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Effect Of Exogenous Leptin On Thrombotic And Metabolic Profiles Of Fvb/b6 Lipodystrophic Mice

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EFFECT OF EXOGENOUS LEPTIN ON THROMBOTIC AND METABOLIC PROFILES OF FVB/B6 LIPODYSTROPHIC MICE

by

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DEDICATION

This thesis is dedicated to my parents, Chaouki and Khadije, whose love and support surrounded me at every moment of my life, my sister, Mona, whose care and hilarity always showed me things from a better window, and my friends, Nadine and Steve, whose loyalty and encouragement constantly helped me feel home and make it through. I can't thank you enough!

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Chapter 1

<u>Introduction</u>

Lipodystrophy

Lipodystrophy is a condition caused by fat deficiency where adipose cells are destroyed or barely present (1). The disease exists in genetic and acquired forms, the former being very rare. Congenital generalized lipodystrophy (the Seip-Berardinelli syndrome) and the familial partial lipodystrophies (the Dunnigan type, the Kobberling type and the mandibuloacral dysplasia type) are the genetic forms. Acquired partial lipodystrophy (the Barraquer- Simons syndrome), acquired generalized lipodystrophy (the Lawrence syndrome) and protease inhibitors-induced lipodystrophy (HIV treatment) are the acquired forms. HIV related lipodystrophies are observed in about 50% of HIV patients, being the most common form of lipodystrophy (2).

There exist two methods through which dysfunctional adipose tissue may play a role in metabolic disease: the first is the reduced capability to store triglycerides which in turn leads to an increase in the levels of circulating fat as well as the deposition of excess lipids in non-adipose tissues. The second is anomalies in the production of hormones and adipokines. In lipodystrophic or obesity-mediated metabolically ill patients, these two pathways typically occur concurrently, rendering it hard to determine the mechanistic and/or pathogenic determinants of these disorders (3).

These patients also suffer from other anomalies including hypertriglyceridemia along with insulin resistance and type II diabetes (4), which

in turn lead to further complications: severe hypertriglyceridemia eventually causes recurrent acute pancreatitis attacks, and severe hyperglycemia increases the risk for diabetic nephropathy and retinopathy. They also develop steatohepatitis which may lead to cirrhosis (5).

Treatment Modalities for Lipodystrophy

The major characteristics of lipodystrophy, insulin resistance and hypertriglyceridemia are usually managed by multiple drugs including lipid-lowering medications along with insulin therapy and/or oral hypoglycemics (5); however, they have been so far resistant to treatment (6). Thiazolidinediones have been shown to be an effective therapy, yet not the perfect one (7). Other treatment modalities have also been attempted. For instance, Wanke et al treated 10 HIV-induced lipodystrophy patients with Recombinant Human Growth Hormone (rhGH) for 3 months and found that this short-term rhGH treatment was an effective therapy for the body shape alterations that accompany the syndrome (8).

Leptin replacement therapy, so far, seems to be the most effective therapy for lipodystrophic patients. This was first demonstrated in 2002 by Oral et al in studies where they administered recombinant leptin subcutaneously to nine lipodystrophic patients over four months. This treatment resulted in an improvement in glycemic control, a decrease in triglyceride levels and a reduction in liver volume, as well as decreased food intake and resting energy expenditure (5). Similarly, Simha et al found in 2003 that leptin replacement therapy reduced intramyocellular and intrahepatic lipid content in three such patients (9). The

reduced food intake, decreased energy expenditure and reduced liver volume in response to leptin treatment were further demonstrated by Moran et al in 2004 (10). Javor et al tested the long-term efficacy of leptin treatment in 15 lipodystrophic patients for 12 months. They also found, in 2005, that leptin administration led to marked improvements in glycemia, dyslipidemia and hepatic steatosis (estimated by liver volume), announcing leptin as the first effective long-term treatment for severe lipodsytrophy (11). Ebihara et al confirmed in 2007 that leptin administered to generalized lipodystrophy patients improves insulin sensitivity as well as lipid metabolism (12).

What is Leptin?

Being a triglyceride storage depot and an endocrine tissue, white adipose tissue plays an important role in energy metabolism as well as lipid and glucose homeostasis. The adipocytes from white adipose tissue produce hormones that regulate food intake and metabolism, mainly leptin and adiponectin (13). The adipocyte-derived hormone leptin was discovered in 1994 by Zhang et al as a protein product of a defective gene in the ob/ob mouse (14). Leptin plays a major role in food intake and energy expenditure regulation (15), as it is known to reduce food intake and increase metabolism. Several studies have shown that its administration to leptin-deficient and normal rodents results in patent reductions in food intake, body weight and body fat. The obese population, on the other hand, has elevated leptin and does not appear to benefit from leptin therapy, a concept typically called "leptin resistance" (16).

Leptin receptors, through which leptin regulates energy homeostasis, are expressed in the hypothalamus (17) and many peripheral tissues (18). The levels of regulation vary: Fatty acid oxidation (19) and glucose uptake in skeletal muscle cells (20) are stimulated by leptin, whereas hepatic gluconeogenesis and lipogenesis are inhibited by leptin (21, 22). Leptin reduces pancreatic B-cell production of insulin (23) and also enhances insulin action (24), which is thought to be controlled via a cascade of reactions (25). Leptin's effect on glucose and lipid metabolism and insulin sensitivity appears to be mostly independent of its regulation of food intake (26). However, various pathways are known to mediate its metabolic effects. For instance, leptin represses the gene stearoyl-CoA desaturase-1 (SCD-1) which plays a role in leptin-induced metabolic changes (27). Leptin also plays a role in growth and reproduction (28), inflammation, hematopoiesis (29) and immunity (28, 29).

Hyperleptinemia is commonly seen in obese patients revealing increased adiposity and leptin resistance. This profile may contribute to the hypertension, impaired glucose metabolism, and pro-atherogenic condition in obese and metabolic syndrome patients (30).

Mouse Models of Lipodystrophy

Excess adiposity in obesity and lack of adipose tissue in lipodystrophy are both often coupled with insulin resistance and related complications. Strangely enough, lipodystrophy shares similar metabolic abnormalities with obesity. For treating lipodystrophy-related metabolic disorders, leptin replacement therapy is a successful approach, yet it does not appear promising in the treatment of

complications associated with obesity (31). However, a well-controlled genetically modified lipodystrophy mouse model can help unravel these mechanisms and also find therapeutic targets for treating/preventing the metabolic syndrome/ metabolic abnormalities related to obesity (3). Mouse models of lipodystrophy are very useful in the sense that they may help understand these mechanisms and possibly uncover new pharmaceutical targets for managing metabolic disorders associated with dysfunctional adipose tissue (31).

Inducible, late onset and early onset mouse models of lipodystrophy have been developed. There are two known inducible models: 1- *Fat apoptosis through targeted activation of caspase 8* (FAT-ATTAC) mice that are transgenic mice suffering from adipose tissue ablation resulting from treatment with a chemical dimerizer (32); and 2- *Protease inhibitor-induced lipodystrophic mice* (3).

There are two late onset models as well: 1- *aP2-diphteria toxin A* mice which are born with a normal phenotype but develop adipose tissue atrophy at five to six months of age (33); and 2- *Ribosomal S6 kinase 2* knockout mice which display partial fat loss, reduced leptin levels, insulin resistance and a propensity for liver steatosis (34).

There exist eight known early onset models: 1- The *aP2-SREBP-1c* mouse is a transgenic mouse model of lipodystrophy expressing an active form of the SREBP-1c transcription factor in its adipose tissue, causing remarkably reduced body fat, hepatic steatosis, insulin resistance, hyperglycemia, hypertriglyceridemia and enlarged organs (35). 2- *Mox2-Cre-floxed PPARγ*

knockout mice are PPARγ deficient mice that are lipodystrophic and insulin resistant. Only a small percentage of these mice survive until adulthood (36). 3- *Adipose-specific PPARγ* knockout mice have only a small amount of adipose tissue with decreased adipokine concentration. In addition, the adipose tissue they have appears prone to inflammation. These mice also have elevated blood lipids and a propensity for hepatic steatosis and insulin resistance (37). 4- *PPARγ-2* knockout mice are more viable than the PPARγ knockout mice, but also have markedly reduced adipose tissue and circulating adipokines (38). 5- *PPARγ hypomorphic* mice are PPARγ-1 deficient due to an alteration in a PPARγ-2-specific exon. These mice also have markedly reduced body fat (39). 6- *PPARγ P465L* mice were made to mimic human patients with the dominant negative PPARγ P465L mutation who experience partial lipodystrophy, liver steatosis, altered lipid metabolism, insulin resistance and hypertension (40). 7- *Fatty liver dystrophy* mice are generated from a lipin1 gene mutation and are also lipodystrophic (41). 8- Last but not least, the A-ZIP/F-1 mouse is a transgenic mouse that lacks white adipose tissue for life via expressing the AZIP/F protein (42).

The AZIP/F-1 Lipodystrophic Mouse

The AZIP/F-1 mouse was produced by adipose-selective expression of a dominant-negative form of the transcription factor C/EBPα thus expressing the A-ZIP/F protein, under the control of the adipose-specific aP2 enhancer/promoter. C/EBPα normally regulates adipocyte differentiation. A-ZIP/F prevents the DNA binding of B-ZIP transcription factors of the C/EBP and Jun families. The mice

therefore have little to no white adipose tissue throughout life and minimal amounts of brown adipose tissue (42).

They firstly encounter delayed development that is then caught up by week 12 when their body weight exceeds that of their non-transgenic littermates. They are also characterized by reduced fertility. They have severe hepatic steatosis as well as enlarged organs in general. They tend to develop type II diabetes as they are hyperinsulinemic (50- to 400-fold) at 1 week of age and hyperglycemic (3-fold) by 4 weeks of age. They have reduced leptin levels (20-fold) and increased free fatty acids (2-fold) and triglycerides (3- to 5-fold). They are finally polyphagic, polydipsic and polyuric (42).

The phenotype of AZIP/F-1 mice is very similar to that of patients with severe lipoatrophic diabetes (1). The A-ZIP/F-1 phenotype therefore may serve as a mouse model for human lipoatrophic diabetes (Seip-Berardinelli syndrome), where the lack of fat contributes to a diabetic phenotype. According to the authors, this model would help tackle the numerous drawbacks of lacking fat throughout growth (42).

General Characteristics of Fatless Mice

The syndrome observed in lipoatrophic mice is markedly comparable to that of lipoatrophic diabetes patients (43). AZIP mice, for instance, have nearly no white adipose tissue, which results in hyperglycemia, insulin resistance, hyperlipidemia, elevated liver triglycerides and hepatomegaly (44, 45). As it is very well known, insulin resistance is the common precursor to type II diabetes (46) and impairments in fat metabolism may promote insulin resistance (47, 48).

Kim et al postulated that insulin resistance in the AZIP fatless mice may be attributed to triglyceride accumulation in the liver and muscle due to the lack of adipose tissue, which in turn alters insulin signaling and action (49). Lipodystrophic mice, such as the AZIP/F-1 mice, are also hypertensive (50). They have also been shown to have a high susceptibility to carcinogenesis that is thought to be related to their insulin resistance and inflammatory state (51) and/or the lack of adipose tissue (52).

