points to look at insulin levels. We could isolate islet cells and do an in vitro GSIS (Glucose-Stimulated Insulin Secretion) assay, which would measure how much insulin the Beta-cells were producing upon the stimulation of glucose and identify Beta-cell autonomous defects. Another collaborator is currently conducting histological studies of the pancreas to examine Beta-cell area and stain for insulin. This will provide us with some data about Beta-cell function and proliferation.

As a result of the 12/15 LOTG study, we can suggest that this enzyme is a main target for insulin resistance. Therefore, a future study could possibly use omega-3 fatty acids in the mouse diet to see if it would reverse the effects that 12/15 lipoxygenase transgenic mice express on a HFD, which is enriched with omega-6 fatty acids. We speculate that the fish oil diet will cause the transgenic mice to be healthier and less insulin resistant than a set of WT mice on the same chow diet. These experiments will help expand our knowledge of lipoxygenase enzymes and the importance of supplementing omega-3 fatty acids into one's diet.

The human diet intervention study targeted a different component to improving insulin resistance, which was to test the applicability of a novel dietary approach engineered to improving insulin resistance associated with metabolic syndrome. The clinical trial set out to find 40 subjects with metabolic syndrome,

and put them on a diet that incorporated low glycemic index foods, fish oils and polyphenols or an isocaloric placebo-controlled diet. After the subjects completed the 12 week study, the groups were compared. On average, both groups lost about the same amount of weight, and their weights significantly decreased from when they had started the diet. Both groups also lost the same amount of fat mass, and their lipid panel improvements were not significantly different. These changes were not expected since the subjects were put on 1500 calorie diets. The aim of the study was to find out if this diet could improve the active group's level of insulin sensitivity compared to the placebo groups. Therefore, a meal tolerance test was performed, and the area under the curve was used as a comparison factor. Since increased area under the curve correlates with increased insulin resistance, we accessed the MTT to see if individuals dropped in glucose AUC after intervention. The placebo group had significantly increased in AUC from baseline to endpoint, the active group had decreased in AUC but it wasn't significant. We evaluated the individual glucose curves of these patients to see if the diet was contributing to increased insulin responsiveness. These curves suggest that there was a difference in responsiveness between the two groups, however, final conclusions can not be made without comparing these results to their corresponding insulin concentrations. Since the diet study has not been completed yet, there are a few predictions that we have make about the results so far. Some of the predictions include, decrease in inflammatory cytokines and CRP in the active group compared to the placebo group, since we also expect to see a decrease in the expression of 12/15 LO. Also an increase in

resolvins and protectins, since they are by-products of omega-3 fatty acids. Lastly, we expect to see a decrease in hyperinsulinemia and hyperglycemia, with an increase in insulin sensitivity.

Success in these two studies will bring us closer to finding innovative, education and application alternatives to improving insulin resistance, and decreasing the risk for Type 2 diabetes, cancer, stroke and cardiovascular diseases.

Figures

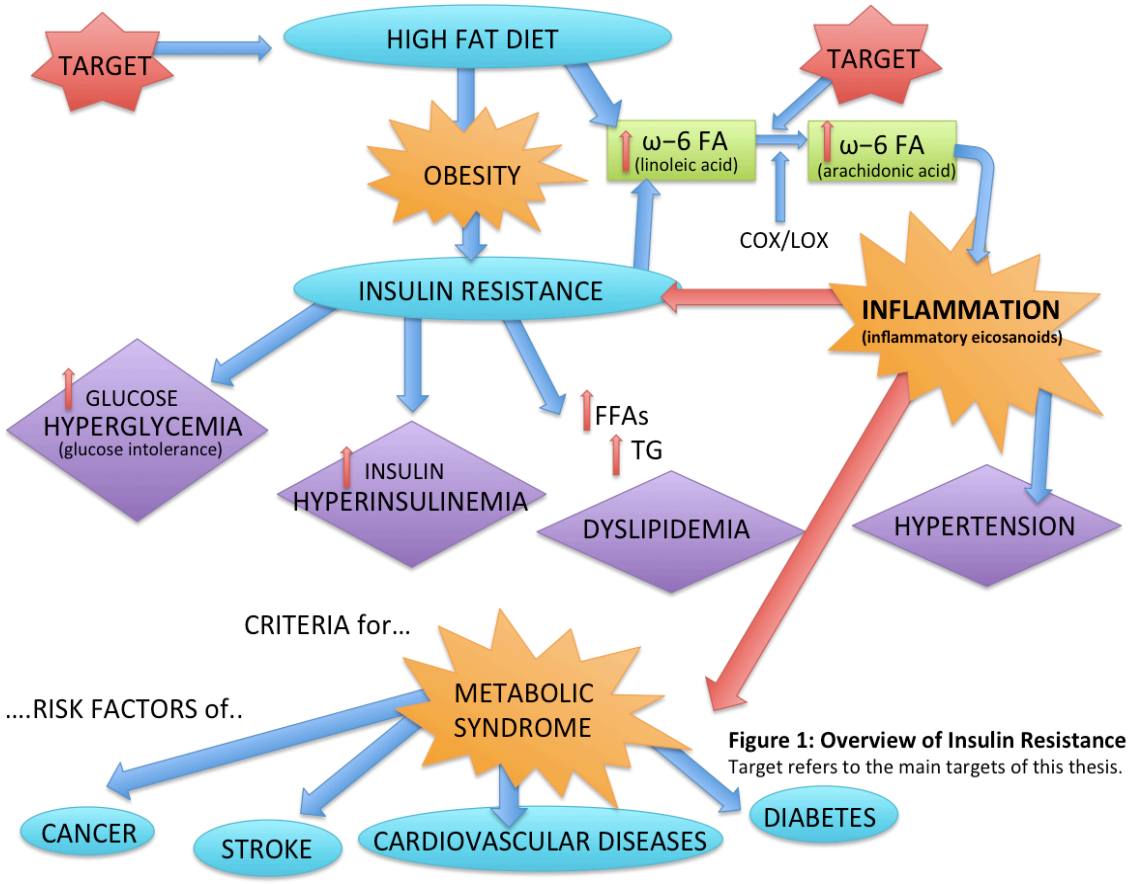


Figure 1: Overview of Insulin Resistance
The “targets” refer to the main targets of this thesis.

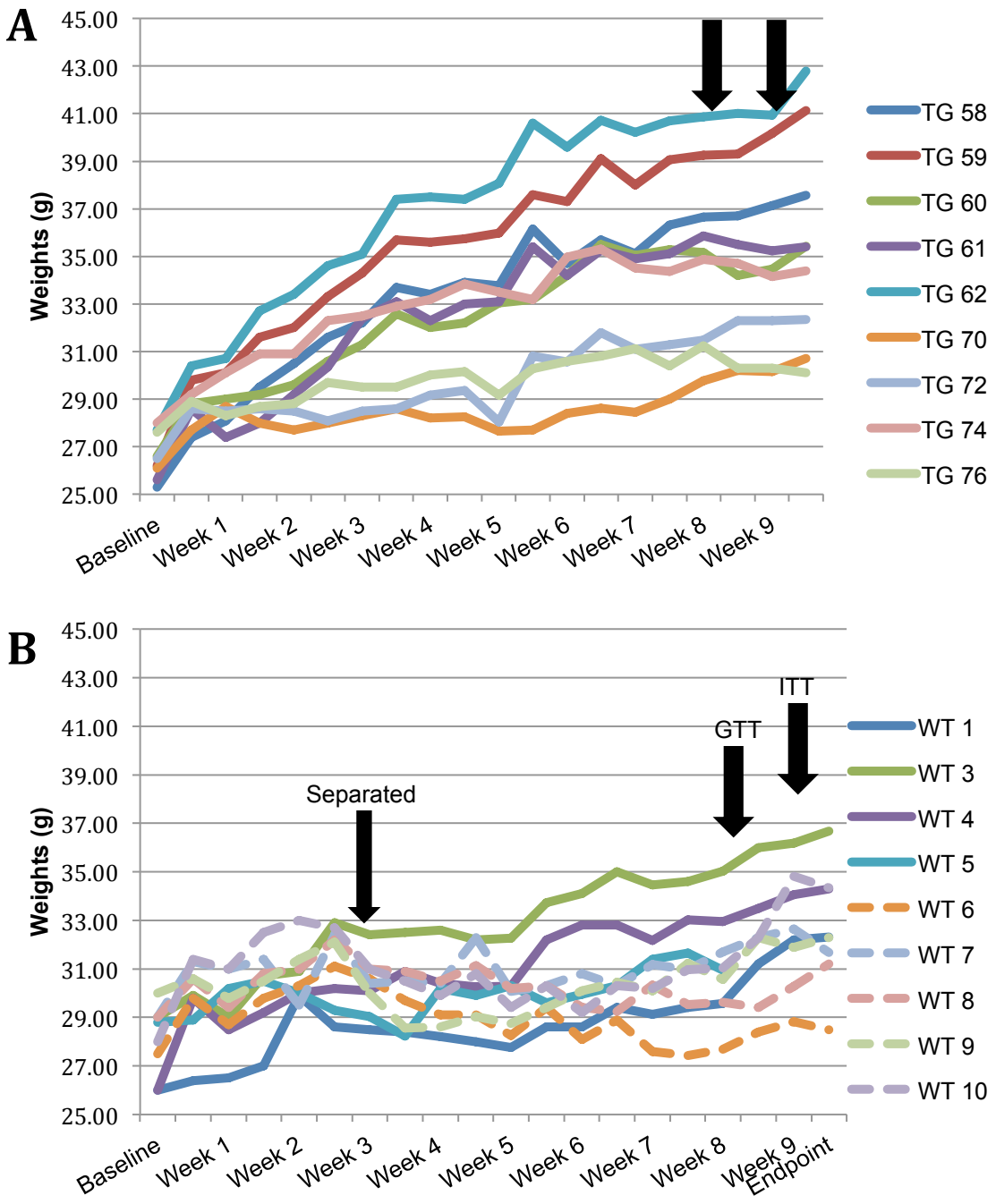
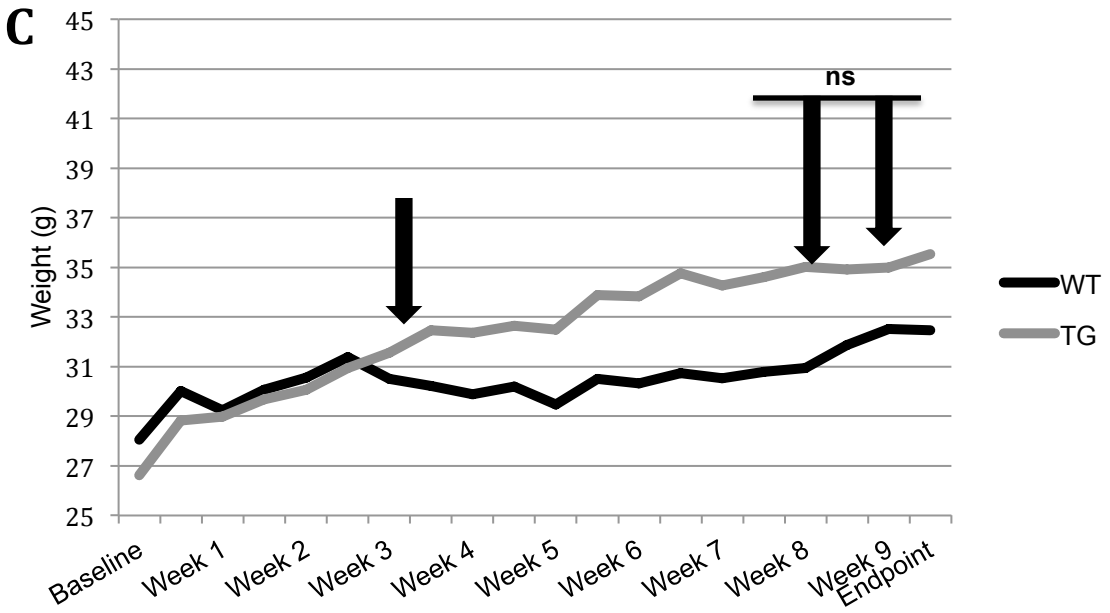