Treatment of Lipodystrophy in Mice: Leptin Replacement Therapy

The metabolic abnormalities associated with lipoatrophy are clearly due to the lack of white adipose tissue since surgical implantation of adipose tissue reversed diabetes in lipoatrophic mice (53). Transplanting adipose tissue lacking leptin into AZIP/F1 fatless mice, however, could not reverse the phenotype of these mice. This evidence suggests that neither adiponectin deficiency nor the lack of triglyceride accumulation into fat per se is responsible for the metabolic abnormalities; it is rather leptin deficiency (54). Ebihara et al produced doubly transgenic mice that are lipodystrophic but over-express leptin. The mice showed hypophagia, improved insulin sensitivity and reduced hepatic steatosis in comparison with pair-fed lipodystrophic mice suggesting that leptin (rather than simply reduced food intake) exerted important metabolic benefits (55). In a study conducted by Shimomura et al, leptin administration to lipodystrophic mice via pumps achieving physiologic levels resulted in a noted improvement in hypertriglyceridemia, insulin resistance, hyperglycemia and hepatic steatosis (56). In another study, leptin reversed hyperglycemia and hyperinsulinemia in

Irs1-/-,Irs3-/- double knockout lipoatrophic mice (57). Furthermore, leptin infused centrally over 6 days to streptozotocin-induced diabetic rats (insulin-dependent diabetes) promoted euglycemia, an effect that was not due to regulating food intake or peripheral insulin levels, it was rather via regulating liver glucose production, energy expenditure and peripheral glucose uptake (58). Leptin replacement therapy also has other physiologic effects, beyond the control of food intake, hypertriglyceridemia, hyperglycemia and liver steatosis. For instance, Suganami et al found marked nephropathy in AZIP fatless mice which was reversed by leptin administration and prevented in the AZIP transgenic/Leptin transgenic mouse that is fatless but produces leptin (59).

Vascular Function/Thrombosis

A procoagulant state has been observed in obese patients with insulin resistance and in type 2 diabetes (60). In addition, inflammation resulting from obesity may play a role in the development of atherosclerosis (61). Plaque disruption in atherosclerosis triggers a thrombotic response characterized by the formation of a thrombus in the atherosclerotic blood vessel thus potentially exposing it to occlusive thrombosis and tissue (e.g. myocardial or cerebral) infarction (62). Acute thrombosis, therefore, often mediates the clinical severity of cardiovascular disease complications (63).

Many circulating substances altered in metabolic disease are thought to influence thrombosis. Adiponectin, a protein produced by adipose cells, seems to be reduced with the increase in adipose stores (64). Thrombus formation is increased in adiponectin-deficient mice, a phenotype that was reversed when

introducing adiponectin via an adenovirus, suggesting that adiponectin plays an antithrombotic role (65). Acute high doses of TNF-α, the inflammatory mediator secreted by inflammatory cells (66), were also shown to have an antithrombotic effect in mice (67). PAI-1 has a detrimental effect on fibrinolysis being the primary inhibitor of tissue plasminogen activator in the blood (68) and it plays a prothrombotic role according to arterial thrombosis studies in mice (69). Another factor that has been shown to play a role in thrombosis is the adipose-derived hormone leptin.

Leptin and Thrombosis

Leptin appears to act as a prothrombotic factor. For example, leptin has been shown to stimulate platelet aggregation via adenosine diphosphate (70). The human platelet has a functional leptin receptor that was identified by Maruyama et al in 2000. High concentrations of leptin promoted platelet aggregation in vitro (71). Two human studies suggested that leptin is an independent risk factor for coronary heart disease (72). Also, plasma leptin levels were significantly associated with coronary artery calcification in a cross-sectional study involving type 2 diabetes patients, after controlling for age, gender and other factors such as adiposity and CRP. This finding led to the suggestion that leptin may promote the proatherosclerotic risk posed by adiposity (73).

Similar effects were also found in mice. Leptin, and especially exogenous leptin, was shown to be positively correlated with vascular thrombosis in various mouse models (30). Bodary et al found that daily leptin administration for 4

weeks promoted thrombosis and atherosclerosis in apolipoprotein E- deficient mice (74). The prothrombotic effect of leptin may be exerted via multiple sites including leptin receptors on platelets, centrally-mediated sympathetic effects of leptin (e.g. on blood pressure), as well as leptin receptor expression on endothelial cells (69).

Consistent with the prothrombotic effect of leptin, the absence of leptin has been shown to reduce thrombus formation. For example, thrombosis is delayed and the thrombi formed are unstable in both leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice. Leptin administration increased platelet aggregation thus enhancing the formation of and stabilizing thrombi in the former, but not in the latter, suggesting a receptor-dependent effect of leptin on platelet function and hemostasis (75). Mice with leptin receptor-deficient bone marrow were protected from photochemical injury-induced arterial thrombosis. Leptin administration has a prothrombotic effect following vascular injury in mice, an effect that was absent in leptin receptor-deficient mice and seemed to be driven by the interaction between leptin and the platelet leptin receptor (76). In addition, inhibiting endogenous circulating leptin delayed arterial and venous thrombosis in mice and rendered the formed thrombi unstable (77).

Chapter 2

<u>Objectives of this Study</u>

Given the background information, the aims of this study were to 1) Observe the thrombotic profiles of FVB/B6 Lipodystrophic mice; 2) Observe the physiologic development and metabolic profiles of these mice via various parameters; and 3) Examine the effect of exogenous leptin on these profiles, while comparing to vehicle-treated and non-transgenic controls.

Chapter 3

<u>Materials and Methods</u>

Animals

AZIP/F-1 transgenic (AZIP-TG) mice (strain name: FVB-Tg(Azip/F)1Vsn/J) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in house to produce AZIP/F1 offspring. AZIP-TG mice express the AZIP/F1 gene under the control of 7.6 kB of the adipocyte specific Fabp4 promoter and therefore have nearly no white adipose tissue and a significantly reduced amount of brown adipose tissue (42). AZIP-TG females are infertile or weakly fertile due to the necessity of leptin and/or adipose tissue for lactation and fertility. Therefore, to obtain a mix of transgenic and wild-type FVB/B6 F1 offspring, several steps were followed: AZIP-WT females were crossed with AZIP-TG males. The offspring of interest were the AZIP-TG males. C57BL/6J (B6) female mice were purchased from Jackson Laboratories as well. The AZIP-TG males were crossed with B6 females (at sexual maturity, i.e. ~6 weeks of age) to obtain FVB/B6 F1 offspring. B6 mice were chosen because they are the best characterized mouse strain for metabolic phenotypes and are genetically not close to the FVB strain (78). In addition, we had previously found FVB AZIP-TG mice to be sickly and poorly viable for use in our photochemical injury model of arterial thrombosis. The FVB x B6 offspring were found to be ideal for our studies as this breeding strategy had reduced mortality among the AZIP-TG offspring.

The offspring of interest for the leptin pump study were the FVB/B6 males, both transgenic and wild-type. Males were chosen rather than females because

they are more biologically stable than the latter and do not encounter estrous cycling, providing more controlled conditions for the experiment. Mice were ear-tagged and tailed (for genotyping purposes) and weaned at two and three weeks of age, respectively.

All laboratory personnel had completed the required animal-handling training offered by the Division of Laboratory Animal Resources (DLAR) under the supervision of Wayne State University's Animal Investigation Committee (AIC) for proper handling of mice. The protocols used in the study were also approved by the AIC prior to the initiation of the study. All mice were housed in an animal room with ~53% relative humidity and a 22°C ambient temperature.

Feeding

Breeders were fed the FormuLabDiet 5008 from LabDiet, which is a high-energy high-protein formula used in breeding colonies of rodents to maximize reproduction. All other mice were fed Laboratory Rodent Diet 5001 from LabDiet, which is a constant formula used to diminish nutritional variables in long-term studies (PMI Nutrition International, Saint Louis, MO). All mice had access to food and water ad libitum.

Genotyping

Genotypes of mice were determined via end-point Polymerase Chain Reaction (PCR). Tail tip amputations were performed and DNA samples were obtained from tail sections. Tail sections were digested using a solution that is 10% tail digestion buffer (50% 1M KCL, 20% 500mM Tris-HCL, 10% 10% Triton X-100, 20% ultra pure water), 4% 10mg/ml Proteinase K enzyme and 86% ultra

pure water. One hundred microliters of the solution was added to each tail sample and the samples were incubated at 55°C overnight, mixed using the Vortex Mixer (Fisher Scientific, Pittsburgh, PA) and spun at 16,100 x g for 10 minutes at room temperature in the Eppendorf Centrifuge 5415 D (Brinkman Instruments, Westbury, NY). PCR reactions were performed using an Eppendorf Mastercycler Gradient PCR machine (Brinkman Instruments) and utilizing four "Azip" primers from Sigma-Genosys (Sigma-Aldrich, St. Louis, MO): Azip Mutant 1981 (sequence 5'-CTGTGCTGCAGACCACCATGG), Azip Mutant 1982 r (sequence 5'-CCGCGAGGTCGTCCAGCCTCA), Azip Wild-Type 0042 (sequence 5'-CTAGGCCACAGAATTGAAAGATCT) and Azip Wild-Type 0043 r (sequence 5'-GTAGGTGGAAATTCTAGCATCATCC), Go Taq Master Mix and Nuclease-Free Water (Promega, Madison, WI). Two and a half percent agarose gel was loaded with amplified product and Exactgene 100 BP DNA Ladder (Fisher Scientific) and run on a Fisher Biotech Electrophoresis System/ Wide Format Horizontal System FB-SB-2318 (Fisher Scientific). Gels were read via the DigiDoc-It Imaging System (UVP Inc., Upland, CA).

Arterial Thrombosis

Blood flow was measured in 6 FVB/B6 (3 WT & 3 TG) male mice at 7-10 weeks of age. The Arterial Thrombosis Protocol described by Bodary et al (76) was modified and followed: The procedure starts by anesthetizing the mice with ~50 mg/Kg body weight intraperitoneal sodium pentobarbital (Meds for Vets, Sandy, UT) then securing them in the supine position and placing them under a dissecting microscope (Nikon SMZ-645, Mager Scientific Inc, Dexter, MI). A

midline cervical incision is then applied in order to isolate the right common carotid artery and apply a flow probe (model 0.5 PSB, Transonic Systems, Ithaca, NY). A flow meter (Transonic model T402, Transonic Systems) is then connected to the probe and blood flow is monitored via the computerized data acquisition software DASYLab9 (Measurement Computing Corporation, Norton, MA). The photochemical injury protocol described by Eitzman et al (79) was the means through which carotid arterial injury and thrombosis were induced. The photochemical used in this procedure is rose bengal (Fisher Scientific) which is diluted in PBS to 10 mg/mL and then injected into the tail vein via a 27-gauge Precision Guide needle with a 1-ml latex-free syringe (Becton Dickinson and Co, Franklin Lakes, NJ) at a dose of 50 mg/kg. Rose Bengal is then activated by exposing the mid common carotid artery to a 1.5-mW green light laser (540 nm, Melles Griot, Carlsbad, CA) from a 6 cm distance. Blood flow in the artery is observed from the time of the injection of rose bengal until the end of the experiment. Two events would determine the end point for the experiment: the cessation of blood flow in the carotid artery for one minute (read via the DASYLab9 software) and the visualization of an occlusive thrombus at the injury site via the dissecting microscope. The observer of both events would be blinded to the mouse group.

Cell and Platelet Counts

Blood cell and platelet counts were obtained from seven FVB/B6 F1 males (4 TG and 3 WT) at 13 weeks of age. Blood was obtained via a cardiac puncture terminal bleed using a 25-gauge Precision Guide needle with a 3-mL latex-free

syringe (Becton Dickinson and Co, Franklin Lakes, NJ) loaded with 3.2% Na-Citrate (Sigma Inc., St. Louis, MO) where fresh circulating blood was obtained from the heart (ratio of Na-Citrate to Blood was 1:10) and diluted with PBS to a 10,000 fold dilution. Ten microliters of diluted blood from each sample was placed in the Hausser Levy Hemacytometer (VWR International Inc, Batavia, IL) where platelets and total blood cells were counted under a Wilovert S Inverted Microscope (Hund Wetzlar, Germany).

Euglobulin Clot Lysis Assay

A Euglobulin Assay was performed on blood samples obtained from five of the mice (2 WT and 3 TG) mentioned above for the determination of fibrinolysis. The protocol described by Smith et al (80) was followed with modifications. The fresh citrated blood was spun and plasma was separated (described later) and mixed with working acetic acid (1.3 ml per 75 ul plasma) in 1.5-2.0 ml polypropylene tubes. The tubes were placed on ice for 10 minutes and spun at 2,000xg for 5 minutes at room temperature. Supernatants were discarded and the tubes were inverted to drain for 5 minutes. The "euglobulin fraction" was resuspended in 100 ul of a solution of 154 mM NaCl and 26 mM sodium borate, stirred, warmed to $37^{o}C$ for 90 seconds, re-stirred and warmed again to $37^{o}C$ for 90 seconds. Samples were then pipetted into a pre-warmed $37^{o}C$ 96-well polystyrene flat-bottom microtiter plate, 90 ul per well. Each sample was pipetted in duplicate and read on the ELx800 Absorbance Microplate Reader at 405 nm. Ninety microliters of 0.025 M $CaCl_2$ was added to one well of each sample while the other served as an individual control. The plate was placed again in the

microplate reader and absorbance was read at 405 nm every minute for the first 10 minutes and then once every 10 minutes for 3 hours. Maximum absorbance was defined as the peak absorbance at 405 nm and lysis time was defined as the time at which the curve, corrected for the individual blank, reaches an absorbance of 0.05 or less.

CBC and PT Measures

Obtaining complete blood counts (CBC) and measuring clotting factors were also steps followed towards trying to uncover the mechanism behind the altered thrombotic profiles of AZIP-TG mice. Seven 28-week-old female FVB/B6 F1 mice (3 WT & 4 TG) were sent to the Division of Laboratory Animal Resources (DLAR) clinical pathology lab (WSU) for CBC and measures of prothrombin time (PT) from fresh blood. Each mouse was placed in a CO_2 chamber for 30-45 seconds and blood was drawn via cardiac puncture. CBC measures were obtained via Heska CBC-Diff Veterinary Hematology System machine (Heska Corporation, Fort Collins, CO) and HeskaView Integrated Software Product Version 2.0.2 data acquisition software (Heska Corporation). PT was measured on the SCA2000 Veterinary Coagulation Analyzer (Symbiotics, San Diego, CA) using IDEXX Coagulation Diagnostics PT Cartridges (IDEXX Laboratories, Westbrook, ME).

a-PTT Measures

Seven female FVB/B6 F1 mice (4 TG, 3 WT) at 41-47 weeks of age were sent to the DLAR clinical pathology lab to obtain levels of activated partial thromboplastin time (a-PTT). The same bleeding protocol (as above) was

followed. The a-PTT was also measured on the SCA2000 Veterinary Coagulation Analyzer (Symbiotics) using IDEXX Coagulation Diagnostics a-PTT Cartridges (IDEXX Laboratories).

Grouping for the Leptin Pump Study

To study the effect of leptin administration on thrombotic and metabolic profiles of fatless mice, leptin was administered to the mice via osmotic pumps. Sixteen FVB/B6 mice were divided into four groups as follows: 4 transgenic mice that received leptin pumps (TG-Tx), 3 transgenic mice that received control pumps (TG-C), 4 wild-type mice that received leptin pumps (WT-Tx) and 5 wild-type mice that received control pumps (WT-C). Reduced fertility in fatless mice along with the difficulty of obtaining healthy male offspring limited the sample size to 16 mice.

Leptin Pumps

Five µg leptin/day was administered to each mouse via an osmotic pump over 12 days. Recombinant mouse leptin was obtained from the National Hormone and Peptide Program (NHPP, Torrance, CA) and reconstituted with PBS pH 7.4 by GIBCO (Invitrogen, Grand Island, NY) into a 0.83 ug/ul concentration. ALZET Osmotic Pumps model 1002, Alza (DURECT Corporation, Cupertino, CA) were used to deliver leptin at a rate of ~0.25 ul/hr. Pumps were filled and primed following the company's instructions.

Surgical Procedure for Pump Implantation

The least invasive procedure, subcutaneous implantation, was used to implant leptin and vehicle pumps in the mice at 8 weeks of age, under sterile

conditions and following the company's protocol. The mouse is first anesthetized with inhaled Attane Isoflourane, USP (MINRAD, Inc., Bethlehem, PA). The lower dorsal area is shaved, slightly posterior to the scapulae and a mid-scapular horizontal incision is made in the skin (≈ 1 cm). Using a hemostat or forceps, a pocket/path is created for the pump all the way along the spine, just before the neck. The filled pump is then inserted in the pocket. Finally, the incision is sealed with Oasis surgical adhesive (Oasis, Mettawa, IL) and the mouse is placed in a separate cage. The pump contents are delivered into the local subcutaneous space and absorbed by local capillaries resulting in systemic administration.

 (Procedure modified from: Alzet technical Information manual, Durect Corporation, Cupertino, CA).

Blood Samples

Blood samples were obtained from mice one week prior to inserting pumps, i.e. at ~7 weeks of age, via retro-orbital bleeds (orbital sinus venipuncture), using Fisherbrand heparinized micro-hematocrit capillary tubes cat # 22-362-566 (Fisher Scientific). Two bleeds were done at 7 weeks of age (2-3 days apart), so that adequate amounts of both plasma and serum could be isolated to measure pre-pump parameters. On the 9th day of having the pump in, during their 9th week of age, the mice were bled again for serum samples, to measure effects of leptin treatment. All blood samples were obtained after a five-hour fast where the mice would have access to water only. For plasma samples, blood was collected in 1.5 ml polypropylene tubes, spun immediately (or kept at $4^{o}C$ until spinning) at 5,000 x g for 10 minutes at room temperature and plasma

was isolated and stored at -20°C. For serum samples, blood was collected in BD Microtainer Serum Separators Ref 365959 (Becton Dickinson and Co), clotted for two hours at room temperature, spun at 2,000 x g for 20 minutes at room temperature and serum was isolated and stored at -20°C. These bleeding and plasma/serum isolation procedures were followed in all other experiments requiring blood samples, unless otherwise indicated.

Measuring Blood Glucose

On day 9 after pump placement, while bleeding for serum samples, fasting blood glucose concentrations (mg/dl) were measured using the Ascensia Elite XL glucometer (Bayer, Tarrytown, NY), to monitor differences between groups.

Monitoring Body Weight Change

During leptin pump experiments, parameters that were to be measured daily were body weight and food intake. The mice were weighed at 10:00 am everyday throughout the study using the Adventurer-Pro digital scale (Ohaus Corporation, Pine Brook, NJ), to monitor weight change in response to leptin treatment. Food remaining in the cage was weighed daily during the first week of the experiment and food intake was estimated by subtraction. However, the measurement was discontinued because food was being chewed but not consumed, making the results very inaccurate.

Arterial Thrombosis (leptin pump study)

Blood flow was monitored on the twelfth day following pump placement, at 10 weeks of age, following the protocol previously described. The maximum duration allowed for observing blood flow was 150 minutes in the FVB/B6 leptin

pump experiment. Again, the observer was blinded to the mouse group (treatment vs control, transgenic vs wild-type).

Tissue Harvesting

The above arterial thrombosis procedure is a non-survival protocol. After disconnecting the mice from the flow probe, while they were still under the effect of anesthesia, they were euthanized via a cardiac puncture terminal bleed for plasma isolation and they were then dissected to harvest organs. The liver was the primary organ of interest. Tissues were weighed while fresh and sections were consistently removed and frozen. Kidneys, hearts, lungs, spleens and fat pads (in WT mice) were also isolated and weighed. All isolated tissue/organ samples were weighed using the Mettler Toledo digital scale Model XS104 (Mettler-Toledo, Columbus, OH) and stored in 1.5-2 ml tubes at -20°C. Blood obtained from the terminal bleed was spun for plasma isolation, following the same isolation technique used in previous bleeds.

Liver Homogenization

Frozen liver tissue was thawed and homogenized in order to be used in assays to measure parameters of interest. Fifty milligrams of each liver sample was homogenized in a BD Falcon Polypropylene Round Bottom Tube (Becton Dickinson and Co) in 2 ml of lysis buffer (150 mM NaCl, 0.1% Triton X-100, 10 mM Tris) using a Model Pro200 Double-Insulated Homogenizer (PRO Scientific, Oxford, CT). The resulting 5-fold diluted livers were spun and supernatants were used in assorted assays as described.

Measuring Leptin Levels

Leptin levels were measured in serum samples of mice pre and post pump treatment, via a leptin ELISA. The Mouse Leptin ELISA Kit Catalog # 90030 (Crystal Chem, Downers Grove, IL) was used with provided standards and controls.

Measuring Insulin Levels

To test the effect of leptin administration on insulin sensitivity in our samples, serum insulin levels were measured by ELISA as well. The Ultra Sensitive Mouse Insulin ELISA Kit Catalog # 90080 (Crystal Chem) was utilized with the provided wide range standards.

Measuring Triglyceride Levels

Plasma and liver triglyceride levels were measured using the Serum Triglyceride Determination Kit Catalog # TR0100 (Sigma-Aldrich, Saint Louis, MO) with provided reagents. Liver triglyceride concentrations were then normalized for liver protein concentrations. Liver samples used in the assay were diluted 40-fold with deionized water.

Measuring Cholesterol Levels

Plasma and liver cholesterol levels were measured using a cholesterol assay following the Wako Cholesterol E Microtiter Procedure Catalog # 439-17501 (Wako Diagnostics, Richmond, VA). Liver cholesterol concentrations were also normalized to liver protein concentrations. Livers samples used were diluted five-fold with deionized water.

Measuring Liver Protein Concentration

Liver protein concentration was measured using Bio-Rad RD DC Protein Assay dye reagent (Bio-Rad, Hercules, CA) and bovine serum albumin standards. Absorbance was read at 570 nm. Livers were 2000x diluted for this purpose.