Figure 2. Weight Changes on HFD

(A) Wildtype (WT) weight changes and (B) Transgenic Mice (TG) weight changes for 10 weeks, weights were taken twice a week. The TG and WT are not significantly different in weight during the GTT, ITT and tissue harvesting (week 8, 9, 10), represented with arrows. WT Mice in cage #2 (6-10) were separated after 3 weeks. (C) The average total weight differences was calculated for TG and WT at every time point (D) Overview of final average weight differences are plotted, data are shown as averages +/- standard error, no significant differences (ns),*p<0.05



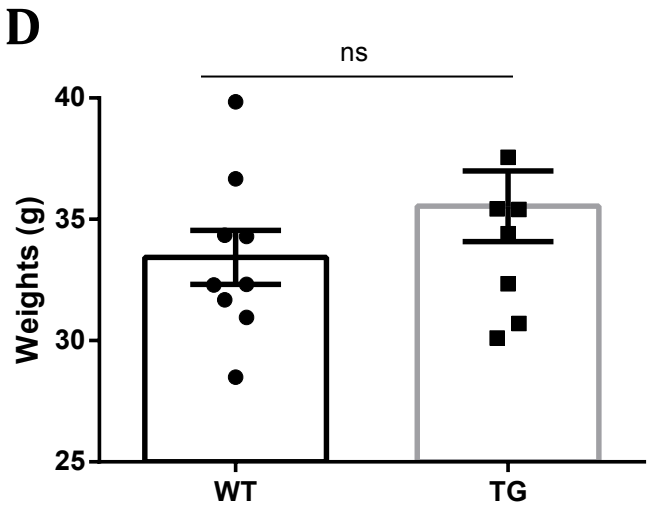


Figure 2. Weight Changes on HFD, continued.

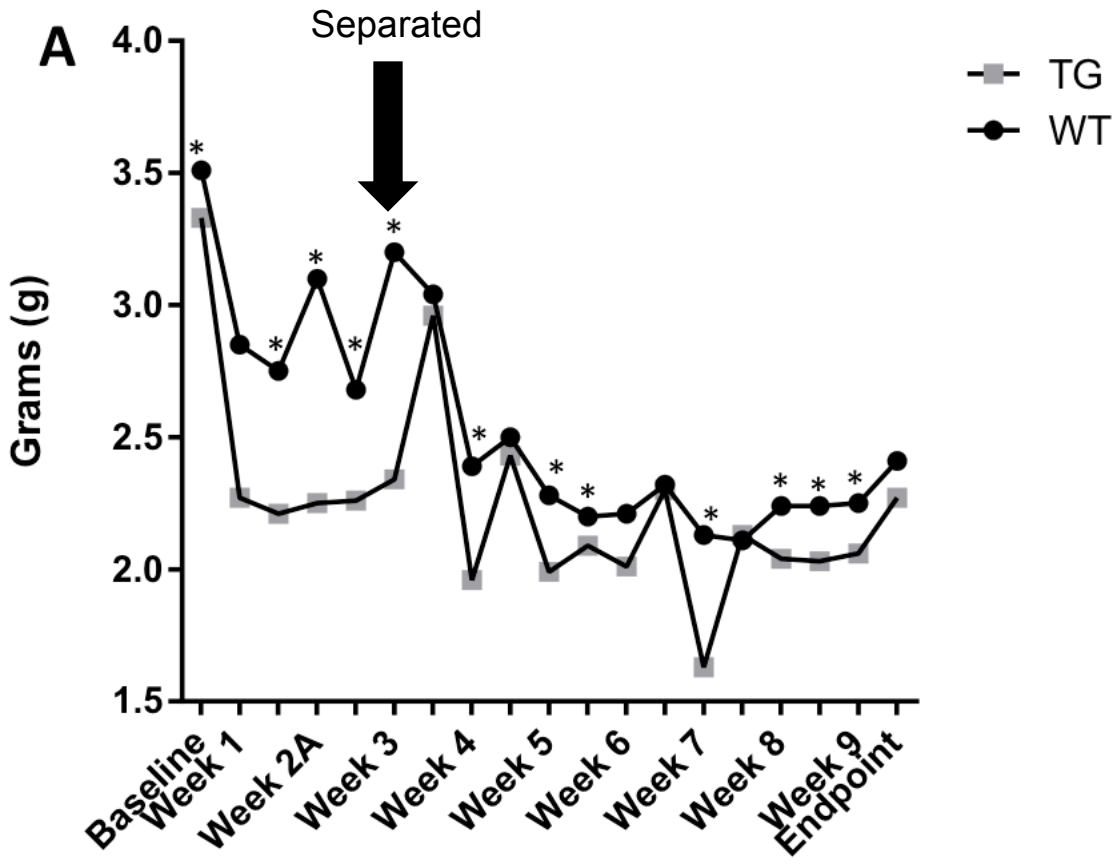


Figure 3. Eating Patterns for Wildtype (WT) and 12/15 Lipoxygenase (TG)

(A) Eating pattern during a 10 week 45% HFD study. Arrow represents week 3 when Cage #2 (WT 6-10) were separated due to aggressive behavior. At all time points, the WT eat more grams/per day/per mouse than the TG, and 12/20 of those time points are significant.
*p<0.05
Data are shown as averages +/- standard error