All assays were read using the ELx800 Absorbance Microplate Reader (Bio-Tek Instruments, Winooski, VT) and the KC4 Software (Bio-Tek).

Statistical Analyses

Results were analyzed via SPSS 15.0 for Windows (SPSS Inc., Chicago, IL) using one way Analysis of Variance (ANOVA), two way ANOVA, paired samples t-test and independent samples t-test. Post-hoc analyses were also used to compare outcomes using $p \leq 0.05$ as a cutoff point for statistical significance (significant effect). Due to the limited mice available for these analyses (and the high risk of a type 2 statistical error), a $p \leq 0.1$ is noted to identify comparisons that should be examined more closely in the future. Mean and standard error of the mean were calculated for each dependent variable.

Chapter 4

<u>Results</u>

A- Prior to Leptin Pump Study

Preliminary Thrombosis Data

Our thrombosis trial reveals a tendency of lipodystrophic mice to have a delayed clotting in response to photochemical arterial injury. When compared to WT littermates, TG mice had significantly longer time to arterial occlusion ($p<0.05$) revealing delayed formation of a thrombus (**Figure 1**). This observation prompted us to conduct several investigations.

Hematology

Aiming towards finding factors responsible for the altered thrombotic profile observed in fatless mice, blood cell and platelet counts were obtained from TG mice and WT controls. The small differences observed were not statistically significant: blood cell counts (x 10^9/L) were 8.95 $\pm$ 0.34 and 8.03 $\pm$ 0.21 in TG and WT mice, respectively. Platelet counts (x 10^9/L) were 1.93 $\pm$ 0.13 in TG mice and 1.53 $\pm$ 0.21 in WT mice.

Also within the search for underlying factors behind delayed clotting in fatless mice, a Euglobulin Assay was performed on blood samples obtained from the same mice mentioned above for the determination of plasma fibrinolytic activity. As shown in **Figure 2**, the TG and WT mice had similar trends and therefore no significant differences in fibrinolysis.

Furthermore, PT and a-PTT were measured in lipodystrophic mice and wild-type controls and also, no significant differences were observed. PT (sec)

was 21.75 $\pm$ 0.63 in the TG group and 20 $\pm$ 1.53 in the WT group. a-PTT (sec) was 59.5 $\pm$ 9.56 and 59.66 $\pm$ 1.45 in TG and WT mice, respectively.

CBC measures were obtained from TG mice and WT littermates and no significant differences were found between the two groups, as shown in **Table 1**.

The above findings, therefore, reveal no significant differences between WT and TG mice in hematologic and fibrinolytic factors that might explain the unexpected anti-thrombotic trend of the AZIP-TG mice. We thus proceeded to leptin administration.

B- Leptin Pump Study

Arterial Thrombosis

The arterial thrombosis data are tabulated in **Table 2**. A major complication was encountered during the arterial thrombosis protocol: death of 4 TG mice before occlusion (> 50% of the TG mice). The TG Tx mice that survived (2 mice), however, did not have the faster clotting that was hypothesized. An occlusive thrombus was not formed and the experiment was discontinued at 150 minutes. The same non-clotting phenotype was observed in the only TG C mouse that survived, consistent with the preliminary data of the TG mice. The TG group had a significantly prolonged time to clotting when compared to the WT group ($p<0.05$), as demonstrated in **Figure 3**. As for WT mice, the difference in clotting time between the treated and the untreated groups was not significant.

Body Weights

At baseline, TG mice were significantly heavier than their WT littermates ($p=0.01$). Leptin treatment significantly reduced the body weight of both the TG mice ($p<0.005$) and the WT mice ($p<0.05$) (**Figure 4**).

Figure 5 depicts the percent body weight change in response to treatment. The values obtained reveal a significant difference in the changes in body weight between the TG C and the TG Tx groups ($p=0.001$).

Blood Parameters

Baseline leptin differences between WT mice and TG mice were huge. WT mice had remarkably higher levels, as leptin levels in TG mice were nearly undetectable. **Figure 6** shows that serum leptin concentration increased with treatment in both the TG mice ($p<0.01$) and the WT mice ($p<0.05$). Control TG mice had no change in leptin concentration.

Even though the leptin-treated TG mice had lower blood glucose concentration (88.5 ± 30.1) compared to the vehicle-treated TG mice (139.6 ± 42.5), this difference ($p=0.1$) did not reach statistical significance. Values for WT mice were also not significantly different (C: 126.4 ± 6.9; Tx: 106.8 ± 7.5).

Figure 7 refers to the change in serum insulin concentration observed in the WT and TG groups. At baseline, the TG mice had remarkably higher insulin concentration than the WT mice ($p=0.001$), as expected. The treatment of TG mice with leptin resulted in a significant reduction in insulin concentration ($p<0.05$). The same effect was seen in the WT mice. No significant changes were observed in control mice.

Homeostasis Model Assessment for estimated insulin resistance (81), HOMA-IR, was computed to analyze the difference in insulin resistance between the groups. HOMA-IR scores revealed a significantly higher insulin resistance in the TG C group when compared to the TG Tx group ($p=0.01$) and either of the WT groups ($p=0.005$), as follows: TG C 131.2 $\pm$ 65.9 > TG Tx 17.4 $\pm$ 15.1 = WT C 6.65 $\pm$ 2.15 = WT Tx 3.39 $\pm$ 1.68.

Prior to treatment, TG mice had significantly higher levels of triglyceride in their plasma when compared to WT mice ($p=0.005$). Plasma triglyceride concentration did not change significantly after treating the mice with leptin, in either the TG or WT mice, as shown in **Figure 8**. However, there was an unexpected increase in triglyceride in the WT control group.

Plasma cholesterol concentration, as **Figure 9** reveals, significantly dropped in the TG mice upon treatment with leptin ($p<0.05$). In addition, both the treatment and control WT mice had a reduction in plasma cholesterol ($p<0.05$). The vehicle-treated TG mice did not encounter any significant changes.

Liver Parameters

Protein concentration in livers of the mice was computed for the purpose of normalizing liver parameters to protein levels.

The liver triglyceride to protein ratio, shown in **Figure 10**, reveals a significant difference in triglyceride concentrations between the control TG mice and the control WT mice *($p<0.05$)*, with the former being higher as expected for "fatless mice". A trend was evident (*$p=0.089$)* for the leptin-treated TG mice to have a lower liver triglyceride to protein ratio than the vehicle-treated TG mice.

No significant differences were observed in liver cholesterol to protein ratios (mg:mg) between groups (TG C 0.0046 $\pm$ 0.0016 = TG Tx 0.0031 $\pm$ 0.001 = WT C 0.0041 $\pm$ 0.001 = WT Tx 0.0042 $\pm$ 0.001).

Harvested Organ and Tissue Weights

Weights of livers of the mice involved in the study are displayed in **Figure 11** which shows that leptin-treated TG mice had significantly smaller livers than vehicle-treated TG mice ($p<0.05$). Moreover, TG-C mice had significantly larger livers than WT-C mice did ($p<0.0001$). Livers of the leptin-treated TG mice were still significantly heavier than either of those of the WT mouse groups ($p<0.01$) i.e. the control WT mice and the treated WT mice. The differences observed were significantly affected by both the genotypes of the mice ($p<0.0001$) and the treatment they received ($p<0.05$).

Results for the other organs that were weighed are shown in **Figure 12**: Organ weights were significantly affected by genotype. However, the difference in these weights between the control TG mice and the treated TG mice was not significant. TG mice had heavier spleens, hearts and kidneys than WT mice ($p<0.05$), consistent with previous observations. Neither genotype nor treatment showed a significant effect on differences in lung weights.

Figure 13 graphs the fat pad weights obtained from the mice. Upon dissection, fat pads from different areas of the mouse body were acquired, i.e. dorsal, perirenal and gonadal fat. The weight obtained is a combination of all the above mentioned fat pads weighed together. No visible adipose tissue was found in the TG mice, whether treated or untreated. The leptin-treated WT mice had

significantly less fat following the intervention than their control littermates (*p=0.005*).

Figure 14 displays two livers, one obtained from a TG mouse and another obtained from a WT mouse. The evident difference lies mostly in the size of the liver where that of the TG mouse is radically larger than that of the WT mouse (hepatomegaly). A difference in the liver color can also clearly be seen with the fatty TG mouse liver having a lighter color (due to increased fat deposition).

Figure 15 shows a dorsal view of 2 skinned mice: a TG and a WT. Hepatomegaly which is a characteristic property of lipodystrophic mice is evident in this TG mouse, in addition to the absence of fat pads. The WT mouse, on the contrary, has a normal dorsal appearance.

Chapter 5

<u>Discussion</u>

Significant Findings Prior to the Leptin Pump Study

The absence of adipose tissue results in several detrimental effects on the metabolic and physiological profiles of patients with lipodystrophy, most of which have been explored. However, even though "better" treatments exist, the "ideal" or "ultimate" treatment is yet to be found (5-12). Moreover, the mechanisms underlying the observed alterations need to be more clearly understood, which may be a key to advancing treatment options. The AZIP/F-1 mouse model, as mentioned previously, shares many characteristics with lipodystrophic patients and therefore provides an animal model of severe lipodystrophy on which studies can be performed to unlock the mysteries of this disease and assist in finding better treatment options (1, 42). It is a mouse model with a complex phenotype. The metabolic aspects of this phenotype have been fairly well studied; however, we were curious about other aspects, such as thrombotic function.

Our arterial thrombosis trial revealed a remarkable and surprising phenotype in the fatless mice: prolonged time to arterial occlusion/clotting when exposed to photochemical arterial injury, compared to wild-type littermate mice. Impaired vascular function has been previously seen in these mice though in a different way. According to Takemori et al, A-ZIP/F1 lipoatrophic mice are hypertensive, likely secondary to the lack of perivascular fat tissue and an upregulation of vascular Ang II type 1 receptors (82). Lamounier-Zepter et al later

suggested that the high blood pressure observed in these mice may be due to metabolic-vascular alterations including changes in adrenocortical cells and the hypercorticosteronemia seen in the mice (83). Thrombosis, however, has not been examined in these mice. The fatless mice phenotype described above, therefore, prompted us to investigate about the etiology through examining blood counts and clotting factors in these mice compared to WT controls. We first started with the blood cell and platelet counts that were not significantly different between the TG and WT mice. This meant that there must be a factor beyond cell and platelet counts that may still be related to blood/thrombotic profiles of the mice. The next step was determining fibrinolysis that we thought might be altered in the transgenic mice. However, the euglobulin clot lysis assay that we performed resulted in similar fibrinolysis trends between the TG and the WT mice, although the baseline absorbance was different between the groups. We rendered that to the turbidity that we observed in the plasma samples of the TG mice, which in turn can be explained by the previously demonstrated hyperlipidemia in the blood of these mice (42, 44, 45). We then proceeded to obtaining CBC's and PT tests from TG mice and WT controls where again, differences between the two groups were not evident. Our last step in evaluating hematologic and fibrinolytic differences was measuring a-PTT which also was not different in the TG mice when compared to their WT littermates. Our conclusion was that the delayed clotting observed in these mice must be the result of a mechanism or an anomaly that is not related to hematologic and fibrinolytic factors but rather to a different characteristic of lipodystrophic mice.

Arterial thrombosis trials performed in the past have shown a positive correlation between leptin and blood clotting, i.e. in other words, leptin has been shown to promote platelet aggregation and therefore blood clotting in humans (70, 71) as well as in mice (74-77). Leptin is very low or undetectable in AZIP-TG mice (42) and at the same time our mice had normal blood counts and clotting factor profiles as previously stated. These findings combined led to the hypothesis that the absence of leptin may be behind the altered thrombotic phenotype seen in our lipodystrophic mice. By providing these mice with leptin, we could test the above hypothesis and examine the metabolic effects of leptin administration specifically on the FVB/B6 lipodystrophic mice. We therefore provided our four mouse groups with leptin or control solutions exogenously and compared the parameters and changes of interest at different time intervals.

Arterial Thrombosis

Although we had a minimal number of successful thrombotic experiments to rigorously test our hypothesis, our data do not support an effect of leptin on thrombosis in the A-ZIP mice. Regardless of whether or not they were receiving leptin, we observed an extremely delayed occlusion time after photochemical injury to the carotid artery of A-ZIP TG mice. We have not been able to attribute this surprising alteration to enhanced fibrinolysis, deficient clotting factors, or abnormal blood/platelet counts. Although we regret that we were unable to obtain an adequate number of animals to carefully test our hypothesis, we feel relatively confident that leptin does not "rescue" the altered thrombotic phenotype of the A-ZIP mice. Our poor success in attaining the

arterial thrombosis endpoint in these studies (with mortality of more than half of our TG mice during the procedure), is a function of the difficulty in delivering anesthesia to these mice. Maintaining their depth of anesthesia at an adequate level (without overdose) is very difficult, as previously mentioned in studies of the AZIP-TG mouse (42).

Body and Organ/Tissue Weights

TG mice weighing more than WT mice was not surprising. This was seen previously (42) and can be explained by organ enlargement that fatless mice encounter as they mature, especially hepatomegaly (42, 44, 45). As expected, treatment with leptin led to a decrease in body weight in both the TG and WT mice. This finding is not surprising since leptin is known to control weight via controlling energy intake and metabolism (15, 16). The reduction in body weight may further be attributed to increasing energy expenditure in the WT mice though this may be unlikely in the TG mice since leptin therapy has been associated with a reduction in energy expenditure in lipodystrophic subjects (5, 10).

The trends of organ and tissue weights were also as we had expected. Livers of TG mice were significantly larger than those of WT mice, which has been previously shown (42, 44, 45). This difference in size is attributed to several factors, mainly the fact that the liver in lipodystrophic animals is acting as a storage depot that also stores what would normally be stored in adipose cells, i.e. triglycerides (42), which leads to the previously proven hepatic steatosis in lipodystrophic mice (34-37, 42, 84). Moreover, the inflammatory state that is seen in these animals (51) may be playing a role in contributing to organomegaly,

mainly hepatomegaly, knowing that hepatic steatosis and inflammation have generally been shown to be associated (84). The livers of leptin-treated TG mice, on the other hand, were significantly smaller than those of the control TG mice, a result that was also predictable and supported by the literature (42, 44, 45). Leptin administration clearly ameliorated the hepatomegaly through enhancing metabolism. It attenuated the steatotic state, an effect that has been seen in both lipodystrophic patients (11) and mice (56). This may partly explain the described alterations in body weight observed in our TG mice.

Spleens, hearts and kidneys were heavier in TG mice than in WT mice. This is consistent with previous findings that reported organomegaly in lipodystrophic mice (42, 44, 45). These organs were not lighter in leptin-treated TG mice when compared to vehicle-treated TG mice. This may imply that some of the anomalies caused by lipodystrophy are irreversible.

The most exciting part of the dissections was the extensive search for fat pads. None were found in the TG mice. This coincides with previous findings (42, 49) and explains most of the physiological and metabolic abnormalities. Importantly, leptin-treated WT mice had less fat than control WT mice did. This implies that leptin treatment was effective and that these WT mice were "leptin sensitive".

Blood and Liver Parameters

At baseline, TG mice had very low levels of leptin (< 0.5 ng/ml), i.e. below normal physiological levels (85). This was anticipated due to the lack of fat in TG mice and therefore a lack of this adipocyte-derived hormone. Consistent with

this, serum leptin concentration has been observed to be directly proportional to fat mass (5). As expected, the WT mice had significantly higher leptin concentrations at baseline, which were in the normal physiologic range. Upon treatment, serum leptin levels in the leptin-treated TG mice increased robustly (almost 7-fold), while vehicle-treated TG littermates encountered no change in leptin concentration. These findings demonstrate that our leptin treatment (via osmotic pump) of TG mice effectively increased the serum leptin concentration into a normal physiologic range.

Blood glucose (BG) levels of untreated TG mice were higher than those of treated mice where the former met the criteria for hyperglycemia and the latter fell within the normoglycemia range; however, these differences were not significant. This is likely due to the variability of the measure, the limited number of mice, and the modest elevation of glucose in this relatively young cohort. In future studies, it will be helpful to obtain BG levels at several time points to more accurately observe the effect of treatment on the mice. This was not done in this study in order to avoid excessive blood withdrawal from the mice and therefore additional stress.

TG mice, which were expected to be diabetic/ insulin resistant at the time of the blood draw, did have significantly more circulating insulin than did their WT controls, at baseline. In addition, compared to the TG control-treated mice (which became worse during the intervention period), the TG leptin-treated mice had significantly reduced fasting insulin concentration. This is consistent with previous findings where insulin resistance became progressively worse from 4

weeks of age to 10 weeks of age in the Azip fatless mice (49), and where leptin enhanced insulin sensitivity in fatless mice (55-57). We also observed a reduction in insulin concentration in leptin-treated WT mice suggesting that a deficit of leptin at baseline was not required for a decrease in fasting insulin during leptin treatment.

HOMA-IR scores confirmed that the TG C group was extremely insulin resistant and that this effect was ameliorated following leptin treatment. This is also consistent with the known insulin resistant phenotype of lipodystrophic mice (44, 45) that is improved with leptin therapy (56).

High circulating levels of lipids in the blood are expected in fatless mice that lack the normal mammalian fat storage depot (42). This was confirmed in our study when comparing these mice to controls at baseline. Triglyceride levels obtained from plasma samples did not change significantly in the TG mice. They seemed to remain almost the same upon treatment, which might imply that treatment at least prevented further increase in triglyceride concentration in the blood. The only significant change that was seen is an increase in triglyceride levels in the untreated WT mice. This may be a normal trend related to age. As for liver triglyceride measures, as expected, levels were higher in TG mice than in WT mice (vehicle-treated). This has been previously documented (49) as AZIP-TG mice are known to develop hepatic steatosis (42). Moreover, the leptin-treated TG group had lower levels than did the vehicle group, which is supported by previous studies both in humans (5, 9) and in mice (56). These results are explained by 1) the fact that the liver in the TG mice acts as the major triglyceride

storage depot in the absence of adipose cells, causing triglyceride accumulation and hepatic steatosis; and 2) the fact that leptin improves triglyceride metabolism and thereby reduces hepatic triglyceride accumulation.

In both plasma and liver, there were no significant differences in cholesterol levels of TG versus WT mice. This was surprising as previous studies have reported hyperlipidemia in lipodystrophic mice (42, 44, 45). What was expected, however, is the reduction in plasma cholesterol concentration in the leptin-treated TG mice. This coincides with previous suggestions that leptin improves lipid metabolism in both lipodystrophic humans (11, 12, 86) and mice (55, 56).

Of interest, the A-ZIP phenotype has been demonstrated to be affected by genetic background. For example, A-ZIP mice on the FVB background have higher circulating triglyceride and fatty acid levels and more hyperglycemia than those on the B6 background according to Colombo et al in 2003 (43). This may explain some of the unexpected findings listed above.

Chapter 6

<u>Conclusion</u>

The United States has been facing a remarkable increase in obesity over the last two decades, associated with several disorders (87-92). Nonalcoholic fatty liver disease is rapidly spreading mainly in the form of hepatic steatosis which in turn is a known component of obesity and the metabolic syndrome along with insulin resistance and hyperlipidemia (93). Insulin resistance and dyslipidemia can be caused by either excessive adiposity or lack of adipose tissue (94). Lipodystrophy, therefore, carries many of the same characteristics as more common metabolic diseases. This disease is being actively researched and effective mouse models have been produced, with the Azip-transgenic model being very similar in physiology and metabolism to some lipodystrophic patients (1, 42). Ongoing and future studies using these models should help explore the vague aspects of lipodystrophy and provide more effective treatment options for lipodystrophic patients.

Adipose tissue serves as an endocrine organ through secreting several bioactive compounds (adipokines) whose dysregulation is thought to contribute to insulin resistance and metabolic disease (94). Leptin, a major adipokine, has an essential role in energy homeostasis (5). Low leptin levels appear to be a characteristic abnormality in lipodystrophic patients that can serve as an effective therapy for reducing their metabolic disease. However, as previously introduced, there are many forms of lipodystrophy, so a clear mechanistic understanding of each disease will lead to the most effective treatment strategies.

In addition, the administration of adipokines other than leptin might be a promising future research option since these adipokines may turn out to be key players in many of the metabolic and physiological aspects of lipodystrophy, obesity and/or the metabolic syndrome.

Aside from the metabolic and physiological characteristics, the transgene possessed by the fatless mice may be leading to an altered thrombotic function via mechanisms that are unrelated to the lipodystrophy or metabolic disturbances of this model. Therefore, adipose tissue transplantation in the A-ZIP lipodystrophic mice may help determine whether it is truly the absence of adipose tissue that induces the observed thrombotic alterations or whether the effect is a by-product of the transgenic strategy. Additionally, transplanting adipose tissue that lacks leptin may further aid in understanding and dissociating these factors.