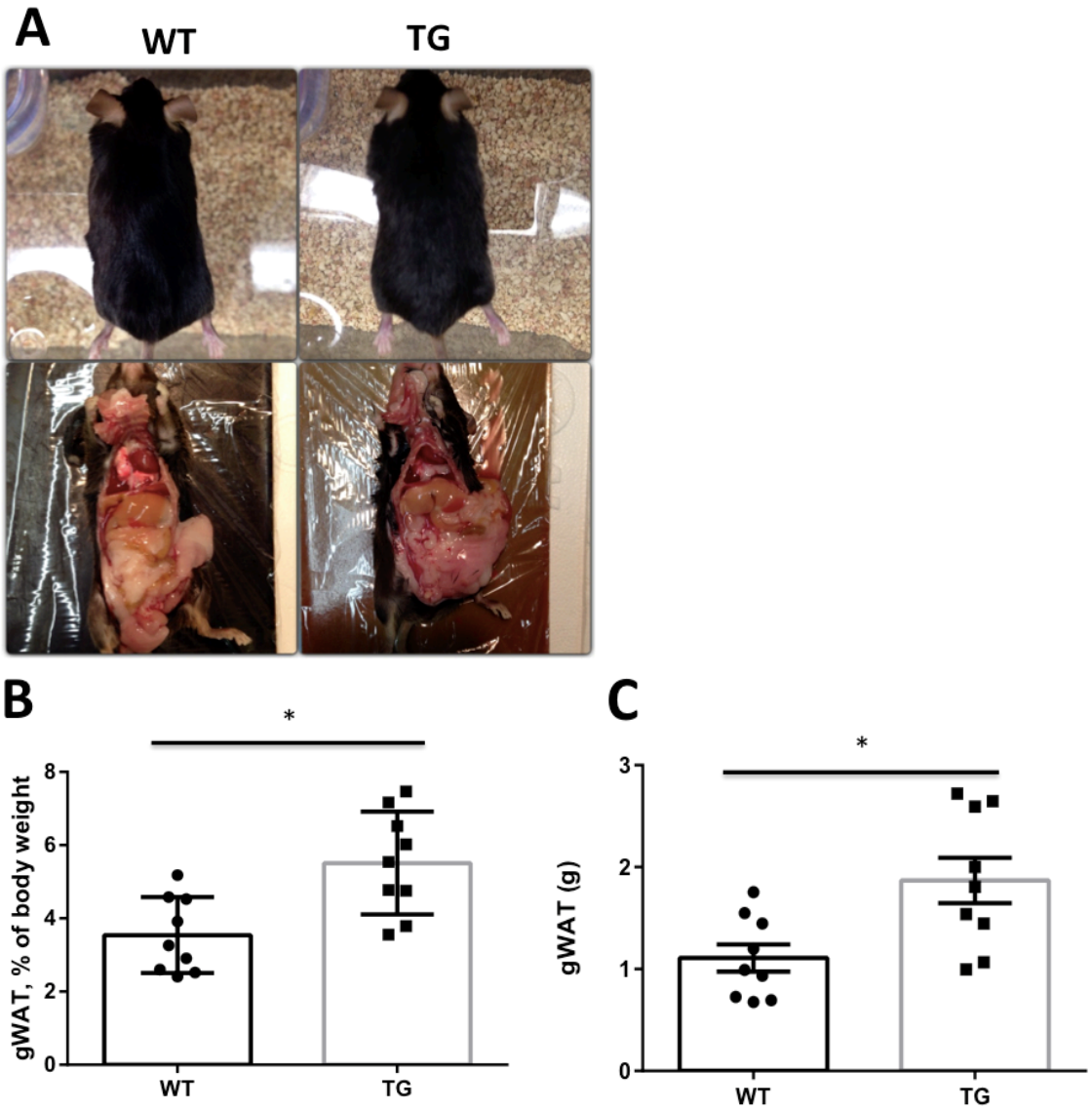


Figure 4. Photos, gWAT Percentage of Body Weight, Fat Pads

(A) Eating pattern during a 10 week 45% HFD study. Arrow represents week 3 when Cage #2 (WT 6-10) were separated due to aggressive behavior. At all time points, the WT eat more grams/per day/per mouse than the TG, and 12/20 of those time points are significant.
*p<0.05
Data are shown as averages +/- standard error

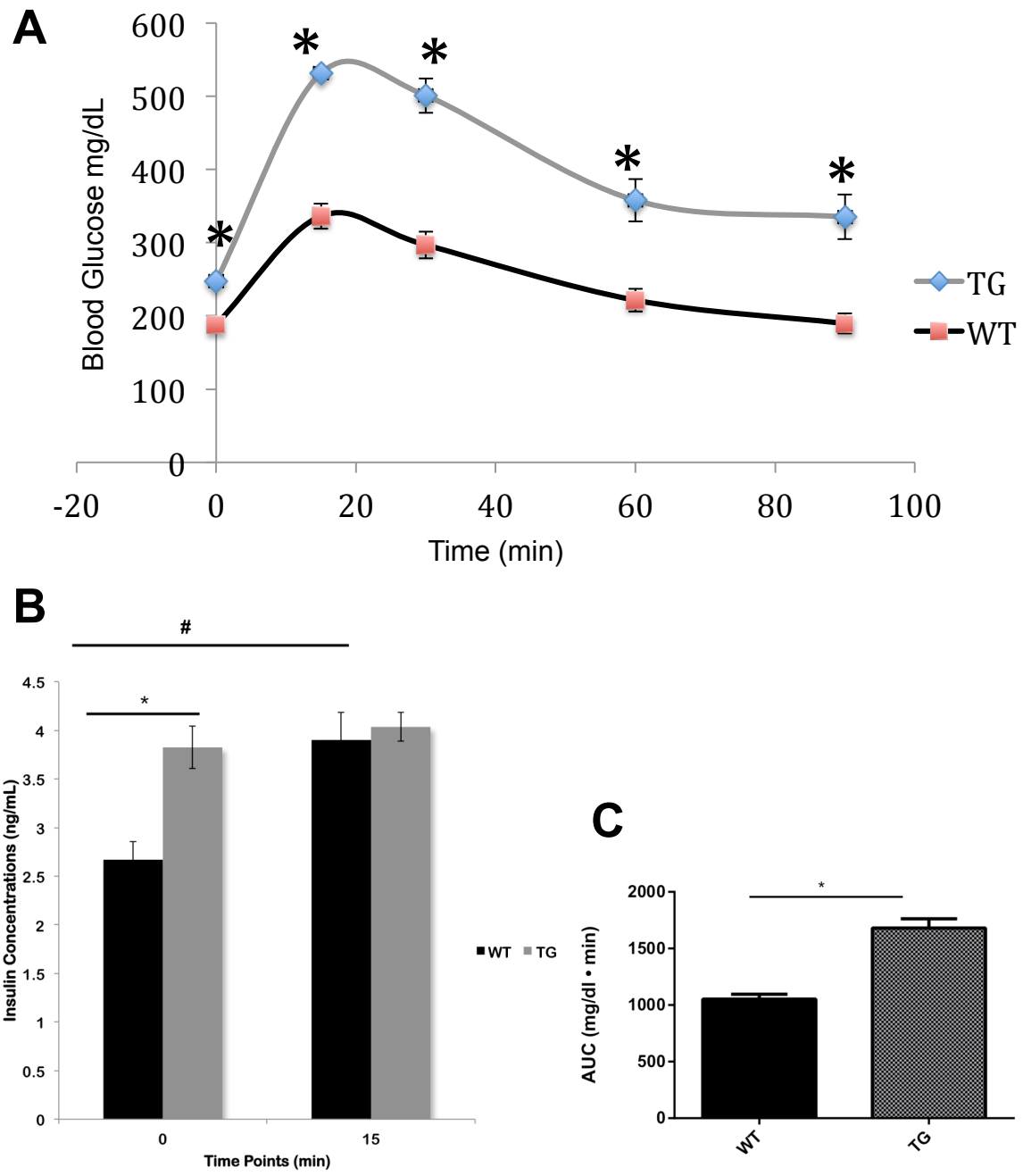


Figure 5. Glucose Tolerance Test (GTT) at Week 8

(A) The glucose tolerance test curve (GTT)
(B) Plasma Insulin Concentrations at 0 min and 15 min of GTT
(C) Area Under the Curve (AUC) of glucose concentrations in WT and TG during the GTT
*p<0.05 (WT vs. TG)
#p<0.05 (0 min vs. 15 min)
Data are shown as averages +/- standard error

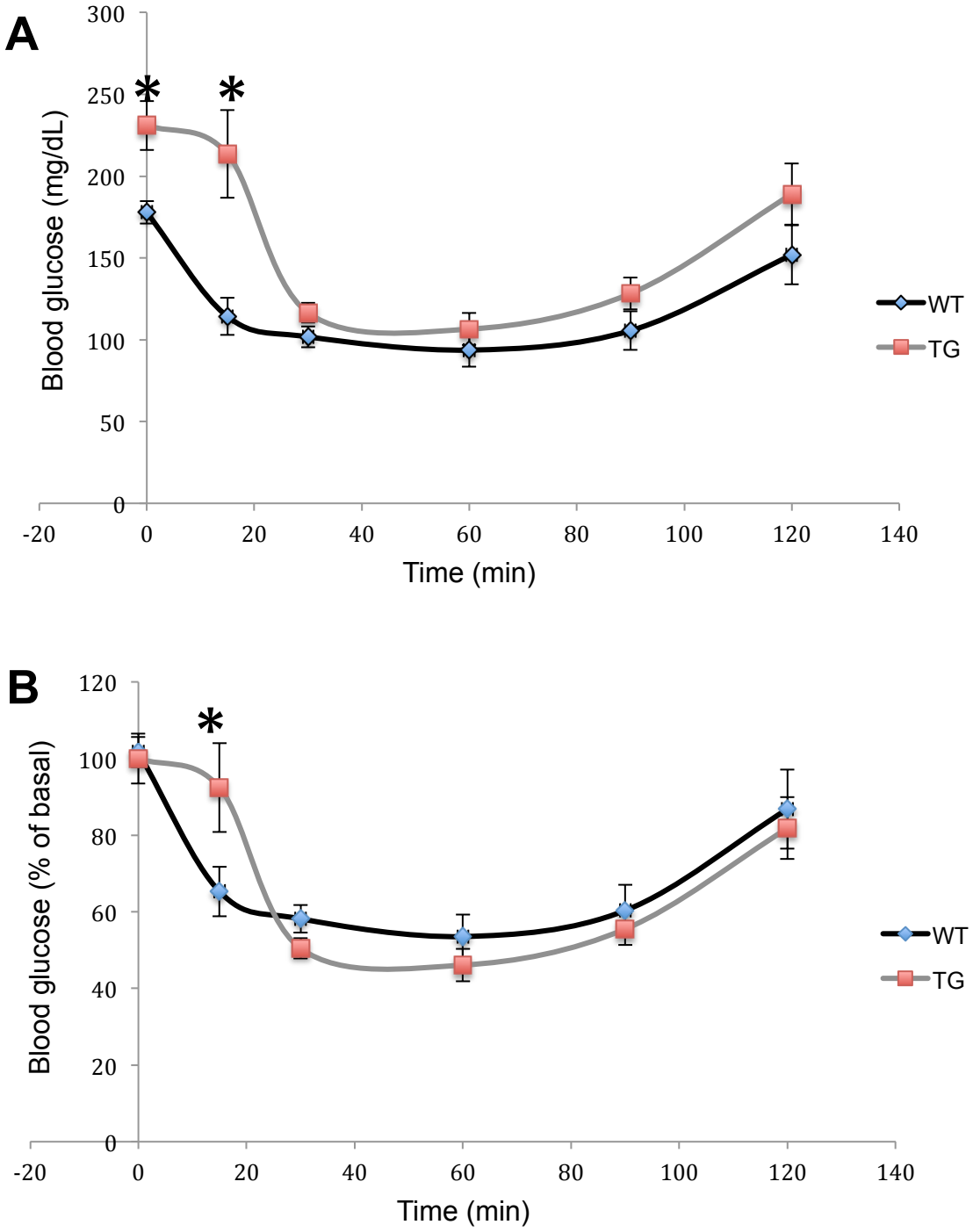


Figure 6. Glucose curve for Insulin Tolerance Test (ITT) at Week 9

(A) Glucose curve for ITT, absolute glucose concentrations (mg/dL)

(B) Glucose curve for ITT, percent change from baseline (%)

*p<0.05 (WT vs. TG), Data are shown as averages +/- standard error

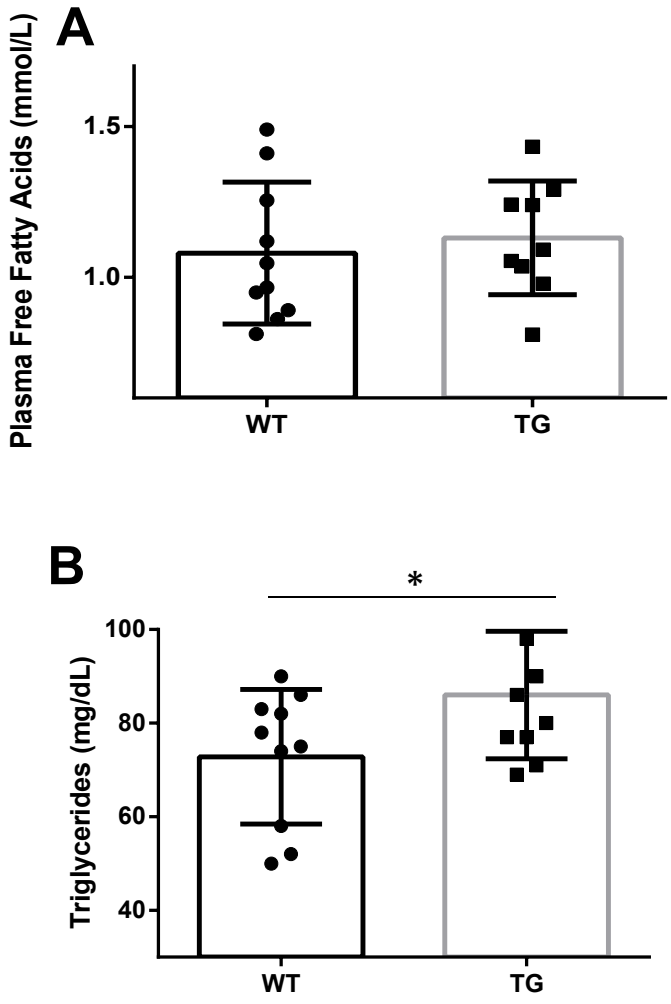


Figure 7. Triglycerides (TGs) and Free Fatty Acids (FFAs)

(A) Plasma Free Fatty Acid concentrations (mmol/L) at Week 8

(B) Plasma Triglyceride concentrations (mg/dL) at Week 10

*p<0.05 (WT vs. TG), Data are shown as averages +/- standard error

no significant differences (ns)

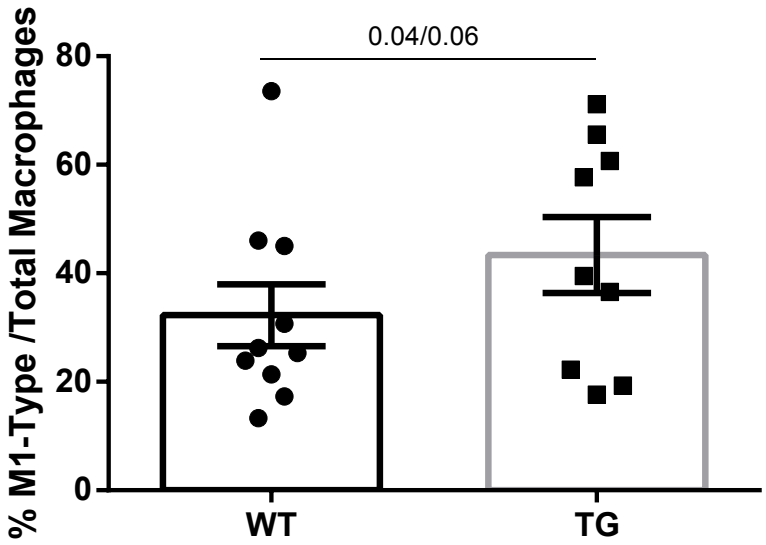


Figure 8. Adipose Tissue Inflammation

Percentage of M1 cells in total amount of macrophages found in the SVCs for WT and TG mice.

*p<0.05 (WT vs. TG), Data are shown as averages +/- standard error

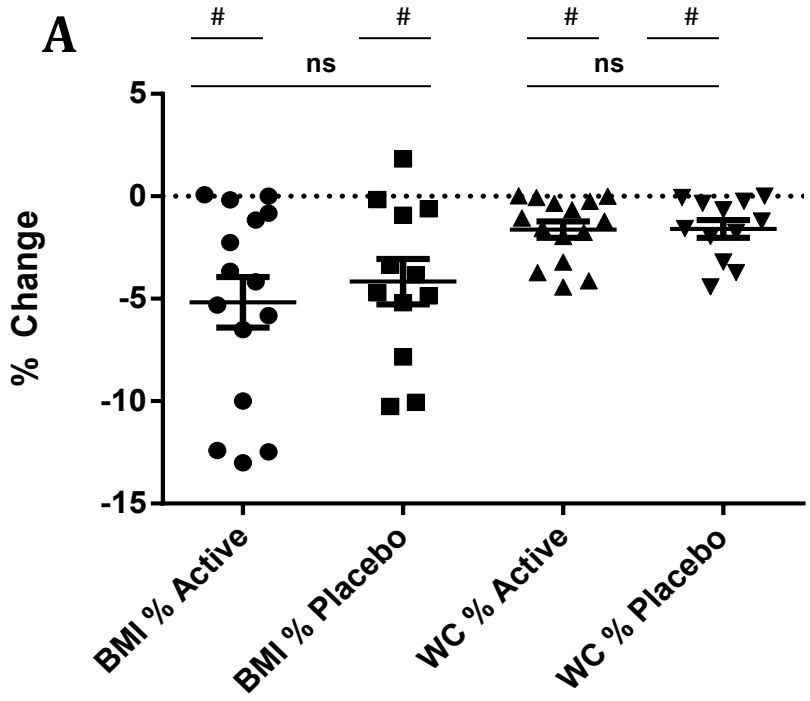


Figure 9. Percent Change in Metabolic Syndrome Risk Factors

(A) Average percent change (%) from baseline for Body Mass Index (BMI) and waist circumference (WC). Significant difference within the groups, but not between active and placebo groups.

(B) Average percent change (%) from baseline for Hemoglobin A1C. There were no significant changes within or between the groups

(C) Average percent change (%) from baseline for fasting glucose concentrations. If the outlier is removed, the change will be significant.

(D) DEXA scan represents the percent change in the fat and lean mass

#$p<0.05$ (baseline vs. endpoint), no significant differences (ns)