In the future, in a study like ours, a larger sample size and a longer duration would likely produce more significant outcomes with a reduced variability. These study-design improvements combined with the above research suggestions may help solve unanswered questions about the lipodystrophies and possibly find treatment targets not only for these disorders, but also for other chronic metabolic conditions such as diabetes.
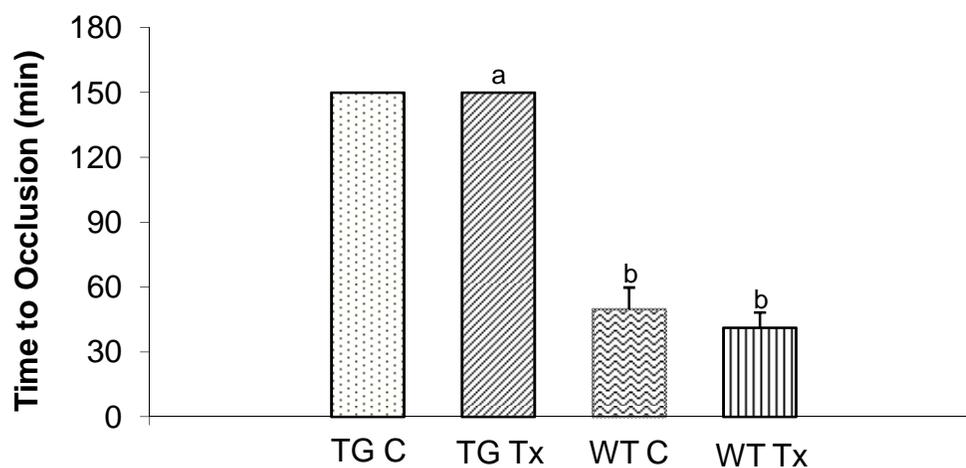
Figure 1
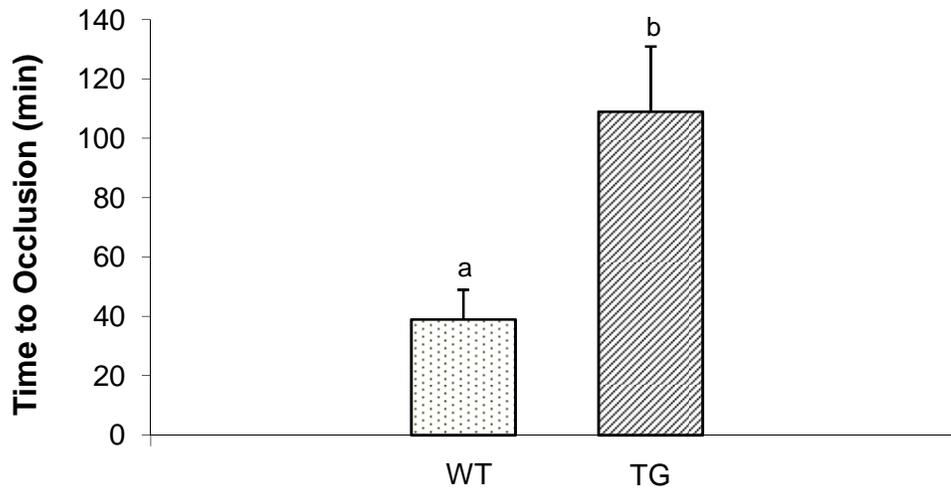
Bars with different superscripts are significantly different from each other ($p < 0.05$)

Figure 1- Formation of Occlusive Thrombus in Wild-type (WT) and Transgenic Fatless (TG) FVB/B6 Mice. TG mice have a delayed time to arterial occlusion, compared to WT mice.
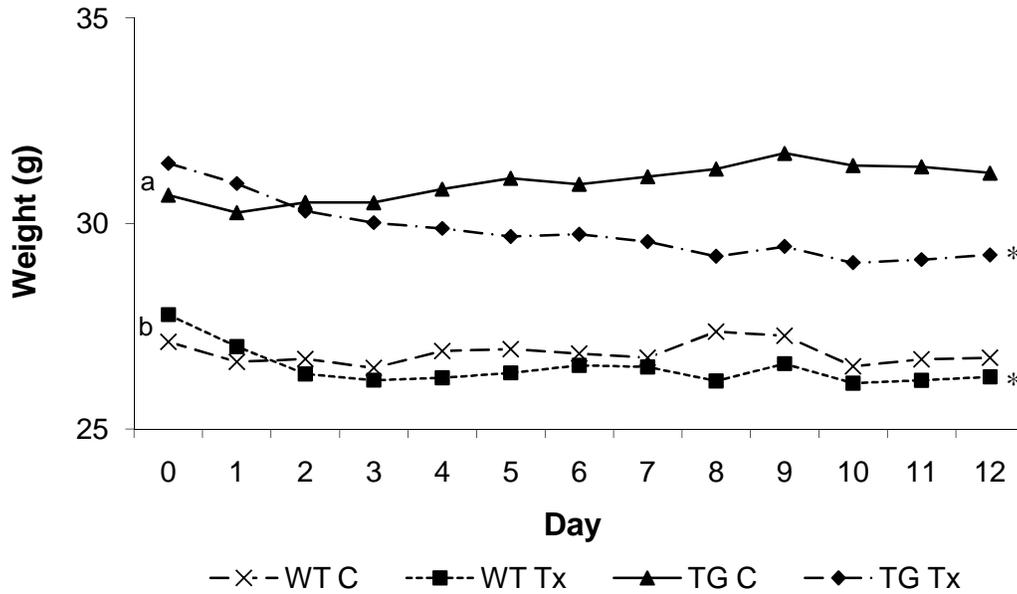
Figure 2
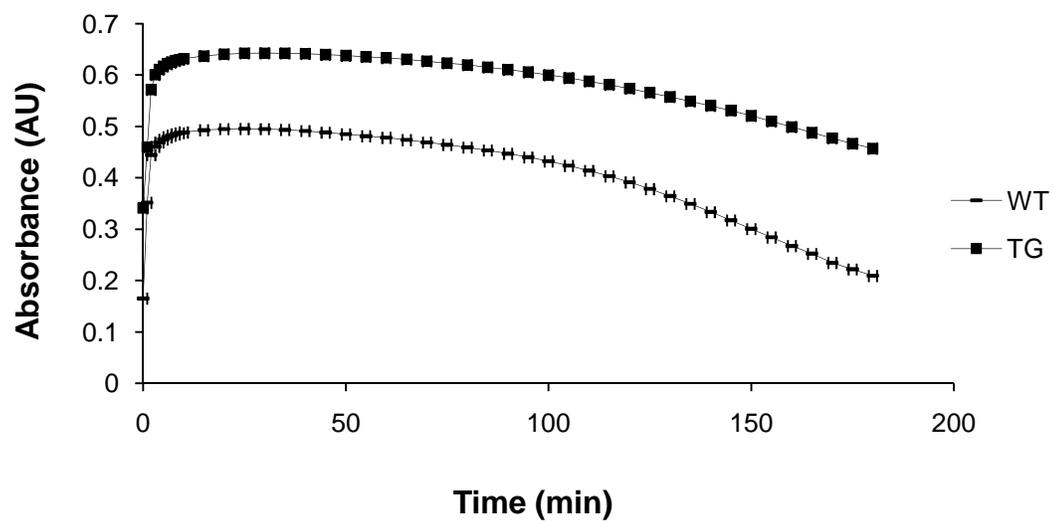
Figure 2- Euglobulin Clot Formation and Lysis in FVB/B6 Mice.
Similar trends in fibrinolysis are observed in TG and WT mice.

Figure 3

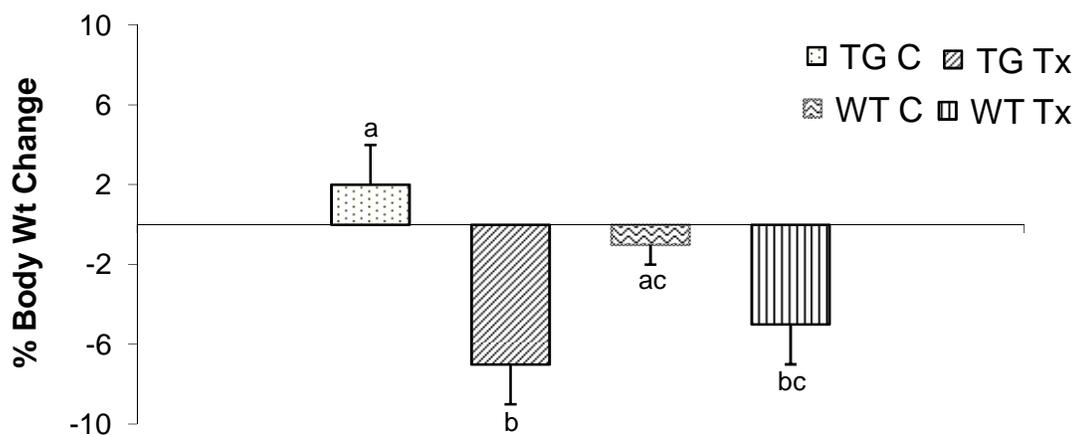Bars with different superscripts are significantly different from each other ($p < 0.05$)

Figure 3- Time to Formation of Occlusive Thrombus per Group. TG mice have a prolonged time to arterial occlusion, compared to WT mice. When comparing TG mice that survived, TG C (n=1) and TG Tx (n=2) are not different.

Figure 4

* Asterisks indicate significant pre-post change; Letters indicate significant TG vs WT baseline difference ($p <0.05$)

Figure 4- Day by Day Body Weights per Group. TG mice are heavier than WT mice at baseline. Following the intervention, both TG and WT leptin-treated mice lost weight, comparing pre-treatment to post-treatment weights.

Figure 5

Bars with different superscripts/subscripts are significantly different from each other ($p < 0.05$)

Figure 5- Percent Body Weight Change per Group. TG C mice continued to gain weight as they mature while leptin-treated mice (TG and WT) lost weight.

Figure 6
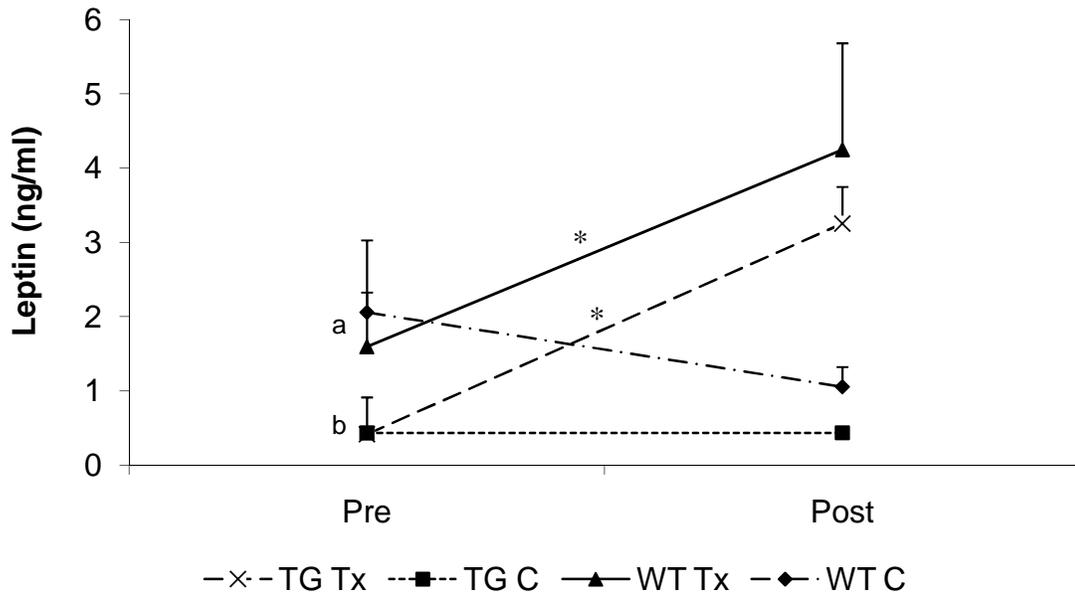
* Asterisks indicate significant pre-post change; Letters indicate
  significant TG vs WT baseline difference (*p* <0.05)