Data are shown as averages +/- standard error

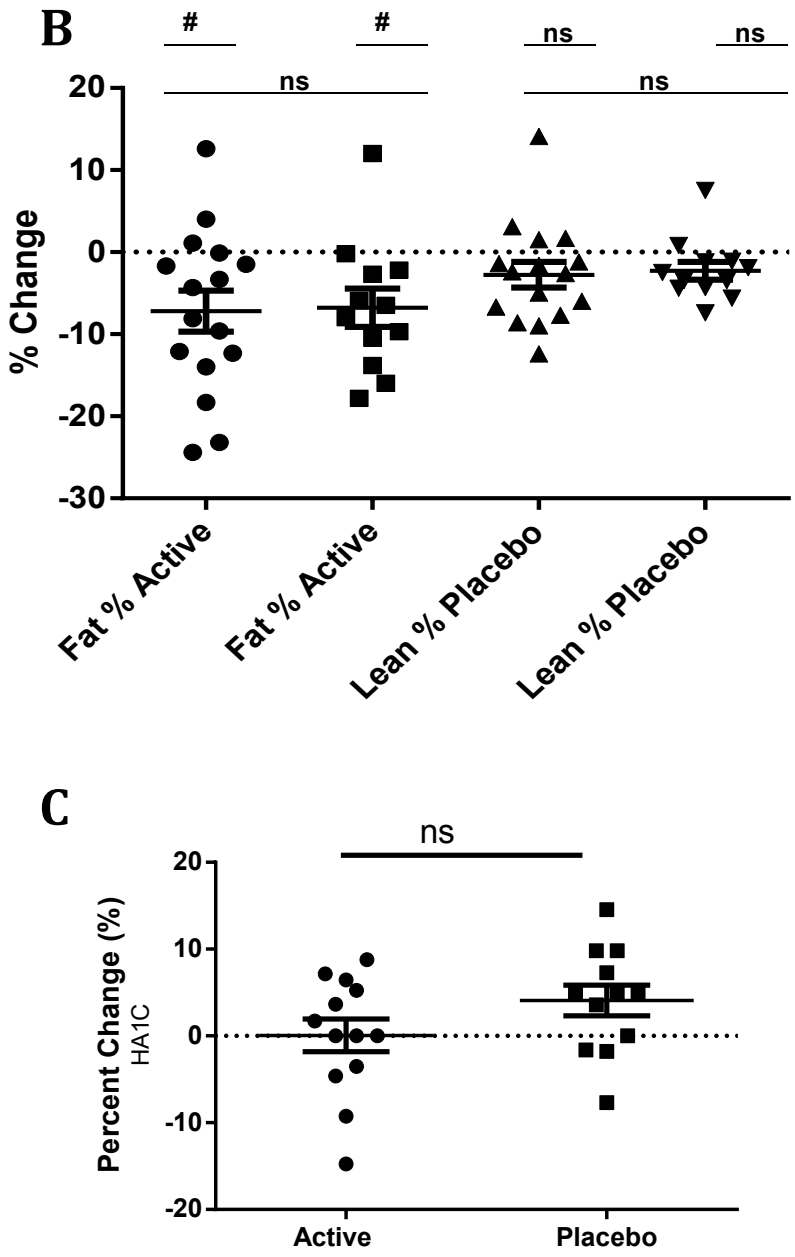


Figure 9. Percent Change in Metabolic Syndrome Risk Factors continued.

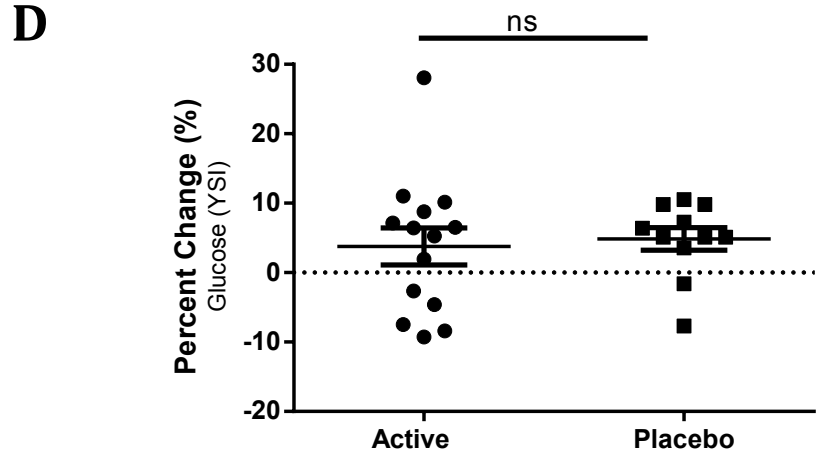


Figure 9. Percent Change in Metabolic Syndrome Risk Factors continued.

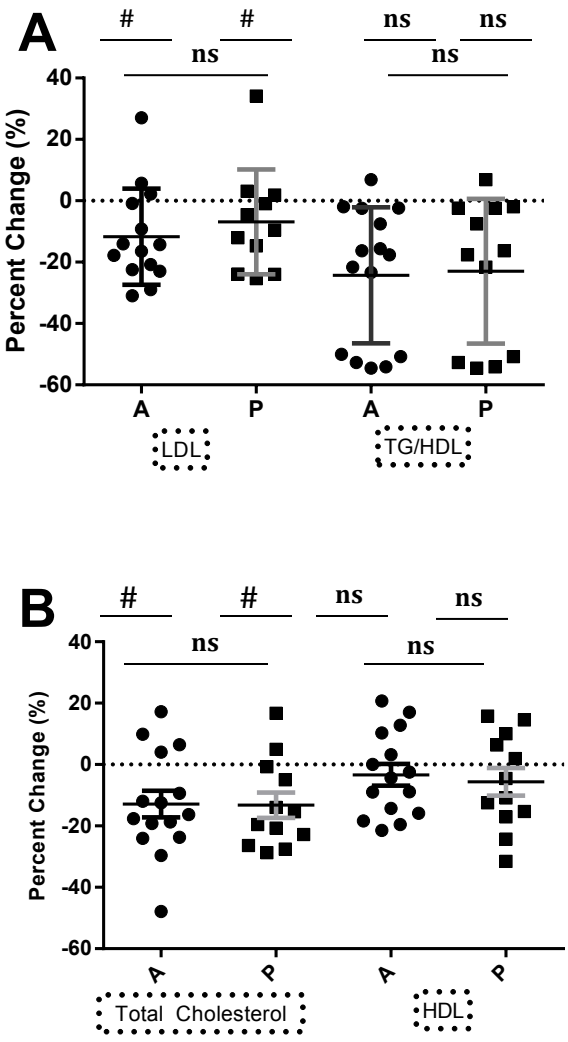


Figure 10. Lipid Panel Assessment

(A) Average percent change (%) from baseline for LDL and TG/HDL.
(B) Average percent change (%) from baseline for Total Cholesterol and HDL.
(C) Average percent change (%) from baseline for Triglycerides and Non-HDL concentrations. If the outlier is removed, the change will be significant.
#p<0.05 (baseline vs. endpoint), no significant differences (ns)
Data are shown as averages +/- standard error

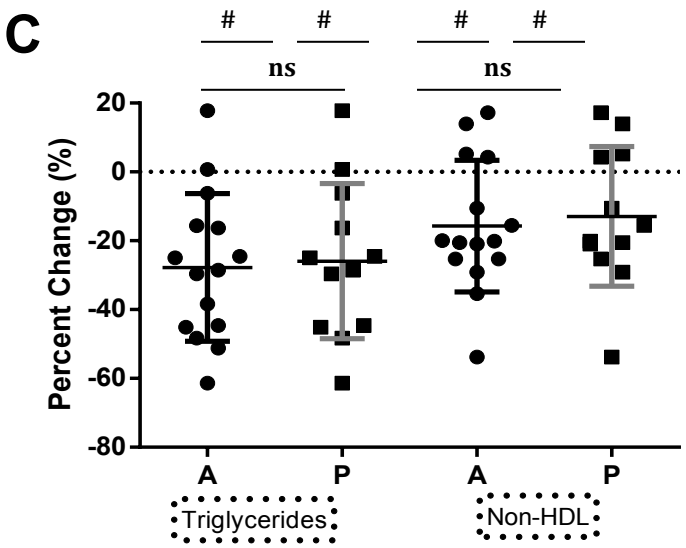


Figure 10. Lipid Panel Assessment continued.

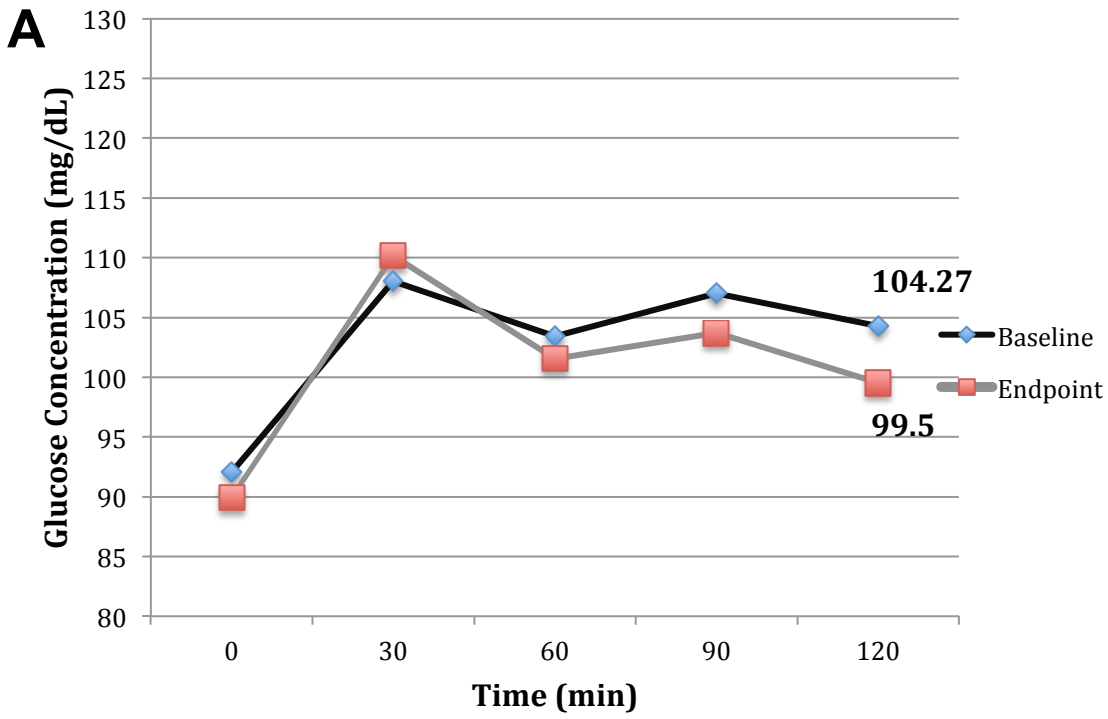


Figure 11. Glucose Excursion After Meal Tolerance Test (MTT)

(A) Line graphs comparing before and after of the Glucose Excursion of the Active group
(B) Line graphs comparing before and after of the Glucose Excursion of the Placebo group
(C) Glucose AUC at baseline and endpoint of both groups. Active group is slightly significant because if an outlier was removed it would be 0.09 from 0.66.
(D) Before and After graph shows relationship between individual AUC from baseline and endpoint. For active diet, 5/15 increased AUC, but 10/15 decreased in AUC. For placebo group, 10/12 increased AUC, but 2/12 decreased in AUC
(E) Change in AUC (endpoint-baseline) for individual subjects, with an outlier present in active group
(F) Average change in AUC (endpoint-baseline)
#p<0.05 (baseline vs. endpoint), no significant differences (ns),),*p<0.05
Data are shown as averages +/- standard error

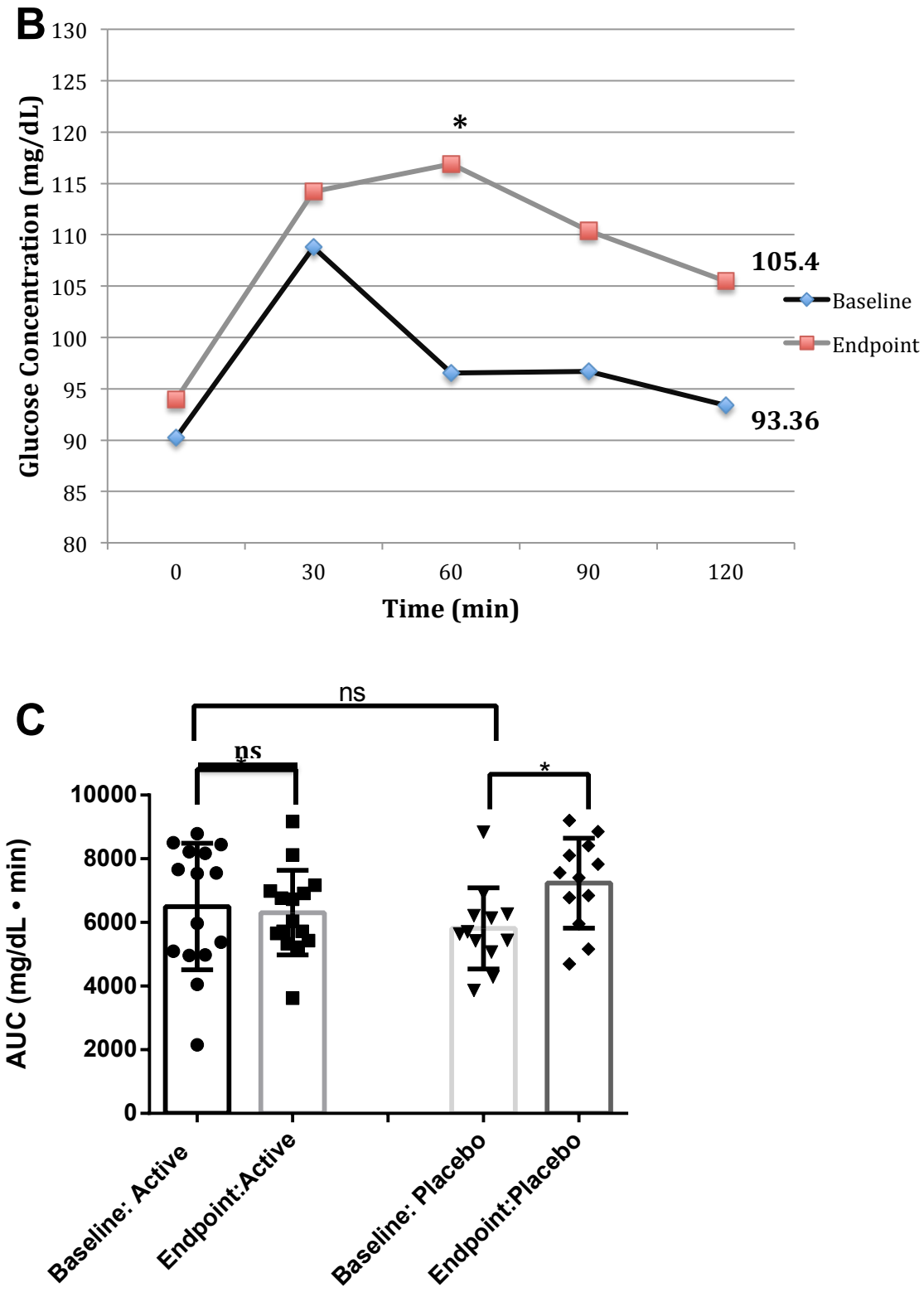


Figure 11. Glucose Excursion After Meal Tolerance Test (MTT) continued.

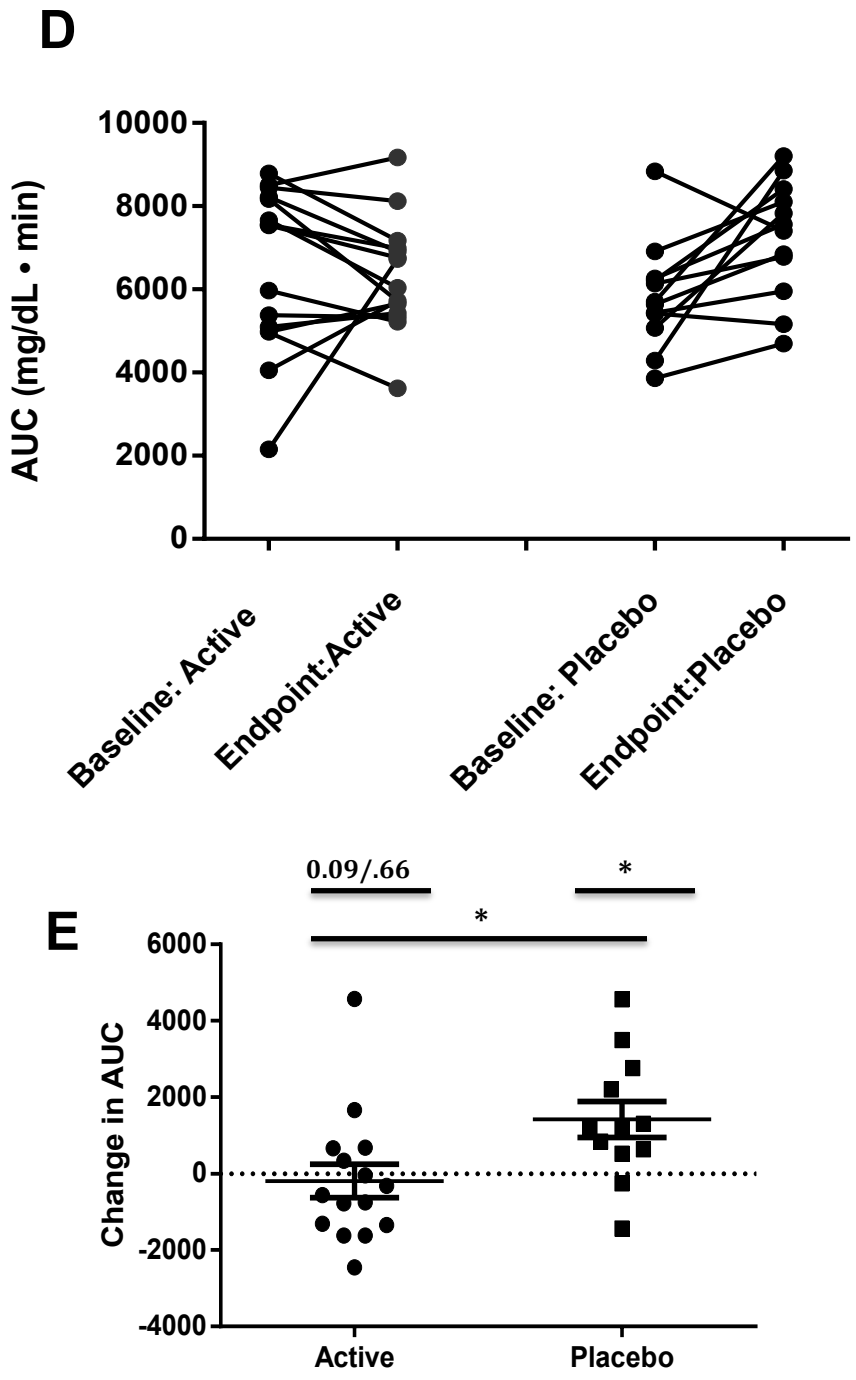


Figure 11. Glucose Excursion After Meal Tolerance Test (MTT) continued.