Figure 6- Change in Mean Serum [Leptin] per Group. Baseline
serum leptin levels are higher in WT mice where TG mice have nearly
undetectable levels. Both the TG and the WT leptin-treated groups
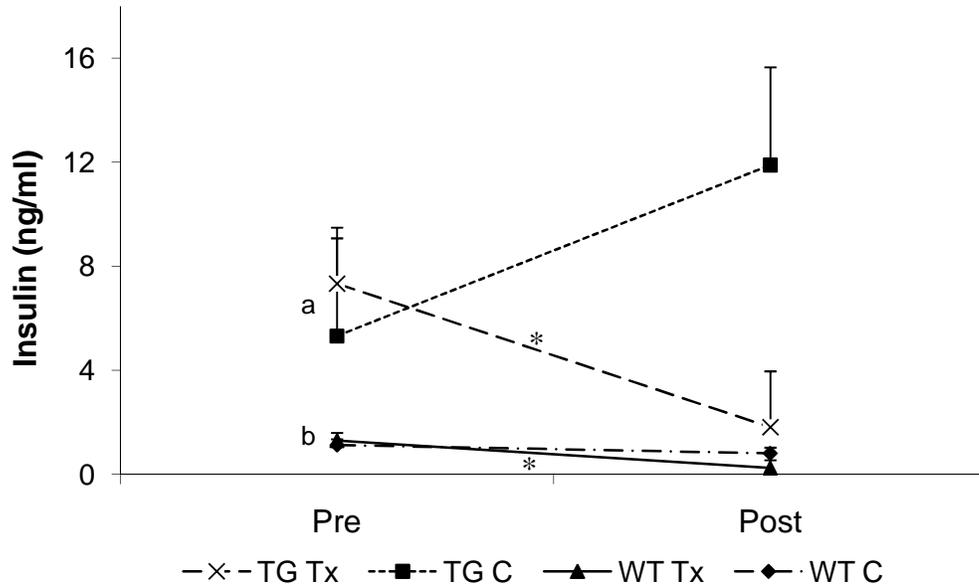had an increase in circulating leptin.

Figure 7

* Asterisks indicate significant pre-post change; Letters indicate
  significant TG vs WT baseline difference (p <0.05)

Figure 7- Change in Mean Serum [Insulin] per Group. Baseline
serum insulin levels are higher in TG mice compared to WT mice.
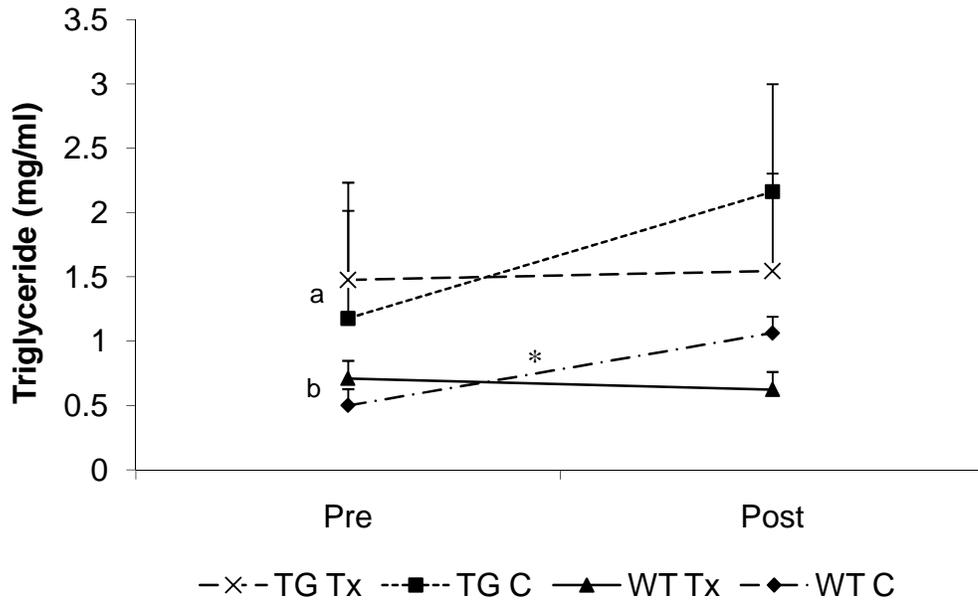Both the TG and the WT leptin-treated groups had a reduction in
insulin levels.

Figure 8

* Asterisks indicate significant pre-post change; Letters indicate
  significant TG vs WT baseline difference (*p* <0.05)

Figure 8- Change in Mean Plasma [Triglyceride] per Group.
Baseline plasma triglyceride levels are higher in TG mice compared to
WT mice. No significant reductions were observed upon treatment
with leptin.

Figure 9
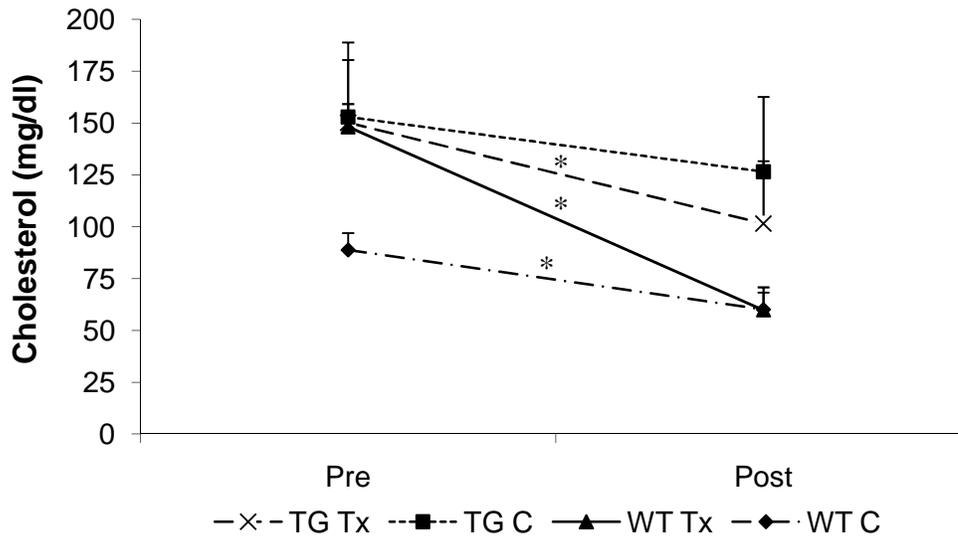
* Asterisks indicate significant change (p <0.05)

Figure 9- Change in Mean Plasma [Cholesterol] per Group.
A significant reduction was observed in all groups except in the
TG C group.

Figure 10
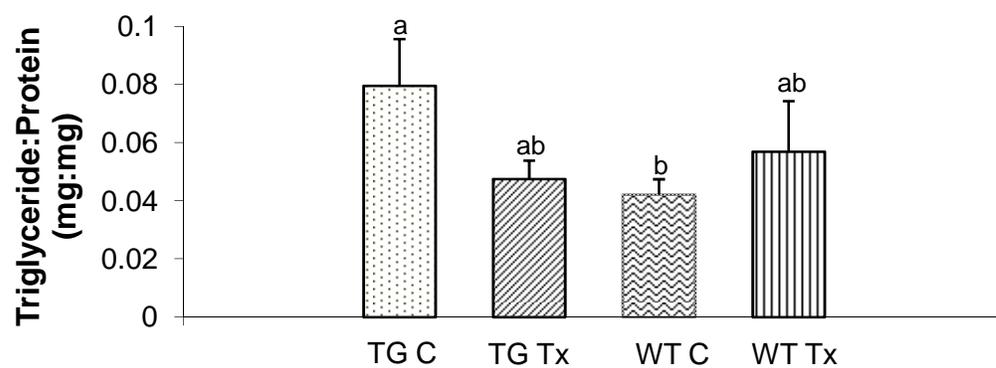
Bars with different superscripts are significantly different from each other ($p < 0.05$)

Figure 10- Mean Liver [Triglyceride:Protein] per Group. TG C mice have higher levels of liver triglyceride than WT C mice.

Figure 11
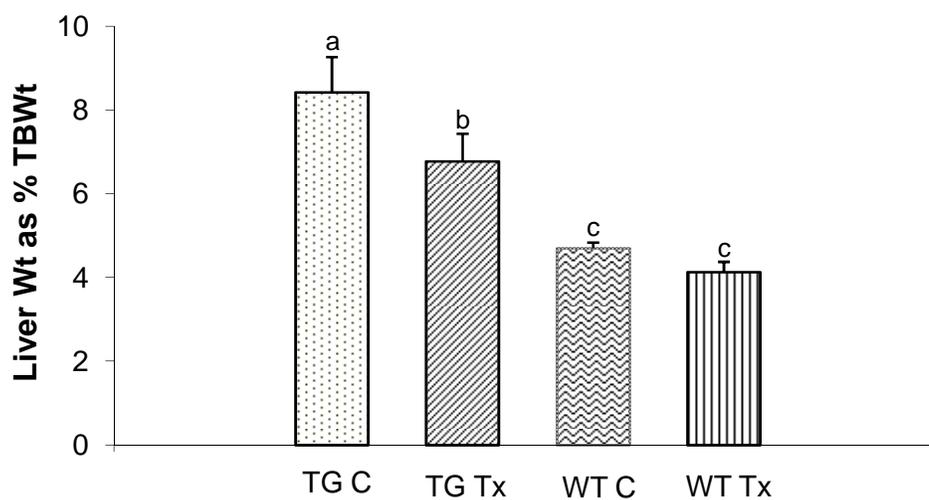
Bars with different superscripts are significantly different from each other
(*p* <0.05)

Figure 11- Mean Liver Weight as % Total Body Weight per Group.
Livers of TG mice are heavier than those of WT mice. Livers of leptin-
treated TG mice are lighter than those of vehicle-treated TG mice.

Figure 12
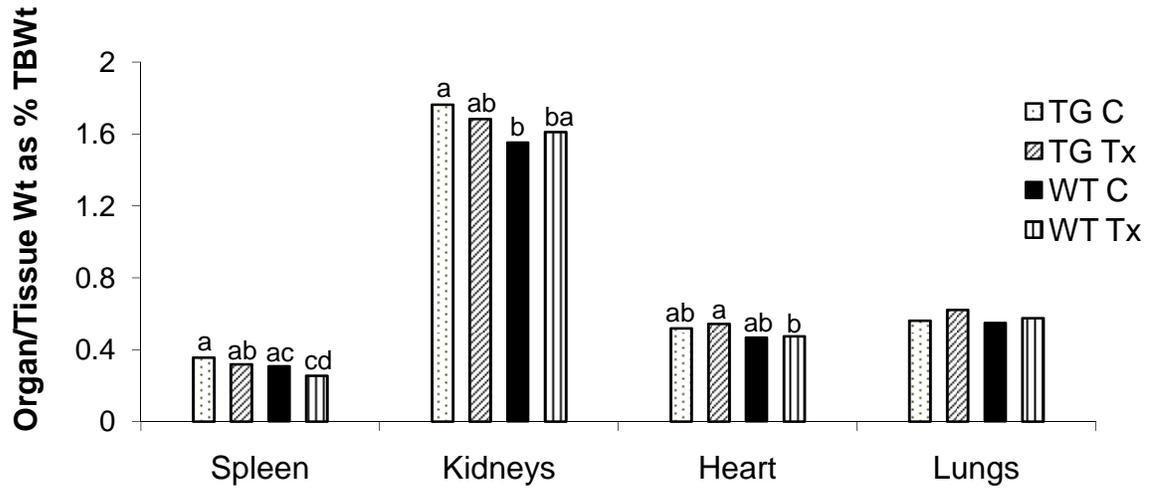
Bars with different superscripts are significantly different from each other (p <0.05)

Figure 12- Mean Organ/Tissue Weights as % Total Body Weight per Group. TG mice have larger organs compared to WT mice. No significant effect of treatment is seen.