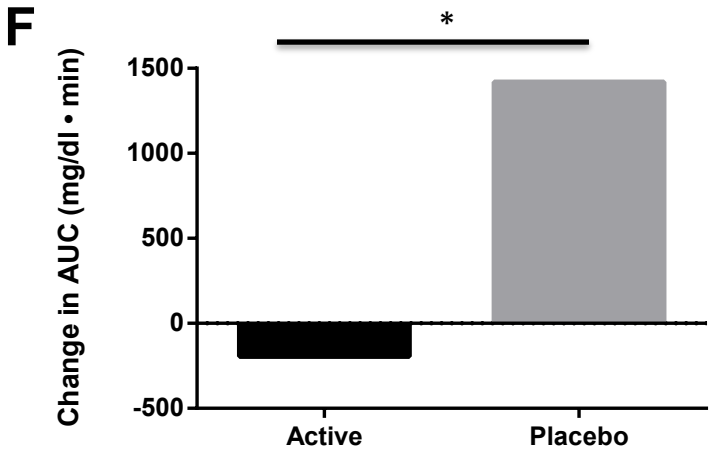


Figure 11. Glucose Excursion After Meal Tolerance Test (MTT) continued

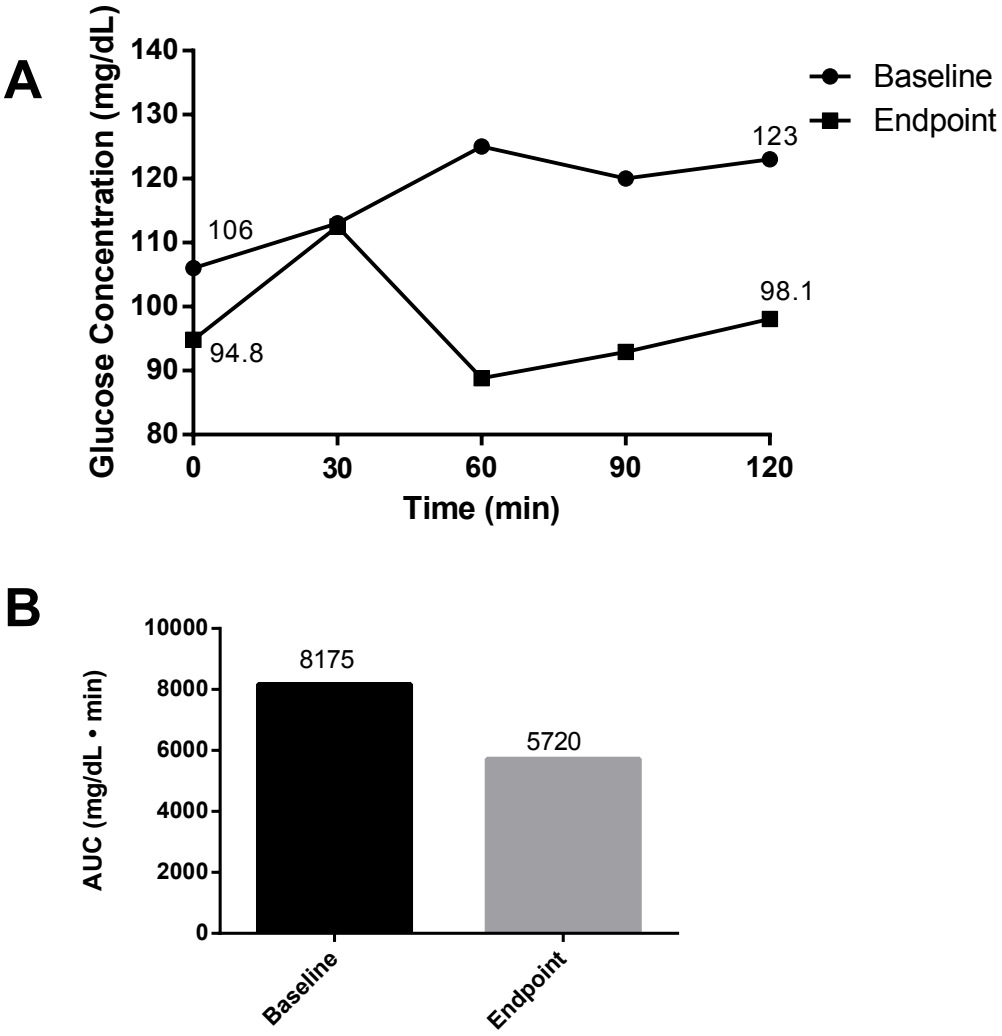


Figure 12. Glucose Excursion After Meal Tolerance Test Glucose Curve
(A) MS 34's glucose tolerance test curve and (B) AUC
(C) MS 25's glucose tolerance test curve and (D) AUC

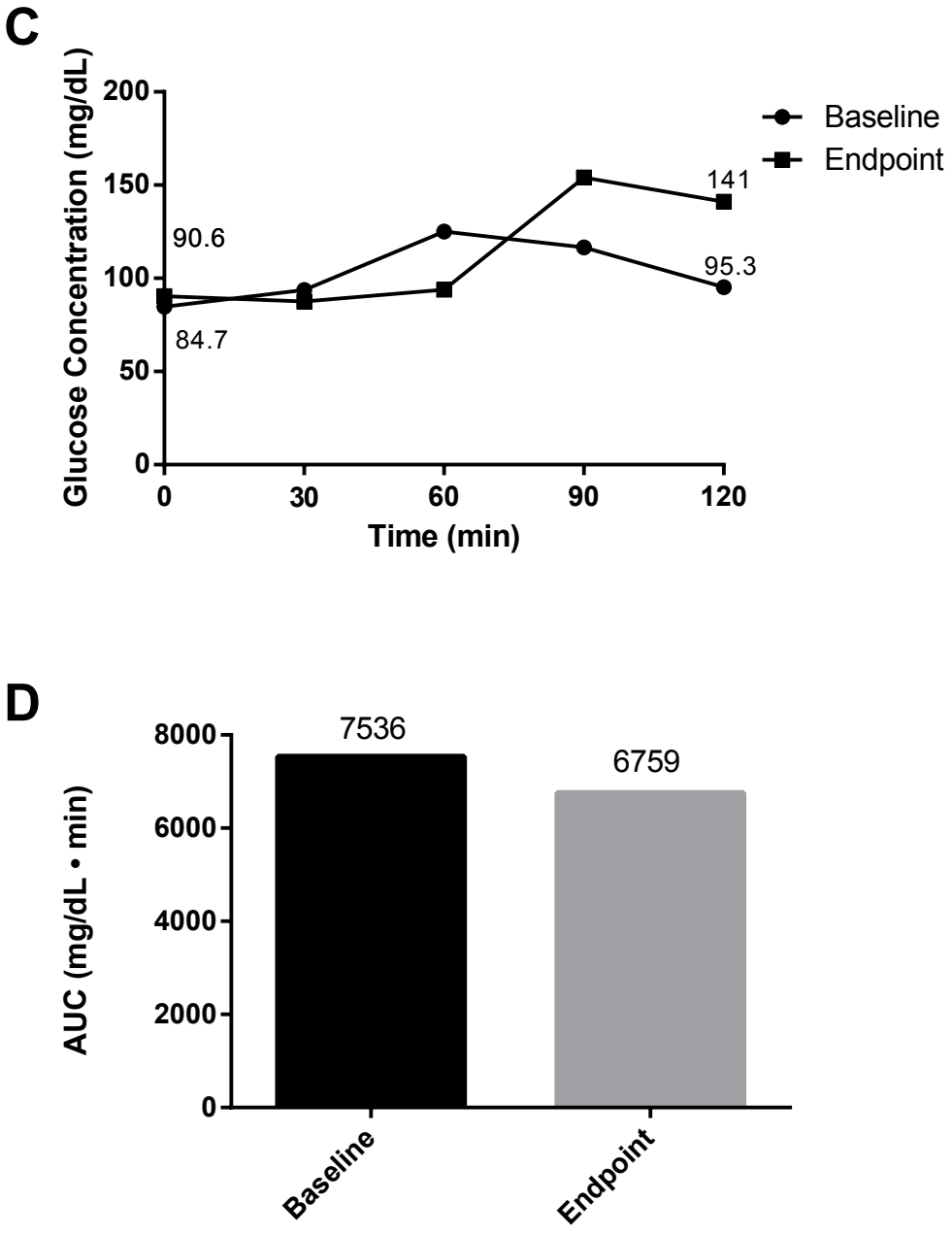


Figure 12. Glucose Excursion After Meal Tolerance Test Glucose Curve

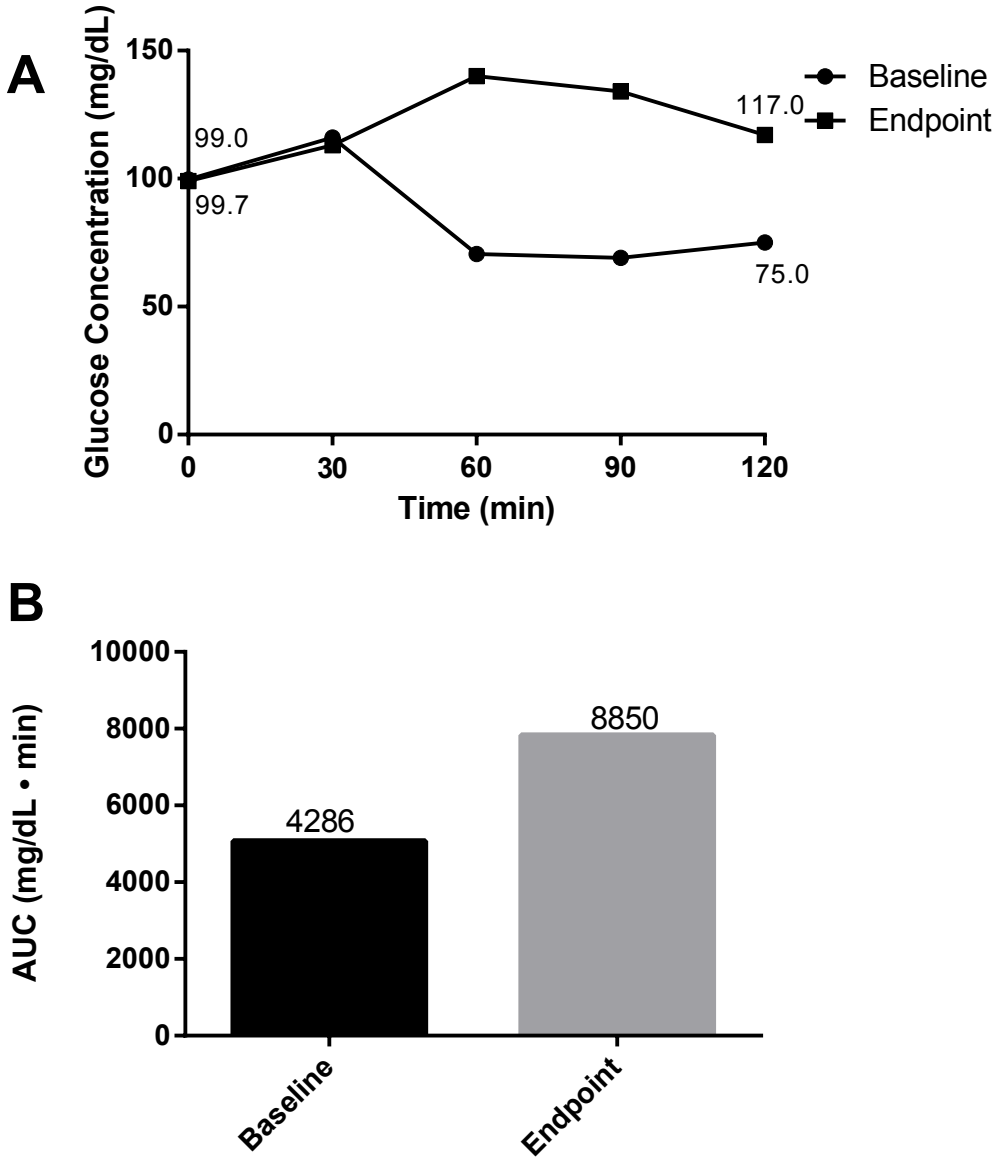


Figure 13. Glucose Excursion After Meal Tolerance Test Glucose Curve

(B) MS 20's glucose tolerance test curve (B) AUC for MS 20
(C) MS 5 glucose tolerance test curve (D) AUC for MS 5

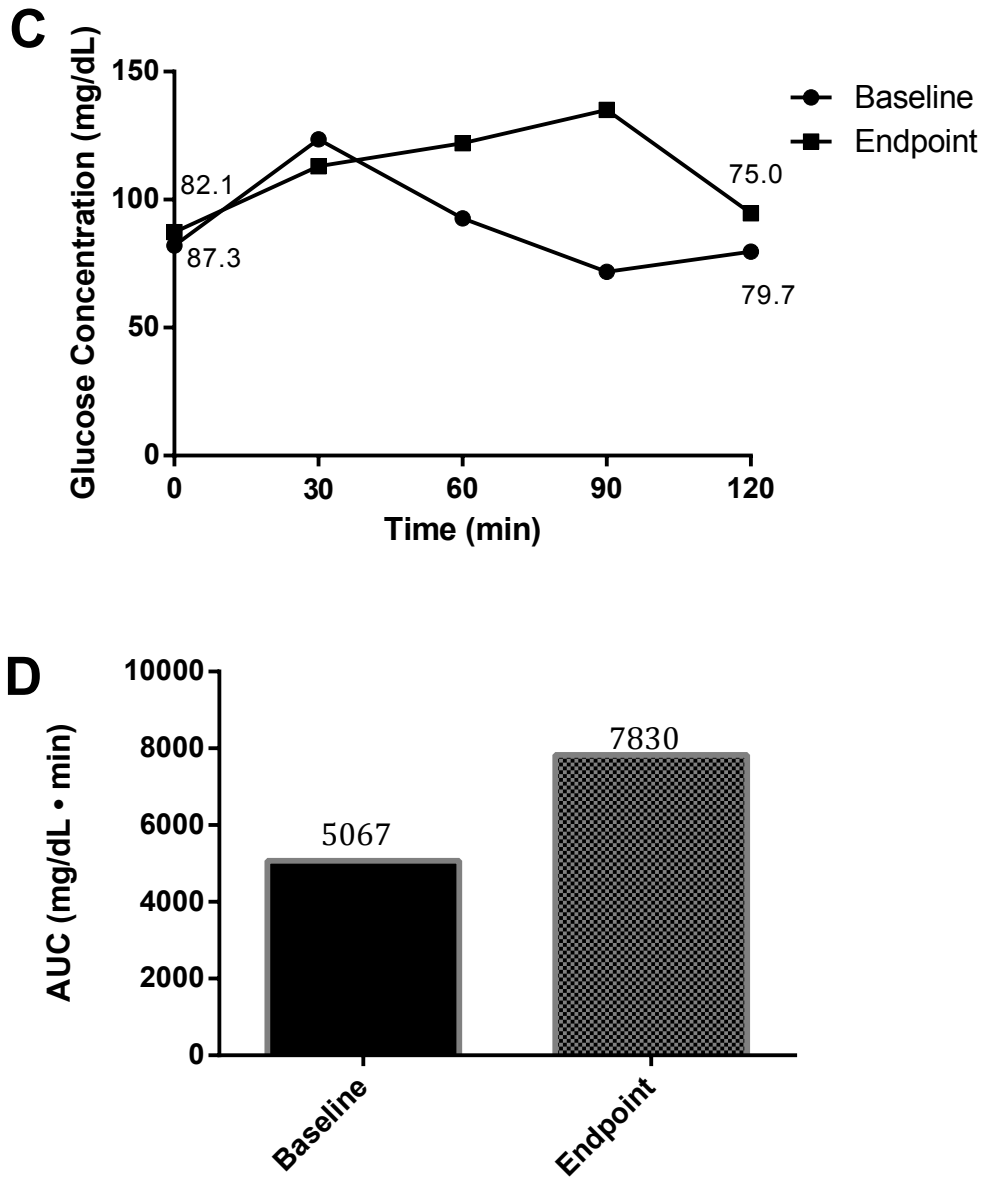


Figure 13. Glucose Excursion After Meal Tolerance Test Glucose Curve continued.

Tables

Table 1: Mouse Strain Characteristics
General Characteristics of two strains of mice, 12/15-Lipoxygenase transgenic and wildtype mice, on a 45% HFD for 10 weeks. Data collected during weeks 8,9 and 10.
Data are shown as averages (mean) with standard deviation
*p<0.05

MOUSE STRAIN CHARACTERISTICS		
	WILDTYPE (WT) MICE	**12/15 LO TRANSGENIC MICE**
NUMBER OF MICE	9	9
TYPES	Male	Male
ENDPOINT WEIGHT (g)	33.43 (1.11)	35.54 (1.46)
gWAT of BODY WEIGHT (%)	3.56 (0.35)	5.51 (0.71)*
gWAT FAT PADS (g)	1.10 (0.13)	1.87 (0.22)*
FASTING GLUCOSE (mg/dL)	202.3 (5.71)	238.7 (9.87)*
FASTING INSULIN (ng/mL)	2.67 (0.18)	3.83 (0.21)*
FREE FATTY ACIDS (mmol/L)	0.99(.41)	1.13 (.18)
TRIGLYCERIDES (mg/dL)	70.8 (13.8)	86 (14.5)*
M1-CELLS/TOTAL MACROPHAGES(%)	27.7 (11.3)	43.4 (20.9)

Table 2: Subject Enrollment and Clinical Characteristics
Clinical Characteristics of diet intervention study are presented according to intervention group and sex. Baseline age, weight, body mass index, waist circumference, fasting glucose concentration, hemoglobin A1c, triglycerides, non-density lipoprotein (non-HDL), high density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides/high-density lipoprotein (TG/HDL) are presented for males and females along with averages in each category after 12 weeks of diet intervention. Data shown only for subjects who have completed the study.
*p<0.05 (active vs. placebo at baseline), #p< 0.05 (% change within the group),
Data are shown as averages (mean) with standard deviation

			Active (F)	Active (M)	Placebo (F)	Placebo (M)	Active (M+F)	Placebo (M+F)
Number of Subjects		**Age**	39	31	44	43	**37 (10.9)**	42(8)
Enrollees	41	**Wt (kg)**	99.3	96.5	96.3	99.3	98.7(13.6)	98.4 (10.5)
Completed	27	**BMI**	36.5	33.4	35.2	35.2	36.0 (2.9)	35.0 (3)
In-Progress	2	**WC (cm)**	116.9	118	114.3	116	117.0 (6.9)	123.0 (7.4)
Placebo Diet	12	**Glucose (mg/dL)**	90.9	96.6	92.7	88.5	92(10.1)	94 (6.0)
Active Diet	15	**HbA1c**	5.9	5.5	5.9	5.6	5.8 (.29)	5.7 (.43)
Female	17	TC	201	185	229	181	198(47)	231 (42)
Male	10	non-HDL	154	143	178	138	152(45)	190 (41)
		HDL	47	42	52	43	46 (10)	41 (10)
		LDL	108	113	151	111	109(24)	168 (35)
		TG	204	199	131	135	203 (102)	112 (40)
		TG/HDL	4.5	4.8	2.6	3.3	4.5 (2.2)	2.7 (1.2)

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