Figure 13
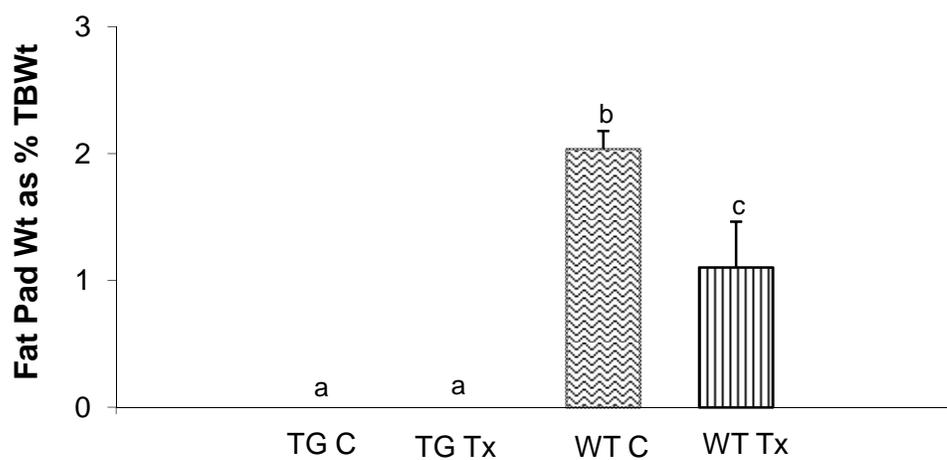
Bars with different superscripts are significantly different from each other (p <0.05)

Figure 13- Total Adipose Tissue Weight as % Total Body Weight per Group. Both groups of TG mice lack visible adipose tissue. WT Tx mice have less fat than WT C mice.

Figure 14
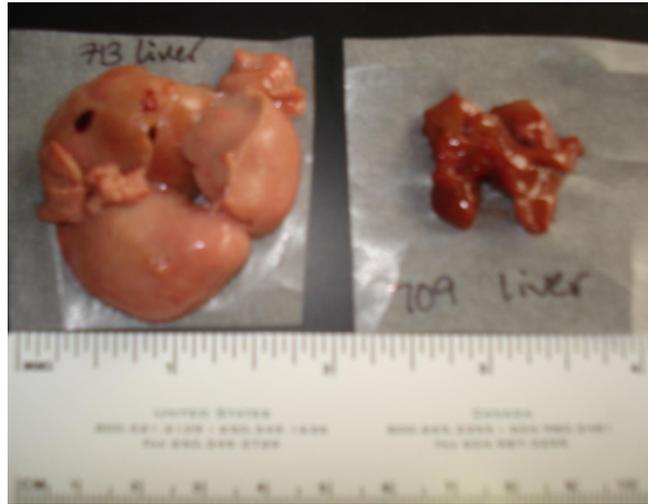
Figure 14- Mouse Livers Following Dissection. A TG mouse liver with hepatomegaly and steatosis (left) vs a healthy WT littermate liver (right).

Figure 15
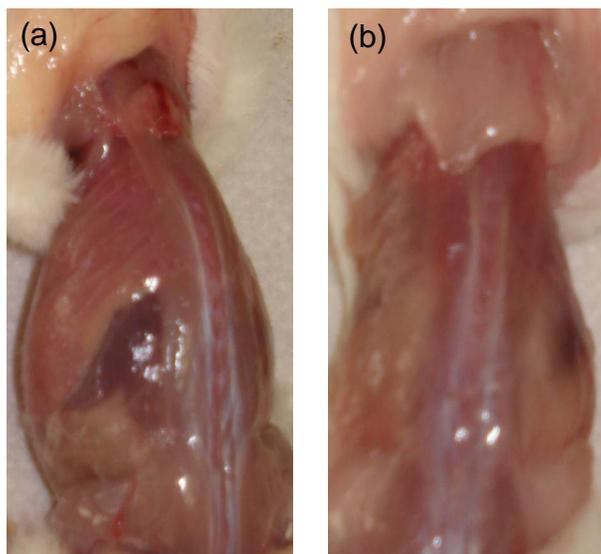
Figure 15- Dorsal View of Skinned Mice with Intact Organs and Tissues. (a) The TG mouse has a large liver occupying most of the abdominal cavity and lacks visible fat pads. (b) The WT mouse has a normal dorsal view with the absence of organomegaly and the presence of fat pads.

Table 1: Complete Blood Counts (CBC) in Wild-Type Vs Transgenic FVB/B6 Mice (Mean ±SEM)

Blood Parameter	Genotype	
	Wild-type	**Transgenic**
Plt (10^3/ul)	492.33 $\pm$ 65.9	567.25 $\pm$ 55.1
WBC (10^3/ul)	4.87 $\pm$ 0.67	4.825 $\pm$ 0.85
RBC (10^6/ul)	9.77 $\pm$ 0.44	9.70 $\pm$ 0.66
MCV (fl)	43.93 $\pm$ 0.88	46.08 $\pm$ 0.27
RDW	18.67 $\pm$ 0.59	17.9 $\pm$ 0.34
MCHC (g/dl)	35.2 $\pm$ 0.75	34.78 $\pm$ 0.28
MCH (pg)	15.4 $\pm$ 0.10	16 $\pm$ 0.04
MPV (fl)	6.33 $\pm$ 0.07	6.4 $\pm$ 0.04
HGB (g/dl)	15.07 $\pm$ 0.73	15.53 $\pm$ 1.08
% HCT	42.97 $\pm$ 2.63	44.73 $\pm$ 3.24
% Lym	81.33 $\pm$ 3.37	78.35 $\pm$ 2.29
% Mon	6.4 $\pm$ 1.07	6.6 $\pm$ 0.31
% Gra	12.27 $\pm$ 2.44	15.05 $\pm$ 1.99

Table 2: Leptin Pump Thrombosis Data: Time to Occlusion

Mouse Group	Time to Occlusion (m)	Status
TG C	48	Expired
TG C	150+	Discontinued
TG C	60	Expired
TG Tx	150+	Discontinued
TG Tx	87	Expired
TG Tx	150+	Discontinued
TG Tx	19	Expired
WT C	53	Completed
WT C	17	Completed
WT C	47	Completed
WT C	52	Completed
WT C	80	Completed
WT Tx	29	Completed
WT Tx	31	Completed
WT Tx	46	Completed
WT Tx	59	Completed

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ABSTRACT

**EFFECT OF EXOGENOUS LEPTIN ON THROMBOTIC AND
METABOLIC PROFILES OF FVB/B6 LIPODYSTROPHIC MICE**

by

HODA KADOUH

May 2010

Advisor: Dr. Peter F. Bodary

Major: Nutrition and Food Science

Degree: Master of Science

Lipodystrophy caused by fat deficiency contributes to metabolic disease for which several treatment modalities have been implemented, with leptin therapy being the most effective to date. In addition to playing a role in energy homeostasis and metabolism, leptin was also shown to play a pro-thrombotic role in mice. This role was not examined in fatless mice, neither was thrombosis measured. The AZIP/F-1 (FVB) lipodystrophic mouse appeared to have a prolonged arterial occlusion time ($p<0.05$) in a trial done in our lab, with clotting factors being normal. The present study was designed to observe the thrombotic and metabolic characteristics of fatless mice and examine the effect of leptin therapy on these traits. 16 FVB/B6 mice were produced in-house for the study, to receive recombinant mouse leptin or saline via osmotic pumps over 12 days. Transgenic (TG) mice were randomly divided into two groups: Treatment (Tx, n=4) and control (C, n=3); and their wild-type (WT) littermates were similarly

divided into Tx (n=4) and C (n=5). TG mice had a prolonged time to formation of occlusive thrombus compared to WT mice (p<0.05). Leptin treatment did not have an effect on arterial thrombosis in our mice. At baseline/control, TG mice were heavier than WT mice (p<0.05), had larger livers (p<0.0001), larger kidneys (p=0.01), higher serum insulin (p<0.01), higher plasma and liver triglyceride (p<0.01 and p<0.05, respectively) and less leptin (p<0.0001). TG-Tx mice decreased in weight (p<0.05) and had smaller livers than those of TG-C mice (p<0.05) while having higher levels of circulating leptin (p=0.01) and reduced levels of serum insulin (p<0.05) and plasma cholesterol (p<0.05). TG C mice had higher HOMA-IR scores (p<0.05) than all other groups verifying insulin resistance that is ameliorated by leptin therapy. These findings confirm the hepatomegaly, hyperinsulinemia/ insulin resistance and hyperlipidemia seen in fatless mice and the effectiveness of leptin therapy while also suggesting that lipodystrophy, at least in the AZIP/F-1 mouse, is associated with delayed thrombosis independent of the lack of leptin. Lipodystrophic mice remain a useful model to study current epidemic metabolic disorders.

AUTOBIOGRAPHICAL STATEMENT

Hoda Kadouh received a Bachelor of Science Degree in Nutrition and Dietetics with a minor in Biology from the American University of Beirut (AUB), Beirut, Lebanon, in 2005 followed by a Dietetic Internship which she completed at the Rafik Hariri University Hospital, Beirut, Lebanon In 2006. In 2007, she joined Wayne State University (WSU) where she has completed her graduate study towards the accomplishment of a Master of Science degree and has recently decided to pursue her studies toward a Doctor of Philosophy degree in Nutrition and Food Science.

While completing her graduate degree, Hoda also fulfilled the requirements for the Coordinated Program in Dietetics at WSU and acquired her credentials as a Registered Dietitian in 2008. Since then, she has been working as a clinical dietitian at Oakwood Healthcare System, Dearborn, MI. she also worked as a part time dietetics instructor at WSU during the academic year 08-09. Since 2007, Hoda has been an active member of dietetic associations, namely the American Dietetic Association (ADA), Michigan Dietetic Association (MDA) and Southeastern Michigan Dietetic Association (SEMDA) in which she has been serving as a board member for two consecutive years in the Bylaws (co-chair), Financial Resources (co-chair) and Nominating committees. Throughout her course of study, she received several academic and professional awards, mainly ADA-MDA's 2010 Recognized Young Dietitian of the Year, SEMDA's 2010 Graduate Scholarship, the WSU Graduate Professional Scholarship for the academic years 07-08 and 08-09 and placement on the AUB Dean's Honor list in 2004 and 2005.

Hoda was an author in an article that was published in Diabetologia, March 2009: "Inhibition of the chemokine (C–C motif) ligand 2/chemokine (C–C motif) receptor 2 pathway attenuates hyperglycaemia and inflammation in a mouse model of hepatic steatosis and lipoatrophy" and an abstract in the American Diabetes Association's 68th Scientific Sessions, June 2008: "Elevations in monocyte chemotactic protein-1 are associated with insulin resistance and inflammation in a mouse model of liver steatosis and lipoatrophy".